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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

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.

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.

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.

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).

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′-ACGTACGTACGTACGT-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′-CTTCCCTCAGATCACTCTTTGGCAA-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).

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

		RFMP		Direct sequencing	
HIV-1 RNA copies/ml	No. tested	No. detected	Per cent detected	No. detected	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).

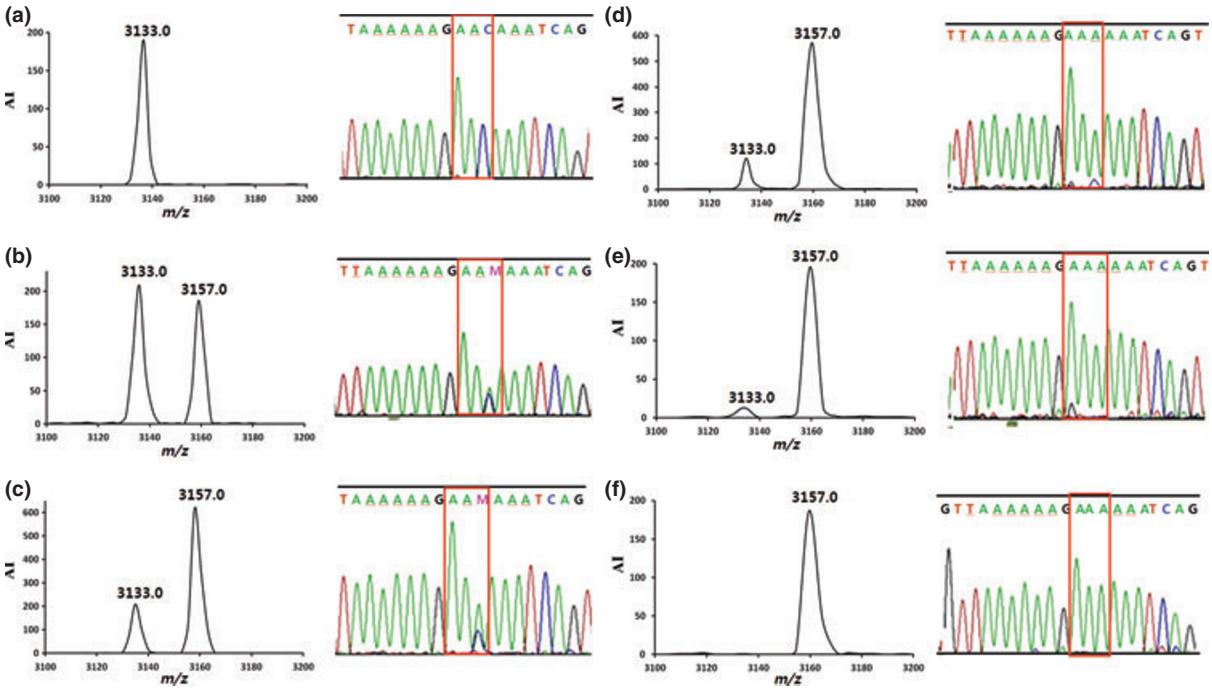

FIG. 1. Evaluation of the sensitivity of the RFMP assay for detection of minor amounts of virus with a defined mixture of K103N. The MALDI-TOF MS spectra and direct sequencing chromatograms shown are representative of experiments repeated three times using mixed populations of wild-type (K103) and NNRTI 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.

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

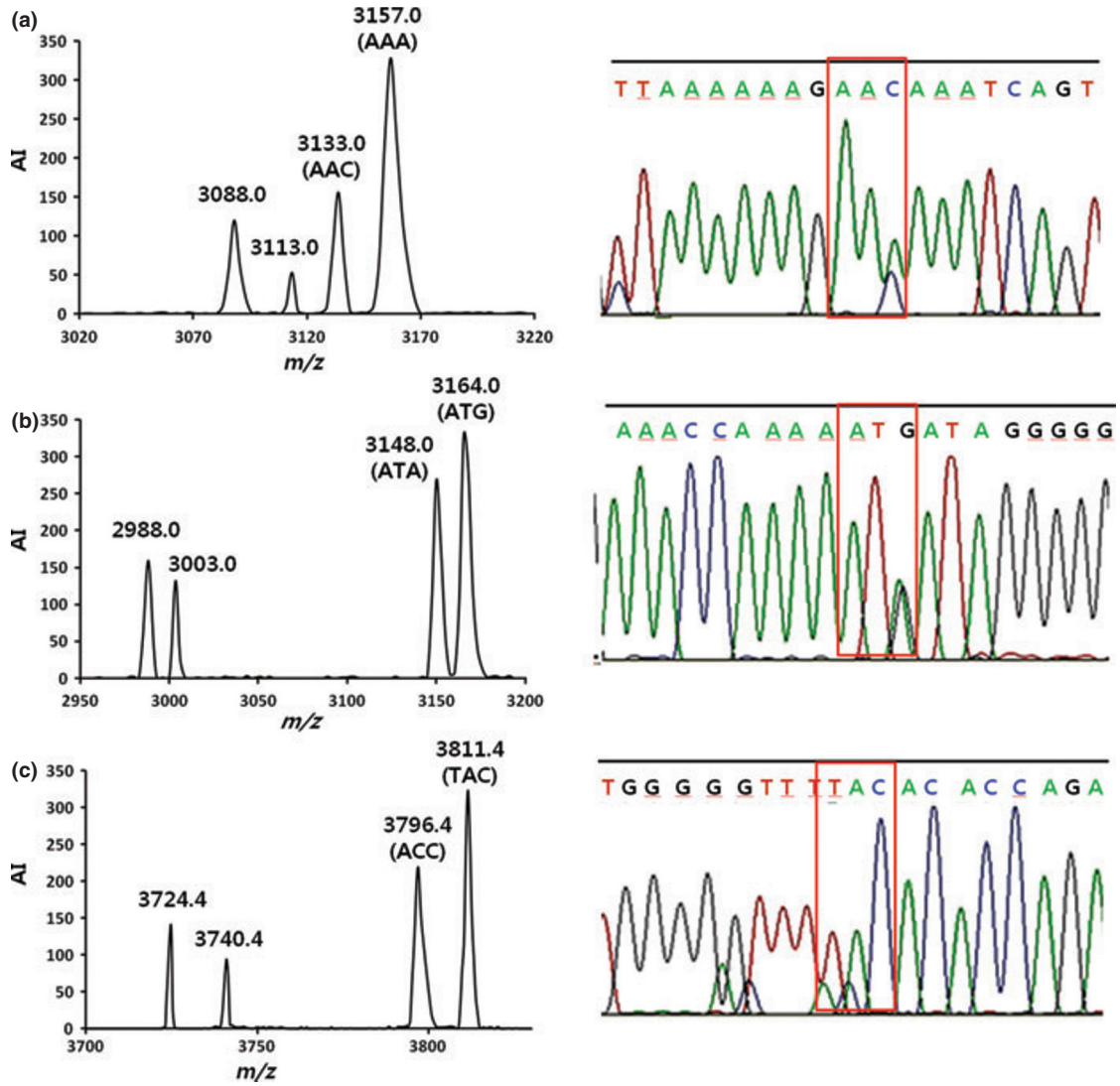

FIG. 2. Comparison of the RFMP and direct sequencing assays for detection of mixed genotypes. Sera were taken from patients infected with HIV-1 carrying ARV drug-resistant mutations and examined by the RFMP and sequencing assays. (a) For codon 103, molecular masses of 3088.0/3157.0 and 3113.0/3133.0 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

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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