Rilpivirine (TMC-278) is a second-generation NNRTI that is high potent against both wild-type and drug-resistant HIV-1 strains (4). Consequently, rilpivirine is expected to treat therapy-experienced patients who failed to use current drugs due to the emergence of drug-resistant HIV mutants. In addition, rilpivirine shows a favourable safety profile (5-7). The recommended dose of rilpivirine is 25 mg (one tablet) once daily in combination with other antiretroviral agents. No dose adjustment is required in patients with moderate hepatic or renal impairment. However, rilpivirine is primarily metabolized by cytochrome P450 (CYP)3A. Therefore, co-administration of rilpivirine and CYP3A inducer may result in decreased plasma concentrations of rilpivirine, loss of virologic response, and possible resistance to rilpivirine. To avoid these risks, therapeutic drug monitoring of rilpivirine is essential.

Else *et al.* (8) recently determined plasma rilpivirine concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, more simple and easy system to determine plasma rilpivirine concentrations has been required. Now we have a routine system, by which antiretroviral drug plasma concentrations are easily determined by HPLC (9). According to our preliminary HPLC application, the sensitivity of LC-MS method must at least be essential for quantification of plasma rilpivirine. In this study, we intended to develop a conventional method for determining plasma rilpivirine concentrations by LC-MS.

MATERIALS AND METHODS

Chemicals and Reagents

Rilpivirine was supplied by Janssen Pharmaceutica (Turnhoutseweg, Beerse, Belgium) and the internal standard (IS), 6,7-Dimethyl-2,3-di (2-pyridyl)-quinoxaline, was purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol, *n*-hexane, ethyl acetate, and acetonitrile (Kanto Chemical, Tokyo, Japan) were HPLC grade. Ammonium acetate, EDTA and acetic acid were purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q® system (Millipore Corp., Bedford, MA, USA). 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, USA), 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 0.1 mM EDTA in 0.1% acetic acid (A), 100% acetonitrile (B) and 100% methanol (C). An isocratic mobile phase consisting of A-B-C (65 : 15 : 20) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A-B-C (30 : 50 : 20) for the next 8 min. The final conditions were maintained for the final 5 min. The system was then reequilibrated for an additional 25 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 rilpivirine and the internal standard. The source temperature was 120°C and the desolvation temperature was 350°C. The desolvation and cone gas flow-rates were set to 600 and 50 L/h, respectively. The acquisition mass range is *m/z* 200-800 at 0.5 s per scan with a 0.1 s interscan delay. All mass spectra are acquired in centroid mode.

Quantitative analysis, carried out in selected-ion recording (SIR) mode, detected rilpivirine at *m/z* 367, and the IS, at *m/z* 313, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions

Stock solutions of rilpivirine and the IS were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 143.0 μg/ml of rilpivirine and 588.0 μg/ml of the IS. 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 rilpivirine concentrations of 18, 72, 143, 358 and 715 ng/ml.

Sample Preparation

Two milliliters of ethyl acetatate/*n*-hexane (50 : 50, v/v) containing the IS (177.5 ng/ml) and 1.0 ml of 0.2 M ammonium acetate were added to a 500 μl plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3,500 g

for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 50 μl of a mobile phase solution. 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 rilpivirine five times on the same day and on three separate days to obtain the relative standard deviation (RSD). The measured value was calculated as the peak area ratio of rilpivirine to the internal standard. The extraction recovery was determined by comparing the peak areas obtained from the extracted samples in plasma with those of direct injected standards, at the same concentrations. The mean recoveries were determined in triplicate. Accuracy was determined as the percentage of the nominal concentration.

RESULTS

LC-MS Chromatograms

Figures 1A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 143.0 ng/ml of rilpivirine and 177.5 ng/ml of the IS. Under the described chromatographic conditions, retention times were 5.3 min for rilpivirine and 10.0 min for IS. 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 the baseline part of Fig. 1B. These peaks did not affect the quantification of the IS. Figures 1E and F show chromatograms of a plasma sample from an HIV-1-infected patient treated with rilpivirine. There were no interfering peaks affecting quantification of rilpivirine in this chromatogram. Anticoagulants of heparin and EDTA did not hinder the selected-ion recording chromatograms for rilpivirine and the IS.

Validation : Linearity, Precision, Accuracy and Recovery

Calibration curves of rilpivirine appeared linear in the concentration range of 18 to 715 ng/ml with a correlation of 0.995.

Precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of rilpivirine covers the expected plasma concentrations found in the patients. The RSDs calculated for rilpivirine in the inter- and intraday

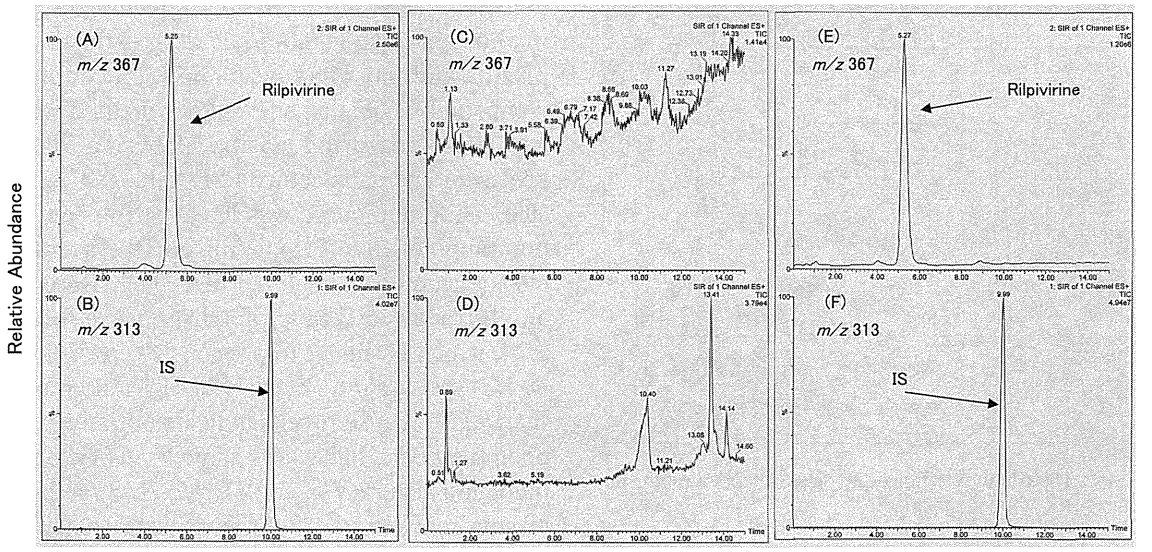

Figure 1. Selected-ion recording chromatograms for rilpivirine and the internal standard.
(A) and (B) were obtained from a spiked plasma containing 143 ng/ml of rilpivirine and 178 ng/ml of the internal standard (IS). (C) and (D) were obtained from a blank plasma sample. (E) and (F) were obtained from a plasma sample from an HIV-1-infected patient on rilpivirine at 16 h after orally administration. (A), (C) and (E) were monitored with *m/z* 367. (B), (D) and (F) were monitored with *m/z* 313. (C) and (D) are the expanded figures of the baselines in (A) and (B), respectively.

Table 1. Intraday and interday precision and accuracy for rilpivirine

	Intraday	(n=5)	Interday	(n=15)		
Expected (ng/ml)	Measured (ng/ml)	RSD (%)	Measured (ng/ml)	RSD (%)	Accuracy (%)	Recovery (%)
18	18.1± 0.2	1.0	18.0± 0.4	2.4	100.3± 1.0	85.1± 1.3
72	72.3± 1.4	1.9	72.0± 2.3	3.3	100.4± 1.9	82.0± 3.7
143	143.7± 3.0	2.1	143.9± 3.1	2.2	100.5± 2.1	87.6± 0.6
358	360.2± 2.9	0.8	357.8± 5.2	1.5	100.6± 0.8	88.3± 7.2
715	715.0± 8.6	1.2	716.3± 6.1	0.8	100.0± 1.2	84.0± 8.6

RSD, relative standard deviation
Means± SD

assays ranged from 0.8 to 3.3%, which are similar to values reported by LC-MS/MS method (8). Accuracies ranged from 100.0 to 100.6%. Recoveries from plasma ranged from 82.0 to 88.3%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

Clinical application

Figure 2 shows the distribution of plasma rilpivirine concentrations in 6 Japanese HIV-1 infected patients. Rilpivirine plasma concentrations were measured at trough level (14-22 h after orally administration). Mean rilpivirine plasma concentration was 49± 22 ng/ml (n=6, range : 23-90 ng/ml). Rilpivirine has been just approved at May 2012 in Japan. This result is the first rilpivirine concentration data for Japanese HIV-1 infected patients. These rilpivirine concentrations were similar to values reported by foreign healthy volunteers (12).

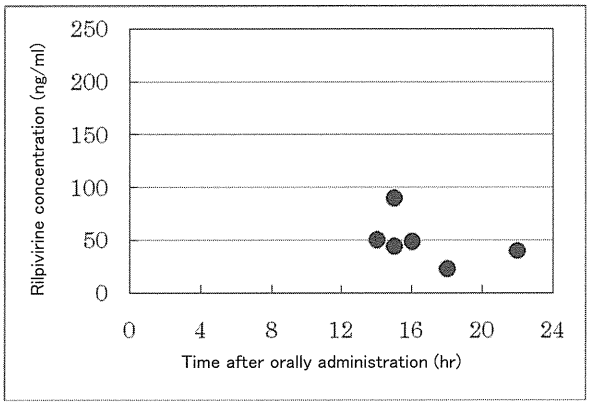

Figure 2. Distribution of rilpivirine plasma concentrations in 6 Japanese HIV-1 infected patients.

DISCUSSION

In NNRTI-based regimens, efavirenz is recommended as an initial combination regimen for antiretroviral-naïve patients, because no regime has proven superior to efavirenz-based regimens with respect to virologic responses. However, efavirenz-based regimens are associated with rash and central nervous system adverse effects (1-3). Clinical trials of rilpivirine (TMC-278) have showed the same efficacy compared with efavirenz, with a slightly increased incidence of virological failures, but a more favourable safety and tolerability profile (10, 11). Therefore, rilpivirine can be an alternative NNRTI-based regimen for antiretroviral therapy-naive patients infected with HIV-1.

Rilpivirine is a substrate of CYP3A4 and its pharmacokinetics is likely to be modulated by inhibitors and inducers of these enzymes. To manage these drug interactions and ensure optimal drug efficacy, monitoring plasma rilpivirine concentrations is essential. For this purpose, we developed a method for determining plasma rilpivirine concentrations using LC-MS. The principal advantages of our method are rapid liquid-liquid drug extraction from plasma and use of an available IS, a commercial compound. Validation showed our method was successful in measuring plasma rilpivirine with high precision and satisfactory RSD values. The rilpivirine calibration curve was linear at the concentration range of 18 to 715 ng/ml, and the average accuracy ranged from 100.0 to 100.6%. Both inter- and intraday RSDs for rilpivirine were less than 3.3%, which is similar to previously reported values by LC-MS/MS (8). Recovery of rilpivirine was more than 82.0%. These results indicate our newly developed method achieves the same level of reproducibility and accuracy as the LC-MS/MS method. As plasma concentrations of rilpivirine are expected in the 67 to 204 ng/ml range when rilpivirine is administered at single dose of 25 mg for healthy volunteers (12), our method successfully covers this region with good precision and accuracy. In clinical practice, mean rilpivirine plasma concentration

at trough was 49 ng/ml. This level compared favourably with trough concentrations of about 50-80 ng/ml seen in ECHO and THRIVE trials (10, 11, 13).

In conclusion, our LC-MS method provides a conventional, accurate and precise way to determine rilpivirine in human plasma. This method can be used in routine clinical application for HIV-1 infected patients, and permits management of drug interactions and toxicity for rilpivirine.

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Short Communication

TRIM5 genotypes in cynomolgus monkeys primarily influence inter-individual diversity in susceptibility to monkey-tropic human immunodeficiency virus type 1

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TRIM5α restricts human immunodeficiency virus type 1 (HIV-1) infection in cynomolgus monkey (CM) cells. We previously reported that a *TRIMCyp* allele expressing TRIM5–cyclophilin A fusion protein was frequently found in CMs. Here, we examined the influence of *TRIM5* gene variation on the susceptibility of CMs to a monkey-tropic HIV-1 derivative (HIV-1mt) and found that TRIMCyp homozygotes were highly susceptible to HIV-1mt not only *in vitro* but also *in vivo*. These results provide important insights into the inter-individual differences in susceptibility of macaques to HIV-1mt.

Considering the global human immunodeficiency virus type 1 (HIV-1) epidemic, development of prophylactic vaccines is strongly desired. In order to evaluate the efficacy of the vaccines, a suitable animal model is also indispensable. However, HIV-1 does not grow in Old World Monkeys (OWMs) such as rhesus monkeys and cynomolgus monkeys (CMs). One of the restriction factors of OWMs is ApoB mRNA editing catalytic subunit 3G (APOBEC3G) (Sheehy *et al.*, 2002). APOBEC3G modifies the minus-strand viral DNA during reverse transcription, resulting in impairment of HIV-1 replication. This activity can be counteracted by the viral protein Vif of simian immunodeficiency virus (SIV) but not by that of HIV-1 (Mariani *et al.*, 2003). Another restriction factor is tripartite motif-containing protein 5α (TRIM5α), which recognizes the viral core and facilitates premature uncoating (Stremlau *et al.*, 2004). To establish a feasible model of HIV-1 infection, monkey-tropic HIV-1 (HIV-1mt) clones were constructed, which were expected to escape from these restriction factors (Hatziioannou *et al.*, 2006; Kamada *et al.*, 2006). In CMs, we reported previously that a modified HIV-1mt, MN4-5S, in which *vif* and the loops of α-helices 4 and 5 (L4/5) and α-helices 6 and 7 of the capsid protein (CA) of HIV-1 were replaced with those of SIVmac239, a pathogenic molecular clone of rhesus macaque SIV, showed enhanced virus replication *in vitro* (Kuroishi *et al.*, 2009) and *in vivo* (Saito *et al.*, 2011).

Accumulating evidence indicates intra-species variations in human and macaque *TRIM5* genes (Johnson & Sawyer, 2009). TRIMCyp is an alternatively spliced isoform of the *TRIM5* gene in which the PRYSPRY domain of TRIM5α is

One supplementary figure is available with the online version of this paper.

replaced with a retrotransposed cyclophilin A (*cypA*) gene (Brennan *et al.*, 2008; Liao *et al.*, 2007; Newman *et al.*, 2008). We recently reported that the frequency of TRIMCyp alleles was >0.8 in Philippine CMs, which is in contrast to the situation in Indochina CMs (Saito *et al.*, 2012a, 2012b). CM TRIMCyp, also known as Mafa TRIMCyp2 (Ylinen *et al.*, 2010), can restrict HIV-1, but fails to do so in SIVmac and HIV-1mt NL-DT5α with L4/5 derived from SIVmac (Saito *et al.*, 2012a), as the CypA domain of CM TRIMCyp binds to L4/5 of HIV-1, but not that of SIVmac (Price *et al.*, 2009; Ylinen *et al.*, 2010).

We recently reported that a new proviral HIV-1mt construct, MN4Rh-3, carrying a glutamine-to-aspartic acid substitution at position 110 (Q110D) of CA in the parental HIV-1mt MN4-8S (Fig. 1), exhibited further enhanced growth properties in a macaque T-cell line (Nomaguchi *et al.*, 2013a, b). In the present study, we investigated whether TRIMCyp alleles in CMs could influence the susceptibility to HIV-1mt infection.

First, we analysed the replication kinetics of HIV-1mt MN4Rh-3 in $CD8^+$ cell-depleted PBMCs from 26 CMs comprising nine TRIM5α homozygotes, eight TRIM5α/TRIMCyp heterozygotes and nine TRIMCyp homozygotes. Prior to this experiment, we confirmed the expression of TRIM5α and/or TRIMCyp in PBMCs from monkeys by reverse transcription-PCR (RT-PCR). We found that the mRNA expression was consistent with the *TRIM5* genotype of each monkey, i.e. the TRIM5α or TRIMCyp homozygotes expressed the respective mRNA, and the heterozygotes expressed both TRIM5α and TRIMCyp mRNAs (Fig. S1, available in JGV Online). Virus stocks for infection experiments were prepared by transfecting HIV-1mt MN4Rh-3 and HIV-1mt MN4-8S clones into HEK293T cells (Saito *et al.*, 2011). Preparation of $CD8^+$ cell-depleted PBMCs and evaluation of viral growth were performed as described previously (Saito *et al.*, 2011). In Fig. 2(a), representative viral kinetics in PBMCs from animals with each *TRIM5* genotype are presented. For comparison, the replication kinetics of HIV-1mt MN4Rh-3 in cells from all 26 animals is shown at the bottom of the figure. Furthermore, the impact of each *TRIM5* genotype on HIV-1mt MN4Rh-3 and MN4-8S replication was evaluated by plotting the peak p24 levels during the observation period (Fig. 2b). HIV-1mt MN4Rh-3 grew significantly better in the PBMCs from TRIMCyp homozygotes or heterozygotes than in those from TRIM5α homozygotes, whilst there was no significant difference between TRIMCyp homozygotes and heterozygotes (Fig. 2a, b). Our results on heterozygotes were consistent with previous findings that co-expression of TRIM5α variants with a distinct antiviral activity interferes with the antiviral activity of the wild-type TRIM5α (Javanbakht *et al.*, 2005; Lim *et al.*, 2010; Nakayama *et al.*, 2006; Perez-Caballero *et al.*, 2005; Stremlau *et al.*, 2004). In addition, HIV-1mt MN4Rh-3 grew better in PBMCs of both TRIMCyp homozygotes and the heterozygotes than HIV-1mt MN4-8S (Fig. 2a, b), which was in agreement with our recent data obtained in a CM-derived T-cell line (Nomaguchi *et al.*, 2013b). Of note, there was no significant difference between each *TRIM5* genotype in the susceptibility to SIVmac239 infection (Fig. 2c), suggesting that the CM *TRIM5* genotypes specifically influence susceptibility to HIV-1mt infection.

We finally investigated whether *TRIM5* genotypes could influence the growth of HIV-1mt MN4Rh-3 *in vivo*. Healthy adult CMs seronegative for B virus and simian retrovirus were housed in individual isolators in a Biosafety Level 3 facility and maintained according to National Institute of Biomedical Innovation guidelines. All experiments were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation, as well as by Kyoto University. Bleeding and virus inoculation were performed under ketamine hydrochloride anaesthesia. Viral stocks propagated in $CD8^+$ cell-depleted PBMCs were inoculated intravenously into TRIMCyp homozygotes (n=6) or TRIM5α homozygotes (n=3) at a dose of HIV-1mt corresponding to 10 ng CA per head. The profiles of plasma viral loads and anti-HIV-1 antibody responses were evaluated as described previously (Saito *et al.*, 2011). We found that HIV-1mt MN4Rh-3 growth was readily observed in all

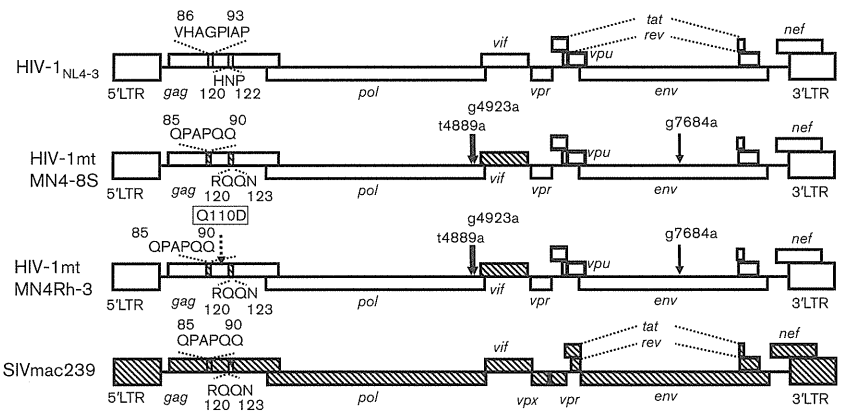

Fig. 1. Structure of the HIV-1mt clones (MN4-8S and MN4Rh-3) used in this study. Open boxes denote HIV-1 (NL4-3) and hatched boxes denote SIVmac239 sequences. Black arrows show adaptive mutations that enhance viral growth potential in CM T-cell lines. Dotted arrows show the CA Q110D mutation.

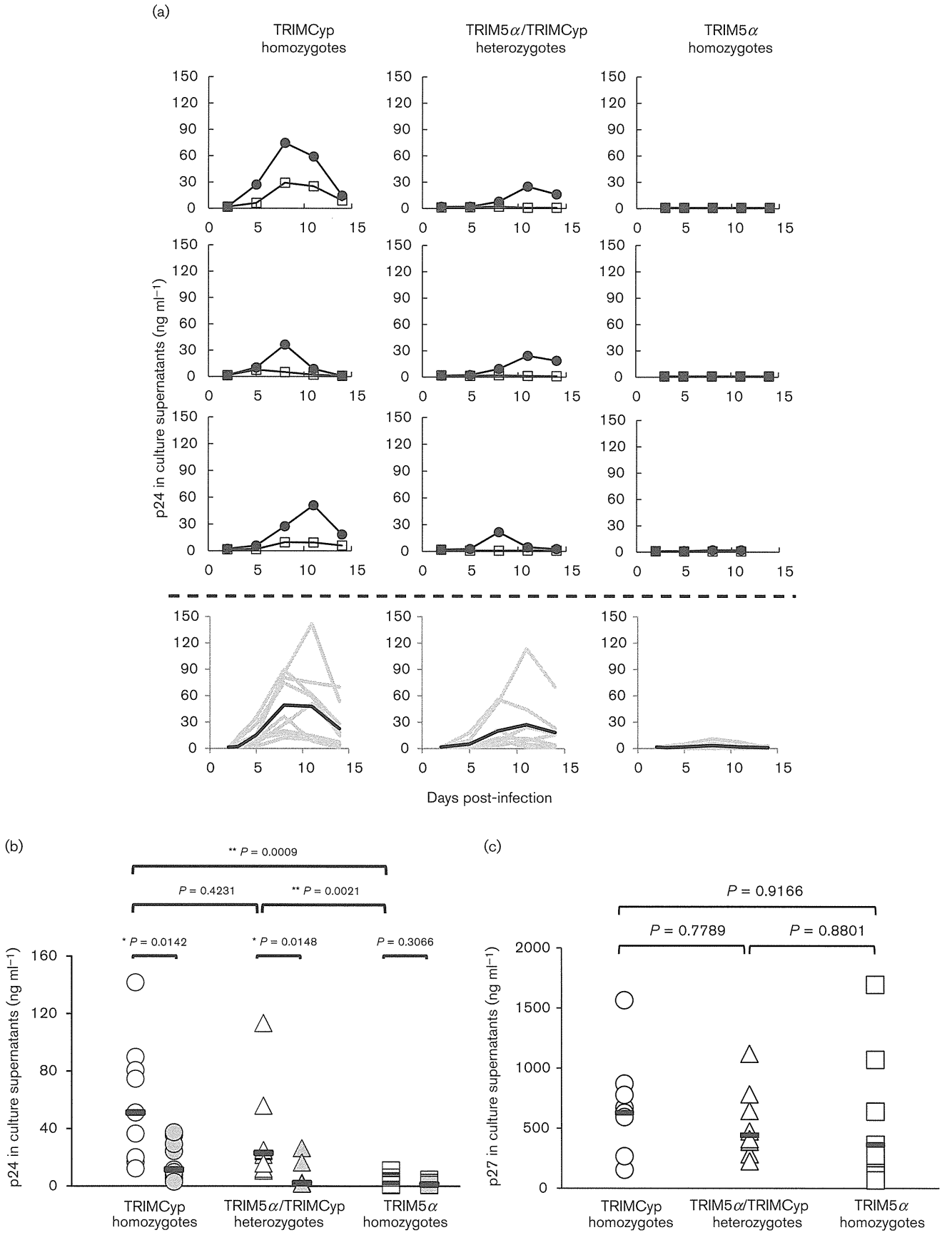
(a)
TRIMCyp homozygotes
TRIM5α/TRIMCyp heterozygotes
TRIM5α homozygotes
p24 in culture supernatants (ng ml−1)
Days post-infection
(b)
** P = 0.0009
P = 0.4231
** P = 0.0021
* P = 0.0142
* P = 0.0148
P = 0.3066
p24 in culture supernatants (ng ml−1)
TRIMCyp homozygotes
TRIM5α/TRIMCyp heterozygotes
TRIM5α homozygotes
(c)
P = 0.9166
P = 0.7789
P = 0.8801
p27 in culture supernatants (ng ml−1)
TRIMCyp homozygotes
TRIM5α/TRIMCyp heterozygotes
TRIM5α homozygotes

Fig. 2. (a) Growth properties of HIV-1mt derivatives in CM PBMCs. $CD8^+$ cell-depleted PBMCs were infected with HIV-1mt MN4Rh-3 (●) or HIV-1mt MN4-8S (□). Culture supernatants were collected periodically and virus replication was assessed using a HIV-1 p24 antigen capture assay kit. These experiments carried out on the PBMCs of each of the 26 macaques were done once. Representative results of virus replication kinetics in the PBMCs prepared from three animals of each *TRIM5* genotype are shown. For comparison, the replication kinetics of HIV-1mt MN4Rh-3 in the PBMCs from the 26 animals are shown at the bottom of the figure (indicated as grey lines). The mean values of the viral growth kinetics in each genotype are indicated in black lines. (b) Influence of *TRIM5* genotypes on the replication of HIV-1mt derivatives in PBMCs. $CD8^+$ cell-depleted PBMCs were infected with HIV-1mt MN4Rh-3 (open symbols) or HIV-1mt MN4-8S (shaded symbols). The cells were derived from TRIMCyp homozygotes ($n=9$), TRIM5α/TRIMCyp heterozygotes ($n=8$) and TRIM5α homozygotes ($n=9$). The peak p24 levels of the virus replication kinetics as shown in Fig. 2(a) were plotted. Thick horizontal bars indicate the median values. Differences in the mean values were assessed using the Wilcoxon rank-sum test (for HIV-1mt MN4Rh-3 and HIV-1mt MN4-8S viruses in each monkey group) and using the Steel–Dwass multiple comparison procedure (for HIV-1mt MN4Rh-3 in the three monkey groups). $^*P<0.05$; $^{**}P<0.01$. (c) Influence of *TRIM5* genotypes on the replication of SIVmac239 in PBMCs. $CD8^+$ cell-depleted PBMCs were infected with SIVmac239. Virus replication was monitored by detecting p27 antigen in the culture supernatants, and the p27 level on the peak day during the observation period (14 days) was plotted. Thick horizontal bars indicate the median values. Differences in the mean values were assessed by the Wilcoxon rank-sum test.

TRIMCyp homozygotes, with plasma viral loads reaching a peak at 2–4 weeks post-inoculation (p.i.) and ranging from 1.1×10^4 to 1.5×10^5 copies ml^{-1} (mean 4.2×10^4 copies ml^{-1}; Fig. 3a). In contrast, HIV-1mt MN4Rh-3 scarcely replicated in TRIM5α homozygotes (mean 1.9×10^3 copies ml^{-1}; Fig. 3a). Accordingly, HIV-1-specific antibodies were also detected in plasma from 3 to 9 weeks p.i. in the TRIMCyp homozygotes but minimally in TRIM5α homozygotes (Fig. 3b), suggesting that the strength of antibody response reflected the level of virus replication. Notably, although TRIMCyp homozygotes had a higher viraemia compared with TRIM5α homozygotes, none developed persistent viraemia (Fig. 3a). As our present HIV-1mts were focused on evasion of TRIM5- and APOBEC3-mediated restrictions, it is reasonable to assume that additional modifications of the viral genome, especially in order to overcome bone marrow stromal antigen 2 (BST-2)-mediated (Jia *et al.*, 2009; Neil *et al.*, 2008; Van Damme *et al.*, 2008) and SAM domain and HD domain-containing protein 1 (SAMHD1)-mediated restriction (Hrecka *et al.*, 2011; Laguette *et al.*, 2011), may be required to establish persistent viraemia *in vivo*. Moreover, Bitzegeio *et al.* (2013) recently suggested the existence of unidentified, type I interferon-inducible antiviral host factors in macaque PBMCs that inhibit HIV-1 replication.

In humans, several genetic factors related to HIV-1 susceptibility have been reported (reviewed by Chatterjee, 2010; Shioda & Nakayama, 2006). A polymorphism in the chemokine (C–C motif) receptor-5 (*CCR5*) gene is an eminent example; thus, individuals carrying a 32 bp deletion in *CCR5* (CCR5-Δ32) are resistant to CCR5-tropic HIV-1 infection and show delayed progression to AIDS (Dean *et al.*, 1996; Samson *et al.*, 1996). In addition to *CCR5*, polymorphisms in the genes encoding IL-4 and IL-10 (Shin *et al.*, 2000) and human leukocyte antigen (Carrington & O'Brien, 2003), as well as *TRIM5* (Sawyer *et al.*, 2006), have also been suggested to affect disease progression in HIV-1-infected individuals. One of the single-nucleotide polymorphisms (SNPs) in human *TRIM5* is a C127T nucleotide substitution, corresponding to an H43Y amino acid substitution in the RING domain. A correlation between this SNP and rapid disease progression has been suggested (van Manen *et al.*, 2008), although this remains controversial (Nakayama *et al.*, 2007; Speelmon *et al.*, 2006). In macaques, an effect of polymorphisms in *TRIM5* on SIV infection has been reported (Kirmaier *et al.*, 2010; Lim *et al.*, 2010); thus, rhesus macaques with TFP residues at positions 339–341 of TRIM5α show greater resistance to SIVsmE041 and SIVsmE543–3 compared with animals with a single glutamine residue at position 339 (Kirmaier *et al.*, 2010). However, it remains elusive as to whether genetic diversity might affect HIV-1mt infection in macaques. In this study, we found for the first time that the *TRIM5* genotypes of CMs primarily influenced inter-individual diversity in terms of susceptibility to HIV-1mt. Our results will provide an important insight into the divergent susceptibility of macaques to HIV-1mt. In particular, the finding that the TRIMCyp homozygotes exhibited a greater susceptibility to HIV-1mt infection will make it possible to identify the susceptibility of each CM by pre-screening for *TRIM5* genotypes, which will be invaluable in establishing a pre-clinical non-human primate model of HIV-1mt infection using CMs. It is noteworthy that our result is consistent with the findings that pig-tailed macaques, a macaque species that is thought to possess TRIMCyp exclusively instead of TRIM5α, shows higher susceptibility to HIV-1 infection (Agy *et al.*, 1992). For this reason, pig-tailed macaques are expected to be a promising model animal for HIV-1mt infection. Indeed, it was reported previously that these macaques developed persistent viraemia following HIV-1mt challenge (Hatziioannou *et al.*, 2009; Igarashi *et al.*, 2007; Thippeshappa *et al.*, 2011).

Moreover, our findings, in which CM *TRIM5* genotype was shown to influence susceptibility to retroviral infection, may imply that the marked geographical variation in the genotypes (Berry *et al.*, 2012; Dietrich *et al.*, 2011; Saito *et al.*, 2012a; Saito *et al.*, 2012b) is a consequence of selective pressures driven by some external factors. As both TRIM5α and TRIMCyp are thought to be associated with retrovirus replication, it is reasonable to speculate that a geographically diverse prevalence of some pathogen(s) such as exogenous or endogenous retroviruses might

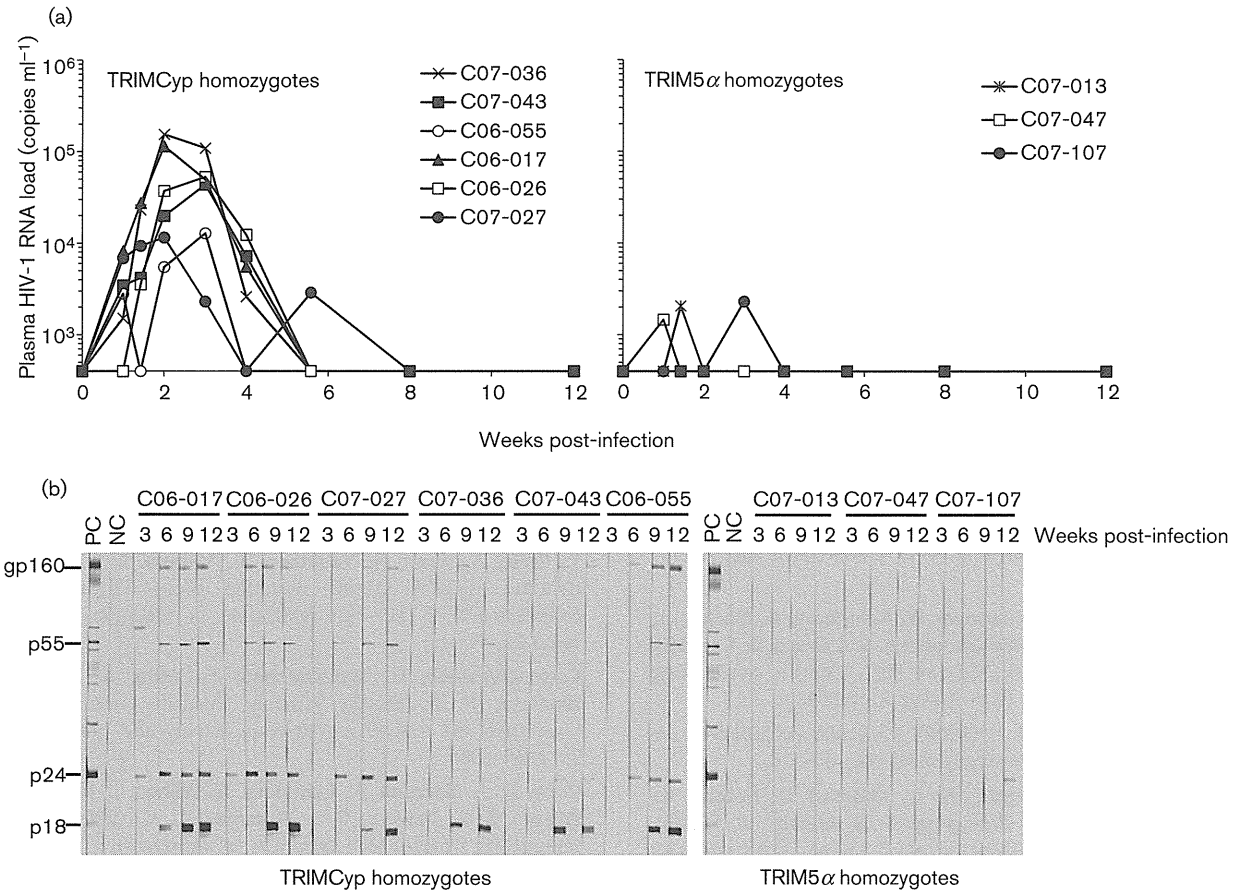

Fig. 3. Growth properties of HIV-1mt derivatives in CMs. (a) Monkeys were infected with 10 ng p24 of HIV-1mt MN4Rh-3 intravenously and bled periodically. Plasma viral RNA load was evaluated by quantitative RT-PCR. (b) Commercially available diagnostic HIV-1 Western blotting strips were reacted with 100-fold-diluted plasma from each monkey. Plasma from HIV-1-infected and uninfected individuals were used as positive (PC) and negative (NC) controls, respectively. Individual monkey numbers are indicated.

contribute to the variation in *TRIM5* genotypes. We are now seeking to identify pathogen(s) that have played a critical role in the diversity of CM *TRIM5* genotypes.

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HIV-1 Drug-Resistance Surveillance among Treatment-Experienced and -Naïve Patients after the Implementation of Antiretroviral Therapy in Ghana

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Abstract

Background

Limited HIV-1 drug-resistance surveillance has been carried out in Ghana since the implementation of antiretroviral therapy (ART). This study sought to provide data on the profile of HIV-1 drug resistance in ART-experienced and newly diagnosed individuals in Ghana.

Methods

Samples were collected from 101 HIV-1-infected patients (32 ART-experienced cases with virological failure and 69 newly diagnosed ART-naïve cases, including 11 children), in Koforidua, Eastern region of Ghana, from February 2009 to January 2010. The *pol* gene sequences were analyzed by in-house HIV-1 drug-resistance testing.

Results

The most prevalent HIV-1 subtype was CRF02_AG (66.3%, 67/101) followed by unique recombinant forms (25.7%, 26/101). Among 31 ART-experienced adults, 22 (71.0%) possessed at least one drug-resistance mutation, and 14 (45.2%) had two-class-resistance to nucleoside and non-nucleoside reverse-transcriptase inhibitors used in their first ART regimen. Importantly, the number of accumulated mutations clearly correlated with the duration of ART. The most prevalent mutation was lamivudine-resistance M184V (n = 12, 38.7%) followed by efavirenz/nevirapine-resistance K103N (n = 9, 29.0%), and zidovudine/stavudine-resistance T215Y/F (n = 6, 19.4%). Within the viral protease, the major nelfinavir-resistance mutation L90M was found in one case. No transmitted HIV-1 drug-resistance mutation was found in 59 ART-naïve adults, but K103N and G190S mutations were observed in one ART-naïve child.

Conclusions

Despite expanding accessibility to ART in Eastern Ghana, the prevalence of transmitted HIV-1 drug resistance presently appears to be low. As ART provision with limited options is scaled up nationwide in Ghana, careful monitoring of transmitted HIV-1 drug resistance is necessary.

Figures

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Introduction

The number of people worldwide living with HIV/AIDS in 2010, according to the latest report from the United Nations Programme on HIV/AIDS, was estimated to be 34.0 million [1]. Although the highest prevalence of HIV/AIDS remains in sub-Saharan Africa, current massive and rapid scaling up of antiretroviral therapy (ART) has resulted in the decline of the epidemic in this region [1]. Indeed, HIV prevalence in Ghana gradually declined from a peak of 3.6% in 2003 to 2.1% in 2011 due to the National AIDS Control Programme implementing a strategy for achieving universal access to ART. The program has been continuously expanding since 2003, and the coverage of ART in 2011 was estimated to be 26.6% (59,007/221,884) and 63.6% (8,057/12,661) for total HIV-infected individuals and for HIV-positive pregnant women, respectively [2].

The first-line regimen of ART recommended in Ghana is the combination of two nucleoside reverse-transcriptase inhibitors (NRTIs) and a non-nucleoside reverse-transcriptase inhibitor (NNRTI) [3]. Specifically, the two NRTIs selected are lamivudine (3TC) and either zidovudine (AZT) or stavudine (d4T), then either nevirapine (NVP) or efavirenz (EFV) as the NNRTI [3]. For the second-line regimen in Ghana, two protease inhibitors (PIs) are available, nelfinavir (NFV) or lopinavir/ritonavir (LPV/r), either of which is recommended to use with two NRTIs, abacavir (ABC) and either tenofovir (TDF) or didanosine (ddI) [3].

Drug-resistant HIV variants selected during ART have the potential to be transmitted to others. Indeed, drug-resistant HIV has been widely described in ART-naïve individuals. For example, a recent systematic review revealed that the overall prevalence of drug-resistant HIV-1 transmission reached 12.9% in North America, 10.9% in Europe, 6.3% in Latin America, 4.7% in Africa, and 4.2% in Asia [4]. Thus, the higher prevalence of drug-resistant HIV-1 transmission has been reported in

higher ART coverage areas, mostly in developed countries. It is important to note that, along with ART scale-up, the prevalence of transmitted HIV-1 drug-resistance increased from 2.8% before 2001 to 5.3% after 2003 in African countries [4]. As the transmission of drug-resistant HIV may seriously affect the efficacy of first-line ART, surveillance to monitor the prevalence of transmitted HIV drug-resistance has become an important issue in African countries. The prevalence of transmitted HIV-1 drug resistance in Ghana was reported in two studies. One was conducted in 2003 in the Greater-Accra Region of Ghana [5], and the other one was conducted between 2002 and 2004 in Accra and two sites of the Eastern region, Agomanya and Atua [6]. Both studies reported no case of drug-resistant HIV-1 transmission [5], [6]. As at December 2009, the national response had established programs for the provision of ART in hospitals and health centers in several districts in the ten regions of Ghana [7]. However, since ART was expanded in Ghana, the situation of transmitted HIV-1 drug-resistance has not been reported.

To clarify the prevalence, pattern, and spectrum of HIV-1 drug resistance in the era of scaled up ART in Ghana, particularly in ART-experienced patients and transmission to new individuals, we surveyed HIV-1 drug resistance among ART-experienced and -naïve patients enrolled between 2009 and 2010 in Koforidua, the capital of the Eastern region, Ghana. Concomitantly, we analyzed HIV-1 subtypes in detail to further understand the epidemiology of HIV-1 infections in Ghana.

Methods

Patients

HIV-infected patients who visited the Koforidua Regional Hospital (KRH) from February 2009 to January 2010 were enrolled in the study. KRH is the main HIV/AIDS clinic in the capital of the Eastern region of Ghana. This hospital is responsible for HIV prevention and intervention programs in the area and provides free ART with care and support to HIV-infected patients. The Institutional Review Board of the Noguchi Memorial Institute for Medical Research granted ethical approval for this study. All patients or their caregivers gave written consent to participate in the study.

$CD4^+$ T-cell Count and Plasma HIV-1 Viral Load Monitoring

For an indication of immune status, $CD4^+$ T-cells were measured using a FACSCount flow cytometer (Becton Dickinson, San Jose, California, USA). Plasma HIV-1 viral loads (pVLs) were quantified using an in-house real-time reverse-transcription and polymerase chain reaction (RT-PCR) assay as previously reported [8]. ART-experienced patients with pVL>150 copies/mL were considered as virological failures.

HIV-1 Drug-resistance Genotyping

HIV-1 drug-resistance genotyping was performed as previously reported with some modifications [9]. In brief, viral RNA was extracted from 200 µL of plasma samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). RT-PCR was performed with QIAGEN one-step RT-PCR kit (Qiagen), and nested PCR was subsequently performed using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, USA). Specific primers known as DRPRO5, DRPRO2L, DRPRO1M, and DRPRO6 were used for the protease (PR) region (424 bp, positions 2,168 to 2,591 in the reference HXB2 sequence), and DRRT1L, DRRT4L, DRRT7L, and DRRT6L primers for the reverse transcriptase (RT) region (838 bp, positions 2,510 to 3,347) [9]. Details of the primers used in the study are shown in Table 1. Nucleotide sequencing was performed using ABI 3730 auto-sequencer followed by editing with SeqScape software v2.5 (Applied Biosystems). HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. In addition, transmitted HIV-1 drug-resistance mutations were defined using the mutation list proposed by Bennett et al. [11].

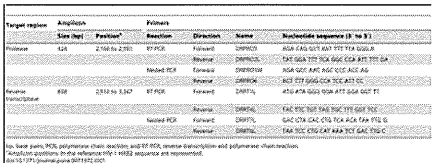


Table 1. List of primers used in HIV-1 genotypic drug-resistance testing.
doi:10.1371/journal.pone.0071972.t001

HIV-1 Subtyping

HIV-1 subtyping was performed using the *pol* gene sequences (1,095 bp, positions 2253 to 3347). Phylogenetic tree was constructed with the references of subtypes A-D, F-H, J, K, and all circulating recombinant forms (CRFs) 01 to 51, except 30, 41, and 50, obtained from the HIV Sequence Database at the Los Alamos National Laboratory. In addition, HIV-1 sub-subtype A3 (DDI579, DDJ360, and DDJ369) and A4 (97CD_KCC2, 97CD_KTB13, and 02CD_KTB035) isolates were added to the phylogenetic tree analysis, as these sub-subtypes have been reported as circulating in several African countries [12], [13]. Multiple sequences were aligned using the MUSCLE program, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v5.05 [14]. Phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap replicates. In similarity plotting and boot-scanning analyses, nine HIV-1 subtypes, A-D, F-H, J, and K, and three CRFs, CRF02_AG, CRF06_cpx, and CRF09_cpx, were used as references. Similarity plotting and boot-scanning were performed using SimPlot software v3.5.1 with window and step sizes of 250 and 20 nucleotides, respectively [15]. One HIV-1 isolate identified with an unknown mosaic pattern both in similarity plotting and boot-scanning analyses was considered as a unique recombinant form (URF).

Statistical Analysis

The Fisher's exact test and the Mann-Whitney U-test were used in SYSTAT software v10.2 (SYSTAT Software, Chicago, USA) for analysis of statistical significance between categorical variables and quantitative valuables, respectively. All tests were two-sided and the level of significance was set at $P<0.05$.

Accession Numbers

Nucleotide sequences have been registered as #AB751399 to AB751499 in the DNA databank of Japan.

Results

CRF02_AG is the Predominant HIV-1 Strain in Koforidua, Ghana

During the study period, 101 HIV-1-infected patients were enrolled in this study. As shown in Table 2, 90 cases were adults (≧15 years old), including 59 newly diagnosed ART-naïve cases and 31 ART-experienced cases. The remaining 11 cases were children (<15 years old), among which were 10 newly diagnosed ART-

naïve cases while one child was ART-experienced (Table 3). To understand the molecular epidemiology of HIV-1 infections in Ghana, we analyzed the *pol* gene sequences in detail through the construction of phylogenetic trees, similarity plotting, and boot-scanning analyses. Among the 101 cases, 75 (74.3%) were identified as HIV-1 subtypes and CRFs (Fig. 1A); 67 were CRF02_AG (66.3%), 4 were sub-subtype A3 (4.0%), 2 were CRF06_cpx (2.0%), and 2 were CRF09_cpx (2.0%). Thus, our analyses clearly showed the predominance of HIV-1 CRF02_AG in Koforidua.

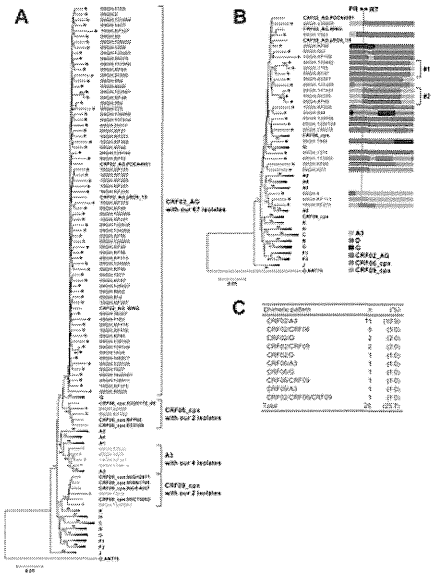


Figure 1. Molecular epidemiology of HIV-1 infections in Koforidua, Ghana.
HIV-1 subtypes of 101 isolates were determined through the construction of phylogenetic trees, similarity plotting, and boot-scanning analyses. (A) Phylogenetic tree containing our 75 isolates classified into known subtypes and CRFs. (B) Phylogenetic tree containing our 26 URF isolates identified with unknown mosaic patterns of the *pol* gene. Two clusters of URF isolates are represented by #1 and #2. (C) Summary on the chimeric patterns of 26 URF isolates. The trees were constructed by the neighbor-joining method. Bootstrap values were calculated from 1,000 analyses, and values greater than 70% are shown at tree nodes. Our isolates are represented by colored circles, and subtype reference isolates are represented by their subtype and name. Scale bar represents nucleotide substitutions per site. HIV-1 group O isolate, ANT70, was used as the outgroup. CRF, circulating recombinant form; PR, protease; RT, reverse transcriptase; and URF, unique recombinant form.
doi:10.1371/journal.pone.0071972.g001

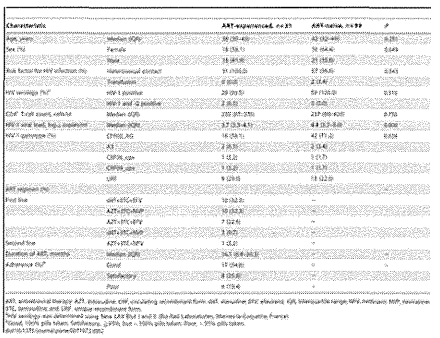


Table 2. Demographic and clinical characteristics of ART-experienced and -naïve HIV-1-infected patients ≧15 years old (*n* = 90).
doi:10.1371/journal.pone.0071972.t002

Characteristic		Value
Age, years	Median (IQR)	5.0 (1.5–8.0)
Sex (%)	Female	6 (54.5)
	Male	5 (45.5)
CD4$^+$ T-cell count, cells/μl	Median (IQR)	747 (474–1152)
HIV-1 viral load, log_{10} copies/ml	Median (IQR)	4.3 (3.4–4.8)
HIV-1 genotype (%)	CRF02_AG	7 (63.6)
	URF	4 (36.4)
ART (%)	Naïve	10 (90.9)
	d4T+3TC+EFV[b]	1 (9.1)

ART, antiretroviral therapy; CRF, circulating recombinant form; d4T, stavudine; EFV, efavirenz; IQR, interquartile range; 3TC, lamivudine; and URF, unique recombinant form.
[a]All were HIV-1 seropositive alone, and their risk factor for infection was mother-to-child transmission.
[b]Only one case had been on treatment for 9.6 months.
doi:10.1371/journal.pone.0071972.t003

Table 3. Demographic and clinical characteristics of HIV-1-infected patients <15 years old (*n* = 11)[a].
doi:10.1371/journal.pone.0071972.t003

Interestingly, the remaining 26 cases (25.7%) were identified as HIV-1 URFs (Fig. 1B). The most prevalent chimeric pattern was CRF02/A3 (*n* = 11, 10.9%), followed by CRF02/CRF06 (*n* = 5, 5.0%), CRF02/G (*n* = 2, 2.0%), CRF02/CRF09 (*n* = 2, 2.0%), and 6 other patterns (Fig. 1C). Of note, two interesting clusters were found in the phylogenetic tree of URF isolates (Fig. 1B). Cluster #1 with three isolates, 09GH.120495, 09GH.1765, and 09GH.KF47, shared the same mosaic *pol* gene comprising a large PR and RT fragment of CRF02 and a short RT fragment of A3 (cluster #1, Fig. 1B). Cluster #2 with the other three isolates, 09GH.107421, 09GH.60390, and 09GH.KF43, shared the same mosaic *pol* gene comprising the PR fragment of A3 and two RT fragments of CRF02 and A3 (cluster #2, Fig. 1B). Our data suggest that the two URF clusters are candidates for a new CRF spreading in this area of Ghana.

HIV-1 Drug-resistance Mutations are Highly Frequent among ART-experienced Cases with Virological Failure Status

Demographic and clinical characteristics of 31 ART-experienced adult cases are shown in Table 2. All patients except one (96.8%, 30/31) were treated with the first-line ART regimen of 2 NRTIs+NNRTI, and the remaining one (3.2%) with the second-line ART regimen of 2 NRTIs+PI. Their median duration of ART was 16.1 months (IQR, 6.8–30.3 months), and most cases maintained their adherence at a "good" or "satisfactory" level (80.6%, 25/31).

Among these ART-experienced adult cases, 22 cases (71.0%) possessed one or more HIV-1 drug-resistance mutations (Table 4). The most prevalent drug-resistance pattern was 2-class resistance to NRTI and NNRTI (*n* = 13, 41.9%), followed by 1-class resistance to NNRTI (*n* = 8, 25.8%). Of note, 3-class resistance was identified in one case (3.2%) treated with the second-line regimen AZT+3TC+NFV. This case possessed HIV-1 RT mutations M41L, V90I, A98G, M184V and T215Y, and the major NFV-resistance mutation L90M in PR. As shown in Table 4, the most prevalent drug-resistance mutation among the 31 cases was M184V (*n* = 12, 38.7%), followed by K103N (*n* = 9, 29.0%), and T215Y/F (*n* = 6, 19.4%). No drug-resistance mutation was detected in the remaining 9 cases (29.0%, Table 4), suggesting that acquisitions of drug resistance was not the primary cause of their virological failure. The cases with and without resistance did not differ significantly in their demographic characteristics.

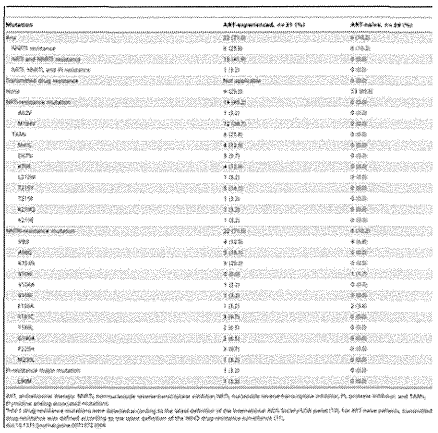
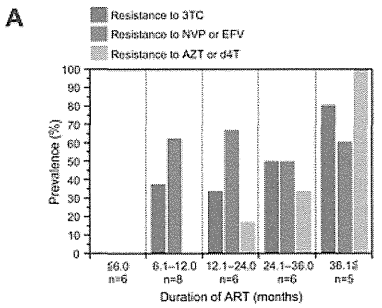


Table 4. Frequency of HIV-1 drug-resistance mutations in ART-experienced and -naïve adult patients (≧15 years old) (*n* = 90)[a].
doi:10.1371/journal.pone.0071972.t004

Furthermore, we analyzed the chronological order of acquiring drug resistance to 3TC, NVP, EFV, AZT, and d4T. As shown in Fig. 2A, no mutation was found in any patients, even with viremia, who had received ART for ≦6.0 months (0%, 0/6). However, M184V mutation was detected in 37.5% (3/8) of patients with 6.1–12.0 months of ART, and the prevalence increased to 80.0% (4/5) at ≧36.1 months of ART (red bars in Fig. 2A and 2B). In the case of NVP and EFV resistance, K103N, V106A, V108I, Y181C/L, G190A, P225H, and M230L mutations were detected in more than half of patients after 6.0 months of ART (blue bars in Fig. 2A and 2B). Importantly, the prevalence and accumulation of thymidine analog-associated mutations (TAMs) appeared to be higher with longer duration of ART; 16.7% (1/6) at 12.1–24.0 months to 100% (5/5) at ≧36.1 months of ART (green bars in Fig. 2A and 2B).



B

Case	ART regimen	Duration of ART (months)	Amino acid mutations conferring resistance to		
			3TC	NVP or EFV	AZT or d4T
KF375	AZT, 3TC, EFV	6.5		V106A, Y181C	
KF49	AZT, 3TC, NVP	6.8	M184V	K103N, P225H, M230L	
KF43	AZT, 3TC, EFV	8.2		K103N	
9	AZT, 3TC, NVP	8.4	M184V	G190A	
19	AZT, 3TC, EFV	8.9	M184V	K103N	
KF351	AZT, 3TC, NVP	16.1		A98G, K103N	
KF327	d4T, 3TC, EFV	16.3	M184V	K103N, P225H	K70R, K219E
KF347	d4T, 3TC, EFV	17.5		K103N, Y188L	
KF372	AZT, 3TC, EFV	23.3	M184V	K103N, P225H	
KF205	AZT, 3TC, NVP	29.4	M184V	K103N	D67N, K70R, T215Y
15	AZT, 3TC, NVP	30.1	M184V	Y181C	M41L, T215Y
KF229	d4T, 3TC, EFV	34.5	M184V	Y188L	
KF131	AZT, 3TC, NVP	39.9	M184V	A98G, K103N	T215Y
KF102	d4T, 3TC, EFV	41.5			M41L
KF128	AZT, 3TC, NVP	44.6	M184V	V108I, Y181C	D67N, K70R, T215F, K219Q
KF367*	AZT, 3TC, NFV	50.6	M184V	V90I, A98G	M41L, T215Y
KF250	AZT, 3TC, EFV	54.4	M184V	A98G, G190A	M41L, D67N, K70R, K219W, T215Y

Figure 2. Prevalence of 3TC-, NVP-, EFV-, AZT-, and d4T-resistance mutations by duration of ART in 31 HIV-1-infected patients ≧15 years old.
(A) Bar graph and (B) details of 17 patients identified with 3TC-, NVP-, EFV-, AZT-, and d4T-resistance mutations. HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. Amino acid mutations responsible for drug resistance are shown in

bold and color coded with bar graph in A. *Major NFV-resistance mutation L90M was found in the protease in the case of KF307. ART, antiretroviral therapy; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; NFV, nelfinavir; NVP, nevirapine; and 3TC, lamivudine.
doi:10.1371/journal.pone.0071972.g002

Low HIV-1 Drug-resistance Transmission in ART-naïve Cases

The general demographics of the 59 adult ART-naïve cases were similar to those of the treated cases, however pVL was significantly higher in the naïve cases (P = 0.006) (Table 2). Among the ART-naïve cases, no transmitted HIV-1 drug-resistance mutation was found (Table 4). However, polymorphisms at NNRTI-resistance mutation loci, V90I, E138A, and V106I, were found in 6 cases (10.2% in Table 4). Our data indicated that drug-resistant HIV-1 transmission events are still low in Koforidua, Ghana.

Eleven children infected with HIV-1 through mother-to-child transmission were also analyzed in our study (Table 3). Their median age was 5.0 years (IQR, 1.5–8.0 years), and 10 of these cases were ART naïve. The remaining case had been treated with d4T+3TC+EFV for 9.6 months but had become viremic. In this case, both 3TC-resistance (M184V) and EFV-resistance (V108I and G190S) mutations were detected (Table 5). Importantly, among the 10 ART-naïve children, a 1.5-year-old case had K103N and G190S NNRTI-resistance mutations (Table 5), suggesting the importance of HIV-1 drug-resistance testing in infants.

ART	n	Amino acid mutations associated with	
		NNRTI resistance	NRTI resistance
Naive	5	–	–
	2	V90I, V106I	–
	2	V90I	–
	1	**K103N, G190S**	–
d4T+3TC+EFV	1	K101E, V106I, V108I, G190S	M184V

ART, antiretroviral therapy; d4T, stavudine; EFV, efavirenz; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; and 3TC, lamivudine.
[a]HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. For ART-naive patients, transmitted drug-resistance (shown in bold and underlined) was detected according to the latest definition of the WHO drug-resistance surveillance [11].
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Table 5. HIV-1 drug-resistance mutations in patients <15 years old (n = 11)[a].
doi:10.1371/journal.pone.0071972.t005

Discussion

Our results present a profile of the circulating subtypes and prevalence of drug resistance for HIV-1 infections in Koforidua, Ghana. The data clearly demonstrate the predominance of HIV-1 CRF02_AG (66.3%, n = 67) in the region (Fig. 1A). Our results, combined with three previous reports on the domination of CRF02_AG in Ghana between 1994 and 2004 [5], [6], [16], indicate that CRF02_AG has stabilized and maintained its predominance in the region for nearly 12 years. However, our study identified 26 isolates (25.7%) as URFs (Fig. 1B), indicating that active viral recombinations are ongoing in Ghana. Interestingly, a similar prevalence (25.1%) of HIV-1 URFs was reported from other cities in Ghana, Accra, Agomanya, and Atua [6]. Taken together, these data thus highlight the importance of HIV-1 URFs in understanding the dynamics of the HIV-1 epidemic in Ghana.

Regarding the situation of HIV-1 drug resistance in Ghana, most of the 31 patients on treatment with virological failure (n = 22, 71.0%) had HIV-1 drug-resistance mutations, suggesting that drug-resistant HIV-1 is the major risk factor for virological failure. Furthermore, nearly half of the cases (45.2%, 14/31) had both NRTI- and NNRTI-resistance mutations (Table 4), a pattern that is consistent with that observed in a recent systematic review on treatment-failure cases in sub-Saharan Africa [17], where M184V/I, K103N, and T215Y/F mutations predominate.

Regarding the timing of drug-resistance acquisition, our data demonstrated that 3TC-, NVP-, and EFV-resistance mutations were selected earlier (6.1–12.0 months) than AZT- and d4T-resistance mutations (12.1–24.0 months). Importantly, the prevalence of TAMs increased from 16.7% (1/6) at 12.1–24.0 months to 100% (5/5) at ≧36.1 months. As the accumulation of TAMs confers cross-resistance not only to the first-line NRTIs (AZT, d4T, and 3TC), but also to the second-line NRTIs (ABC, TDF, and ddI) to some extent [18], their accumulation should be avoided by conducting drug-resistance testing earlier and appropriately switching the regimen, once virological failure is suspected.

As no transmitted HIV-1 drug-resistance mutation was found among the 59 newly diagnosed treatment-naïve adult cases, the transmission of drug-resistant HIV-1 appeared to be a rare event in Koforidua, Ghana. Comparing our data with that from other African countries with a similar background, roll-out time of ART, and coverage rate of ART (26.6% in Ghana) [2], the low prevalence of transmitted HIV-1 drug resistance is not surprising and understandable. However, we cannot exclude the possibility of low levels of transmitted HIV-1 drug resistance in our 59 ART-naïve adult cases. The results of our study are limited by using direct sequencing, which may not have been sensitive enough to detect minority drug-resistant variants hidden among the wild-type strains. Indeed, several studies have reported 2- to 3-fold higher prevalence of drug-resistance transmission with ultra-deep sequencing than with direct sequencing [19], [20], which can detect 1% minority populations [21]. Furthermore, as ultra-deep sequencing can better detect the presence of dual or multiple infections of HIV-1 subtypes compared with direct sequencing [22], [23], using such new technology may identify subtypes of 26 URFs.

Finally, an eventual increase of transmitted drug-resistance cases is anticipated in Ghana as well. Thus, access to HIV-1 genotypic drug-resistance testing should ideally be expanded along with the scale-up of ART programs. In addition, vertical transmission of drug-resistant HIV-1 was found in one of 10 newly-diagnosed treatment-naïve children, suggesting that expanded access to HIV-1 genotypic drug-resistance testing is also needed for programs to prevent mother-to-child transmission in Ghana.

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Author Contributions

Conceived and designed the experiments: NIN SI JSB KI JAMB SBO SY WKA WS. Performed the experiments: NIN SI JSB KI JAMB. Wrote the paper: NIN SI WS.

Organized the study team: KI SY WKA WS. Enrolled patients into the study: SBO. Prepared a clinical database: NIN JSB KI SBO. Revised the manuscript critically JSB KI JAMB SBO SY WKA.

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Seroprevalence of Kaposi's Sarcoma-Associated Herpesvirus Among Men Who Have Sex With Men in Japan

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Kaposi's sarcoma-associated herpesvirus (KSHV), the etiologic agent of Kaposi's sarcoma, causes malignancies frequently in patients with acquired immunodeficiency syndrome. In the United States and Europe, KSHV infection is common among men who have sex with men. However, the seroprevalence of KSHV among men who have sex with men in Japan is unknown. In the present study, the seroprevalence of KSHV was investigated among 230 men who have sex with men and 400 age- and area of residence-matched men (controls) using a mixed-antigen (KSHV-encoded K8.1, open reading frame 59, 65, and 73 proteins) enzyme-linked immunosorbent assay and an immunofluorescence assay. Among the Japanese men who have sex with men, serological assays revealed that 27 (11.7%) were seropositive for KSHV; 20 (5%) of the men in the control group were also KSHV seropositive. The seroprevalence of KSHV among men who have sex with men was significantly higher than in the control group (odds ratio = 2.52, 95% confidence intervals = 1.38–4.62, $P = 0.0019$, Chi-square test). Infection with the human immunodeficiency virus, *Treponema pallidum*, or hepatitis B and C virus did not correlate with KSHV infection. Furthermore, the association of KSHV seropositivity with specific sexual activities was not statistically significant. In conclusion, a higher KSHV seroprevalence was found among Japanese men who have sex with men than among the controls, suggesting that the circulation of KSHV infection is more efficient among men who have sex with men in Japan than among men who do not engage in such sexual activities. ***J. Med. Virol. 85:1046–1052, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: KSHV; seroprevalence; men who have sex with men

INTRODUCTION

Kaposi's sarcoma (KS) is a malignancy observed frequently in patients with acquired immunodeficiency syndrome (AIDS). KS occurs not only in human immunodeficiency virus 1 (HIV-1)-positive men who have sex with men, but also in immunocompromised hosts like transplant patients, elderly people in the Mediterranean region, and young African patients [Antman and Chang, 2000]. Kaposi's sarcoma-associated herpesvirus (KSHV) has been detected in all cases of KS, and the serum of KS patients is positive for anti-KSHV antibodies [Antman and Chang, 2000; Ganem, 2005]. Thus, it is clear that KSHV is associated with the pathogenesis of KS, but its infection route and mechanism remain unknown. Among the general

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