infectivity relative to the value of the wild-type virus without the expression of APOBECs.

Editing of HIV-1 proviral DNA and genomic RNA

The cells were harvested 48 h postinfection, and total DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). A 408 bp of the HIV-1 *pol* region was amplified with the high-fidelity DNA polymerase (Takara, Ohtsu, Japan) subsequent to the digestion with *DpnI*. Amplified fragments were subsequently gel purified, then cloned into pCR-Blunt vector (Invitrogen) and sequenced. The viral genomic RNA in cell-free virions was purified using QIAamp viral RNA Mini Kit (Qiagen) and converted to cDNA *in vitro* using a High Capacity cDNA Archive kit with random primers, sebsequent to the treatment with DNase. The *pol* region was amplified by PCR, cloned and sequenced as described above.

Western blot analysis

The encapsidation of APOBEC proteins into HIV-1 or MLV virions were detected by pelleting the supernatant of 293T cells transfected with viral DNA and HA-epitope tagged forms of the APOBEC expression vector through a 20% sucrose cushion. The pellets were solubilized in 50 µl of 1% Triton-containing buffer and the equivalent p24 of each solubilized virions was subjected to immunoblot analysis. Cell lysates, virion lysates and immunoprecipitates were subjected to SDS–PAGE, and then transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were probed with the anti-HA epitope (HA.11; Covance, Princeton, NJ) or anti-β-actin (AC-74; Sigma, Saint Louis, MO) antibody. A monoclonal antibody recognizing the p24 CA (18) and a goat anti-MLV p30 serum (ViroMed Biosafety Labs, Camden, NJ) was used for detection of HIV-1 and MLV CA, respectively. Reactive proteins were detected using biotin-conjugated rabbit immunoglobulin (Sigma), streptoavidin-conjugated peroxidase (Sigma) and developed using Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan). The plasmids expressing HIV-1 Gag or the deletion mutants were described previously (22). Bands in western blots were quantified on a VersaDoc 5000 imager (BIO-RAD, Hercules, CA).

Nucleotide sequence accession numbers

The sequences determined in this study have been submitted to GenBank and assigned accession no. AB425821.

RESULTS

Molecular cloning and phylogenetic analysis of mammalian A1s

We cloned, sequenced and compared the predicted amino acid sequences of A1 cDNAs from primate (human), carnivora (ferret), lagomorphs (rabbit) and rodents (hamster, rat, mouse). Sequence analysis revealed that the clones obtained encoded genes that were identical to the GenBank sequences of A1s (rabbit;U10695, hamster; AF176577, rat;NM012907 and mouse;NM031159), except for one amino acid residue (M80I) substitution in human A1 (NM001644). Results showed that human A1 cDNA encodes an open reading frame of 236 amino acids that has a 78.0% amino acid sequence identity with ferret, 75.8% with rabbit and 70–72% with rodent orthologs. A short C-terminal extension was found in human and rabbit as previously reported (19), and also in ferrets but not in rodents. In particular, the active site motif, designated as His-X-Glu-$(X)_{23-28}$-Pro-Cys-X_2-Cys (X can be any amino acid) (20), was well conserved (Figure 1A). Phlyogenetic tree analyses revealed that the rabbit A1 gene is related to primate A1 genes, while A1s from rodents form a single separate cluster (Figure 1B).

A1s from small animal species inhibit HIV-1 virion infectivity

To examine the anti-HIV activities of A1s from these small animal species, single-round infectivity assay with VSV-G pseudotyped wild-type and *Δvif* HIV-1 luciferase reporter viruses (5) produced in the presence of the influenza HA epitope-tagged A1 proteins was performed. hA3G was used as a control in these experiments. To examine the effect on HIV-1 infectivity quantitatively, virions were normalized based on p24 content. Virus-induced intracellular luciferase activity, which is directly proportional to the infectivity of the virus, was measured 48 h after infection and calculated relative to the APOBEC-negative control virus (Figure 2A, Mock). As expected, hA3G caused a modest decrease in infectivity of the wild-type and a more pronounced decrease in infectivity of the *Δvif* HIV-1virus (Figure 2A). A1 from human intestine did not showed any antiviral activity, while, in agreement with previous observations (12,13), A1 from rat caused a relatively small (2- to 3-fold) decrease in infectivity of the *Δvif* HIV-1virus and a >5-fold decrease in infectivity of the wild-type virus (Figure 2A). In contrast with previous findings however (5,12,13), the A1s from rodents, such as hamster and mouse were equally active against wild-type and *Δvif* HIV-1 viruses, reducing their infectivity >10-fold in some experiments. A modest reduction in infectivity of wild-type and *Δvif* viruses was also found with A1 from ferrets, but of more interest are the findings with A1 from rabbits. Over 100-fold decrease in the *Δvif* HIV-1 virus and a more pronounced 300-fold decrease in infectivity of the wild-type virus was seen with this cytidine deaminase from rabbits. Identical results were obtained when rabbit A1 was expressed with an alternate expression vector, pcDNA3.1 (Figure S1), ruling out an effect specific to the pCAGGS construct used in these experiments. Dose titration studies showed that as little as 0.05 µg of rabbit A1 was sufficient to achieve significant inhibition against HIV-1 (Figure 2B), 10- to 20-fold more potent than APOBECs from other small animal species. For further confirmation, the anti-HIV activity of A1s from small animal species was examined with the use of a HIV-1-based the green fluorescent protein (GFP)-expressing reporter virus. As shown in Figure S2, the results obtained with the HIV-GFP vector are consistent with those seen using the HIV-Luc vector. These multiple lines of investigations indicate that, similar to hA3G, A1s from small animal species can function on HIV-1.

The cytidine deaminase domains of APOBEC proteins contains an active site with conserved consensus motifs in

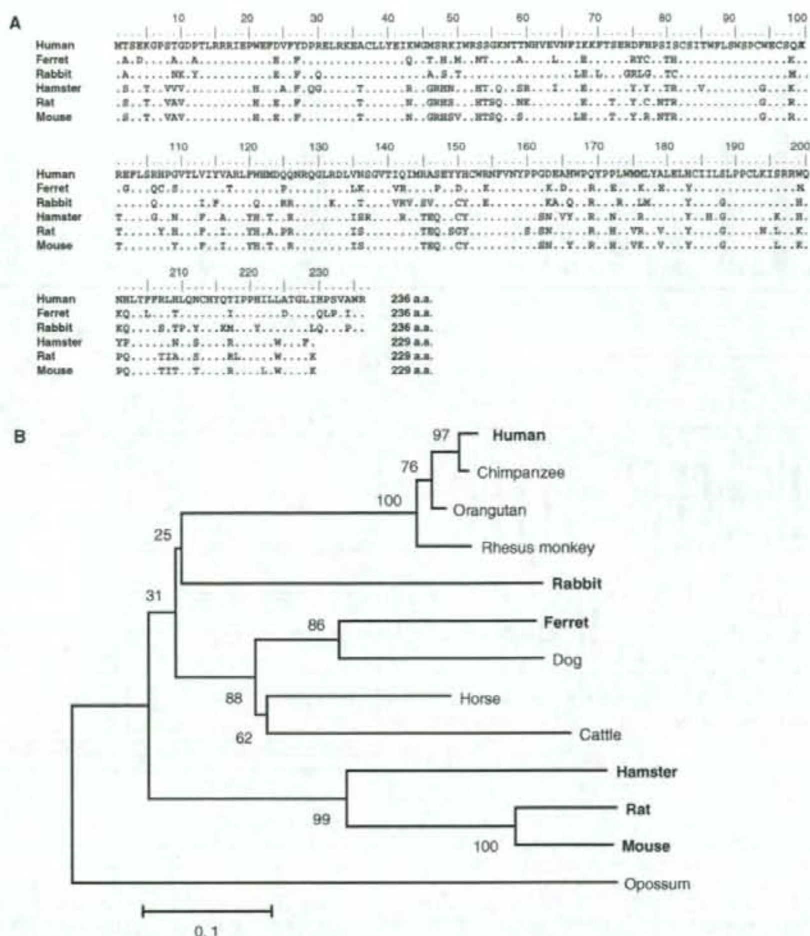

Figure 1. Alignment of the amino acid sequence and phylogenetic analysis of mammalian A1 proteins. (**A**) Amino acid sequence alignment of A1 from human, ferret, rabbit, hamstar, rat and mouse. The predicted amino acid sequences of these cloned A1 molecules are aligned with Clustal W software to the previously identified sequences of mammalian A1s. The numbers are amino acid residue positions. (**B**) Phylogenetic analysis of the protein sequences of full-length mammalian A1s. The tree was reconstructed by neighbor-joining method with protein *p*-distances using MEGA 3.1. Shown interior nodes are bootstrap percentages derived from 2000 replications. Branch lengths represent the number of substitution per site. Opossum is a separate group. The species used for further experiments in this study are indicated in bold letters.

which the His–Cys–Cys residues coordinate a Zn^{2+} ion, and the Glu residue serving an essential role in catalysis as a proton shuttle (21). Therefore, we generated the catalytic site mutant forms of rabbit A1 in which the critical Glu-63 of active site was changed to Ala or Gln (E63A, E63Q), and examined their antiviral activity. Results showed that the ability of the mutant proteins to restrict the infectivity of wild-type and *Δvif* viruses were severely, but not completely impaired (Figure 2C), suggesting the existence of an albeit weak deaminase-independent restriction mechanism by A1.

A1s are packaged into HIV-1 virions

It is now well established that human and murine A3s inhibit HIV-1 infectivity by being packaged into progeny virions. Therefore, we verified whether A1 proteins (which are epitope tagged) would indeed be selectively packaged into HIV-1 virions. For these studies, transfected cells were harvested, and proteins in whole-cell lysates were analyzed by western blotting with anti-HA, anti-HIV-1 CA p24 and anti-β-actin monoclonal antibodies with the β-actin blot serving as a loading control. Data showed comparable amounts of A1 proteins and HIV-1 Gag precursor protein (p55) expression, but minor effects on the processing efficiency into p24 CA with some A1 proteins (e.g. human and rabbit, Figure 2D). Cell-free virus was concentrated by pelleting through a 20% sucrose cushion, and then equivalent amount of solubilized virions was subjected into the western blotting. A1 proteins from small animal species were

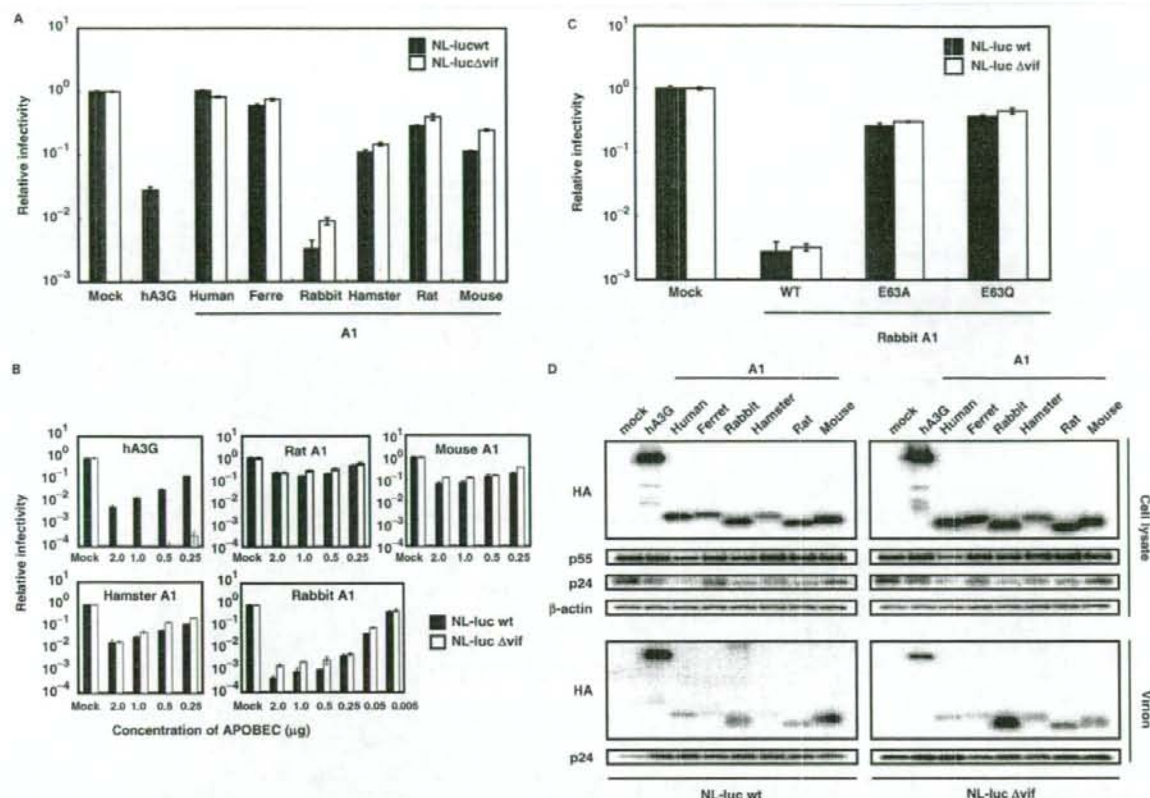

Figure 2. Inhibition of HIV-1 infection by A1s. (**A**) VSV-G pseudotyped wild-type and *Δvif* HIV-1 luciferase viruses were produced in 293T cells transfected with 1.5 μg of luciferase reporter viruses, 1.0 μg of pVSV-G and 0.5 μg of HA-tagged APOBEC expression vector or empty vector. Virus-containing supernatants were normalized for equal p24 content and used for the infection of the fresh 293T cells. Virus-induced intracellular luciferase activity was measured. Data are presented as a percentage of the level of luciferase activity detected in cells infected with virions derived from cells that did not express an exogenous APOBEC protein. The average of three experiments with standard deviation is indicated. (**B**) Wild-type and *Δvif* HIV-1 luciferase reporter viruses pseudotyped were produced in 293T cells transfected with decreasing amounts of HA-tagged APOBEC expression vector. The amount of expression vector plasmid transfected is shown in micro gram on the *X*-axis. (**C**) The catalysis domain of A1 is required for the efficient anti-HIV-1 potency of the rabbit A1. The HA-tagged rabbit A1 (WT), A1 mutated in the active site motif (E63A and E63Q) were used. (**D**) A1 proteins from small animal species are encapsidated into HIV-1 virions. The producer cells were collected and lyzed, while the released virus in the supernatants were collected by ultracentrifugation. The cells and virion lysates were then subjected to Western analysis using an antibody specific for the HA tag and HIV-1 Gag CA. The immunoblot probed with anti-β-actin antibody of the proteins present in the cell lysates is shown.

found to be packaged into HIV-1 virions, but variations in the efficiency of the incorporation of each A1 protein were seen. Rabbit A1 was incorporated efficiently, which could explain for its potent antiviral activity. The incorporations into HIV-1 virions of A1s from small animal species were not affected by expression of the Vif protein (Figure 2D). We therefore conclude that A1s like hA3G are specifically packaged into HIV-1 virions. However, unlike hA3G, this incorporation is not inhibited with coexpression of HIV-1 Vif. The mutant rabbit A1 E63A E63Q proteins were correctly expressed within the producer cells and also incorporated into the HIV-1 virions efficiently, as judged by western blot analysis (data not shown).

The HIV-1 Gag NC domain is required for A1 packaging

We next determined whether parts of Gag were dispensable for A1 packaging into virions, by use of previously described Gag mutants (22). Two Gag deletion mutants, δ10-110 mutant lacking residues 10 through 110, δ10-277 mutant lacking residues 10 through 277 or the Zwt-p6 mutant, in which NC is replaced by a leucine zipper from GCN4 were tested (Figure 3A). A1s from human, rabbit and rat as well as hA3G were efficiently incorporated into virus like particles (VLPs) formed by both Gag deletion δ10-110 and δ10-277 mutants (Figure 3B). Consistent with previous observations (22), hA3G was not incorporated into VLP formed by Zwt-p6 mutant. A1 from human and rabbit did not exhibit any detectable incorporation, while, A1 from rat caused a relatively small amount of incorporation into VLP formed by Zwt-p6 mutant (Figure 3B). Thus, almost all matrix and the amino-terminal two-thirds of capsid appear to be largely dispensable for A1 packaging, similar to those observed in hA3G packaging.

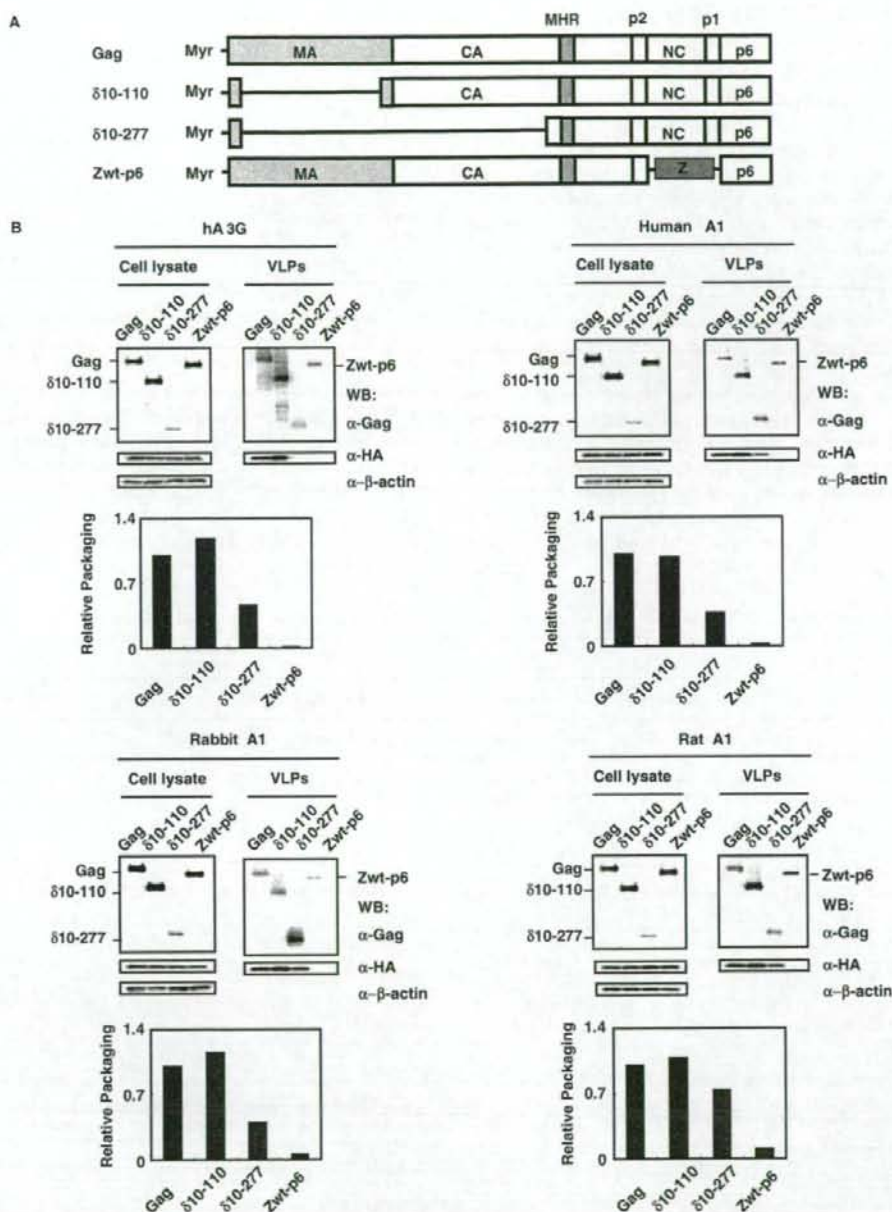

Figure 3. A1 proteins are encapsidated into HIV-1 virions via through the interaction with NC, but not the majority of Gag. (A) Schematic representation of the intact HIV-1 Gag precursor and its derivatives used in this study. The Gag are myristoylated (Myr-) on the N-tereminus of MA. The positions of the major homology region (MHR) and the various Gag cleavage sites are shown. (B) Western blot analysis of cell lysates and extracellular VLPs generated following transfection of 293T cells with plasmids expressing HIV-1 Gag or its derivatives shown in (A) and the HA-epitope tagged forms of the indicated APOBEC proteins. The producer cells were collected and lyzed, while the supernatants were harvested and released virus were collected by ultracentrifugation. The cells and virion lysates were then subjected to Western analysis using antibodies specific for the HIV-1 Gag CA and HA tag. The immunoblot probed with anti-β-actin antibody of the proteins present in the cell lysates is shown. Results were normalized to those obtained from the transfection of cells with the full-length Gag and APOBEC constructs, and were graphically shown as relative packaging.

Editing of HIV-1 proviral DNA and genomic RNA by A1 proteins

A3-mediated antiretroviral activity has been shown to be associated with cytidine deamination of nascently transcribed viral cDNAs following infection of target cells. Such dC-to-dU deamination in minus-strand DNA is detected as replacement of dG-to-dA in integrated proviral genomes. To address whether A1 functions in a similar manner, accumulation of dG-to-dA changes in the proviral DNA during reversetranscription was examined. Viral DNA was prepared from cells infected with wild-type or *Δvif* HIV-1 viruses produced in the presence of A1s from small animal species, and the 3′-end of the *pol* gene was analyzed for evidence of hypermutation. Consistent with previous reports, significant dG-to-dA hypermutation was observed for hA3G serving as a control for these studies (Table 1) and no dG-to-dA mutation was observed when the virus was prepared in the absence of an APOBEC protein (data not shown). In contrast, the predominant mutations induced by rabbit A1 that exhibited the most prominent antiviral activity were dC-to-dT changes (22 and 14 events in 23 664 and 24 480 bases in wild-type and *Δvif* viruses, respectively), which could have arisen through deamination of unpaired plus-stranded cDNA or virion RNA. To address the latter possibility, cell-free virions were purified and the genomic RNA was converted to cDNA for *pol* gene analyses. As can be seen in Table 2, there was a clear accumulation of dC-to-dT (U in the template RNA) changes in the RNAs of HIV-1 produced in the presence of rabbit A1 (39 and 51 events in 9792 and 8976 bases in wild-type and *Δvif* viruses, respectively), a frequency that is 4- to 5-fold higher than those observed in DNA sequencing. Thus, HIV-1 genome RNA as well as reversetranscribed proviral DNA could be a substrate for A1-mediated deamination.

The preferential sites of hypermutations induced by rabbit A1 were also examined (Figure 4). Consistent with previous observations (23–26), hA3G prefers to edit dC (marked by an asterisk) in the viral target DNA sequence C/TCC*. In constrast, rabbit A1, similar to mouse A3 (27) and human A3F (13,28,29), show preference for TTC* (Figure 4A). These results suggest that rabbit A1 inhibits HIV-1 infection by a deamination-dependent mechanism through targeting of proviral DNA sequences that are different from hA3G. Sequence context of the cytidine in viral RNA sequences mutated by rabbit A1 showed a clear

Table 1. Sequence analysis of reverse transcribed second-strand proviral DNA in the presence of mammalian A1s

	hA3G		A1													
			Human		Rabbit		Rabbit E63A		Rabbit E63Q		Hamster		Mouse		Rat	
	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*
Clones sequenced	34	36	24	24	58	60	24	23	24	23	34	36	24	24	24	24
Total base pair sequenced	13 872	14 688	9792	9792	23 664	24 480	9792	9384	9792	9384	13 872	14 688	9792	9792	9792	9792
Clones with G to A	15	22	2	1	13	24	0	0	0	2	1	1	0	6	0	0
Number of G to A mutations	71	318	5	7	69	118	0	0	0	2	1	1	0	24	0	0
Number of G to A mutations per 1 kb	5.12	21.65	0.51	0.71	2.92	4.82	0	0	0	0.21	0.07	0.07	0	2.45	0	0
Clones with C to T	0	0	0	0	20	10	1	0	1	0	3	3	0	5	6	6
Number of C to T mutations	0	0	0	0	22	14	2	0	1	0	3	4	0	5	7	7
Number of C to T mutations per 1 kb	0	0	0	0	0.93	0.57	0.20	0	0.10	0	0.22	0.27	0	0.51	0.71	0.71
Number of Other mutations	0	1	0	0	1	3	0	0	0	0	0	0	0	0	0	0

A 408 bp fragment of HIV-1 *pol* region was amplified from reverse transcripts infected with wt or *Δvif* NL-Luc viruses produced in the presence of hA3G or mammalian A1s. The number in the clones of each group are shown (wt, wild-type). All mutations are designated using the conventional plus-strand nomenclature.

Table 2. Sequence analysis of HIV-1 genomic RNA in the presence of mammalian A1s

	Mock		hA3G		A1							
					Rabbit		Rabbit E63A		Rabbit E63Q		Rat	
	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*	wt	*Δvif*
Clones sequenced	23	23	21	24	24	22	21	23	24	24	22	24
Total base pair sequenced	9384	9384	8568	9792	9792	8976	8568	9384	9792	9792	8976	9792
Clones with C to T	0	0	0	0	15	17	0	0	0	0	6	13
Number of C to T mutations	0	0	0	0	39	51	0	0	0	0	13	17
Number of C to T mutations per 1 kb	0	0	0	0	3.98	5.68	0	0	0	0	1.45	1.74
Number of Other mutations	0	2	1	0	1	0	0	1	1	0	0	0

This experiment was performed as described in the legend for Table 1, except that the genomic RNA of HIV-1 was amplified (wt, wild-type).

A hA3G

	−2	−1	C	1	2
A	18.3%	1.4%	0.0%	28.2%	33.8%
C	**40.8%**	**87.3%**	**100.0%**	33.8%	18.3%
G	0.0%	0.0%	0.0%	2.8%	11.3%
T	**40.8%**	11.3%	0.0%	**35.2%**	**36.6%**
	C/T	**C**	**C**	**T**	**T**

	−2	−1	C	1	2
A	20.4%	0.9%	0.0%	23.9%	**43.1%**
C	23.6%	**80.5%**	**100.0%**	29.2%	20.4%
G	0.3%	0.0%	0.0%	0.6%	6.0%
T	**55.7%**	17.9%	0.0%	**46.2%**	30.5%
	T	**C**	**C**	**T**	**A**

Rabbit A1

	−2	−1	C	1	2
A	34.8%	0.0%	0.0%	24.6%	27.5%
C	13.0%	2.9%	**100.0%**	20.3%	2.9%
G	11.6%	0.0%	0.0%	2.9%	5.8%
T	**40.6%**	**97.1%**	0.0%	**52.2%**	**63.8%**
	T	**T**	**C**	**T**	**T**

NL-luc wt

	−2	−1	C	1	2
A	28.0%	0.0%	0.0%	22.0%	38.1%
C	19.5%	14.4%	**100.0%**	37.3%	12.7%
G	0.0%	0.0%	0.0%	1.7%	6.8%
T	**52.5%**	**85.6%**	0.0%	**39.0%**	**42.4%**
	T	**T**	**C**	**T**	**T**

NL-luc Δvif

B Rabbit A1

	−2	−1	C	1	2
A	30.8%	41.0%	0.0%	**51.3%**	12.8%
C	**35.9%**	0.0%	**100.0%**	10.3%	20.5%
G	15.4%	0.0%	0.0%	5.1%	28.2%
T	17.9%	**59.0%**	0.0%	33.3%	**38.5%**
	C	**A/T**	**C**	**A/T**	**T**

NL-lucwt RNA

	−2	−1	C	1	2
A	31.4%	35.3%	0.0%	**54.9%**	19.6%
C	**47.1%**	0.0%	**100.0%**	0.0%	11.8%
G	9.8%	0.0%	0.0%	0.0%	**35.3%**
T	11.8%	**64.7%**	0.0%	45.1%	33.3%
	C	**A/T**	**C**	**A/T**	**G**

NL-luc ΔvifRNA

Figure 4. Comparison of the preferred sequence context for cytidine deamination by rabbit A1 in first strand cDNA and the genomic RNA of wild-type or*Δvif* viruses. (**A**) TTC*T in the HIV-1 minus-strand cDNA was the preferred tetranucleotide target of rabbit A1. Shown are percentages of each nucleotide found at the −2, −1, +1 and +2 positions relative to the dC residue targeted for deamination (position zero). The consensus DNA sequence is shown at the bottom of each minitable by bold. (**B**) WC*W sequence in the HIV-1 genome was the preferred RNA target of rabbit A1. Comparison of the preferred sequence observed in the viral genomic RNA produced in the presence of rabbit A1. All of the mutations in HIV-1 genomic RNA aligned with respect to the cytidine (**C**) targeted for deamination (position zero). The frequency with which each of the four bases found at positions adjacent to the deaminated C is indicated. The consensus RNA sequence is shown at the bottom of each minitable by bold.

WCW (W is A or T) trinucleotide preference on both the wild-type and *Δvif* HIV-1 genomic RNA (Figure 4B). The cytosine residue within WCW sequences was reported to be highly conserved as the apoB mRNA-editing site sequence by A1 protein among divergent of mammalian species (30). Rabbit A1 deaminated the cytosines in the genomic RNA but with different frequencies; some cytosines were changed at high frequencies, whereas others were not changed in any of the clones (Figure S4). Taken together, analysis of these mutations confirms that the inhibitory effects observed in rabbit A1s were based on, at least in part, cytosine-deaminating activity on viral genomic RNA, and the analysis of mutational hot spots indicated that the molecular mechanisms for editing of HIV-1 genome RNA and apoB mRNA overlap.

A1s from small animal species exhibit broad antilentiviral activity

To assess the breath of A1-mediated antiviral activity, the activities of these proteins on SIV infectivity were examined. A single-round assay was used to measure the infectivity of wild-type and *Δvif* SIVmac, and SIVagm luciferase reporter viruses (5), in the presence of A1s. VSV-G pseudotyped viral supernatants were collected, normalized for SIV CA p27 and used to infect 293T cells. As expected, hA3G caused a profound decrease in infectivity of both wild-type and *Δvif* SIV viruses (Figure 5A and C). A1 from rodents were found to be moderately active, but rabbit A1 significantly (>100-fold) reduced the infectivity of wild-type and *Δvif* SIV viruses. In contrast to A1 from small animal species, A1 from human intestine showed no antiviral activity. Expression of catalytic site mutant forms of rabbit A1 (E63A, E63Q) has little effect on the infectivity of SIV (Figure 5B and D), suggesting that the deaminase activity is also important for SIV repressive activities.

To measure the activities of A1 proteins on FIV infectivity, plasmids expressing APOBECs were cotransfected with an FIV (pFIV-H1/U6-copGFP) genome along with the packaging pFIV-34N plasmid and pVSV-G.

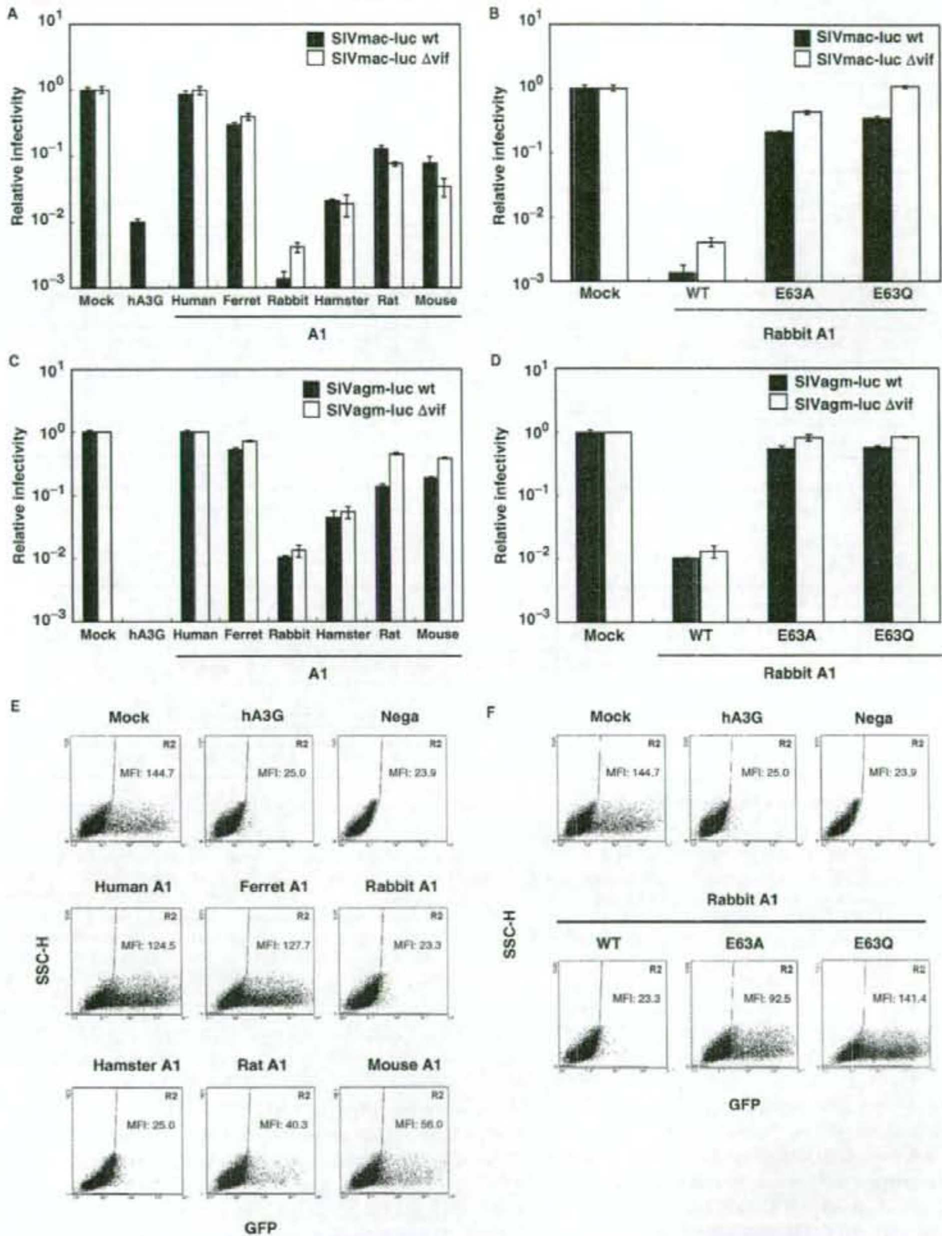

Figure 5. Inhibition of SIV and FIV infection by A1s. Wild-type and *Δvif* SIVmac (**A** and **B**), wild-type and *Δvif* SIVagm (**C** and **D**) luciferase reporter viruses pseudotyped were prepared. 293T cells were transfected with 1.5 µg of luciferase reporter viruses, 1.0 µg of pVSV-G and 0.5 µg of HA-tagged APOBECs (A and C) or rabbit A1 with catalytic site mutations (B and D). Virus-containing supernatants were normalized for equal p27 content and used for the infection. At 48 h postinoculation, virus-induced luciferase activity was measured and presented as described. VSV-G pseudotyped FIV GFP reporter viruses were produced in 293T cells transfected with 1.5 µg of pFIV-H1/copGFP and pFIV-34N, 1.0µg of pVSV-G and 0.5 µg of HA-tagged APOBECs (**E**) or rabbit A1 with catalytic active site mutations (**F**). At 48 h postinoculation with FIV-GFP virions normalized for equal RT activity, cells exhibiting GFP fluorescence of target 293T cells were analyzed on flow cytometry. The level of GFP MFI detected within the GFP-positive windows are indicated. Comparable results were obtained in three additional experiments.

Viral supernatants of pseudotyped virions were collected, normalized for RT activity and used to infect 293T cells. The GFP expression was analyzed by flow cytometry at 48 h postinfection. Large proportion of of 293T cells were positive for GFP expression, with a MFI of 144.7, while in the cells transduced with hA3G containing FIV virions, only a few green cells were present, with 5.7-fold lower MFI of 25.0 (Figure 5E). Interestingly, similar to primate lentiviruses, the infectivity of FIV was significantly reduced in the presence of rabbit A1, with a 6.2-fold lower MFI of 23.3. We also detected reduced GFP signals in the cells transduced with rodent A1-containing virions, while human and ferret A1s caused minimal reduction in the number of GFP positive cells (Figure 5E). As seen in Figure 5F, rabbit A1 with catalytic site mutation E63A caused a slight decrease in FIV infectivity, but the E63Q mutation had no effect. Thus, the results using HIV-1, SIVmac, SIVagm and FIV reporter viruses combined suggest that the A1s from small animal species have a relatively broad lentivirus restriction potential that is mainly mediated through deaminase-dependent mechanism.

A1s affect MLV virion infectivity

Evidence is mounting that some APOBEC proteins can target a variety of retroviral substrates, such as various oncovirus and spumavirus. The A3 orthologs from artiodactyls; cattle, pigs and sheep, as well as hAB and hA3G have been reported to exert antiviral activity on MLV (6,13,23,24). These findings suggest that A3s in artiodactyls could function as barriers of cross-subspecies transmission of MLV from mice. Interestingly, this simple oncovirus is resistant to the mouse A3, explaining the absence of a Vif-like activity in MLV (27,31), but the underlying mechanism is currently unknown (32). Therefore, we examined whether the A1s expressed in small animal species affect MLV infection. MLV-based reporter viruses were produced by transient transfection of pFB-Luc, encoding the luciferase gene, into the MLV packaging cell line GP293 with pVSV-G, in the presence of APOBEC proteins. As shown in Figure 6A, hA3G was able to restrict the infectivity of MLV, consistent with previous reports (13,23,24). Interestingly, MLV infectivity was inhibited ~7-fold by rabbit A1, while A1s from other mammalian species had none or only moderate effect. Similar results were obtained with another murine retroviral vector, pFB-hrGFP (data not shown). Deaminase-defective rabbit A1s retained only partial antiviral activity (Figure 6B) despite comparable levels of MLV virion incorporation (Figure 6C). These data suggest that A1 from small animal species functions as potential barriers of cross-species transmission of this gammmaretroviruses from mice.

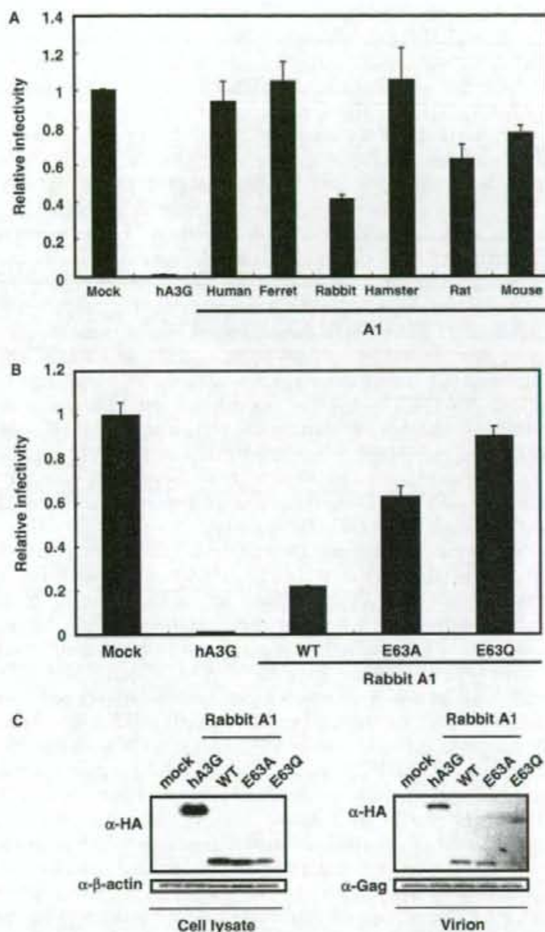

Figure 6. Inhibition of MLV infection by A1s. (**A** and **B**) MLV packaging cell line GP293 were transfected with 1.5 μg of luciferase pFB-Luc reporter plasmids, 1.0 μg of pVSV-G and 0.5 μg of HA-tagged APOBECs or rabbit A1 with catalytic site mutations. Virus-containing supernatants were normalized for equal MLV p30 CA content and used for the infection of the MDTF cells. Virus-induced intracellular luciferase activity was measured and presented as described. (**C**) Rabbit A1 proteins are encapsidated into MLV virions. After transfection, released virion was collected by ultracentrifugation, while the producer cells were collected and lyzed. The cells and virion lysates were then subjected to Western analysis using antibodies specific for the HA tag and MLV Gag CA. An immunoblot probed with anti-β-actin antibody of the proteins present in the cell lysates is also shown. While only the immunoblot of p30 CA performed with the disrupted virions is presented, closely similar results were also obtained using the cell lysates (data not shown).

DISCUSSION

In this study, we showed that single domain cytidine deaminase A1 from rodents (mouse, rat and hamster) and largomorphs (rabbit) are capable of inhibiting the infectivity of various lentiviruses in tissue culture models. A rank order in anti-HIV potency was seen, with rabbit A1 showing the greatest activity. The finding of more efficient virion incorporation of rabbit as compared to other small animal species A1 proteins may be a contributing factor. Catalytic site mutant analysis suggested a deaminase-dependent restriction mechanism, with genomic RNA as well as reversetranscribed proviral DNA serving as substrates for A1-mediated deamination.

A clear accumulation of C-T changes in the genomic RNAs of HIV-1 produced in the presence of rabbit A1 was observed, with G-A changes in the proviral DNA. Furthermore, expression of catalytic site mutant forms of rabbit A1 has little effect on the viral infectivity, supporting the importance of the deaminase activity for these repressive activities. Cytidine deaminase-defective A3 mutants have been shown to exhibit significant antiviral activity (33), implying that antiviral and deaminase activities can be uncoupled. Further evidence in support of editing-independent antiviral mechanism comes from studies on the enzymatically inactive, high-molecular mass complex of hA3G (34) as well as on the antiviral activity against hepatitis B virus (HBV) (35). Nevertheless, more recent studies using deaminase-defective A3 mutants show that efficient inhibition of HIV-1 or retroelements requires catalytically active A3 (36,37). In this regard, although deaminase-defective rabbit A1 mutants were shown to inhibit various lentivirus and MLV, these antiviral activities were significantly lower than those seen with wild-type A1. The suppressive activity of A1, therefore, is principally associated with its cytidine deaminase activity.

We demonstrated that the molecular mechanism for A1 editing of the HIV-1 genomic RNA and apoB mRNA overlapped. The C to U editing of apoB mRNA is shown to be a nuclear event (38), mediated by a complex composed of A1 homodimer and an A1 complementation factor (ACF) (39–41). Expression of ACF mRNA in cells such as Caco-2, a human colon cancer-derived cell line demonstrated to edit endogenous apoB mRNA has been documented (42). However, mRNA for ACF could not be detected by RT-PCR in 293T cells used for retorovirus production in this study (Supplementary Figure S5). Thus, the site(s) in virus-producing cells (e.g. nucleus, cytoplasm or both) where the deamination of HIV-1 genomic RNA takes place remains to be identified, and further experiments are needed to fully understand the role of ACF in the editing of retroviral genome observed in the present study.

In our study, A1s were expressed using both a chick β-actin (CAG) and a cytomegalovirus promoter-driven expression vector, and the concentration of A1s required to mediate antiviral activity was titrated carefully. We found that only 0.05 μg of rabbit A1 was required to achieve significant inhibition against HIV-1 (Figure 2B). Moreover, the antiviral activity of rabbit A1 appeared to be more potent than hA3G (Supplementary Figure S1), and this is unlikely to be explained by differences in virion incorporation of the two enzymes (Figure 2D). Taken together, the data suggest that antiretroviral activities of A1s observed in this study were not solely due to using overexpressed protein systems.

We found that human A1 exhibited no antiretroviral activity, consistent with reports of others (12,13,29,43). A1 from hominoids, therefore, appears to exclusively mediate the C to U editing of apoB mRNA, giving rise to two proteins with different sizes in the gastrointesitinal tissues that function in lipid transport and metabolism. As there have been no reports thus far of lentivirus infection in rodents, the antiviral activity of A1 proteins in rodents seen against HIV, SIV and FIV are also unlikely to have evolved originally to restrict infection of these lentiviruses. Nevertheless, the finding that rabbit A1 can restrict MLV raises the possibility that A1s from small animals species evolved to restrict cross-subspecies transmission of oncoviruses from mice. Furthermore, endogenous lentivirus of rabbits has recently been described (44), lending biological significance to the antilentiviral activity in lagomorphs reported here. Further studies of the antiviral activities of A1 proteins from other members of the placentalia super-order of Laurasiatheria; cetartiodactyla (cow, pig, sheep) and carnivore (cat) will be required to fully understand the complex evolutionary history of *APOBEC* genes as an intrinsic resistance mechanism against retroelements.

HIV-1 exhibits a highly restricted host cell tropism. The identification of chemokine receptors as entry cofactors with human CD4 raises the possibility that small animal species, in particular, rodents could be engineered to express these molecules, thereby rendering them able to support a productive HIV-1 infection. However, HIV-1 replication in rodents (45–47) and rabbits (48,49) expressing human versions of the HIV-1 receptors appeared to be limited and variable. Our findings with A1 suggest that this enzyme may be partly responsible for the inefficient replication of HIV-1 observed in rabbit as well as rodent cells. Blocking of antiviral A1 function by RNA interference in cells from small animal species should verify whether A1 acts as an intrinsic resistance factor. Further understanding of these species-nonspecific repressive activities to HIV-1 replication at the late phase, in conjugation with the early block owing to the different classes of activities, such as TRIM5α (50,51), may suggest approaches to the development of small animal models of HIV-1 infection.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Bieniasz,P.D. (2004) Intrinsic immunity: a front-line defense against viral attack. *Nat. Immunol.*, **5**, 1109–1115.
2. Holmes,R.K., Malim,M.H. and Bishop,K.N. (2007) APOBEC-mediated viral restriction: not simply editing? *Trends Biochem. Sci.*, **32**, 118–128.
3. Chiu,Y.L. and Green,W.C. (2008) The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annu. Rev. Immunol.*, **26**, 317–353.
4. Sheehy,A.M., Gaddis,N.C., Choi,J.D. and Malim,M.H. (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*, **418**, 646–650.
5. Mariani,R., Chen,D., Schrofelbauer,B., Navarro,F., Konig,R., Bollman,B., Munk,C., Nymark-McMahon,H. and Landau,N.R. (2003) Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell*, **114**, 21–31.
6. Jonsson,S.R., Hache,G., Stenglein,M.D., Fahrenkrug,S.C., Andresdottir,V. and Harris,R.S. (2006) Evolutionarily conserved and non-conserved retrovirus restriction activities of artiodactyl APOBEC3F proteins. *Nucleic Acids Res.*, **34**, 5683–5694.
7. Munk,C., Zielonka,J., Constabel,H., Kloke,B.P., Rengstl,B., Battenberg,M., Bonci,F., Pistello,M., Lochelt,M. and Cichutek,K. (2007) Multiple restrictions of human immunodeficiency virus in feline cells. *J. Virol.*, **81**, 7048–7060.
8. Munk,C., Beck,T., Zielonka,J., Hotz-Wagenblatt,A., Chareza,S., Battenberg,M., Thielebein,J., Cichutek,K., Bravo,I.G., O'Brien,S.J. *et al.* (2008) Functions, structure, and read-through alternative splicing of feline APOBEC3 genes. *Genome Biol.*, **9**, R48.
9. Chester,A., Scott,J., Anant,S. and Navaratnam,N. (2000) RNA editing: cytidine to uridine conversion in apolipoprotein B mRNA. *Biochem. Biophys. Acta.*, **1494**, 1–13.
10. Keegan,L.P., Gallo,A. and O'Connell,M.A. (2001) The many roles of an RNA editor. *Nat. Rev. Genet.*, **2**, 869–878.
11. Harris,R.S., Petersen-Mahrt,S.K. and Neuberger,M.S. (2002) RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol. Cell*, **10**, 1247–1253.
12. Bishop,K.N., Holmes,R.K., Sheehy,A.M. and Malim,M.H. (2004) APOBEC-mediated editing of viral RNA. *Science*, **305**, 645.
13. Bishop,K.N., Holmes,R.K., Sheehy,A.M., Davidson,N.O., Cho,S.-J. and M. H. Malim. (2004) Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Curr. Biol.*, **14**, 1392–1396.
14. Niwa,H., Yamamura,K. and Miyazaki,J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**, 193–199.
15. Ohsugi,T. and Koito,A. (2007) Human T cell leukemia virus type 1 is resistant to the antiviral effects of APOBEC3. *J. Virol. Methods*, **139**, 93–96.
16. Koito,A., Kameyama,Y., Cheng-Mayer,C. and Matsushita,S. (2003) Susceptibility of mink (*Mustera vision*)-derived cells to replication by human immunodeficiency virus type 1. *J. Virol.*, **77**, 5109–5117.
17. Burns,J.C., Friedmann,T., Driever,W., Burrascano,M. and Yee,J.-K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl Acad. Sci. USA*, **90**, 8033–8037.
18. Koito,A., Hattori,T., Matsushita,S., Maeda,Y., Nozaki,C., Sagawa,K. and Takatsuki,K. (1988) . Conserved immunogenic region of a major core protein (p24) of human and simian immunodeficiency viruses. *AIDS Res. Hum. Retroviruses*, **4**, 409–417.
19. Yamanaka,S., Poksay,K.S., Balestra,M.E., Zeng,G.-Q. and Innerarity,T.L. (1994) Cloning and mutagenesis of the rabbit apoB mRNA editing protein. A zinc motif is essential for catalytic activity, and noncatalytic auxiliary factor(s) of the editing complex are widely distributed. *J. Biol. Chem.*, **269**, 21725–21734.
20. Harris,R.S. and Liddament,M.T. (2004) Retroviral restriction by APOBEC proteins. *Nat. Rev. Immunol.*, **4**, 868–877.
21. Betts,L., Xiang,S., Short,S.A., Wolfenden,R. and Carter,C.W.J. (1994) Cytidine deaminase. The 2.3 A crystal structure of an enzyme: transition-state analog complex. *J. Mol. Biol.*, **235**, 635–656.
22. Zennou,V., Perez-Caballero,D., Gottlinger,H. and Bieniasz,P.D. (2004) APOBEC3G incorporation into human immunodeficiency virus type 1 particles. *J. Virol.*, **78**, 12058–12061.
23. Harris,R.S., Bishop,K.N., Sheehy,A.M., Craig,H.M., Petersen-Mahrt,S.K., Watt,I.N., Neuberger,M.S. and Malim,M.H. (2003) DNA deamination mediates innate immunity to retroviral infection. *Cell*, **113**, 803–809.
24. Mangeat,B., Turelli,P., Caron,G., Friedli,M., Perrin,L. and Trono,D. (2003) Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcript. *Nature*, **424**, 99–103.
25. Zhang,H., Yang,B., Pomerantz,R.J., Zhang,C., Arunachalam,S.C. and Gao,L. (2003) The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature*, **424**, 94–98.
26. Yu,Q., Konig,R., Pillai,S., Chiles,K., Kearney,M., Palmer,S., Richman,D., Coffin,J.M. and Landau,N.R. (2004) Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat. Struct. Mol. Biol.*, **11**, 435–442.
27. Kobayashi,M., Takaori-Kondo,A., Shindo,K., Abudu,A., Fukunaga,K. and Uchiyama,T. (2004) APOBEC3G targets specific virus species. *J. Virol.*, **78**, 8238–8244.
28. Liddament,M.T., Brown,W.L., Schumacher,A.J. and Harris,R.S. (2004) APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. *Curr. Biol.*, **14**, 1385–1391.
29. Wiegand,H.L., Doehle,B.P., Bogerd,H.P. and Cullen,B.R. (2004) A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. *EMBO J.*, **23**, 2451–2458.
30. Fujino,T., Navaratnam,N., Jarmuz,A., Von Haeseler,A. and Scott,J. (1999) C – U editing of apolipoprotein B mRNA in marsupials: identification and characterization of APOBEC-1 from the American opossum *Monodelphus domestica*. *Nucleic Acids Res.*, **27**, 2662–2671.
31. Doehle,B.P., Schafer,A., Wiegand,H.L., Bogerd,H.P. and Cullen,B.R. (2005) Differential sensitivity of murine leukemia virus to APOBEC3-mediated inhibition is governed by virion exclusion. *J. Virol.*, **79**, 8201–8207.
32. Browne,E.P. and Littman,D.R. (2008) Species specific restriction of Apobec3, mediated hypermutation. *J. Virol.*, **82**, 1305–1313.
33. Newman,E.N., Holmes,R.K., Craig,H.M., Klein,K.C., Lingappa,J.R., Malim,M.H. and Sheehy,A.M. (2005) Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. *Curr. Biol.*, **15**, 166–170.
34. Chiu,Y.-L., Soros,V.B., Kreisberg,J.F., Stopak,K., Yonemoto,W. and Greene,W.C. (2005) Cellular APOBEC3G restricts HIV-1 infection in restricting CD4+ T cells. *Nature*, **435**, 108–114.
35. Turelli,P., Mangeat,B., Jost,S.S., Vianin,S. and Trono,D. (2004) Inhibition of hepatitis B virus replication by APOBEC3G. *Science*, **303**, 1829.
36. Miyagi,E., Opi,S., Takeuchi,H., Khan,M., Goila-Gaur,R., Kao,S. and Strebel,K. (2007) Enzymatically active APOBEC3G is required for efficient inhibition of human immunodeficiency virus type 1. *J. Virol.*, **81**, 13346–13353.
37. Schumacher,A.J., Hache,G., MacDuff,D.A., Brown,W.L. and Harris,R.S. (2008) The DNA deaminase activity of human APOBEC3G is required for Ty1, MusD and human immunodeficiency virus type 1 restriction. *J. Virol.*, **82**, 2652–2660.
38. Lau,P.P., Xiong,W.J., Zhu,H.J., Chen,S.H. and Chan,L. (1991) Apolipoprotein B mRNA editing is an intranuclear event that occurs posttranscriptionally coincident with splicing and polyadenylation. *J. Biol. Chem.*, **266**, 20550–20554.
39. Lellek,H., Kirsten,R., Diehl,L., Apostel,F., Buck,F. and Greeve,J. (2000) Purification and molecular cloning of a novel essential component of the apolipoprotein B mRNA editing enzyme-complex. *J. Biol. Chem.*, **275**, 19848–19856.
40. Mehta,A., Kinter,M.T., Sherman,N.E. and Driscoll,D.M. (2000) Molecular cloning of apobec-1 complementation factor, a novel RNA-binding protein involved in the editing of apolipoprotein B mRNA. *Mol. Cell. Biol.*, **20**, 1846–1854.
41. Chester,A., Somasekaram,A., Tzimina,M., Jarmuz,A., Gisbourne,J., O'Keefe,R., Scott,J. and Navaratnam,N. (2003) The apolipoprotein B mRNA editing complex performs a multifunctional cycle and suppresses nonsense-mediated decay. *EMBO J.*, **22**, 3971–3982.

42. Jiao,S., Moberly,J.B. and Schonfeld,G. (1990) Editing of apolipoprotein B messenger RNA in differentiated Caco-2 cells. *J. Lipid Res.*, **31**, 695–700.
43. Zheng,Y.-H., Irwin,D., Kurosu,T., Tokunaga,K., Sata,T. and Peterlin,B.M. (2004) Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. *J. Virol.*, **78**, 6073–6076.
44. Katzourakis,A., Tristem,M., Pybus,O.G. and Gifford,R.J. (2007) Discovery and analysis of the first endogenous lentivirus. *Proc. Natl Acad. Sci. USA*, **104**, 6261–6265.
45. Browning,J., Horner,J.W., Pettoello-Mantovani,M., Raker,C., Yurasov,S., DePinho,R.A. and Goldstein,H. (1997) Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc. Natl Acad. Sci. USA*, **94**, 14637–14641.
46. Sawada,S., Gowrishankar,K., Kitamura,R., Suzuki,M., Suzuki,G., Tahara,S. and Koito,A. (1998) Disturbed $CD4^+$ T cell homeostasis and in vitro HIV-1 susceptibility in transgenic mice expressing T cell line-tropic HIV-1 receptors. *J. Exp. Med.*, **187**, 1439–1449.
47. Keppler,O.T., Welte,F.J., Ngo,T.A., Chin,P.A., Patton,K.S., Tsou,C.-L., Abbey,N.W., Sharkey,M.E., Grant,R.M., You,Y. *et al.* (2002) Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *J. Exp. Med.*, **195**, 719–736.
48. Dunn,C.S., Mehtali,M., Houdebine,L.M., Gut,J.-P., Kirn,A. and Aubertin,A.-M. (1995) Human immunodeficiency virus type 1 infection of human CD4-transgenic rabbits. *J. Gen. Virol.*, **76**, 1327–1336.
49. Speck,R.F., Penn,M.L., Wimmer,J., Esser,U., Hague,B.F., Kindt,T.J., Atchison,R.E. and Goldsmith,M.A. (1998) Rabbit cells expressing human CD4 and human CCR5 are highly permissive for human immunodeficiency virus type 1 infection. *J. Virol.*, **72**, 5728–5734.
50. Stremlau,M., Owens,C.M., Perron,M.J., Kiessling,M., Autissier,P. and Sodroski,J. (2004) The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in old world monkeys. *Nature*, **427**, 848–853.
51. Schaller,T., Hue,S. and Towers,G.J. (2007) An active TRIM5 in rabbits indicates a common antiviral ancestor for mammalian TRIM5 proteins. *J. Virol.*, **81**, 11713–11721.

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Profile of HIV Type 1 Infection and Genotypic Resistance Mutations to Antiretroviral Drugs in Treatment-Naive HIV Type 1-Infected Individuals in Hai Phong, Viet Nam

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Abstract

We evaluated the prevalence and profile of antiretroviral treatment (ART)-associated resistance mutations among HIV-1 strains in northern Vietnam by genotypically analyzing strains isolated from ART-naive individuals in Hai Phong, a city in which HIV-1 is highly prevalent. Plasma samples were collected from injecting drug users (IDU, $n=760$), female sex workers (FSW, $n=91$), seafarers ($n=94$), pregnant women ($n=200$), and blood donors ($n=210$), and screened for HIV-1 antibodies. Plasma viral RNA was extracted from HIV-1-positive samples, amplified by reverse transcriptase (RT)-PCR of protease and RT genes, and analyzed for genotypes and ART-associated resistance mutations. HIV-1 prevalence among IDU, FSW, seafarers, pregnant women, and blood donors was 35.9%, 23.1%, 0%, 0.5%, and 2.9%, respectively. Phylogenetic analyses revealed that the most prevalent HIV-1 subtype was CRF01_AE (98.3%), similar to strains prevalent in southern China. Four (1.4%) subtype B strains and one (0.3%) unique recombinant between subtypes B and C were also identified. We found protease inhibitor-associated major resistance mutations in one of the 294 cases analyzed (0.3%; mutation M46I). We found RT inhibitor-associated major resistance mutations in 7/273 cases (2.6%; one occurrence each of L74I, M184I, and K219E; three cases of K103N; and two cases of G190E). One CRF01_AE strain harboring a protease codon 35 insertion was first identified in Vietnam. Thus, monitoring of drug-resistant HIV-1 and establishment of a database are required for the proper selection of ART in Vietnam.

Introduction

Combination antiretroviral drug therapy (ART) using reverse-transcriptase inhibitors (RTI) and protease inhibitors (PI) has been the gold standard for HIV/AIDS treatment since the late 1990s, and the prognosis for HIV/AIDS patients has correspondingly improved dramatically in developed countries. In contrast, many human immune deficiency virus type 1 (HIV-1)-infected individuals in developing countries were not able to access antiretrovirals (ARV) until early 2000, due mainly to the high price. However, the World Health Organization (WHO), the Joint United Nations Programme on HIV/AIDS (UNAIDS), and other international donors have been promoting the intensive introduction of ART to low- and middle-income countries through "3 by 5" initiatives and similar programs since 2003.

The number of people living with HIV-1 has risen steadily in Vietnam, from 122×10^3 in 2000 to 283×10^3 in 2006.[1] HIV-1 infection was first recognized in southern Vietnam in 1990 and had spread to all of the Vietnamese provinces by 2006 with variable epidemic status.[2] The majority of people infected with HIV-1 in Vietnam are intravenous drug users (IDU) and their sex partners.[1,3,4] A large number of governmental, civilian, and international programs have been implemented to reduce endemic HIV-1 infection in Vietnam, and the availability of treatment, care, and support programs for HIV-1-infected individuals has also increased in scale.[2] Beginning in 2003, ART has been intensively introduced to

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Vietnam by the Vietnamese government, WHO, and international donors, resulting in an increase in ART coverage of HIV-1-infected individuals from 1% in 2003 to 11% in 2005 and 28.4% in 2007.[2,5]

As ART is introduced into resource-limited countries, the appearance and spread of ART-resistant HIV-1 have become an emerging problem. In Ho Chi Minh City (HCM) in southern Vietnam, drug-resistant HIV-1 among ART-naive HIV-infected individuals was reported to be 6.5% in 2003, a time when ART was not yet common in Vietnam.[6] In Hanoi, the capital of Vietnam and located in the northern part of the country, the HIV drug-resistance threshold survey was conducted for the specimens collected in 2006 and showed low prevalence (<5%) of transmitted HIV-1 drug resistance to all drugs and drug classes evaluated.[7] However, little information is available regarding the current status of drug-resistant HIV-1 in Vietnam, where CRF01_AE is reported to be the predominant strain,[6,8–11] though further increases in drug-resistant HIV-1 are expected.

There are several well-established drug-resistance databases for subtype B HIV-1, which accounts for only 10% of the global HIV/AIDS pandemic.[12] However, databases for non-B subtypes, which are prevalent mainly in resource-limited countries, are far from comprehensive. It is important to investigate ARV resistance-associated mutations of non-B subtype HIV-1 strains, and to establish a database so that appropriate ARVs can be selected for individuals infected with ARV-resistant strains of HIV-1.

In the current study, we investigated the prevalence and profile of ARV resistance-associated mutations among ART-naive HIV-1-infected individuals in Hai Phong, a city in northern Vietnam in which HIV-1 is highly prevalent.[5]

Materials and Methods

Study population

Residents of Hai Phong, the largest port city in northern Vietnam, were invited to join this study in 2007. The participants had different risks of HIV infection and were categorized into five groups: (1) IDUs, who were concentrated in rehabilitation centers in Hai Phong ($n = 760$, all male, mean age: 34.1 years old, age range: 19–65); (2) female sex workers (FSW), who had previously been commercial sex workers and were concentrated in a rehabilitation center ($n = 94$, mean age: 24.8 years old, age range: 17–42); (3) seafarers, who worked for marine companies ($n = 91$, all male, mean age: 32.5 years old, age range: 20–56); (4) pregnant women, who attended antenatal clinics ($n = 200$, mean age: 30.8 years old, age range: 15–50); and (5) blood donors ($n = 210$; female/male: 69/140, one person whose sex was not known; mean age: 31.2 years old; age range: 16–58). None of the participants had any previous history of ART.

After thorough ethical clearance and informed consent, we collected blood samples from the participants from April to October in 2007. Plasma samples that were found to be reactive for HIV-1 antibody with an immunochromatography assay kit (Determine HIV ½; Abbott Japan, Tokyo, Japan) were confirmed with Western blotting (New Lab Blot 1, Bio-Rad Laboratories, Tokyo, Japan) and included in this study. The study protocol was reviewed and approved by the ethical committees of Hanoi Medical University in Vietnam and Kanazawa University in Japan.

Extraction and amplification of plasma HIV-1 viral RNA

HIV-1 RNA was extracted from 100 μl of HIV-1-positive plasma using SMITEST EX-R&D nucleotide extraction kit (Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. Amplification of the HIV-1 *pol* gene, which encodes reverse transcriptase and protease, was performed by both one-step RT-PCR (SuperScript III One-step RT-PCR system with Platinum Taq DNA polymerase; Invitrogen, Carlsbad, CA) and nested PCR using AmpliTaq Gold (Applied Biosystems, Japan) and/or KOD FX (Toyobo, Osaka, Japan).

A region of the HIV-1 *pol* gene that includes the protease sequence (*pol-PR*, corresponding to nucleotides 2148–2611 in HIV-1_{HXB2}) was amplified by nested RT-PCR with primers DRPRO5 (5′-AGACAGGYTAATTTTTTAGGGA-3′) and DRPRO2L (5′-TATGGATTTTCAGGCCCAATTTTTGA-3′) in the first round and DRPRO1M (5′-AGAGCCAACAGCCCC ACCAG-3′) and DRPRO6 (5′-ACTTTTGGGCCATCCATT CC-3′) in the second round. A region of the HIV-1 *pol* gene that includes parts of the RT sequence (*pol-RT*, corresponding to nucleotides 2485–3372 in HIV-1_{HXB2}) was amplified by nested RT-PCR with primers DRRT1L (5′-ATGATGGGGGAATTG GAGGTTT-3′) and RTout (5′-ATATACTCCATGCACAGG GGTTTT-3′) in the first round, and DRRT7L (5′-GACCTA CACCTGTCAACATAATTGG-3′) and DRRT6L (5′-TAATC CCTGCATAAATCTGACTTGC-3′) in the second round. For the amplification of HIV-1 *pol-RT*, the primer pairs RT18/K104 and K101/K102[13] were also used in the first and second rounds, respectively.

RT-PCR was performed with one cycle at 55°C for 30 min and one cycle at 94°C for 2 min, then 40 cycles at 94°C for 15 s, 55°C (for DRPRO5/DRPRO2L and RT18/K104 primer pairs) or 50°C (for the remaining primer sets) for 30 s, and 68°C for 1 min, with a final extension of 68°C for 5 min, using the One-step RT-PCR system (Invitrogen). Nested PCR for *pol-PR* was done with one cycle at 95°C for 10 min, followed by 40 cycle at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min, using AmpliTaq Gold. Nested PCR for *pol-RT* was done with one cycle at 94°C for 1 min, and 35 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 1.5 min, using KOD FX (Toyobo, Japan). PCR amplification was confirmed by ethidium bromide staining of samples electrophoresed on an agarose gel. The amplified products were directly sequenced and analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with BigDye Terminator v1.1 (Applied Biosystems).

Genotype and drug-resistance determination

The sample nucleotide sequences were aligned with HIV-1 subtype/CRF reference sequences from the Los Alamos database and previously reported sequences of HIV-1 strains isolated from Southeast Asia and southern China using CLUSTAL W (version 1.83), with minor manual adjustments. Phylogenic trees were constructed and visualized as described previously.[12] Reference HIV-1 strains (accession number) used in this study were as follows: for subtype B, HXB2 (K03455) and China Yunnan RL42 (U71182); for subtype C, India (AF067155); for CRF01_AE, Thai CM240 (U54771), China Fujian (DQ859180), China Guangxi 2F (AY008714), China Guangxi 11F (AY008718), Vietnam HCM vr79 (AY238295 and AY238028), Vietnam HCM vr115 (AY238279

and AY238024), Vietnam HCM vr135 (AY238242 and AY238088), China Yunnan (AB213669), and China Liaoning (EF122521); for CRF15_01B, Thai (AF516184); for CRF 01B, Myanmar CSW (AB097866) and Myanmar IDU (AB097865); for CRF07_BC, China Xinjiang (AF286226) and China Yunnan (AB213675); for CRF08_BC, China Gansu (AF286229) and China Guangxi (AY008716); and as an outgroup, SIVcpz (AF395563). To improve the accuracy of HIV-1 subtyping, we used the National Center for Biotechnology Information (NCBI) genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/), as needed.

The *pol-PR* and a part of *pol-RT* nucleotide sequences (297 bps and 660 bps, respectively) were translated into the corresponding 99 and 220 amino acids, respectively. Using the Stanford University HIVdb sequence analysis program (http://hivdb.stanford.edu/pages/algs/HIVdb.html) and the International AIDS society-USA Spring 2008 list,[14] we analyzed the amino acid sequences for those ARV resistance-associated major and minor mutations that had been previously reported mainly in subtype B strains.

Results

HIV-1 prevalence

Of the 1355 individuals from five different groups in Hai Phong, 301 were positive for HIV-1 antibodies. The prevalence of HIV-1 among IDU, FSW, seafarers, pregnant women, and blood donors was 35.9% (273/760), 23.1% (21/94), 0% (0/91), 0.5% (1/200), and 2.9% (6/210), respectively.

Subtype distribution

Of the 301 HIV-1-positive samples, 272 could be analyzed in both the *pol-PR* and the *pol-RT* regions, 22 could be analyzed in the *pol-PR* region only, and one could be analyzed in the *pol-RT* region only. A total of 295 samples were successfully analyzed in the *pol-PR* and/or *pol-RT* region. The subtype or circulating recombinant form (CRF) of each sample was identified. Of the 295 HIV-1 strains, 290 (98.3%) were CRF01_AE, four (1.4%) were subtype B, and one (0.3%) was subtype B/C recombinant. Of these, 19 of the CRF01_AE strains and three of the subtype B strains were identified based on *pol-PR* sequences, one subtype B strain was identified based on the *pol-RT* sequence, and the remaining strains were identified based on both *pol-PR* and *pol-RT* sequences.

Phylogenetic analyses also revealed that most of the CRF01_AE strains from Hai Phong were similar to one another, and distinct from strains of HCM, Thai, and China Yunnan strains; however, a few of the Hai Phong strains were similar to the HCM strains. It is noteworthy that the CRF01_AE strains from Hai Phong were phylogenetically indistinguishable from the China Guangxi strains (Fig. 1).

The subtype-B/C recombinant strain found in this study was relatively similar to CRF08_BC strains from Guangxi province, in southern China (Fig. 1). However, further analysis with the Recombination Identification Program (RIP; Los Alamos National Laboratory, Los Alamos, NM) showed that a crossover event had taken place in the recombinant at a point in the *pol-RT* region different from that of the Guangxi CRF08_BC strain (data not shown).

PI resistance-associated mutations

Of the 294 cases that we analyzed, one (0.3%) had a strain with a major PI resistance-associated mutation, M46 I (a "flap" mutation); its determined subtype was CRF01_AE (Table 1A).

Minor PI resistance-associated mutations were also observed and are listed in Table 1A. M36I (99.0%) and H69K (99.3%), recently identified minor resistance mutations to the new PI tipranavir, were frequently observed in CRF01_AE strains and are considered to be natural polymorphisms. One strain (0.3%) harbored PR codon 35, a glutamic acid insertion (E35E_E). R41K (99.0%) and L89M (98.6%), which are not known to cause PI resistance, were also frequently observed in the *pol-PR* region of CRF01_AE strains (Table 2).

RTI resistance-associated mutations

Of the 273 cases analyzed, three (1.1%) had strains with major nucleoside reverse transcriptase inhibitor (NRTI)-resistance mutations: one case each with L74I, M184I, and K219E. Five (1.8%) cases had strains with major nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistance mutations: three cases with K103N and two cases with G190E. One case (0.3%) had a strain that harbored both the M184I and the K103N mutation. Hence, the overall prevalence of RTI-resistance mutations was 2.6% (Table 1B and C).

We also observed the minor mutations V90I, V106I, and V179 D/F, which are related to the resistance of the new NNRTI etravirine. Other polymorphisms that are not associated with RTI resistance are summarized in Table 3. We did not identify any strains that harbored both PI-resistance and RTI-resistance mutations together.

All of the HIV-1 strains with major mutations were found to be CRF01_AE and only from the IDU group, which consisted of men.

Discussion

In the current study, we found ARV resistance-associated major mutations in 2.9% of our study population of ART-naive HIV-1-infected individuals in Hai Phong, Vietnam. To our knowledge, this is the first report on the current status of ARV-resistant HIV-1 strains in Hai Phong, northern Vietnam.

The prevalence of HIV-1 was first investigated among various risk groups in Hai Phong. Among IDU, it was found to be 35.9%, which is lower than the percentages reported by UNAIDS of 57.8% in 2005[2] and 65.8% in 2006.[5] This difference may be explained by the fact that the IDU in our study were recruited from rehabilitation centers and were not actively injecting drugs at the time of the study. Among FSW, the prevalence was 23.1%, which is far higher than what was reported by UNAIDS, which was 5.6% in 2005[2] and 7.2% in 2006.[5] These differences may be due to differences in the criteria used for FSW between our study and the study by UNAIDS. In our study, past and present FSW were recruited regardless of their history of injected drug use. It has been reported that around 30% of FSW inject drugs in Vietnam.[3,4,15–17] Therefore, it is very possible that several FSW in our study have had a history of injected drug use. The prevalence of HIV-1 in the pregnant women (0.45%), one of the representatives of the general population in our survey, was similar to that in UNAIDS reports in 2005.[2]

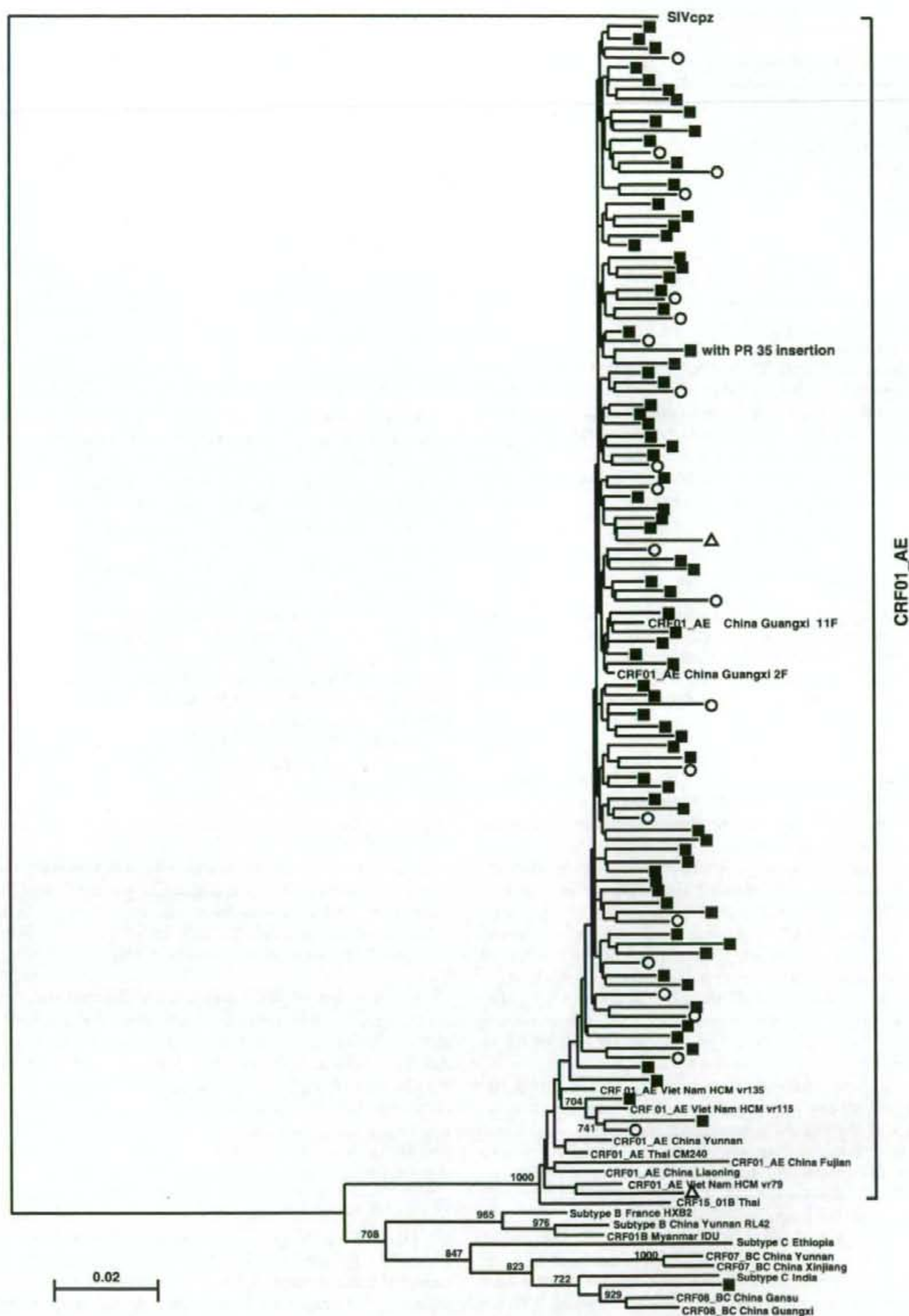

FIG. 1. Phylogenic tree of representative HIV-1 strains from IDU, all 21 strains from FSW, and two strains from blood donors, based on the *pol-PR* and *pol-RT* genes (approximately 957 bases). Filled squares, representative HIV-1 strains from IDU; open circles, HIV-1 strains from FSW; and open triangles, HIV-1 strains from blood donors. Boot strap values greater than 700 are shown.

TABLE 1. AMINO ACID SUBSTITUTIONS ASSOCIATED WITH RESISTANCE TO (A) PROTEASE INHIBITORS (PI), (B) NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTI), AND (C) NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTI)[a]

(A) Position		L10	V11	I13	G16	K20	*E35*	M36	**M46**	D60	I62	L63	I64	H69	A71	V77	V82	I93
01_AE	290	I (15) V (7)	I (1)	V (233)	E (68)	R (28) I (7) T (1)	*D (234)* *N (1)* *E insertion (1)*	I (287) V (1)	**I (1)**	E (6)	V (6)	P (25)	L (1) M (1)	K (288)	T (1) V (1)	I (1)	I (8)	L (45)
B+C	1							V (1)		E (1)				K (1)				L (1)
B	3																	

(B) Position		T69	L74	V75	V118	**M184**	*L210*	**K219**
01_AE	271	N S (2)	I	G	I (1)[b]	**I (1)**[c]	*M*	**E (1)**
B+C	1							
B	1							

(C) Position		V90	K103	V 106	*V 179*	**G190**	*P225*	**T236**
01_AE	271	I (1)	N (3)	I (4)	*D (4)* *F (1)* *E (1)* *A (1)*	**E (2)**	*S (1)*	**S (1)**
B+C	1	I (1)						
B	1							

[a]Text, minor mutations; boldface text, major amino acid mutations associated with drug resistance; italic text, PI- or NRTI-selected mutations, the significance of those substitutions is not known, or atypical substitutions.
[b]The significance of single V118I is unknown.
[c]With K103N.

HIV-1 CRF01_AE strains were found to predominate in Hai Phong in northern Vietnam, the same as was previously reported in both northern and southern Vietnam.[6–12,18–23] Phylogenetic analyses revealed that the majority of these strains were closely related to strains prevalent in Guangxi, southern China, and different from strains from HCM (southern Vietnam), Thailand, and Cambodia. This is consistent with the findings of previous studies, which reported that CRF01_AE strains in the northern provinces along the Vietnam–China border were introduced from Guangxi province and reached Hanoi through heroin-trafficking routes.[10,11,22,23] The CRF01_AE strains in southern Vietnam are believed to be derived from strains in Thailand and Cambodia.[11,18,22,23] In addition, the unique subtype-B/C recombinant strain that we found in our study was relatively similar to the CRF08_BC strain from Guangxi province (Fig. 1). However, it should be noted that we found strains in Hai Phong that were similar to those in HCM, showing that there may have been some mixing of strains from northern and southern Vietnam in this area.

Phylogenetic analysis also showed that the HIV-1 strains from the IDU group formed a cluster together with the strains from the FSW and blood donor groups (Fig. 1), suggesting that the HIV-1 epidemic in Hai Phong has already begun to spread from IDU into the general population through the FSW population, as has been observed in other Asian countries.[24]

We detected major mutations that cause PI and RTI resistance in ART-naive patients at rates of 0.3% and 2.6%, respectively, in Hai Phong as of October 2007. Our result is consistent with the previous findings in Hanoi in 2006,[7] though it is slightly lower than the findings in HCM in 2003.[6] These results are to be expected, because the current first line of ART in Vietnam is a combination of two NRTIs and one NNRTI, and PI use is still limited compared with developed countries. Further monitoring of changes in HIV-1

TABLE 2. POLYMORPHISM AT THE *Pol-PR* REGION NOT ASSOCIATED WITH RESISTANCE TO PROTEASE INHIBITORS

Position		*K14*	*I15*	*Q18*	*L19*	*K20*	*N37*	*P39*	*R41*	*K43*	*K45*	*R57*	*Q61*	*K70*	*A71*	*I72*	*L89*	*T91*	*Q92*
01_AE	290	R(15)	L (1) V (15)	E (1) M (2)	I (1) M (2) Q (4)	R (28) I (7) T (1)	D (13) K (2) S (1) T (1)	Q (1) S (2)	K (287)	R (7)	R (8)	K (14)	E (3) H/P (1) Q (1)	Q (2) R (23)	T (1) V (1)	T (1) V (4)	I (2) M (286)	A (2) I (1) S (2)	K (3)
B+C	1		V (1)		V (1)				K (1)					K (1)			M (1)		
B	3																		

Table 3. Polymorphism at the *Pol-RT* Region and Not Associated with Resistance to Reverse Transcriptase Inhibitors

Position		*E6*	*K11*	*K20*	*V21*	*K20*	*E29*	*V35*	*E36*	*T39*	*E40*	*K43*	*S48*	*V60*	*S61*	*K101*	*K102*	*T107*
01_AE	271	D (257) K (2) N (2)	A (2) Q (1) R (2) S (3) T (219)	R (6)	I (4)	R (28) I (7) T (1)	A (1) K (3)	A (1) I (1) M (1) R (1) T (258) Y (1)		A (6) E (16) G (1) K (174) L (1) N (41) Q (3) R (1) S (2)	D (3)	E (6) Q (13) R (1)		I (3)		Q (2) R (1)	E (1) Q (5) R (4)	S (9)
B+C	1							T (1)	A (1)	D (1)			T (1)	I (1)	I (1)			
B	1																	

Position		*V111*	*G112*	*D121*	*K122*	*K123*	*I132*	*I135*	*T139*	*I142*	*S162*	*Y173*	*Q174*	*D177*	*I178*	*V189*	*E194*
01_AE	271	I (10)	A (3)	A (1) E (1) H (6)	E (260)	E (1) G (4) K (7) N (18) S (237)	L (6)	R (1) T (28) V (3)	A (3)	V (12)	C (255) Y (5)	A (9) I (215) L (2) M (5) R (9) T (25) V (6)	K (262) L (2) N (3) R (3)	E (266) G (1) I (1)	M (136)	I (4)	D (2) G (1) K (1)
B+C	1			Y (1)				T (1)									
B	1																

Position		*G196*	*T200*	*K201*	*I202*	*E203*	*Q207*	*R211*	*E224*	*K238*	*V245*	*K249*	*S251*	*I257*	*Q258*	*L264*	*N265*	*W266*
01_AE	271	E (9) K (1) T (1)	A (58) E (1) I (19) Q (1) R (1) V (2)	R (3)	V (4)	D (3)	A (7) D (2) G (15) K (6) N (24) R (3) S (212)	G (1) H (1) K (1) N (4) S (262) T (1)	D (1) K (2)	R (212)	A (1) E (228) K (3) Q (4)	R (10)	H (1) I (1) N (2)	M (3) V (1)	E (2) R (4)	T (1) V (2)	I (9) K (1) S (4)	C (1) E (1) F (1)
B+C	1										Q (1)							
B	1																	

drug-resistance mutations in different areas of Vietnam is needed for the proper selection of ARV in this country.

Three cases who had HIV-1 with K103N mutation were found in the treatment-naive male IDU group in our study. K103N is selected by nevirapine (NVP) and is likely to be identified among pregnant women previously enrolled in the Prevention of Mother-to-Child Transmission (PMTCT) program.[25] In Vietnam, the PMTCT program using a single-dose NVP, two- or three-combination ARV regimen was first introduced in 2006, and those prophylaxis coverage rates of HIV-infected pregnant women were increased from 9.2% in 2006 to 13.9% in 2007.[5] It would be interesting to know whether those K103N mutations found among the IDU group in Hai Phong in 2007 were from those PMTCT program population or from outside of the country, such as southern China, where HIV-1 strains closely related to the CRF_01AE strains in IDU in Hai Phong were found.

In our study, we confirmed several minor drug-resistance mutations that are considered to be CRF01_AE-specific polymorphisms in the protease gene. We detected I13V (80.0%), G16E (23.4%), M36I (99.9%), and H69K (99.3%) at high frequencies. This profile is similar to those reported in previous studies from Vietnam and Southeast Asia.[6,26–31] Although no clinical survey has shown a significant correlation between natural polymorphisms and the development of ART failure,[13,32–38] the possibility should not be excluded that these polymorphisms might negatively affect the outcome of future ART.[39] Natural polymorphisms of CRF01_AE, which were recently identified as minor resistance mutations to the newly developed PIs tipranavir/ritonavir and darunavir/ritonavir[40,41] and the NNRTI etravirine,[42] highlight the importance of monitoring non-subtype B strains when planning new antiviral drug development.

A CRF01_AE strain harboring a PR codon 35 insertion, which is known to be related to PI treatment,[43–46] was first identified in Vietnam. In Asia, PR codon 35-inserted subtype B strains were reported to be circulating among ART-naive patients in Hong Kong,[47] but not in other countries. Careful monitoring of this mutation is needed to determine whether PR codon 35-inserted strains have begun to circulate more widely in Asian countries.

In conclusion, the most prevalent HIV-1 strains in Hai Phong, northern Vietnam, were CRF01_AE, and the majority were similar to those found in southern China. The prevalence of ARV-resistant HIV-1 among ART-naive individuals in Hai Phong was 2.9% in 2007, which is slightly lower than the

prevalence in HCM in 2003[6] and consistent with the threshold survey in Hanoi in 2006.[7] Further monitoring is necessary to establish a useful database of ARV-resistant HIV-1 in Vietnam. There is also a need for a consensus algorithm, based on what is known about drug-resistance mutations in subtype B strains, which can be used to predict the clinical outcomes of people who are infected with non-subtype B HIV-1 strains.[39]

Sequence Data

The GenBank accession numbers of the sequences reported in this study are as follows: from FJ006949 to FJ007345 for *pol-PR/RT*, FJ007346 to FJ007369 for *pol-PR*, and FJ007370 for *pol-RT*.

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Disclosure Statement

No competing financial interests exist.

References

1. UNAIDS/WHO: ASIA AIDS epidemic update Regional Summary. UNAIDS 2008.
2. UNAIDS/WHO: Epidemiological Fact Sheets on HIV/AIDS and Sexually Transmitted Infections. UNAIDS 2006.
3. Nguyen TA, Hoanh LT, Pham VQ, and Detels R: Risk factors for HIV-1 seropositivity in drug users under 30 years old in Haiphong, Vietnam. Addiction 2001;96(3):405–413.
4. Nguyen AT, Fylkesnes K, Thang BD, *et al.*: Human immunodeficiency virus (HIV) infection patterns and risk behaviors in different population groups and provinces in Viet Nam. Bull WHO 2007;85(1):35–41.
5. UNAIDS: The United Nations General Assembly Special Session on HIV/AIDS Country progress report; Viet Nam. UNAIDS 2008.
6. Nguyen THL, Recordon-Pinson P, Pham VH, *et al.*: HIV type 1 isolates from 200 untreated individuals in Ho Chi Minh City (Vietnam): ANRS 1257 study. Large predominance of CRF01_AE and presence of major resistance mutations to antiretroviral drugs. AIDS Res Hum Retroviruses 2003;19(10):925–928.
7. Nguyen TH, Nguyen BD, Shrivastava R, *et al.*: HIV drug resistance threshold survey using specimens from voluntary counseling and testing sites in Hanoi, Vietnam. Antiviral Ther 2008;13(Suppl. 2):115–121.
8. Tran TTH, Maljkovic I, Swartling S, *et al.*: HIV-1 CRF01_AE in intravenous drug users in Hanoi, Vietnam. AIDS Res Hum Retroviruses 2004;20(3):341–345.
9. Caumont A, Nguyen THL, Nguyen TVU, *et al.*: Sequence analysis of *env* C2/V3, *gag* p17/p24, and *pol* protease regions of 25 HIV type 1 isolates from Ho Chi Minh City, Vietnam. AIDS Res Hum Retroviruses 2001;17(13):1285–1291.
10. Kato K, Shiino T, Kusagawa S, *et al.*: Genetic similarity of HIV type 1 subtype E in a recent outbreak among injecting drug users in northern Vietnam to strains in Guangxi Province of southern China. AIDS Res Hum Retroviruses 1999;15(13):1157–1168.
11. Kato K, Kusagawa S, Motomura K, *et al.*: Closely related HIV-1 CRF01_AE variant among injecting drug users in northern Vietnam: Evidence of HIV spread across the Vietnam-China border. AIDS Res Hum Retroviruses 2001;17(2): 113–123.
12. Hemelaar J, Gouws E, Ghys PD, and Osmanov S: Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 2006;20(16):W13–23.
13. Lwembe R, Ochieng W, Panikulam A, *et al*: Anti-retroviral drug resistance-associated mutations among non-subtype B HIV-1-infected Kenyan children with treatment failure. J Med Virol 2007;79(7):865–872.
14. Jonhson VA, Brun-Vezinet F, Clotet B, *et al.*: Update of the drug resistance mutations in HIV-1: Spring 2008. Top HIV Med 2008;16(1):62–68.
15. Luu TMC, Tran NN, Nai TH, *et al.*: HIV prevalence and risk behaviors among injecting drug users in Ho Chi Minh city, Hai Phong and Hanoi, Viet Nam. XVI International AIDS Conference. Toronto, Canada, 13–18 August 2006. Abstract CDC0320.
16. Tran TN, Detels R, Long HT, and Lan HP: Drug use among female sex workers in Hanoi, Vietnam. Addiction 2005; 100(5):619–625.
17. Ministry of Health Viet Nam: Results from the HIV/STI integrated biological and behavioral surveillance (IBBS) in Viet Nam, 2005–2006. UNAIDS 2006.
18. Nerurkar VR, Nguyne HT, Dashwood WM, *et al.*: HIV type 1 subtype E in commercial sex workers and injection drug users in southern Vietnam. AIDS Res Hum Retroviruses 1996;12(9):841–843.
19. Nerurkar VR, Nguyen HT, Woodward CL, *et al.*: Sequence and phylogenetic analyses of HIV-1 infection in Vietnam: Subtype E in commercial sex workers (CSW) and infection drug users (IDU). Cell Mol Biol (Noisy-le-Grand) 1997; 43(7):959–968.
20. Nguyen THL, Masquelier B, Pham VH, *et al.*: Further characterization of HIV-1 isolates from Ho Chi Minh City, Vietnam. J Acquir Immune Defic Syndr Hum Retrovirol 1999;20(1):93–95.
21. Menu E, Truong TX, Lafon ME, *et al.*: HIV type 1 Thai subtype E is predominant in South Vietnam. AIDS Res Hum Retroviruses 1996;12(7):629–633.
22. Beyrer C, Razak MH, Lisam K, *et al.*: Overland heroin trafficking routes and HIV-1 spread in south and south-east Asia. AIDS 2000;14(1):75–83.
23. Yu XF, Chen J, Shao Y, *et al.*: Emerging HIV infections with distinct subtypes of HIV-1 infection among injection drug users from geographically separate locations in Guangxi Province, China. J Acquir Immune Defic Syndr 1999;22(2): 180–188.
24. Monitoring the AIDS Pandemic Network (MAP): Drug injection and HIV/AIDS in Asia. The MAP Reports 2005.
25. Jackson JB, Becker-Pergola G, Guay LA, *et al.*: Identification of the K103N resistance mutation in Ugandan women

receiving nevirapine to prevent HIV-1 vertical transmission. AIDS 2000;14(11):F111–F115.

26. Liu J, Yue J, Wu S, and Yan Y: Polymorphisms and drug resistance analysis of HIV-1 CRF01_AE strains circulating in Fujian Province, China. Arch Virol 2007;152(10):1799–1805.
27. Ly N, Recordon-Pinson P, Viseth P, *et al.*: Characterization of mutations in HIV type 1 isolates from 144 Cambodian recently infected patients and pregnant women naïve to antiretroviral drugs. AIDS Res Hum Retroviruses 2005;21(11): 971–976.
28. Han X, Zhang M, Dai D, *et al.*: Genotypic resistance mutations to antiretroviral drugs in treatment-naive HIV/AIDS patients living in Liaoning Province, China: Baseline prevalence and subtype-specific difference. AIDS Res Hum Retroviruses 2007;23(3):357–364.
29. Sukasem C, Churdboonchart V, Sirisidthi K, *et al.*: Genotypic resistance mutations in treatment-naïve and treatment-experienced patients under widespread use of antiretroviral drugs in Thailand: Implications for further epidemiologic surveillance. Jpn J Infect Dis 2007;60(5):284–289.
30. Yam WC, Chen JHK, Wong KH, *et al.*: Clinical utility of genotyping resistance test on determining the mutation patterns in HIV-1 CRF01_AE and subtype B patients receiving antiretroviral therapy in Hong Kong. J Clin Virol 2006;35(4):454–457.
31. Liu L, Lu HZ, Henry M, and Tamalet C: Polymorphism and drug selected mutations of reverse transcriptase gene in 102 HIV-1 infected patients living in China. J Med Virol 2007;79(10):1593–1599.
32. Holguín A, Suñe C, Hamy F, *et al.*: Natural polymorphisms in the protease gene modulate the replicative capacity of non-B HIV-1 variants in the absence of drug pressure. J Clin Virol 2006;36(4):264–271.
33. Ode H, Matsuyama S, Hata M, *et al.*: Computational characterization of structural role of the non-active site mutation M36I of human immunodeficiency virus type 1 protease. J Mol Biol 2007;370(3):598–607.
34. Sanches M, Krauchenco S, Martins NH, *et al.*: Structural characterization of B and non-B subtypes of HIV-protease: Insighs into the natural susceptibility to drug resistance development. J Mol Biol 2007;369(4):1029–1040.
35. Hsu LY, Subramaniam R, Bacheler L, and Paton NI: Characterization of mutations in CRF01_AE isolates from antiretroviral treatment-naive and experienced patients in Singapore. J Acquir Immune Defic Syndr 2005;38(1):5–13.
36. Ariyoshi K, Matsuda M, Miura H, *et al.*: Patterns of point mutations associated with antiretroviral drug treatment failure in CRF01_AE (subtype E) infection differ from subtype B infection. J Acquir Immune Defic Syndr 2003;33(3): 336–342.
37. Kantor R, Katzenstein DA, Efron B, *et al.*: Impact of HIV-1 subtype and antiretroviral therapy on protease and reversed transcriptase genotype: Results of a global collaboration. PLoS Med 2005;2(4):325–337.
38. Tee KK, Kamarulzaman A, and Ng KP: Prevalence and pattern of drug resistance mutations among antiretroviral-treated HIV-1 patients with suboptimal virological response in Malaysia. Med Microbiol Immunol 2006;195(2):107–112.
39. Snoeck J, Kantor R, Shafer RW, *et al.*: Discordance between interpretation algorithms for genotypic resistance to protease and reverse transcriptase inhibitors of human immunodeficiency virus are subtype dependent. Antimicrob Agents Chemother 2006;50(2):694–701.
40. Baxter JD, Schapiro JM, Boucher CA, *et al.*: Genotypic changes in human immunodeficiency virus type 1 protease associated with reduced susceptibility and virologic response to the protease inhibitor tipranavir. J Virol 2006;80(21): 10794–10801.
41. De Meyer S, Vangeneugden T, Van Baelen B, *et al.*: Resistance profile of darunavir: Combined 24-week results from the POWER trials. AIDS Res Hum Retroviruses 2008; 24(3):379–388.
42. Picchio G, Vingerhoets J, Staes M, *et al.*: Prevalence of etravirine (ETR; TMC125) resistance-associated mutations in a large panel of clinical isolates. 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 3–6, 2008.
43. Kim EY, Winters MA, Kagan RM, and Merigan TC: Functional correlates of insertion mutations in the protease gene of human immunodeficiency virus type 1 isolates from patients. J Virol 2001;75(22):11227–11233.
44. Winters MA and Merigan TC: Insertions in the human immunodeficiency virus type 1 protease and reverse transcriptase genes: Clinical impact and molecular mechanisms. Antimicrob Agents Chemother 2005;49(7):2575–2582.
45. Paolucci S, Baldanti F, Dossena L, and Gema G: Amino acid insertions at position 35 of HIV-1 protease interfere with virus replication without modifying antiviral drug susceptibility. Antiviral Res 2006;69(3):181–185.
46. Kozisek M, Saskova KG., Rezacova P, *et al.*: Ninety nine is not enough: Molecular characterization of inhibitor resistant HIV-1 protease mutants with insertions in the flap region. J Virol 2008;82(12):5869–5878.
47. Chen JHK, Wong KH, Chan KC, *et al.*: Molecular epidemiology and divergence of HIV type 1 protease codon 35 inserted strains among treatment-naive patient in Hong Kong. AIDS Res Hum Retroviruses 2008;24(4):537–542.

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Rapid detection of human immunodeficiency virus type 1 group M by a reverse transcription-loop-mediated isothermal amplification assay

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ABSTRACT

A rapid one-step reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay targeting the *pol*-integrase gene was developed to detect human immunodeficiency virus type 1 (HIV-1) group M. This HIV-1 RT-LAMP assay is simple and rapid, and amplification can be completed within 35 min under isothermal conditions at 60 °C. The 100% detection limit of HIV-1 RT-LAMP was determined using a standard strain (WHO HIV-1 [97/656]) in octuplicate and found to be 120 copies/ml. The RT-LAMP assay was evaluated for use for clinical diagnosis using plasma samples collected from 57 HIV-1-infected and 40 uninfected individuals in Cameroon, where highly divergent HIV-1 strains are prevalent. Of the 57 samples from infected individuals, 56 harbored group-M HIV-1 strains, such as subtypes A, B, G, F2, and circulating recombinant forms (CRFs) _01, _02, _09, _11, _13; all were RT-LAMP positive. One sample harboring group-O HIV-1 and the 40 HIV-1-uninfected samples were RT-LAMP negative. These findings indicate that HIV-1 RT-LAMP can detect HIV-1 group-M RNA from plasma samples rapidly and with high sensitivity and specificity. These data also suggest that this RT-LAMP assay can be useful for confirming HIV diagnosis, particularly in resource-limited settings.

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1. Introduction

The number of people living with human immunodeficiency virus (HIV) infection was estimated at 33 million as of December 2007, and over 2.7 million people acquired new HIV infections in 2007 (UNAIDS, in press). HIV testing and counseling have been recognized as entry points for prevention, care, treatment, and support (WHO, 2004). Recently, rapid serological HIV tests have been introduced to facilitate radical scaling up of HIV testing and counseling services in many settings, such as in diagnosing and treating sexually transmitted infections, in services providing and linked to the prevention of mother-to-child transmission, and in general medical settings (WHO, 2004). It has been shown that sequential combinations of two or three antibody (Ab) tests (ELISA and/or rapid tests) are reliable for confirming HIV-positivity (WHO, 2004; Aghokeng et al., 2004; Carvalho et al., 1996; Meda et al., 1999). However, considering that the fourth generation HIV ELISA test, which can detect both HIV P24 antigen and HIV antibody in the same sample simultaneously, has been introduced to detect early-stage HIV infection (Meda et al., 1999) and that a combined antigen–antibody rapid test for diagnosing HIV will be introduced soon (Keren et al., 2008), a method for detecting rapidly HIV-1 RNA and/or proviral DNA to confirm HIV diagnosis in these settings would be a valuable diagnostic aid.

HIV-1 is classified into three groups: M, N, and O. Group M, which accounts for the HIV pandemic, is further classified into nine major clades (A–D, F–H, J, and K) and 42 circulating recombinant forms (CRFs; Heeney et al., 2006; Powell et al., 2007; HIV, 2008). The diverse nature of HIV causes difficulties in nucleotide-based diagnoses of HIV infection. In addition, low HIV DNA burden and low concentrations of HIV RNA in plasma often result in failure to detect HIV RNA or DNA in clinical specimens (Zazzi et al., 1995). These two factors, high diversity and low plasma RNA/proviral DNA concentration, limit the ability to diagnose HIV infection reliably and efficiently.

The reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay developed by Notomi is a simple method for nucleotide-based diagnostics that exhibits high sensitivity and specificity (Notomi et al., 2000). This method relies on auto-cycling strand displacement DNA synthesis by a DNA polymerase with high strand displacement activity and a set of two each of specially designed inner and outer primers. The entire RT-LAMP procedure

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