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CASE REPORT

Combination of high-dose dexamethasone and antiretroviral therapy rapidly improved and induced long-term remission of HIV-related thrombocytopenic purpura

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Abstract We present a case of HIV-related thrombocytopenic purpura (HIV-ITP) successfully treated with high-dose dexamethasone and antiretroviral therapy (ART). Although high-dose dexamethasone is regarded as the first-line therapy in adult patients with non-HIV ITP, there is limited information on treatment of HIV-ITP and long-term prednisone therapy is considered the standard therapy. High-dose dexamethasone is preferable to conventional long-term prednisone therapy, because of fewer side effects mainly due to shorter steroid use. The ART helps achieve long-term remission for HIV-ITP, although this therapy lacks an immediate effect. In our patient, administration of high-dose dexamethasone resulted in rapid rise in platelet count and ART maintained long-term remission of HIV-ITP. The combination therapy is potentially suitable strategy for the treatment of patients with HIV-ITP and severe thrombocytopenia or bleeding.

Keywords HIV-related immune thrombocytopenic purpura · High-dose dexamethasone · Antiretroviral therapy · HIV-1 infection

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Introduction

HIV-related thrombocytopenic purpura (HIV-ITP) is the most common cause of low platelet count encountered in patients with HIV-1 infection [1]. It is similar to classic immune thrombocytopenic purpura (ITP) in non-HIV patients, and long-term steroid therapy is regarded the standard treatment [2]. High-dose dexamethasone (HD-DXM) is effective in non-HIV ITP [3–5], however, little is known about its effectiveness in HIV-ITP [6, 7]. We describe a 72-year-old man who presented with HIV-ITP and was effectively treated with HD-DXM combined with antiretroviral therapy (ART).

Case report

A 72-year-old Japanese man was admitted to our hospital with thrombocytopenia. The patient had been diagnosed with HIV-1 infection 10 years earlier and ART was initiated 3 months after the diagnosis. However, adherence to therapy was poor, and the platelet count tended to decrease at times of high HIV-1 RNA viral load during poor adherence. Three months before admission, ART was changed to once-daily ritonavir-boosted darunavir (DRV/r) plus tenofovir/emtricitabine (TDF/FTC) to enhance adherence to therapy. Although repeated HIV-1 resistance testing showed no major mutation, HIV-1 RNA viral load was >1,000 copies/ml over several months. Apart from ART, there was no change in his medications and he had not had any infections during 6 months before admission. On admission, platelet count was 20,000/μl and CD4 count was 168/μl. The patient was alert and oriented with body temperature of 36.2 °C. Physical examination showed no signs of bleeding (e.g., no petechiae, purpura, or mucosal

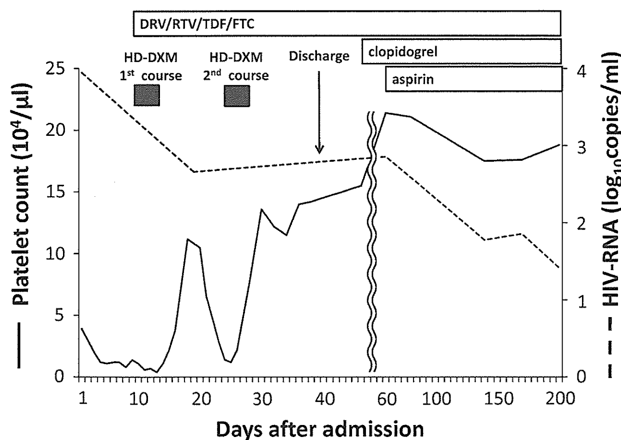

Fig. 1 Clinical course during hospitalization. *DRV* darunavir, *RTV* ritonavir, *TDF* tenofovir, *FTC* emtricitabine, *HD-DXM* high-dose dexamethasone

bleeding). To rule out drug-induced thrombocytopenia, ART, together with clopidogrel and aspirin, which had been administered for years, were discontinued on admission. Although platelet transfusion was initiated for a couple of days, no change in platelet count was noted. Bone marrow examination on day 5 showed hypocellularity with a low number of megakaryocytes. No histopathological findings specific to myelodysplastic syndrome or leukemia were noted. On day 10, the patient developed intermittent epistaxis with a platelet count of 4,000/μl. On that day, a four-day course of orally administered HD-DXM of 40 mg/day was initiated, and ART with DRV/r plus TDF/FTC was reinitiated. The platelet count increased to 66,000/μl on day 10 after the above treatment, but it decreased to 12,000/μl on day 14. A second course of HD-DXM of 40 mg/day was initiated. The platelet count improved to 115,000/μl on day 10 after the second course, and 142,000/μl on day 15. Based on such improvement, no third course was considered necessary. The patient was discharged on day 39 from admission. No adverse event of dexamethasone was observed. The platelet count remained stable after discharge despite the re-initiation of clopidogrel and aspirin (Fig. 1). Three months after re-initiation of ART and thereafter, the HIV-1 viral load was suppressed to <100 copies/ml with good medication adherence. The patient experienced no relapses of HIV-ITP for 9 months.

Discussion

We reported here a patient with HIV-ITP who was treated successfully with a combination with two courses of HD-DXM and ART. The ITP likely relapsed when the platelet count diminished to <90,000/μl on day 10 after the first course of HD-DXM [3], thus justifying the second course of HD-DXM. No additional courses were provided once the platelet count was above 90,000/μl on day 10 after the second course. That platelet count remained stable after re-initiating clopidogrel and aspirin negated any drug-induced thrombocytopenia. To our knowledge, this is the first case describing the use and effectiveness of the combination of HD-DXM and ART in the treatment of HIV-ITP.

In adult patients with non-HIV ITP, HD-DXM is preferred to conventional long-term prednisone [2], because of fewer adverse events, mainly due to the shorter term of steroid administration. The major side effect of steroid is immunosuppression, and it is important to avoid such complication, especially in immunocompromised hosts, such as HIV-1 infected patients. For the treatment of HIV-ITP, HD-DXM is probably as effective as in non-HIV ITP, because both diseases are considered to have a similar etiology [8]. Although about one-fifth of non-HIV ITP patients on HD-DXM treatment relapse by 8 months after treatment [5], the use of ART in patients with HIV-ITP can maintain long-term remission despite the lack of an immediate effect [9, 10]. In our patient, HD-DXM was applied when the clinical condition was severe with bleeding and thrombocytopenia, and it resulted in rapid improvement in platelet count. Thereafter, administration of ART resulted in suppression of viral load, which probably promoted long-term remission of HIV-ITP.

Notably, HD-DXM is cost-effective, compared to other treatments for ITP, such as immunoglobulin or rituximab. Although further studies are needed to confirm the efficacy and safety of the combination therapy, HD-DXM and ART is potentially suitable for treatment of HIV-ITP patients with severe thrombocytopenia or bleeding.

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Conflict of interest All authors declare no conflict of interest.

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Prophylactic Effect of Antiretroviral Therapy on Hepatitis B Virus Infection

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Background. Hepatitis B virus (HBV) infection is common in individuals infected with human immunodeficiency virus, especially in men who have sex with men (MSM). Almost all currently used regimens of antiretroviral therapy (ART) contain lamivudine (LAM) or tenofovir disoproxil fumarate (TDF), both of which have significant anti-HBV activity. However, the prophylactic effect of ART on HBV infection has not been assessed previously.

Methods. Non-HBV-vaccinated HIV-infected MSM were serologically evaluated for HBV infection using stocked serum samples. Cases negative for HBV surface antigen (HBsAg), antibody to HBsAg (anti-HBs), and antibody to HBV core antigen (anti-HBc) in first serum samples were serologically followed until last available stocked samples. HBV genotype and LAM-resistant mutation (rtM204V/I) were analyzed in cases that became HBsAg-positive.

Results. The first stocked samples were negative for all analyzed HBV serological markers in 354 of 1434 evaluated patients. The analysis of their last samples indicated HBV incident infection in 43 of them during the follow-up period. The rate of incident infections was lower during LAM- or TDF-containing ART (0.669 incident infections in 100 person-years) than during no ART period (6.726 incident infections in 100 person-years) and other ART (5.263 incident infections in 100 person-years) ($P < .001$). Genotype A was most prevalent (76.5%), and LAM-resistant HBV was more frequent in incident infections during LAM-containing ART (50.0%) than in those during no ART and other ART (7.1%) ($P = .029$).

Conclusions. LAM- and TDF-containing ART regimens seem to provide prophylaxis against HBV infection, although drug-resistant strains seem to evade these effects.

Keywords. lamivudine; tenofovir disoproxil fumarate; resistant; chronic infection.

Patients with human immunodeficiency virus (HIV) infection are at high risk for both hepatitis B virus (HBV) infection and development of chronic infection [1–4]. Based on information from Western countries, the rate of coinfection varies according to risk categories; the highest rate is in men who have sex with men (MSM), with a slightly lower rate among intravenous drug users, and much lower in individuals infected through heterosexual contacts [5–8]. In Japan, HIV/HBV coinfection is also significantly associated with MSM [9, 10]. The progression of chronic HBV infection to cirrhosis, end-stage liver diseases, and/or hepatocellular carcinoma is more rapid in HIV-infected persons than in those with chronic HBV infection alone [11, 12]. Vaccination of non-HBV-immunized HIV-infected individuals is recommended to prevent HBV infection [13]. However, all current recommended antiretroviral therapy (ART) regimens contain lamivudine (LAM) or tenofovir disoproxil fumarate (TDF), both of which have significant anti-HBV activity [14]. Do these ART regimens provide any prophylaxis against HBV infection? This is an important question, as a positive answer could influence the strategy applied to prevent HBV infection in HIV-infected individuals. To delineate the hepatitis B prophylactic effect of ART, we used stocked samples for serological evaluation of HBV infection in HIV-infected MSM. The present

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study included those patients who had tested negative for hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), and antibody to hepatitis B core antigen (anti-HBc) using their first stocked blood samples, who were followed up serologically to identify new HBV incident infections among them. The other part of the study covered analysis of the relation between the frequency of incident infection and ART regimens.

METHODS

Patients

Since April 1997, we have stocked serum samples taken at routine clinical practice from HIV type 1 (HIV-1)–infected patients who visited the Outpatient Clinic of the AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, under signed informed consent for use in virologic research. Every patient had been interviewed at the first visit by clinical nurse specialists at the HIV outpatient clinic using a structured questionnaire that includes items on sexuality and history of HBV vaccination. Most of the patients regularly visited our clinic every 1–3 months, and we had collected and stored their sera at almost all visits. The ethics committee of the National Center for Global Health and Medicine approved the collection and analysis of the samples. First, we selected HIV-1–infected MSM who met the following inclusion criteria: (1) the first visit to our clinic was between April 1997 and December 2009, (2) they had not received HBV vaccination before the first visit, and (3) at least 2 serum samples were available and collected at least 6 months apart. The first sample was defined as the baseline serum sample, and baseline clinical data were defined as those recorded on the date of sampling of the first stocked serum. Patients' baseline characteristics, including age, race, hepatitis C virus antibody, results of *Treponema pallidum* hemagglutination assay, and $CD4^+$ cell count were collected from the medical records.

HBV Analysis

In order to identify new HBV incident infection, we excluded patients with previously confirmed HBV infection. The baseline samples of the patients who met the inclusion criteria described above were serologically evaluated for HBsAg, anti-HBs, and anti-HBc using ARCHITECT HBsAg QT assay, anti-HBs assay, and anti-HBc assay, respectively (Abbott Laboratories, Chicago, Illinois) [15, 16]. Patients positive for any of HBsAg, anti-HBs, and anti-HBc at baseline were excluded from the serological follow-up. The remaining patients were considered to have never been infected with HBV before the baseline. Their last stocked sample taken before or in December 2010, or before HBV vaccination if performed during the follow-up period, was analyzed for HBsAg, anti-HBs, and anti-HBc. If the last sample was negative for all 3, the patient was considered to have never been infected with HBV up to the sampling date of the last stocked serum. If HBsAg, anti-HBs, or anti-HBc was positive in the last stocked serum, the patient was considered to have HBV incident infection during the follow-up period. In the latter case, the baseline samples were subjected to polymerase chain reaction (PCR) analysis for HBV DNA [17, 18], and all the stocked samples during the follow-up period were serologically analyzed to determine the date of HBV incident infection. The date of incident infection was defined as the sampling date of the first positive serum for any HBV serological marker. The time from the baseline to HBV incident infection was analyzed by the Kaplan-Meier method. The data were censored at the sampling date of the last stocked sample if it was negative for all analyzed HBV serological markers. Patients' age and $CD4^+$ cell count at the date of incident infection and alanine aminotransferase (ALT) values within 3 months of incident infection were collected. If an HBsAg-positive sample was available, HBV genotype and LAM-resistant mutation (rtM204V/I) were analyzed by PCR-invader assay [17–19]. The diagnosis of chronic HBV infection was considered when HBsAg was still positive in sera taken at 6 months or longer after the incident infection.

Antiretroviral Therapy

To determine the type of ART under which HBV incident infection occurred, the regimen information of ART was collected from medical records over the period spanning from the baseline to the incidence infection or to the end of follow-up. The treatment status was divided into 4 categories: (1) No ART, no treatment with any antiretroviral agent; (2) Other-ART, ART with regimens that did not contain LAM, TDF, or emtricitabine (FTC); (3) LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC; and (4) TDF-ART, ART with TDF-containing regimens with or without LAM or FTC. Data were censored on the sampling date of the last stocked sample if it was negative for all analyzed HBV serological markers. When the treatment category was modified, the data were censored on the date of category change for the previous treatment category and a new follow-up as a different case was initiated for the replacement treatment category.

Statistical Analysis

The time from the baseline to HBV incident infection was analyzed by the Kaplan-Meier method. The Cox proportional hazards regression analysis was used to assess the risk of HBV incident infections. The impact of patients' baseline characteristics, year of entry, the use of antiretroviral agents (any antiretroviral, and any of LAM, TDF, or FTC), and the frequency of changing ART regimen during the follow-up period was estimated with univariate analysis, and those with statistical significance were incorporated into multivariate analysis. The

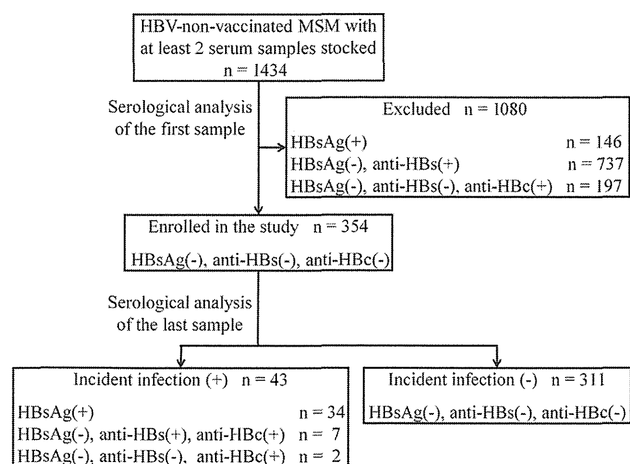

Figure 1. Patient selection process: 1434 patients met the inclusion criteria. Of these patients, 1080 were excluded because of positive hepatitis B virus serology in the first samples. The results of various serological tests are shown. The remaining 354 were enrolled for serological follow-up. Of these, 43 were positive in the last sample analysis. Their stocked samples were analyzed serologically and the results of HBV serology using the first positive samples are indicated. Abbreviations: anti-HBc, antibody to HBV core antigen; anti-HBs, antibody to HBsAg; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

frequency and risk of HBV incident infection during each treatment category was also assessed by univariate Cox proportional hazards regression analysis. We used hazard ratios and 95% confidence intervals to estimate the impact of each variable on incident infection. Patients' age and $CD4^+$ cell count on the date of incident infection, and peak value of ALT within 3 months of incident infection were compared between transient infection and chronic infection with Wilcoxon rank-sum test. The differences in rates of HBV genotype A and rtM204V/I mutation were compared with χ^2 test (ie, the Fisher exact test). Statistical significance of difference was defined as a 2-sided P value of <.05. All statistical analyses were performed with the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, Illinois).

RESULTS

Figure 1 shows the patient selection procedure. A total of 1434 HIV-1–infected MSM met the inclusion criteria described in the Methods section. Of these, 146 patients (10.2%) were positive for HBsAg, 737 (51.4%) were positive for anti-HBs, and 197 (13.7%) were solely positive for anti-HBc using baseline samples. The remaining 354 patients (24.7%; negative for HBsAg, anti-HBs, and anti-HBc at baseline), who were considered to have never been infected with HBV, were enrolled for serological follow-up. Table 1 lists their baseline characteristics. Serological analysis of the last sample of each of these patients showed HBV incident infection during follow-up in 43 (12.1%). Their baseline samples were found to be PCR-negative for HBV DNA, confirming that the incident infection in these patients occurred during the follow-up period. All stocked samples of the 43 patients were analyzed serologically to determine the date of HBV incident infection. HBV incident infections occurred every year between 1997 and 2010 except in 1998. The median time period from the baseline to HBV incident infection was 1.6 years (interquartile range [IQR], 192–1151 days; range, 28–4068 days). The total observation period was 1607 person-years (median, 3.7 years [IQR], 1.9–6.5 years). Figure 2 shows the Kaplan-Meier curve for the HBV incident infection for the whole cohort of enrolled patients.

In order to assess the risk of HBV incident infections, patients' baseline characteristics, year of entry, the use of any antiretroviral agents, the use of any of LAM, TDF, or FTC, and the frequency of changing ART regimen during the follow-up

Table 1. Baseline Characteristics of the 354 Enrolled Patients

		Year of Entry			
Characteristic	Total (n = 354)	1997–2000 (n = 61)	2001–2003 (n = 79)	2004–2006 (n = 112)	2007–2009 (n = 102)
Age, y, median (IQR)	32.0 (27.0–38.0)	32.0 (27.8–37.3)	31.0 (27.0–37.8)	32.0 (27.0–38.0)	35.0 (27.0–42.0)
Race/ethnicity					
Japanese	340 (96.0)	59 (96.7)	78 (98.7)	109 (97.3)	94 (92.2)
Asian other than Japanese	4 (1.1)	0 (0.0)	0 (0.0)	1 (0.9)	3 (2.9)
Caucasian	10 (2.8)	2 (3.3)	1 (1.3)	2 (1.8)	5 (4.9)
HCV antibody, positive	8 (2.3)	1 (1.6)	2 (2.5)	1 (0.9)	4 (3.9)
TPHA positive	101 (28.5)	23 (37.7)	20 (25.3)	30 (26.8)	28 (27.5)
$CD4^+$ cell count, cells/mm^3, median (IQR)	277 (151–404)	277 (169–417)	313 (97–443)	316 (176–413)	252 (129–359)
HIV RNA, $\log_{10}$ copies/mL, median (IQR)	4.6 (3.8–5.2)	4.5 (3.6–5.2)	4.8 (3.9–5.4)	4.4 (3.8–4.9)	4.7 (3.9–5.2)

Data are No. (%) unless otherwise specified.

Abbreviations: HCV, hepatitis C virus; HIV, human immunodeficiency virus; IQR, interquartile range; TPHA, *Treponema pallidum* hemagglutination assay.

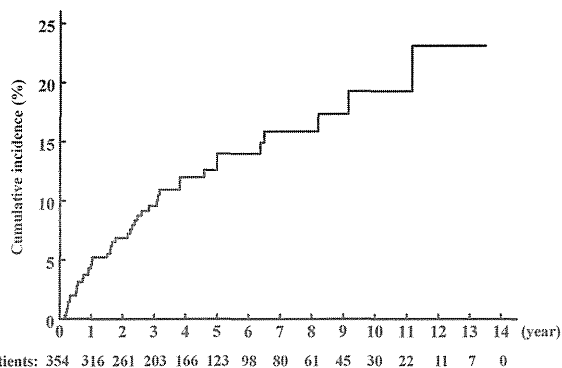

Figure 2. Kaplan-Meier curve showing the time to hepatitis B virus incident infection.

period were estimated using a proportional hazards model (Table 2). Younger age and higher CD4$^+$ cell count correlated positively, and use of any antiretroviral, use of LAM, TDF, or FTC, and the frequency of changing ART regimen correlated negatively with HBV incident infection, with statistical significance in univariate analysis. However, in multivariate analysis, the use of LAM, TDF, or FTC continued to show significant relation. Then, we focused on the relation between treatment status and HBV incident infection. The observation period in each patient was divided into 4 categories by treatment status: No ART, no treatment with any antiretroviral agent; Other-ART, ART with regimens that did not contain LAM, TDF, or FTC; LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC; or TDF-ART, ART with TDF-containing regimens with or without LAM or FTC. No participant received FTC single tablet (Emtriva). All the participants who took FTC received the combination tablet of TDF/FTC (Truvada), and therefore, such treatment status was categorized as TDF-ART. The total categorized observation period of No ART, Other-ART, LAM-ART, and TDF-ART was 446, 114, 814, and 233 person-years, respectively. The number of the HBV incident infections was 30 during the No ART period, 6 during Other-ART period, 7 during LAM-ART period, and 0 during TDF-ART period. No incident infection occurred at the time of changing ART regimen. The proportional hazards model showed a significantly lower frequency of HBV incident infection during LAM- or TDF-ART (0.669 incident infections per 100 person-years) compared with that during No ART (6.726 incident infections per 100 person-years), although there was no significant difference between Other-ART (5.263 incident infections per 100 person-years) and No ART, suggesting that ART regimens with anti-HBV activity can reduce HBV incident infections by 90% (Table 3). During LAM-ART, the HIV-1 load around the period of incident infection remained below the detection limit in all the 7 infected patients, indicating excellent adherence to ART.

Figure 3 shows peak ALT levels for the 43 HBV incident infections. Among the 36 incident infections observed the No ART and Other-ART groups, 16 infections (44.4%) were asymptomatic and not associated with significant increases in ALT (peak ALT, <60 IU/L). We were able to serologically follow 33 of the 36 cases for 6 months after the date of incident infection (TDF-ART was introduced within 6 months of incident infection in the other 3 cases). Among the 33 patients, 13 (39.4%) developed chronic infection (HBsAg was still positive 6 months after the date of incident infection). The median CD4$^+$

Table 2. Cox Proportional Hazards Regression Analysis for the Risk of Hepatitis B Virus Incident Infection

	Univariate Analysis		Multivariate Analysis	
Factors	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value
Year of entry, per 1 y increase	.942 (.860–1.033)	.207		
Baseline characteristics				
Age, per 1 y increase	.921 (.879–.965)	.001	.958 (.917–1.001)	.054
Race (Japanese)	21.243 (.010–45 657.613)	.435		
HCV antibody	.048 (<.001–346.311)	.503		
TPHA	1.475 (.792–2.747)	.220		
CD4$^+$ cell count, per 100 cells/mm^3 increase	1.121 (1.008–1.246)	.035	.882 (.752–1.034)	.121
HIV RNA, per 1 $\log_{10}$ copies/mL increase	1.387 (.999–1.924)	.051		
Antiretroviral use during follow-up period				
Any antiretroviral	.097 (.052–.184)	<.001	.927 (.305–2.818)	.893
LAM, TDF, or FTC	.075 (.039–.146)	<.001	.110 (.031–.390)	.001
Frequency of changing regimen	.245 (.145–.414)	<.001	.700 (.385–1.270)	.240

Abbreviations: CI, confidence interval; FTC, emtricitabine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LAM, lamivudine; TDF, tenofovir disoproxil fumarate; TPHA, *Treponema pallidum* hemagglutination assay.

Table 3. Frequency and Hazard Ratio of Hepatitis B Virus Incident Infection in Each Treatment Status Category

ART	Observation Period (Person-Years)	Incident Infection	Hazard Ratio (95% CI)	*P* Value
No ART	446	30	1	...
Other-ART	114	6	.924 (.381–2.239)	.861
ART containing at least 1 of LAM, TDF, and FTC[a]	1047	7	.113 (.049–.261)	<.001
LAM-ART	814	7		
TDF-ART	233	0		

Abbreviations: ART, antiretroviral therapy; CI, confidence interval; FTC, emtricitabine; LAM, lamivudine; TDF, tenofovir disoproxil fumarate; LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC; Other-ART, ART with regimens that did not contain LAM, TDF, or FTC; TDF-ART, ART with TDF-containing regimens with or without LAM or FTC.

[a] No participant received FTC single tablet (Emtriva) during the observation period. All the participants who took FTC received the combination tablet of TDF/FTC (Truvada), and therefore, such treatment status was categorized into TDF-ART.

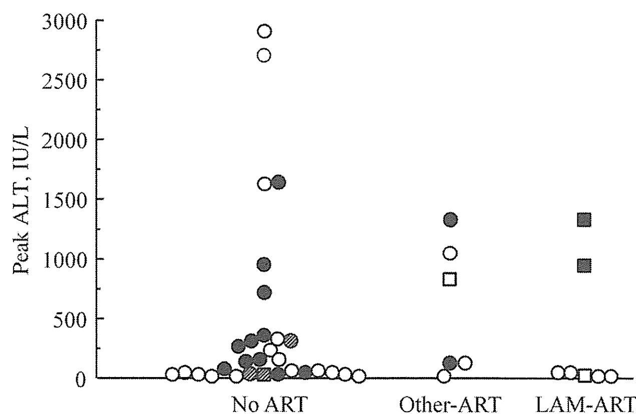

Figure 3. Peak alanine aminotransferase (ALT) values in hepatitis B virus (HBV) incident infections according to treatment regimen. Thirty, 6, and 7 HBV incident infections were observed during No antiretroviral therapy (ART), Other-ART, and lamivudine (LAM)–ART, respectively. No incident infection was identified during tenofovir disoproxil fumarate (TDF)–ART. No participant received emtricitabine (FTC) single tablet (Emtriva) during the observation period. All the participants who took FTC received the combination tablet of TDF/FTC (Truvada), and therefore, such treatment status was categorized into TDF-ART. Data are peak ALT values measured within 3 months of the date of incident infections. LAM-resistant mutation (rtM204V/I) was analyzed in 34 cases using the available hepatitis B surface antigen (HBsAg)–positive samples. Open squares: patients infected with LAM-resistant HBV. Closed circles and squares: patients who developed chronic infection (HBsAg-positive 6 months after the date of incident infection). Checked circles and squares: patients who received TDF-containing ART within 6 months of incident infection. Abbreviations: ALT, alanine aminotransferase; ART, antiretroviral therapy; LAM, lamivudine.

cell count was lower in the patients who developed chronic infection than in those with transient infection, although the difference was not significant ($P = .068$; Table 4), indicating that HIV-related immunodeficiency may play a role in the induction of chronic HBV infection. Among the 7 incident infections observed during LAM-ART, only 2 patients (28.6%) were symptomatic, had significant rise in ALT, and developed chronic HBV infection, and both of these infections were caused by LAM-resistant HBV (Table 5). The other 5 cases were asymptomatic and transient. Three of them were caused by LAM-sensitive strains and 1 was by LAM-resistant strain. HBsAg-positive serum sample was not available in the last case. LAM-resistant HBV was more frequently identified in analyzed incident infections during LAM-containing ART (50.0%) than in those during no ART and other ART (7.1%) ($P = .029$). Considered together, LAM seems to prevent acquisition of HBV infection, progression to symptomatic hepatitis, and development of chronic infection even after the development of infection, although these effects may be less pronounced in patients with LAM-resistant strains.

Among the 43 infection cases observed during total serological follow-up, HBsAg-positive samples were available in 34 cases and their HBV genotype was determined. Genotype A was the most frequent, as reported previously [10, 20–22], and genotypes B, G, and H were also identified. The rate of development of chronic infection was higher in genotype A than in other genotypes as previously reported [23], although the difference was not significant in our study. In the remaining 9 cases, only anti-HBc with (7 cases) or without (2 cases) anti-HBs were detected, although their samples were available and serologically analyzed at least every 3 months around the incident infection.

DISCUSSION

The results of this serological follow-up study indicated that LAM- and TDF-containing ART regimens protect against HBV incident infection. Furthermore, the results also suggested that LAM prevents progression to symptomatic hepatitis and development of chronic infection even after the development of HBV incident infection, provided such infection is caused by LAM-sensitive strains. However, it seems that LAM-resistant strains may evade this protective effect. One previous study that estimated the incidence of acute HBV infection among HIV-infected patients reported similar frequencies in patients receiving ART with and without LAM [5]. However, the authors defined immunoglobulin M anti-HBs positivity as a marker of HBV incident infection and did not exclude anti-HBc–positive patients at study entry. This probably made it difficult to distinguish incident infection from reactivation of chronic infection, as discussed in the report. In this study, we identified a

Table 4. Patient Characteristics and Clinical Features of Hepatitis B Virus Incident Infections in the No Antiretroviral Therapy (ART) and Other-ART Treatment Categories

Factors	Transient (n = 20)	Chronic[a] (n = 13)	Treated[b] (n = 3)	*P* Value[c]
Age, y, median (IQR)	31.0 (28.0–33.0)	29.0 (25.0–38.3)	25.0 (21.0–35.0)[d]	.406
$CD4^+$ cell count, cells/mm^3, median (IQR)	371 (308–518)	320 (235–383)	674 (206–1935)[d]	.068
Peak ALT level[e], U/L, median (IQR)	65 (30–573)	264 (115–774)	31 (15–314)[d]	.162
HBV genotype, No. (%)				.645
Genotype A	9 (45.0)	11 (84.6)	2 (66.7)	
Other genotypes	3 (15.0)	2 (15.4)	1 (33.3)	
Genotype unknown	8 (40.0)	0 (0.0)	0 (0.0)	
HBV rtM204V/I mutation, No. (%)				.480
Positive	1 (5.0)	0 (0.0)	1 (33.3)	
Negative	11 (55.0)	13 (100.0)	2 (66.7)	
Unknown	8 (40.0)	0 (0.0)	0 (0.0)	

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; IQR, interquartile range.

[a] Hepatitis B surface antigen–positive 6 months after the date of incident infection.

[b] Treated cases with tenofovir disoproxil fumarate–containing antiretroviral therapy within 6 months of incident infection.

[c] *P* values between transient and chronic cases calculated with Wilcoxon rank-sum tests for continuous variables and χ^2 tests for proportions.

[d] Minimum and maximum values.

[e] Peak ALT level within 3 months of incident infection.

significant number of isolated anti-HBc–positive patients, a finding in agreement with previous reports [24–27], and excluded them from the serological follow-up to avoid improper inclusion of isolated anti-HBc–positive ones as HBV-naive [28, 29].

Table 5. Patient Characteristics and Clinical Features of Hepatitis B Virus Incident Infections During LAM-ART Treatment

Factors	Transient (n = 5)	Chronic[a] (n = 2)	*P* Value[b]
Age, y, median (IQR)	33.0 (30.3–36.5)	38.0 (33.0–43.0)[c]	.329
$CD4^+$ cell count, cells/mm^3, median (IQR)	430 (267–648)	362 (360–364)[c]	.699
Peak ALT level[d], U/L, median (IQR)	22 (14–51)	1133 (941–1325)[c]	.051
HBV genotype, No. (%)			>.999
Genotype A	3 (60.0)	1 (50.0)	
Other genotypes	1 (20.0)	1 (50.0)	
Genotype unknown	1 (20.0)	0 (0.0)	
HBV rtM204V/I mutation, No. (%)			.400
Positive	1 (20.0)	2 (100.0)	
Negative	3 (60.0)	0 (0.0)	
Unknown	1 (20.0)	0 (0.0)	

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; IQR, interquartile range; LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC.

[a] Hepatitis B surface antigen–positive 6 months after the date of incident infection.

[b] *P* values calculated with Wilcoxon rank-sum tests for continuous variables and χ^2 tests for proportions.

[c] Minimum and maximum values.

[d] Peak ALT level within 3 months of incident infection.

HBV vaccination is recommended for individuals seeking evaluation or treatment for sexually transmitted diseases, HIV-infected patients, sexually active persons with >1 partner, and MSM [13]. However, the response and durability of adequate titers of anti-HBs are often reduced in HIV-infected patients [30–34]. Modified regimens of vaccination have been reported to improve anti-HBs response in HIV-infected patients, although the response rate was still low in those with low $CD4^+$ cell counts [35–37]. Our study demonstrated the HBV prophylactic effects of LAM- and TDF-containing ART regimens, suggesting that ART should be initiated before HBV vaccination, especially in those with low $CD4^+$ cell counts. Early introduction of ART was recommended recently not only for HIV-infected individuals, but also for prevention of transmission to others [38, 39]. Early introduction of treatment may also be recommended to prevent HBV infection to the patients themselves if they are HBV-naive. One randomized clinical trial reported the prophylactic effect of TDF combined with FTC in HIV prevention in seronegative MSM [40]. However, in that trial, HBV vaccination was offered to all susceptible participants, which made it impossible to estimate the prophylactic effect of the treatment on HBV prevention.

Our study carries certain limitations related to its retrospective nature. Patients on ART might have more opportunities to improve their behavior to prevent transmission of HIV to others, which could reduce HBV infection in themselves but

introduce bias in our analysis. However, the results suggest prophylaxis against potential HBV infection by oral medications, which could be useful for nonimmunized medical care providers.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Restriction fragment mass polymorphism (RFMP) analysis based on MALDI-TOF mass spectrometry for detecting antiretroviral resistance in HIV-1 infected patients

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Abstract

Viral genotype assessment is important for effective clinical management of HIV-1 infected patients, especially when access and/or adherence to antiretroviral treatment is reduced. In this study, we describe development of a matrix-assisted laser desorption/ionization-time of flight mass spectrometry-based viral genotyping assay, termed restriction fragment mass polymorphism (RFMP). This assay is suitable for sensitive, specific and high-throughput detection of multiple drug-resistant HIV-1 variants. One hundred serum samples from 60 HIV-1-infected patients previously exposed to nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) were analysed for the presence of drug-resistant viruses using the RFMP and direct sequencing assays. Probit analysis predicted a detection limit of 223.02 copies/mL for the RFMP assay and 1268.11 copies/mL for the direct sequencing assays using HIV-1 RNA Positive Quality Control Series. The concordance rates between the RFMP and direct sequencing assays for the examined codons were 97% (K65R), 97% (T69Ins/D), 97% (L74VI), 97% (K103N), 96% (V106AM), 97% (Q151M), 97% (Y181C), 97% (M184VI) and 94% (T215YF) in the reverse transcriptase coding region, and 100% (D30N), 100% (M46I), 100% (G48V), 100% (I50V), 100% (I54LS), 99% (V82A), 99% (I84V) and 100% (L90M) in the protease coding region. Defined mixtures were consistently and accurately identified by RFMP at 5% relative concentration of mutant to wild-type virus while at 20% or greater by direct sequencing. The RFMP assay based on mass spectrometry proved to be sensitive, accurate and reliable for monitoring the emergence and early detection of HIV-1 genotypic variants that lead to drug resistance.

Keywords: Drug, HIV-1, MALDI-TOF, resistance mutation, RFMP
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Introduction

Highly active antiretroviral therapy (HAART) can dramatically suppress HIV-1 replication, improve immunological response and extend a patient's lifespan. However, less than excellent adherence to HAART or conditions that result in reduced treatment efficacy leads to a higher risk of the emergence of antiretroviral (ARV) drug-resistant viral strains, which eventually leads to increased viral loads, poor immunological response and eventually treatment failure [1]. Especially, women who have received single-dose nevirapine to prevent mother-to-child HIV-1 transmission are at increased risk of virological failure as a result of the replication of low-abundance nevirapine-resistant variants when treated with a subsequent nevirapine-containing regimen [2]. Of importance in the effective management of HIV-1 infections is the timely and efficient detection of

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drug-resistant viral strains and their specific mutations in a patient's clinical samples.

The guidelines for use of ARV drugs in HIV-1-infected adults and adolescents established by the US Department of Health and Human Services (DHHS) (http://www.aidsinfo.nih.gov/guidelines) recommend monitoring viral genotypic changes in patient samples and use of this information to determine which therapeutic regimens are most appropriate for the specific patient [3].

The restriction fragment mass polymorphism (RFMP) method is based on amplification and mass detection of oligonucleotides excised by type-IIS restriction enzyme digestion, using matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF MS). RFMP-based drug-resistance testing and genotyping has been shown to be a sensitive, accurate and reliable method for clinical utility in many fields [4–12]. Especially important is that RFMP enables sensitive detection of mutations without population-based cloning and subsequent sequencing analysis [6].

In this study, we applied the RFMP assay for detection of mutations in the coding sequences for reverse transcriptase (RT) and protease (PR) of HIV-1 that engender resistance to nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Compared with direct sequencing, RFMP is shown to be a sensitive and reliable method for genotypic testing of drug-resistance mutations in HIV-1 infected patients.

Materials and Methods

Specimens

A total of 100 plasma samples were collected from 60 HIV-1 infected patients who had received HAART (including NRTIs, NNRTIs and PIs) at the AIDS Clinical Center, National Center for Global Health and Medicine, Japan, between 1999 and 2009. Written informed consent was obtained from each participant, and the experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval (NCGM-H22-938) by the Ethics Committee of the AIDS Clinical Center, National Center for Global Health and Medicine, Japan. The demographic characteristics are summarized in Table 1.

HIV-1 performance panels

To assess the limit of detection ability of the RFMP assay, the HIV-1 RNA Positive Quality Control Series (ACCURUN® 315) obtained from SeraCare Life Sciences (Milford, MA, USA) was used to measure viral load in HIV-1 performance panels.

TABLE 1. Demographic characteristics of 60 HIV-1-infected patients

Characteristic	Value
Mean age in years (range)	42 (22–67)
No. male (%)	53 (88)
No. female (%)	7 (12)
Race (%)	
Asian	58 (97)
African	2 (3)
Risk factor for HIV (% of patients)	
Heterosexual	14 (23)
Homosexual	27 (45)
Haemophilia (infected blood products)	19 (32)
CDC clinical stage (%)	
A1/A2/A3	0/0/0
B1/B2/B3	17/23/13
C1/C2/C3	5/12/20
Unknown	10
Mean CD4 cell count (No. of cells/uL [range])	320 (12–759)
No. of unknown (%)	3 (5)
Mean HIV-1 RNA (No. of RNA copies/mL [range])	43 000 (50–1 200 000)
No. of unknown (%)	6 (10)
History of actual treatment (No. of patients)	
With NRTI	2
With NRTI plus NNRTI	23
With NRTI plus PI	21
With NNRTI plus PI	1
With NRTI plus NNRTI plus PI	33
With NRTI plus PI plus INI	1
With NRTI plus INI	2
With NRTI plus PI plus INI plus FI	1
With NRTI plus NNRTI plus PI plus INI plus FI	1
Interruption	15

NRTI, nucleoside reverse transcriptase inhibitor; lamivudine, abacavir, emtricitabine, tenofovir, stavudine, didanosine, zidovudine; NNRTI, non-nucleoside reverse transcriptase inhibitor; efavirenz, nevirapine; PI, protease inhibitor; atazanavir, ritonavir, lopinavir, darunavir, fosamprenavir, amprenavir, nelfinavir; INI, integrase inhibitor; raltegravir; FI, fusion inhibitor; enfuvirtide.

Construction of recombinant HIV-1 clones

Recombinant infectious HIV-1 clones with various mutations in the RT region were constructed using site-directed mutagenesis. Briefly, the mutations were introduced into the *Xma*I-*Nhe*I fragment (759 bp) of pTZNX1, which encodes Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by oligonucleotide-based mutagenesis [13]. The *Xma*I-*Nhe*I fragment was inserted into a pNL4-3-based plasmid, generating various molecular clones with the desired mutations. Each molecular clone (10 /mL as DNA) was transfected into human 293T cells (4×10^5 cells/100-mm-diameter dish) with Fugene transfection reagent (Roche Diagnosis, Basel, Switzerland). After 48 h, culture supernatants were harvested and stored at −80°C until use. Viral loads were determined using the COBAS® Amplicor HIV-1 Monitor Test, v1.5.

RNA extraction and cDNA amplification

HIV-1 RNA was extracted from 200 μL of plasma using the High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Purified viral RNA was dissolved in 50 μL elution buffer (nuclease-free, sterile, double distilled water). cDNA was synthesized using only the reverse transcription step component of the RNA PCR kit (TaKaRa, Otsu, Japan).

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RFMP assay

PCR reactions were performed in 25 μL reaction mixtures containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM deoxynucleoside triphosphates (dNTPs), 10 pmol of primers and 0.4 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The initial denaturing phase of 5 min at 94°C was followed by a 35-cycle amplification phase containing a denaturation step at 94°C for 15 s, annealing step at 50°C for 15 s and elongation step at 72°C for 15 s, and completed with a final extension phase at 72°C for 5 min. For the RFMP analysis of codons 65, 69, 74, 103, 106, 151, 181, 184 and 215 in the HIV-1 RT region and codons 30, 46, 48, 50, 54, 82, 84 and 90 in the HIV-1 PR region, each of the forward and reverse primers contained the viral target sequence and the *Fok*I recognition sequence ggatg (Table S1). Restriction enzyme digests were performed by mixing the PCR reaction with 10 μL of buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM dithiothreitol) and 1 U of *Fok*I enzyme (New England Biolabs, Beverly, MA, USA). The reaction mixtures were incubated at 37°C for 2 h. Subsequently, the digest desalting and mass analysis were performed as described previously [14].

MALDI-TOF instrumentation and calibration

Mass spectra were acquired on a Biflex IV linear MALDI-TOF MS (Bruker Daltonics) workstation equipped with a 337 nm nitrogen laser and a nominal ion flight path length of 1.25 m. The samples were analyzed in the negative-ion mode with a total acceleration voltage of 20 kV, extraction voltage of 18.25 kV, laser attenuation of 55, and delayed extraction of long time delay mode. Typically, time-of-flight data from 20 to 50 individual laser pulses were recorded and averaged on a transient digitizer with a time base of 2 ns and delay of 24 000 ns, after which the averaged spectra were automatically converted to mass by the accompanying data-processing software. Using these settings the instrument typically provided mass accuracy of 40–80 ppm (10^{-6}), mass resolution of 1500–2000 and sensitivity of 10–50 fmol in the 2- to 6-kDa mass range for oligonucleotides. Oligonucleotide standards of 6mer (5′-ACGTAC-3′; 1762.2 Da) and 16mer (5′-AC GTACGTACGTACGT-3′; 4881.2 Da) with no terminal phosphate were used for mass calibration of the instrument. The presence of metal cations produces salt adducts, leading to reduced resolution and low sensitivity, so C18 reverse phase micro-column chromatography was used for desalting oligonucleotides. Non-homogeneous crystallization is obtained with the classic dried droplet preparation, and a search for a ‘sweet spot’ is required. Re-crystallization of sample DNAs on matrix-prespotted anchorchip plates allowed robust formation of small single crystals.

Direct sequencing assay

To amplify the HIV-1 RT and PR regions for analysis by direct sequencing, PCR was performed with the following primers: 5′-AACAATGGCCATTGACAGAAGAAA-3′ (2614–2637 bp of HXB2), 5′-CTGTATGTCATTGACAGTCCAGCT-3′ (3299–3323 bp of HXB2) for the RT region and 5′-CTTCCCTCA GATCACTCTTTGGCAA-3′ (2248–2273 bp of HXB2), 5′-AGGGCTAATGGGAAAATTTAAAGT-3′ (2238–2561 bp of HXB2) for the PR region. PCR products were sequenced using the BigDye Terminator (version 3.1) Cycle Sequencing kit and an ABI PRISM 310 Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistics

A limit of detection test was performed by probit analysis to compare sensitivity between the RFMP and direct sequencing assays using the statistical package SAS (version 8; SAS Institute Inc., Cary, NC, USA).

Results

RFMP assay strategy

The RFMP assay is based on mass spectrometric analysis of small DNA fragments that include sites of mutation (Fig. S1). The first step requires PCR amplification with forward and reverse primers that introduce the *Fok*I enzyme site, ggatg (Table S1). The diagnostic fragments released by enzymatic digestion consist of various sizes from 8 nt oligomers to 14 nt oligomers for nine codons in the RT region and eight codons in the PR region, leading to facile identification of sequence variation by mass spectrum analysis. Genotypic analysis of mutations at codons 65, 69, 74, 103, 106, 151, 181, 184 and 215 in the RT region and codons 30, 46, 48, 50, 54, 54, 82, 84 and 90 in the PR region, as assessed by the RFMP assay, was determined for 100 plasma samples. The RFMP results showed distinct peaks relevant to each codon, with the mass values for each diagnostic fragment being exactly as predicted (Supplementary Material Table S2).

Estimation of limit of detection and ability to detect mixed genotype populations

The detection limit was estimated using replicates of each of nine dilutions of HIV-1 RNA Positive Quality Control Series (ACCURUN® 315) material ranging between 10 and 5000 copies/ml. Analysis of various calibrated HIV-1 RNA dilution series determined the lower detection limit to be 223.02 copies/mL for the RFMP and 1268.11 copies/mL for the direct sequencing assays by probit analysis. The probit analysis predicts a 95% CI: 132.64–693.00 for the RFMP and 863.09–3656.80 for the direct sequencing assays (Table 2).

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TABLE 2. Limit of detection of the RFMP and direct sequencing assays

HIV-1 RNA copies/ml	No. tested	RFMP No. detected	RFMP Per cent detected	Direct sequencing No. detected	Direct sequencing Per cent detected
5000	10	10	100%	10	100%
2500	10	10	100%	10	100%
1000	10	10	100%	9	90%
500	10	10	100%	4	40%
250	10	10	100%	1	10%
100	10	8	80%	0	0%
50	10	3	30%	0	0%
25	10	1	10%	0	0%
10	10	1	10%	0	0%
Limit of detection		223.02 copies/mL (95% CI, 132.64–693.00)		1268.11 copies/mL (95% CI, 863.09–3656.80)	

Defined dilutions of HIV-1 RNA Positive Quality Control Series were made from 10 copies to 5000 copies/mL and limit of detection abilities were calculated by probit analysis at a 95% detection level.

To evaluate the ability of the RFMP assay to determine small amounts of mutant virus in mixed populations, assays were performed with recombinant HIV-1 clones composed of different ratios of wild-type (K103 in the RT region) and mutant genotypes (N103 in the RT region). Defined mixtures were prepared with the following percentages of K103N mutant virus in the total virus population: 100%, 50%, 20%, 10%, 5% and 1%. The K103N mutant virus could be detected in concentrations as low as 5% of the total virus by RFMP, whereas direct sequencing assays were able to detect mutant virus only when present in 20% or more of the total virus population (Fig. 1).

Comparison of RFMP with direct sequencing analyses

All 100 clinical samples from 60 patients were analysed by the RFMP and direct sequencing assays for the presence of drug resistance-related mutations: nine codons in the RT region and eight codons in the PR region of the HIV-1 *pol* gene (a total of 17 codons).

The overall concordance rates between RFMP and sequencing assays were excellent, irrespective of PR and RT regions (Table 3). Concordance rates in the RT region were 97% (97/100) at codons 65, 69, 74, 103, 151, 181 and 184, 96% (96/100) at codon 106, and 94% (94/100) at codon 215 (Fig. 2a).

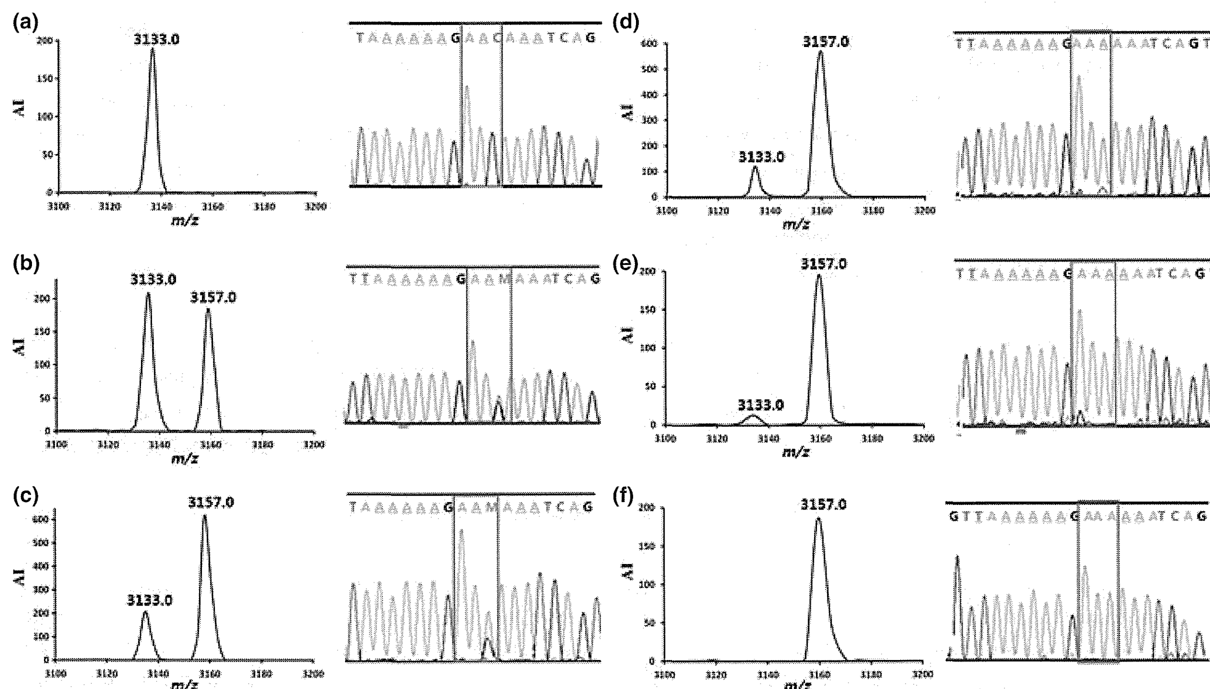

FIG. 1. Evaluation of the sensitivity of the RFMP assay for detection of minor amounts of virus with a defined mixture of K103N. The MALDI-TOF MS spectra and direct sequencing chromatograms shown are representative of experiments repeated three times using mixed populations of wild-type (K103) and NNRTI mutant (N103). The wild-type plasmids were mixed with mutant type at various ratios as follows: (a) 100%, (b) 50%, (c) 20%, (d) 10%, (e) 5% and (f) 1%. Molecular masses of 3133.0 and 3157.0 correspond to N103 and K103, respectively. AI is absolute intensity; m/z is mass-to-charge ratio.

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TABLE 3. **Comparison of the results obtained by the RFMP and direct sequencing assays in 100 clinical specimens**

				Compatible (n)		Amino acid	
pol gene	Codon	Discordant (n)	Concordant (n)	RFMP only	Direct sequencing only	RFMP	Direct sequencing
RT	65	3	97	–	–		
	69	3	97	–	–		
	74	3	97	–	–		
	103	3	97	–	–		
	106	3	96	1	–	V/A	V
	151	3	97	–	–		
	181	3	97	–	–		
	184	3	97	–	–		
	215	3	94	3	–	T/F	T/F or I/S or T/I/F
						T/Y	T/Y or N/S or T/S/Y
						T/Y	T/Y or N/S or T/S/Y
PR	30	–	100	–	–		
	46	–	100	–	–		
	48	–	100	–	–		
	50	–	100	–	–		
	54	–	100	–	–		
	82	–	99	1	–	V/A	V
	84	–	99	1	–	I/V	V
	90	–	100	–	–		

RT, reverse transcriptase; PR, protease.

Similarly, concordance rates in the PR region were 100% (100/100) at codons 30, 46, 48, 50, 54 and 90, and 99% (99/100) at codons 82 and 84 in the PR region (Fig. 2b). Both assays showed identical base substitution and amino acid composition in these positions. Rate of compatible cases were observed 1% (1/100) at codons 106, and 3% (3/100) at codon 215 in the RT region and 1% (1/100) at codons 82 and 84 in the PR region, respectively. Three samples (mixed-type) at codon 215 containing double mutations in a single codon were identified only by RFMP, as a result of the inability of the direct sequencing assay to determine the variants present in the clinical samples. As shown in Fig 2(c), 215T/Y mixtures detected by RFMP could be scored as 215T (ACC) plus 215Y (TAC), or 215N (AAC) plus 215S (TCC), or 215T (ACC) plus 215S (TCC) plus 215Y (TAC), by direct sequencing. A compatible single nucleotide mixture at one position was observed in three samples at three codons (codon 106 in the RT; codon 82, 84 in the PR), respectively. Of these, the RFMP assay detected more mixed samples than the direct sequencing assay. The details of mixtures detected by both assays are shown in Table 3. Discordances between the two assays occurred for three samples at RT region codons, which had undetectable viral loads; the correct viral genotypes were identified only by RFMP assay.

Discussion

ARV drug-resistance is a major obstacle in the effective clinical management of HIV-1-infected patients [3,15] and therapeutic strategies must maximize the early detection of drug resistance mutations. Having a sensitive genotyping assay that can detect with high accuracy and reliability, drug-resistance mutations that emerge during HAART can be very important for the optimization of ARV regimens, improvement of patient treatment and outcome of therapy. The RFMP assay has been demonstrated to be a sensitive, accurate and reliable method for genotyping and detecting drug-resistance mutations in several viruses, including hepatitis and papillomavirus [4,6,8–12].

In the present study, we validated use of the MALDI-TOF MS-based RFMP assay to detect oligonucleotides containing 8–14 nucleotides for codons implicated with ARV drug-resistance mutations. Specifically, we established successful detection at codons 65, 69, 74, 103, 106, 151, 181, 184 and 215 in the HIV-1 RT coding region, and 30, 46, 48, 50, 54, 82, 84 and 90 in the HIV-1 PR coding region. These codons address resistance to all approved NRTI and NNRTI inhibitors [16–18]: mutation at RT codon 65 (K65R) confers resistance to tenofovir, didanosine and abacavir; RT mutation L74V confers resistance to didanosine and abacavir; the K103N RT mutation engenders resistance to the NNRTIs efavirenz and nevirapine; the Q151M RT mutation causes resistance to AZT, D4T, didenosine and abacavir through the decreased incorporation mechanism; Y181C causes resistance to nevirapine, etravirine and rilpivirine; M184V/I confers resistance to 3TC and FTC, and also affects resistance to rilpivirine; and finally, T215Y causes resistance to AZT and D4T through the excision mechanism. In addition, mutations at the 30, 46, 48, 50, 54, 82, 84 and 90 sites of the HIV-1 PR coding region cause resistance to all known protease inhibitors: specifically, D30N causes resistance to nelfinavir; M46I/L causes resistance to nelfinavir and indinavir; G48V/M causes resistance to atazanavir, nelfinavir and saquinavir; I50L/V causes resistance to atazanavir, darunavir, fosamprenavir and lopinavir; and mutations at

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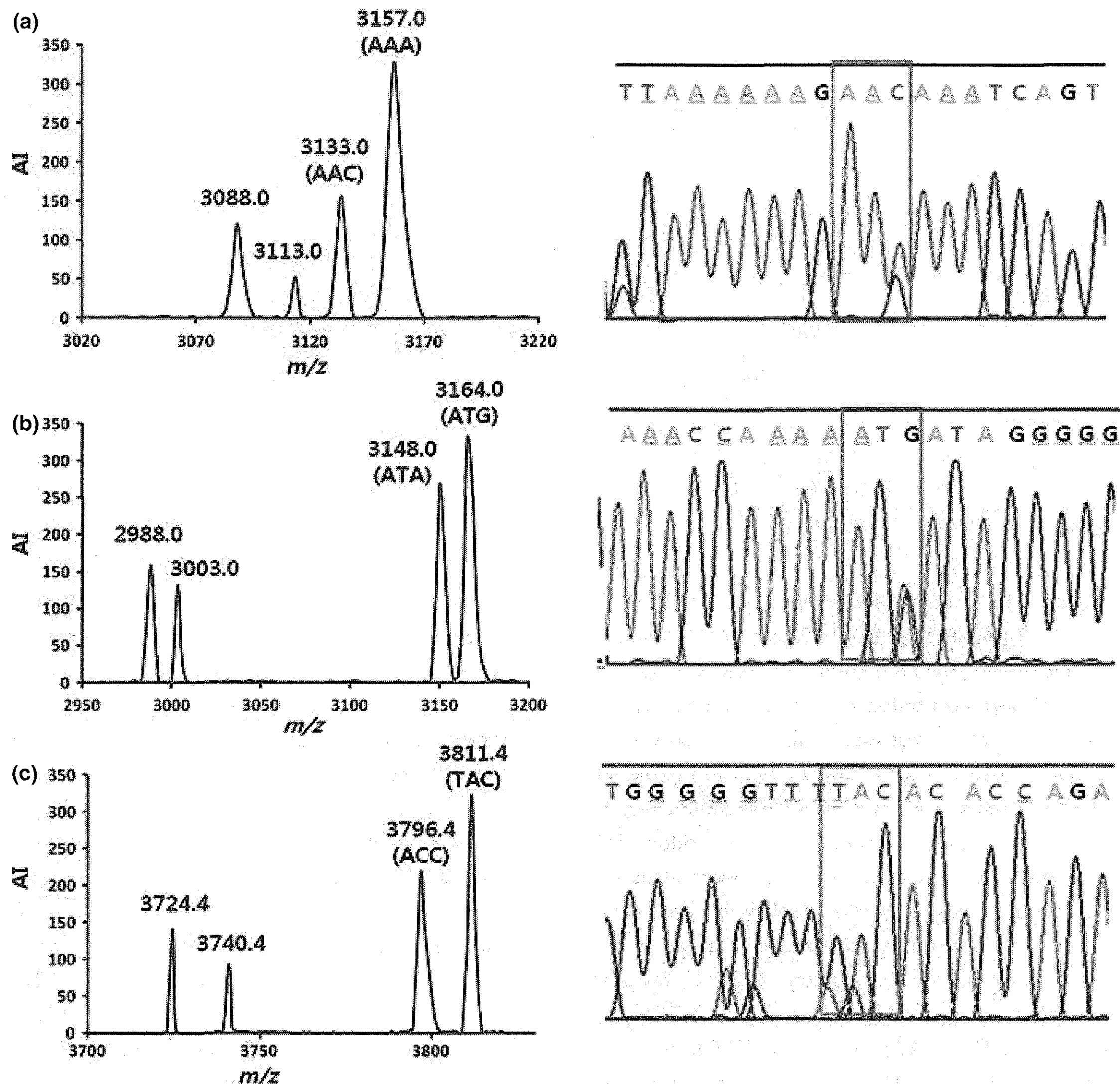

FIG. 2. Comparison of the RFMP and direct sequencing assays for detection of mixed genotypes. Sera were taken from patients infected with HIV-1 carrying ARV drug-resistant mutations and examined by the RFMP and sequencing assays. (a) For codon 103, molecular masses of 3088.0/3157.0 and 3113.0/3133.0 represent Lys (AAA) and Asn (AAC), respectively. (b) For codon 46, molecular masses of 2988.0/3164.0 and 3003.0/3148.0 represent Met (ATG) and Ile (ATA), respectively. (c) For codon 215, molecular masses of 3740.4/3796.4 and 3724.4/3811.4 represent Thr (ACC) and Tyr (TAC), respectively. Each codon was indicated by a red box in the sequencing chromatogram. AI is absolute intensity; m/z is mass-to-charge ratio.

residues 54, 82, 84 and 90 cause resistance to all protease inhibitors to a varying extent (http://hivdb.stanford.edu/DR/PIResiNote.html).

The detection limit of the RFMP assay was determined to be 223.02 copies/mL and able to identify a minority mutant at a concentration as low as 5% of the circulating mixed populations, whereas the detection limit of the direct sequencing method was 1268.11 copies/mL and able to detect variants only when present at >20% of the total population (Table 2 and Fig. 1). A clear correlation was observed between peak ratios and relative genotype concentration of mixed populations.

The performance of the RFMP assay in detecting mutations related to ARV drug-resistance was compared with direct sequencing assays for 100 clinical samples from 60 HIV-1-infected patients who experienced HAART therapy. The RFMP assay successfully identified genotypic changes at all 17 codons tested in clinical samples. Compared with direct sequencing, the RFMP assay exhibited 96.6% concordance in the RT region and 99.8% concordance in the PR region (Table 3 and Fig 2). The PR data were not significantly more concordant than the RT data (the difference was only two cases). The reason for the slight difference is not clear, thus further investigation is required with more samples. Notably, the RFMP assay

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outperformed direct sequencing for the detection of single and double mutations in compatible samples. The RFMP assay detected 1% (1/100) more mutant viruses in codons 106, 82 and 84 and 3% (3/100) more mutant viruses in codon 215. All discordances between the two assays were due to the inability of the direct sequencing assay to identify the residues at nine RT codons in three patients. Hence, the discrepancy among the two assays may be due to a lower sensitivity of direct sequencing.

HAART can clearly extend the life expectancy of HIV-1 patients. However, as adherence is usually imperfect, continuous ARV drug-resistance testing can be an important management tool. There are two major methods of assessing ARV drug-resistance: phenotypic assays and genotypic assays that provide complementary information and be preferable to the other [19,20]. Current treatment guidelines define as treatment failure the detection of more than 200 copies/mL of HIV-1 RNA [3]. However, existing genotypic assays, such as direct sequencing and the reverse hybridization-based assay, do not reliably detect fewer than 1000 copies/ml of HIV-1 RNA, nor do they enable detection of sequence variants present at <20% of minority variants in mixed populations [21,22]. This performance does not allow facile interpretation of ratios between multiple virus mixtures, especially when a double mutation is present in a single codon. While the reverse hybridization-based assay has somewhat higher sensitivity than direct sequencing it gives rise to false-positive and false-negative results more frequently than direct sequencing [21–26].

With the advent of Next Generation Sequencing methods it is possible to detect, by 454 pyrosequencing or an Illumina Genome Analyzer, minor viral variants whose prevalence is <1%. 454 pyrosequencing, also called ultra-deep pyrosequencing (UDPS), relies on fixing nebulized and adapter-ligated DNA fragments to small DNA-capture beads in a water-in-oil emulsion. The DNA fixed to these beads is then amplified by PCR. An advantage of UDPS in the case of viral genomic analysis is that it yields long sequence information for each sample (average ~800 bases). However, UDPS has some technical challenges. A major limitation of the UDPS relates to resolution of homopolymer-containing DNA segments, such as AAA and GGG [27]. Because there is no terminating moiety preventing multiple consecutive incorporations at a given cycle, pyrosequencing relies on the magnitude of light emitted to determine the number of repetitive bases. This is prone to greater error than misincorporation. Hence, the dominant error type for the 454 platform is insertion-deletion, rather than substitution. The decrease in single read accuracy from 99.4% for test fragments to 96% for genomic libraries is primarily due to a lack of clonality in a fraction of the genomic templates in the emulsion [28]. Moreover, based on current list prices for the UDPS, the current cost for all the reagents, including the picotiter plate, library preparation kits and emulsion PCR kits, to perform a single experiment is $1000–7000 [29].

By combining the merits of unique assay chemistry and the mature nature of MALDI-TOF mass spectrometry, the RFMP assay can be used to screen for HIV drug-resistance mutations in a robust high-throughput manner (e.g. 96 samples can be analysed in 3 h with Bruker software (flexcontrol 3.0), which is faster than existing hybridization or sequencing-based methods). In terms of cost-effectiveness, the direct cost per test (reagents and labour) of the RFMP assay can be <$30, including viral DNA extraction, PCR, restriction digestion, desalting and matrix for mass analysis, which is cheaper than the sequencing or hybridization assays that are *c.* $50–100 per test. These costs do not include the equipment costs, which are slightly greater for the RFMP method. However, with the advent of many diagnostic assays operating on a mass spectrometer platform, such as clinical genotyping and microorganism identification, and the gradual spread of compact mass spectrometers into laboratories as medical devices (i.e. Bruker Microflex), the burden of the amortization cost should be substantially reduced [30]. A limitation of the RFMP assay is that it determines only molecular mass and is thus applicable only to known resistance mutations and may fail to detect DNA sequence changes that do not affect molecular mass. Moreover, the occurrence of a novel resistance mutation with sequence alterations that cause a deviation from the predicted molecular mass pattern should be addressed by periodic updating of mass patterns for unambiguous result interpretation from up-to-date HIV databases.

We demonstrate here that the mass spectrometry-based RFMP assay is highly sensitive and able to successfully detect HIV-1 carrying drug-resistant mutations that are present in <5% of the total virus population. Hence, this assay can be used for the efficient assessment of genotype dynamics of viral quasispecies. Therefore, this simple procedure can be easily adapted to a high-throughput format, should enable earlier detection of drug-resistant viruses and help elucidate mechanisms of HIV-1 resistance, as well as guide and optimize treatment decisions.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic representation of the RFMP strategy.

Table S1. Primers used in PCR amplification for the RFMP assay.

Table S2. Expected masses of oligonucleotides resulting from restriction enzyme digestion of PCR products.

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