and E. The analysis revealed that 12 HBV strains of the samples that were cloned belonged to genotype A, 2 to genotype D and 4 to genotype E (fig. 2). Those obtained through direct sequencing revealed segregation of subtypes into A genotype, with both subgenotypes A1 and A2 present. 18 of these sequences clustered closely with reference A2 and A1 strain from South Africa, 6 with reference A from Uganda, 6 with A2 from Zanzibar and 1 with reference A from Somalia and 4 sequences clustering closely with reference A from Brazil. The nucleotide sequences of the 52 isolates obtained in this study have been deposited in the GeneBank. The accession numbers for the sequences reported are as follows: DQ460641 to DQ460665 for HBV *preS1* cloned samples and direct sequencing; EU514582 to EU514615.

There are 360 million people in the world with chronic HBV infections, 65 million (18%) of those infected live in Africa; however, there has been limited information into the type of HBV genotypes circulating in the region. In Kenya, particularly, very little information is available on the molecular epidemiology of HBV. In our study, we found that HBV genotype A (88.5%) was the dominant strain in Kenya, followed by genotype E (7.7%) and D (3.8%). HBV genotype A has been reported to be the most predominant subtype in sub-Saharan Africa [10], subtype E restricted to Africa [11] and subtype D in the Mediterranean countries. Our findings in this study suggest that the majority of infections in Kenya could be of subtype A and its variants. The clinical significance of HBV genotypes is a subject of discussion. It has been suggested that infection by HBV genotype A could be more frequently associated with chronic infection than genotype D [12, 13]. While genotype A appears to respond better to interferon treatment, compared to genotype D, it also generates a higher rate of viral resistance during treatment [14]. This genotype has also been shown to be the most prevalent among patients with acute hepatitis B [15]. Thus, the findings of our study suggest that most people in Kenya may develop chronic HBV infections and high viral resistance to treatment.

Hepatitis B screening is not a readily available test in Kenya for routine patients; most testing is done for safety of blood for transfusion. Normally, donors detected as positive for HBV infection during blood screening are not followed up and therefore no management of the infection is sought. Currently, interferon-a (IFN-a), lamivudine and adefovir have been licensed globally for the treatment of HBV. Thymosin-a1 has also been approved in more than 30 countries, mainly in Asia. Peginterferon-α-2a has been granted approval in some Asian and European countries and the approval process is underway in other countries [16]. Thus, even if treatment could be available, access to the treatment is limited due to lack of testing.

Most genotype A strains belong to subgroup or subgenotype A1; however, in our study 14 isolates (fig. 2) clustered closely with A2 subgenotype from South Africa suggesting that subgenotype A2 could be a prevalent subgenotype in Kenya. Six isolates clustered closely with subgenotype A2 reference from Zanzibar probably reflecting the effect of the coastal migratory route. Six genotypes clustered closely with genotype A from Uganda and the rest of the sequences (fig. 1, 2) could not be immediately placed to either subgenotype; however, some of these isolates clustered closely with A reference strain from out of Africa (D, E and some A genotypes). These isolates could not cluster with references from African countries, thus suggesting a distant link with Africa.

Genotype D was also detected in our study, this suggests that a significant proportion of this genotype could be circulating in Kenya. Genotype D is the most widely distributed genotype and has been found universally [13, 17] with highest prevalence stretching from Southern Europe and North Africa [18, 19] to India, in West and South Africa [20] and among intravenous drug users on all continents [21–23]. Thus, the finding of our study further seems to confirm this observation. Nevertheless, further studies are required to highlight HBV subtypes distribution in Kenya.

In our study 4 isolates were of genotype E as HBV genotype E strains are found exclusively in West and South Africa [5]. This genotype is restricted to Africa and little information is available on its clinical significance. However, our findings suggest that this genotype is also circulating in the eastern parts of Africa. From the results of this study, it appears that the HBV subtypes circulating in Kenya reflect the distribution of the genotypes in Africa.

It is important to note that all our isolates were from self-selected 'healthy' individuals in blood donor settings. It would be interesting to find out if the situation is different within the clinical settings and further elucidate the impact of diversity in HBV infections and disease management in Kenya and in this region. Cloned isolates gave different genotypes while the direct sequencing only grouped isolates into one genotype, thus cloning could be required for future work to further understand the complexity of genotypes in Kenya.

In conclusion, we have established the HBV genotypes and the existence of their variants in Kenya for the first

time. These findings suggest the existence of different genotypes and subgenotypes in Kenya. There is need to further monitor the diversity of HBV in the region since the cross-border effect, presence of refugees and selective presence of certain genotypes could have an impact on viral evolution, transmission and disease management. Because genotype may impact disease progression and response to treatment, additional studies are needed to add to these findings and improve treatment and prevention in our region.

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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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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.

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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can be completed in a single step at a constant temperature without a programmed thermal cycler. LAMP provides highly efficient DNA amplification, up to 10^9–10^{10} times in 15–60 min, and the concentration of the LAMP product is much higher than that generated by conventional polymerase chain reaction (PCR). Conventional PCR is relatively time consuming (3–4 h) and much more complicated than RT-LAMP, requiring several amplification steps and the use of a high-precision thermal cycler. The RT-LAMP assay has been validated and applied to the rapid detection of a number of RNA viruses, such as rubella virus (Mori et al., 2006), Japanese encephali-

Table 1
HIV-1 genotype data for 57 infected individuals from eastern Cameroon and the results of HIV-1 RT-LAMP.

Sample ID	Genetic subtype[a]				LAMP	
	gag	*pol*	*env*-C2V3	gp41	Tt[b]	EP
01CM2213	CRF_01.AE	na[c]	CRF_01.AEA	na	19.2[d]	P[e]
01CF2214	G	U	U	na	25.8	P
01CM2215	CRF_02.AG	na	CRF_02.AG	na	28.7	P
01CM2216	A	na	A	na	21.2	P
01CM2217	CRF_11.cpx	na	CRF_11.cpx	na	26.5	P
01CM2218	CRF_11.cpx	CRF_11.cpx	nd	U	31.0	P
01CM2219	CRF_11.cpx	na	CRF_02.AG	na	No Tt	P
01CM2220	CRF_02.AG	na	A	na	29.2	P
01CM2222	CRF_02.AG	na	CRF_02.AG	na	29.2	P
01CM2223	CRF_01.AE	na	CRF_02.AG	na	26.2	P
01CM2224	CRF_02.AG	na	CRF_02.AG	na	28.8	P
01CM2225	B	na	A	na	24.3	P
01CM2226	CRF_02.AG	na	CRF_02.AG	na	26.4	P
01CM2227	CRF_02.AG	na	CRF_02.AG	na	27.2	P
01CM2228	CRF_02.AG	na	CRF_02.AG	na	30.9	P
01CM2229	CRF_11.cpx	na	CRF_11.cpx	na	27.0	P
01CM2230	A	na	A	na	22.7	P
01CM2231	CRF_02.AG	na	A	na	23.4	P
01CM2232	B	U	A	U	No Tt	P
01CM2234	CRF_11.cpx	na	CRF_02.AG	na	26.0	P
01CM2235	B	U	nd	U	21.9	P
01CM2236	CRF_02.AG	na	CRF_02.AG	na	25.2	P
01CM2237	F2	na	F2	na	25.1	P
01CM2238	CRF_13.cpx	na	CRF_01.AE	na	22.2	P
01CM2239	CRF_13.cpx	na	CRF_11.cpx	na	26.2	P
01CM2240	CRF_02.AG	na	CRF_13.cpx	na	29.6	P
01CM2241	CRF_01.AE	CRF_11cpx	nd	U	27.5	P
01CM2242	CRF_02.AG	na	CRF_02.AG	na	24.8	P
01CM2243	CRF_11.cpx	CRF_11cpx	nd	CRF_11.cpx	24.7	P
01CM2244	CRF_01.AE	na	CRF_11.cpx	na	23.1	P
01CM2246	B	na	CRF_01.AE	na	23.6	P
01CF2247	CRF_11.cpx	na	CRF_01.AE	na	24.1	P
01CM2248	CRF_01.AE	na	A	na	21.9	P
01CM2249	A	na	A	na	23.6	P
01CM2250	CRF_02.AG	CRF_02.AG	nd	U	30.5	P
01CM2252	CRF_02.AG	U	nd	U	28.6	P
01CM2253	CRF_01.AE	U	nd	A	21.7	P
01CM2256	CRF_01.AE	na	A	na	21.6	P
01CM2257	CRF_01.AE	na	A	na	21.9	P
01CM2260	CRF_13.cpx	U	A	CRF_13.cpx	23.7	P
01CM2262	B	na	CRF_02.AG	na	27.8	P
01CF2268	CRF_02.AG	CRF_02.AG	nd	CRF_02.AG	32.5	P
01CM2269	CRF_11.cpx	CRF_11.cpx	nd	CRF_11.cpx	26.7	P
01CM2270	CRF_02.AG	CRF_02.AG	nd	U	31.9	P
01CM2271	CRF_11.cpx	CRF_02.AG	nd	CRF_11.cpx	23.9	P
01CM2272	CRF_11.cpx	na	CRF_11.cpx	na	21.2	P
01CM2273	CRF_11.cpx	na	CRF_11.cpx	na	25.5	P
01CM2274	CRF_02.AG	na	CRF_02.AG	na	22.6	P
01CM2275	CRF_09.cpx	CRF_02.AG	nd	CRF_09.cpx	24.5	P
01CM2276	CRF_11.cpx	na	CRF_11.cpx	na	23.9	P
01CM2277	CRF_11.cpx	CRF_11.cpx	nd	CRF_11.cpx	21.4	P
01CM2278	B	na	CRF_02.AG	na	24.2	P
01CM2280	CRF_11.cpx	CRF_02.AG	nd	CRF_02.AG	29.8	P
01CM2281	CRF_02.AG	CRF_02.AG	nd	CRF_02.AG	23.4	P
01CM2284	CRF_11.cpx	CRF_11.cpx	nd	CRF_11.cpx	24.5	P
01CM2287	CRF_11.cpx	na	CRF_01.AE	na	33.2	P
02CM319	nd[f]	O[g]	nd	O	No Tt	N[h]

[a] Genotyping based on part of *gag*-p24 (460 bp), *env*-C2V3 (approximately 550 bp), *pol*-integrase, and *env*-gp41 (approximately 405 bp) regions.
[b] Threshold time by LA-200.
[c] Not available.
[d] Agarose gel electrophoresis.
[e] Positive.
[f] Not detected.
[g] Group O.
[h] Negative.

tis virus (Toriniwa and Komiya, 2006), influenza virus (Ito et al., 2006), mumps virus (Okafuji et al., 2005), West Nile virus (Parida et al., 2004), severe acute respiratory syndrome corona virus (Hong et al., 2004; Poon et al., 2005), measles virus (Fujino et al., 2005), dengue virus (Parida et al., 2005), respiratory syncytial virus (Ushio et al., 2005), and HIV-1 (Curtis et al., 2008).

In the present study, another RT-LAMP assay was developed for the rapid detection of HIV-1 RNA. Its intended application is on-site confirmation of HIV diagnosis.

2. Materials and methods

2.1. Standard serum

WHO standard 97/656 (10^5 international units (IU) per vial, National Institute for Biological Standards and Control, Herts, UK) was used to determine the detection limit of the RT-LAMP assay (Davis et al., 2003; Holmes et al., 2001). The assay was carried out in octuplicate. The lowest concentration of genome copies with all octuplicate samples confirmed as positive was considered the detection limit.

2.2. Human plasma samples

Plasma samples were collected from 57 HIV-1-infected individuals in eastern Cameroon in 2001 (Ndembi et al., 2004) and 40 HIV-1-uninfected antenatal clinic attendees in western Cameroon in 2003. These samples were used to evaluate the sensitivity and specificity of HIV-1 RT-LAMP. In a previous study (Ndembi et al., 2004), phylogenetic analysis of genomic DNA samples from the 57 infected individuals revealed the presence of highly divergent strains of HIV-1 circulating in eastern Cameroon (Table 1). The 40 samples from uninfected individuals collected in 2003 were confirmed HIV-negative by HIV-Ab testing (AxSYM HIV1/2 and/or Determine HIV-1/2; Abbott Japan, Tokyo, Japan) and conventional PCR, as described previously (Ndembi et al., 2004).

2.3. RNA preparation

HIV RNA was extracted from plasma as follows: 200 μl of plasma was incubated with 400 μl of lysis buffer consisting of 10 mM Tris–HCl (pH 8.0), 68% (w/v) guanidine isothiocyanate, 3% (w/v) dithiothreitol, and 4 μl of co-precipitant (10 mg/ml amylopectin azure) at 25 °C for 10 min. HIV RNA was precipitated by adding 600 μl of isopropanol and centrifuging at 20,000 × *g* for 15 min. The RNA pellet was washed with 70% ethanol and resuspended in 10 μl of RNAse-free and DNAse-free water.

2.4. Primer design

A set of primers that recognizes eight distinct target sites in the HIV-1 *pol*-integrase gene, a well-conserved region of HIV-1 genome, was designed based on the HIV-1 genome sequence (GenBank accession number K02013) using a primer-designing software program for LAMP (Primer Explorer ver. 2.0; Net laboratory, Japan, http://venus.netlaboratory.com; Table 2). The set consisted of the six following primers: a forward inner primer (FIP), backward inner primer (BIP), two outer primers (F3 and B3), and two loop primers (loop F and loop B). Two additional inner primers comprise the combination of two functionally different primer parts: FIP consists of F1c (complementary to F1) and F2 and BIP consists of B1c (complementary to B1) and B2. The sequences of the two loop primers are complementary to the primers located between regions corresponding to F1 and F2 primer sequences.

2.5. RT-LAMP assay

The RT-LAMP reaction was carried out in 25 μl using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan) containing FIP (40 pmol), BIP (40 pmol), F3 (5 pmol), B3 (5 pmol), loop F (40 pmol), loop B (40 pmol), *Bst* DNA polymerase (16 U), AMV reverse transcriptase (2 U), and 5 μl of target RNA. The reaction mixture was incubated at 60 °C for 60 min in a Loopamp real-time turbidimeter (LA-200; Teramecs, Kyoto, Japan; Fig. 1A). A turbidity value of more than 0.1 was considered positive. The amplified products of RT-LAMP were resolved by 2% agarose gel electrophoresis (Agarose S; Wako Pure Chemical Industries, Ltd., Osaka, Japan); the gel was stained with ethidium bromide and visualized using an ultraviolet (UV) transilluminator (Fig. 1B). The turbidity of the amplified products was also ascertained by naked eye. The amplified products were inspected further under UV irradiation with or without adding ethidium bromide, an intercalating dye, when RT-LAMP assay was carried out in the presence of Fluorescent Detection Reagent (Eiken Chemical Co., Ltd., Tokyo, Japan; Fig. 1C).

3. Results

3.1. Development of the HIV-1 RT-LAMP assay

Using the primer sets targeting the HIV-1 *pol*-integrase gene (Table 2), a one-step RT-LAMP assay for the rapid detection of HIV-1 RNA was standardized. The success of amplification was assessed using a real-time turbidimeter (LA-200; Fig. 1A). Threshold time (Tt), the time required for the turbidity value to exceed 0.1, is shown in Table 1. Amplification was also detected by the presence of a ladder-like pattern on a 2% agarose gel. The ladder-like pattern results from a mixture of stem-loop DNAs of various stem lengths and cauliflower-like structures with multiple loops (formed by annealing between alternately inverted repeats of the target sequence in the same strand; Fig. 1B). Furthermore, amplification was detected by naked eye inspection of turbidity; visual detection was enhanced further by the addition of Fluorescent Detection Reagent and/or the intercalating dye under UV irradiation (Fig. 1C).

3.2. Sensitivity and specificity of the HIV-1 RT-LAMP assay

The sensitivity of the RT-LAMP assay for detecting HIV-1 RNA was determined using RNA from WHO standard HIV-1 97/656 (10^5 IU/vial) diluted to 6000, 600, 240, 120, 90, and 60 copies/ml. One IU was reported to be equivalent to 0.62 genome copies (Davis et al., 2003). The assay was carried out in octuplicate using viral RNA extracted from the equivalent of 100 μl of diluted serum. The reproducible 100% detection limit of the RT-LAMP assay was 120 copies/ml.

Of the 57 HIV-1-positive samples, 54 were positive for RT-LAMP in 19.2–33.2 min as assessed by turbidity using the LA-200 detec-

Table 2
Sequences of primers used for HIV-1 RT-LAMP.

Primer name	Sequence	Genome position[a]
F3	5′-GGTAAGAGATCAGGCTGAACATC-3′	4721–4743
F2	5′-AGACAGCAGTACAAATGGCA-3′	4747–4766
Loop F	5′-TTAAAATTGTGGATGAAT-3′	4786–4769
F1c	5′-CCCCAATCCCCCCTTTTCTT-3′	4806–4787
B1c	5′-AGTGCAGGGGAAAGAATAGTAGAC-3′	4812–4835
Loop B	5′-GCAACAGACATACAAACTAAAG-3′	4842–4863
B2	5′-CTGCTGTCCCTGTAATAAACCC-3′	4921–4900
B3	5′-GCTGGTCCTTTCCAAAGTGG-3′	4945–4926
FIP	F1c+F2	
BIP	B1c+B2	

[a] In HIV-1_{HXB2}.

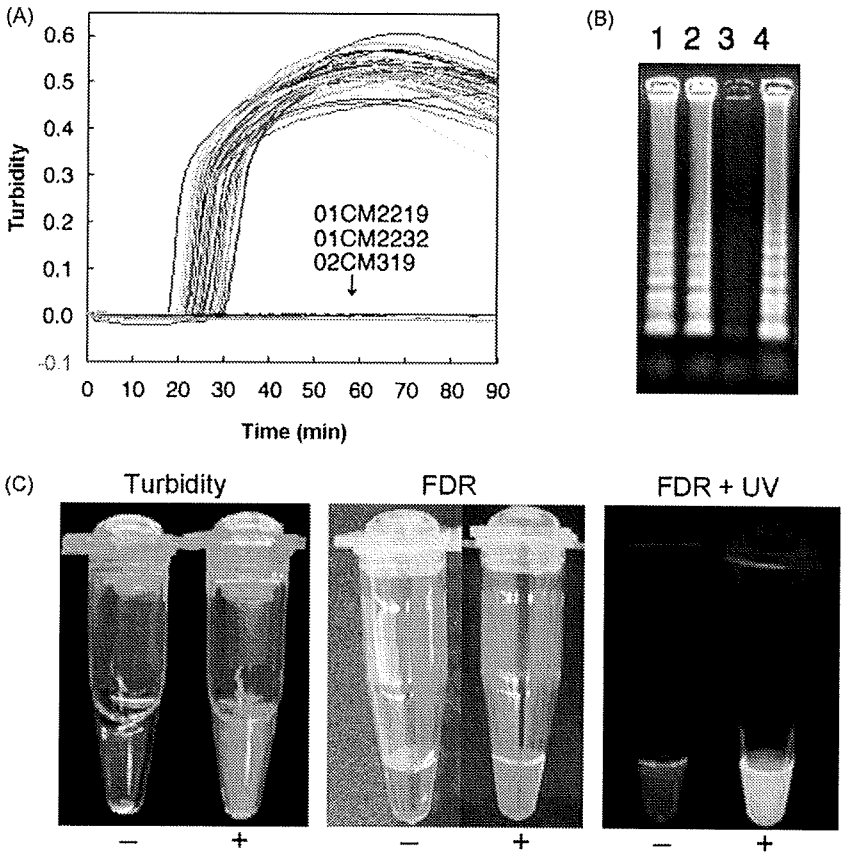

Fig. 1. Real-time detection of HIV-1 RT-LAMP products of 57 HIV-1-positive samples from Cameroon by turbidimeter (LA-200). (A) Agarose gel electrophoresis of HIV-1 RT-LAMP products that were undetectable by LA-200. A turbidity value of more than 0.1 was considered positive. Turbidity of three samples (01CM2219, 01CM2232, and 02CM319) was less than 0.1. (B and C) Representative pictures of HIV-1 RT-LAMP products with (B) and without (C) Fluorescent Detection Reagent. (B) Lane 1: 01CM2219; lane 2: 01CM2232; lane 3: 02CM319; and lane 4: 01CM2213 (positive control). (C) HIV-1 RT-LAMP positive (+) and negative (–). FDR: Fluorescent Detection Reagent; UV: ultraviolet irradiation.

tion system (Table 1 and Fig. 1A). HIV-1 RT-LAMP products of the two samples that were not detected by the real-time turbidimeter (01CM2219 and 01CM2232) could be detected by agarose gel electrophoresis (Fig. 1B) and by the naked eye after adding the intercalating dye under UV irradiation in the presence of Fluorescent Detection Reagent (data not shown). The remaining sample (02CM319) containing HIV-1 group-O RNA was RT-LAMP negative (Table 1 and Fig. 1B). Thus, all 56 samples that harbored HIV-1 group-M were positive by HIV-1 RT-LAMP assay.

Plasma specimens obtained from 40 pregnant women without HIV infection were also subjected to RT-LAMP and all were confirmed negative.

4. Discussion

An RT-LAMP assay was developed to detect HIV-1 RNA. This method was simple, rapid, and highly sensitive and specific for group-M HIV-1. Therefore, the HIV-1 RT-LAMP assay can be used as a rapid confirmatory test for HIV-1 group-M infection.

The HIV genome is usually detected by RT-PCR and PCR performed on plasma RNA and proviral DNA, respectively. These methods require at least 2–3 h despite the implementation of real-time PCR. In this study, the HIV-1 RT-LAMP assay was completed within 35 min, considerably faster than by RT-PCR or PCR. In addition, unlike RT-PCR and PCR, a simple apparatus such as a water bath can be used to maintain the constant incubation temperature at 60 °C.

The RT-LAMP reaction yields a white precipitate of magnesium pyrophosphate in the reaction mixture, indicating a positive result. This white precipitate is easily detected by the naked eye (Fig. 1C); thus, the results of the assay can be assessed without a turbidimeter. Although the amount of HIV-1 RT-LAMP products was monitored by a real-time turbidimeter (LA-200) in the current study, the results of visual inspection were consistent with those determined by turbidimeter (data not shown). According to the manufacturer's instructions for the Loopamp DNA amplification kit, visual detection can be enhanced by the addition of Fluorescent Detection Reagent to the reaction mixture. Interestingly, HIV-1 RT-LAMP products of the two samples that were undetectable by LA-200 (01CM2219 and 01CM2232) could be visualized by adding the intercalating dye under UV irradiation, when the assay was carried out in the presence of Fluorescent Detection Reagent. Thus, the HIV-1 RT-LAMP assay has the advantage of enabling the amplification of HIV-1 RNA and/or DNA in resource-limited settings in which sophisticated machines such as the thermal cycler and real-time turbidimeter are unavailable. In the two samples that were not detected by LA200, the production of magnesium pyrophosphate was prevented by unknown inhibitor(s). The cause and frequency of this phenomenon are under investigation.

RT-LAMP assay exhibits high specificity due to its use of multiple primers, including two loop primers, that recognize eight distinct regions of the target sequences. Previous studies in which RT-LAMP was used to detect various viral RNAs have documented the high specificity of RT-LAMP (Mori et al., 2006; Toriniwa and Komiya, 2006; Ito et al., 2006; Okafuji et al., 2005; Parida et al., 2004, 2005; Hong et al., 2004; Poon et al., 2005; Fujino et al., 2005; Ushio et al., 2005). Similarly, HIV-1 RT-LAMP analysis of 40 sero-negative

and PCR-negative samples showed 100% specificity, making the RT-LAMP assay ideal for confirming diagnosis.

The 100% detection limit of the HIV-1 RT-LAMP assay was found to be 120 copies/ml (12 copies/100 μl/assay). This sensitivity is inferior to the quantification limit (50 copies/ml) of the UltraSensitive Assay of the COBAS AMPLICOR HIV-1 MONITOR test, v 1.5 (Roche), but superior to the detection limit of the Standard Assay in the kit (400 copies/ml), and typical RT-PCR assays. Furthermore, the sensitivity of the current HIV-1 RT-LAMP could be improved to reach or exceed that of the UltraSensitive Assay by using a larger initial plasma sample (more than 240 μl) for extracting viral RNA.

The HIV-1 RT-LAMP assay was evaluated using 57 HIV-1 strains belonging to nine different group-M subtypes/CRFs and one group O based on *gag* and *pol* sequences, respectively (Table 1): subtypes A ($n=3$), B ($n=6$), F2 ($n=1$), G ($n=1$), CRF_01AE ($n=8$), CRF_02.AG ($n=17$), CRF_09.cpx ($n=1$), CRF_11.cpx ($n=16$), CRF_13.cpx ($n=3$), and group O ($n=1$; Ndembi et al., 2004). This assay system identified all of the 56 group-M HIV-1 strains despite their diversity, but did not detect the group-O strain, indicating that the primers used in the current HIV-1 RT-LAMP assay were group-M specific. Thus, in order to detect not only all of the HIV-1 groups but also HIV type-2 strains as well, the design of universal primer set will be necessary.

Although the viral RNA extraction method used in this study is relatively easy and cheap as compared to conventional methods, it still requires knowledge and training not usually available in resource-limited settings. Therefore, it will be necessary to revise and simplify the extraction method in order to use this assay as a confirmatory test for HIV diagnosis in the field. Future evaluation of the direct use of plasma or serum after heating as a test material is warranted (Curtis et al., 2008).

In conclusion, a one-step RT-LAMP assay for detecting group-M HIV-1 has been developed. The RT-LAMP assay is simple, rapid, and highly sensitive and specific for group-M HIV-1; therefore, this assay can be used to confirm group-M HIV-1 diagnosis. Once the RNA extraction method is simplified, the group-M HIV-1 RT-LAMP assay will be ideal for use in resource-limited settings.

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High-Risk HPV Types in Lesions of the Uterine Cervix of Female Commercial Sex Workers in the Philippines

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In order to prevent cervical cancer, vaccines against human papilloma virus types 16 (HPV-16) and 18 (HPV-18) have been implemented worldwide. However, the HPV types that cause cancer can differ according to geographical area and ethnicity. In this new era of the HPV vaccine, it is important to elucidate the prevalent HPV types in each area. Therefore, the prevalence of HPV infection and cervical abnormalities among 369 female commercial sex workers in the Philippines were examined. HPV *L1* gene was amplified by polymerase chain reaction (PCR) using modified GP5+/6+ primers, and genotyping was performed by sequencing cloned PCR products. HPV DNA was detected in 211 (57.2%) women, among whom 46 HPV types were identified. HPV-52 was most common and multiple-type infection was observed in 44.5%. Among 56 women with abnormal cervical cytology (low- and high-grade squamous intraepithelial lesions and adenocarcinoma in situ), HPV-52 was most common (23.2%), followed by HPV-16 (19.6%), -58 (10.7%), and -67 (10.7%). Only 27% of these women were positive for HPV-16 and -18. Multivariate analysis revealed that HPV-16, -39, -52, -67, and -82 were significantly associated with abnormal cytology. Repeated analysis of HPV-52 single-positive samples using the original GP5+/6+ PCR primers produced negative results in 57% of cases, suggesting that the prevalence of HPV-52 infection may have been underestimated in previous studies, and the current vaccines may not be sufficient for preventing infection and the development of premalignant lesions of the cervix in women in the Philippines. ***J. Med. Virol. 81:545–551, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HPV high-risk type; cervical cytology; Philippines; female commercial sex workers

INTRODUCTION

Human papillomavirus (HPV) is the most important risk factor for cervical cancer [Muñoz et al., 2003], which is the second most common malignancy and the third most common cause of cancer-related death in women [Parkin et al., 2005]. The incidence and mortality of cervical cancer are very high in women of reproductive age, especially in developing countries [Parkin et al., 2005; Frain et al., 2006]. Cervical cancer screening using cytological testing and HPV vaccination are paramount for preventing cervical cancer in young women.

More than 40 HPV types have been identified in the mucosal epithelia of the human genital tract; these are classified into high-risk and low-risk types according to their ability to cause cancer [Muñoz et al., 2003]. HPV type 16 (HPV-16) is the most common high-risk type and is detected in 50–60% of high-grade squamous

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intraepithelial lesions and invasive cervical cancers [Muñoz et al., 2003; Wheeler, 2007]. HPV-18 is identified in 10–20% of cancers [Muñoz et al., 2003; Wheeler, 2007]. Thus, HPV-16 and -18 are thought to be responsible for about 70% of cervical cancer cases in many countries [Bosch et al., 1995; Brown et al., 1999; Muñoz et al., 2003].

To reduce the incidence of cervical cancer, vaccines against HPV-16 and -18 have been developed and have been found 100% effective in preventing infection by these HPV types [Harper et al., 2006; Wheeler, 2007]. However, the distribution of common HPV types may vary depending on the geographic area and ethnicity of the population. Thus, the impact of these HPV vaccines on the prevention of infection and cancer may differ in different areas. In Japan, the prevalence of HPV-16 and -18 in cancers and high-grade squamous intraepithelial lesions is approximately 50% and 33%, respectively [Sasagawa et al., 2001]. HPV-52 is more common than HPV-18 in Japan, Taiwan, and eastern Africa [de Sanjosé et al., 2007]. Thus, in order to estimate the effectiveness of the current HPV vaccines for preventing cervical cancer, it is essential to determine the predominant cancer-causing HPV type in each area.

Degenerate and/or consensus primers for polymerase chain reaction (PCR) have been used to amplify a variety of HPV types from clinical specimens. The GP5+/6+ primers that target the HPV *L1* gene have been considered one of the best primer sets for HPV PCR and have been used in many epidemiological studies. However, it has been reported that some HPV types, such as HPV-52, may not be amplified by GP5+/6+ PCR as effectively as HPV-16 and -18 because of sequence mismatches between the target gene and the primers [Matsukura and Sugase, 2004]. Therefore, the GP5+/6+ primers have been modified to broaden the spectrum of detectable HPV types [Yamada et al., 2008].

In a case–control study of the cause of cervical cancer in the Philippines [Ngelangel et al., 1998], the most common HPV type in women with squamous cell carcinomas was HPV-16 (42.9%), followed by HPV-18 (25.3%) and -45 (13.5%). HPV-45 (17.2%) was the most common type found in cytology-normal women (controls), followed by HPV-16 (14.3%) and -18 (14.3%). However, the original GP5+/6+ PCR primers were used in this study. Little other information about the prevalence of HPV types in premalignant lesions of the cervix in the Philippines is available.

In the current study, the prevalence of HPV types and their associations with abnormal cervical cytology among female commercial sex workers in the Philippines were examined. HPV types were detected by PCR using modified GP5+/6+ primers. The goal was to ascertain whether the current HPV vaccine is sufficient for preventing infection and the development of premalignant lesions of the cervix in the Philippines. The prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections and their association with HPV infection were also investigated.

SUBJECTS AND METHODS

Subjects and Sample Collection

Three hundred seventy female commercial sex workers who were attending the Makati Social Hygiene Clinic or its mobile clinic at a night bar in Manila, Philippines, for a regular check-up in January or July 2006 were enrolled in this study. Written informed consent was obtained from all participants.

Specially trained technicians used cervical brushes to collect two cervical specimens from each participant (Honest Uterine Cervical Brushes Type S, Honest Medical, Tokyo, Japan). The first sample was smeared onto a microscope slide, fixed with alcohol solution (Rapid Fix, Muto, Tokyo, Japan), and stained according to the Pap test. The second sample was suspended in 1 ml of cell lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 2% SDS) and stored at −80°C for DNA extraction.

Classification of Cervical Cytology

Cervical cytology was diagnosed according to the Bethesda system [Solomon et al., 2002] and classified as normal (negative for intraepithelial lesion or malignancy), atypical glandular cells/atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, or adenocarcinoma in situ.

Detection and Typing of HPV DNA

DNA was extracted from cervical cells using a DNA extraction kit (SMI test; Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. The quality of the extracted DNA was evaluated by amplifying the glyceraldehyde-3-phosphate dehydrogenase gene (primers: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′) [Fujimori et al., 2002]. All but one of the samples, (n = 369 of 370) were confirmed as adequate for HPV, *C. trachomatis*, and *N. gonorrhoeae* testing.

HPV DNA detection was carried out using three pairs of modified GP5+/6+ primers: GP5+M1-2 (5′-TTTRTTACTGTTGTWGATACTAC-3′); GP5+M2-2 (5′-TGTWACTGTTGTWGATACCAC-3′); GP5+M3-2 (5′-GTWACTGTTGTRGACACCAC-3′); GP6+M1-2 (5′-AATTGAAAWATAAACTGTAAWTCATATTC-3′); GP6+M2-2 (5′-GAAACATAAAYTGTAAATCAWATTC-3′); and GP6+M3 (5′-GAAAATYTGCAAATCAWACTC-3′).

These primers were designed to amplify a 140-bp fragment of the HPV *L1* gene. Amplification was performed as follows: one cycle at 95°C for 10 min followed by 45 cycles at 95°C for 30 sec, 45°C for 30 sec, and 74°C for 30 sec, with a final extension at 74°C for 10 min. The presence of HPV DNA was confirmed by ethidium bromide staining of the PCR products following agarose gel electrophoresis. HPV DNA-negative samples were retested using the original GP5+/6+ primers [de Roda Husman et al., 1995; van den Brule et al., 2002]. The PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and

sequenced as described previously [Ndembi et al., 2003]. The similarity between *L1* sequences obtained by PCR and those of various HPV genotypes in the GenBank database was determined by BLAST analysis (http:www.ncbi.nlm.nih.gov/BLAST/). Ten clones from each sample were analyzed. HPV types were classified as high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82), low-risk (HPV-6, -11, -40, -42, -43, -44, -54, -61, and -81), unknown risk (HPV-26, -30, -32, -34, -53, -55, -62, -66, -67, -70, -74, -83, -84, -86, -87, -90, -91, and -102), epidermodysplasia verruciformis (HPV-9 and -38), and unclassified types (JEB2 and unclassified), according to previous reports [Muñoz et al., 2003; Schiffman et al., 2005].

Detection of *C. trachomatis* and *N. gonorrhoeae*

C. trachomatis and *N. gonorrhoeae* were detected using the LAMP method as described elsewhere [Hong et al., 2004; Poon et al., 2005].

Statistical Analysis

Statistical analysis was performed using SPSS Version 15.0 J for Windows. Odds ratio (ORs) and 95% confidence intervals (CIs) were calculated as approximations of relative risks. Univariate analyses were performed to assess the association between HPV infection and demographic factors and between abnormal cervical cytology and HPV types. Any variables shown significant in univariate analysis were analyzed by a multivariate model. The level of statistical significance was set at $P < 0.05$.

Nucleotide Sequence Accession Numbers

GenBank accession numbers of the sequences reported in this study are EU911006–EU911930.

RESULTS

Risk Factors for Cervical HPV Infection

This study evaluated the presence of HPV, *C. trachomatis*, and *N. gonorrhoeae* DNA in cervical samples from 369 commercial sex workers (mean age ± SD: 24.5 ± 5.1 years; range: 18–40 years) working in Manila, Philippines. HPV DNA was detected in 198 of the 369 women by PCR using modified GP5+/6+ primers and in 13 of the remaining 171 women using the original GP5+/6+ primers. Ultimately, 211 (57.2%) women were positive for HPV DNA. *C. trachomatis* and *N. gonorrhoeae* DNA were detected in 84 (22.8%) and 24 (6.5%) women, respectively.

In order to determine the risk factors for cervical HPV infection, multivariate analysis using a logistic regression model was performed. Being 25 years of age and younger (OR: 2.5; 95% CI: 1.4–4.3) and having worked for at least 6 months to 2 years (OR: 3.3; 95% CI: 1.9–5.8) were significantly associated with HPV infection (Table I). Cervical *C. trachomatis* and *N. gonorrhoeae* infections,

TABLE I. Demographic Factors Associated With HPV Infection

Demographic factors	No. of subjects	No. of cases	%	Univariate analysis		Multivariate analysis		
				OR	95% CI	OR[a]	95% CI	*P*-value
Age (years)								
>25	127	59	47	1		1		
≤25	239	150	63	1.9	1.3–3.0	1.9	1.1–3.4	0.028
No answer	3	2	67					
Marital status								
Married	37	19	51	1		1		
Single	326	189	58	1.3	0.7–2.6	1.3	0.6–2.7	0.484
No answer	6	3	50					
Duration of sex work (months)								
≤6	120	58	48	1		1		
7–24	137	100	73	2.9	1.7–4.9	3.3	1.9–5.8	<0.0001
>24	97	45	46	0.9	0.5–1.6	1.5	0.8–2.8	0.212
No answer	15	8	53					
Age at first coitus (years)								
≥20	103	56	54	1		1		
<20	261	153	59	1.2	0.8–1.9	1.0	0.6–1.6	0.885
No answer	5	2	40					
C. trachomatis								
Negative	285	161	57	1		1		
Positive	84	50	60	1.1	0.7–1.9	1.2	0.7–2.1	0.507
N. gonorrhoeae								
Negative	345	195	57	1		1		
Positive	24	16	67	1.5	0.6–3.7	1.3	0.5–3.2	0.609

OR, odds ratio; CI, confidence interval.
[a]Adjusted for all other variables in the table.

marital status, and age at sexual debut were not significantly associated with HPV infection. Interestingly, having worked longer than 24 months did not increase the risk when compared with working at least 6 months (OR: 1.5; 95% CI: 0.8–2.8).

Profile of HPV Infection

Of the 211 women with HPV infection, 117 (55.5%) had a single-type infection and 94 (44.5%) had multiple-type infection. Among the infected women, 46 different HPV types were detected; HPV-52 (16.1%) was most prevalent, followed by HPV-66 (12.3%), -16 (11.8%), -45 (10.0%), and -67 (9.5%). One hundred eighty-two women (86.3%) were infected with high-risk types, 38 (18.0%) were infected with low-risk types, and 28 (13.3%) were infected with unknown-risk types. Among those with high-risk HPV infection, HPV-52 (18.7%) was the most common, followed by HPV-16 (13.7%) and -45 (11.5%). HPV-16 and -18 comprised only 20.3% of the high-risk HPV types (Fig. 1).

Risk Factors for Abnormal Cervical Cytology

Among the 369 women, 239 (64.8%) had normal cytology, 74 (20.1%) had atypical glandular cells/atypical squamous cells of undetermined significance, and the remaining 56 (15.2%) had abnormal cytology (low-grade squamous intraepithelial lesion, n = 42; high-grade squamous intraepithelial lesion, n = 12; adenocarcinoma *in situ*, n = 2). HPV DNA was detected in 91 (38.1%) of the 239 women with normal cytology, in 61 (82.4%) of the 74 women with atypical glandular cells/atypical squamous cells of undetermined significance, and in all (100%) of the 56 women with abnormal cytology. Stepwise regression analysis revealed that HPV infection was only the factor significantly associated with abnormal cytology ($P < 0.0001$; OR: 18; 95% CI: 7.6–52).

Twenty-five different HPV types were detected in the 56 women with abnormal cervical cytology. Of these types, HPV-52 was most prevalent (23.2%), followed by HPV-16 (19.6%), -58 (10.7%), -66 (10.7%), and -67 (10.7%). Multivariate analysis using a logistic regression model revealed that HPV-16, -39, -52, -67, and -82 were significantly associated with abnormal cytology (Table II).

PCR Using Modified Versus Original GP5+/6+ Primers

In order to evaluate the efficacy of the modified primers, HPV DNA samples from women with single-type infection according to PCR with modified GP5+/6+ primers were re-analyzed using the original GP5+/6+ primers (Table III). None (0%) of the seven women with HPV-16 infection, eight (57%) of 14 women with HPV-52 infection, and one (20%) of five women with HPV-67 infection tested negative for HPV DNA using the original GP5+/6+ PCR.

DISCUSSION

In this study, the prevalence of HPV infection among female commercial sex workers in the Philippines was 57.2%. The reported prevalences of HPV infection in this

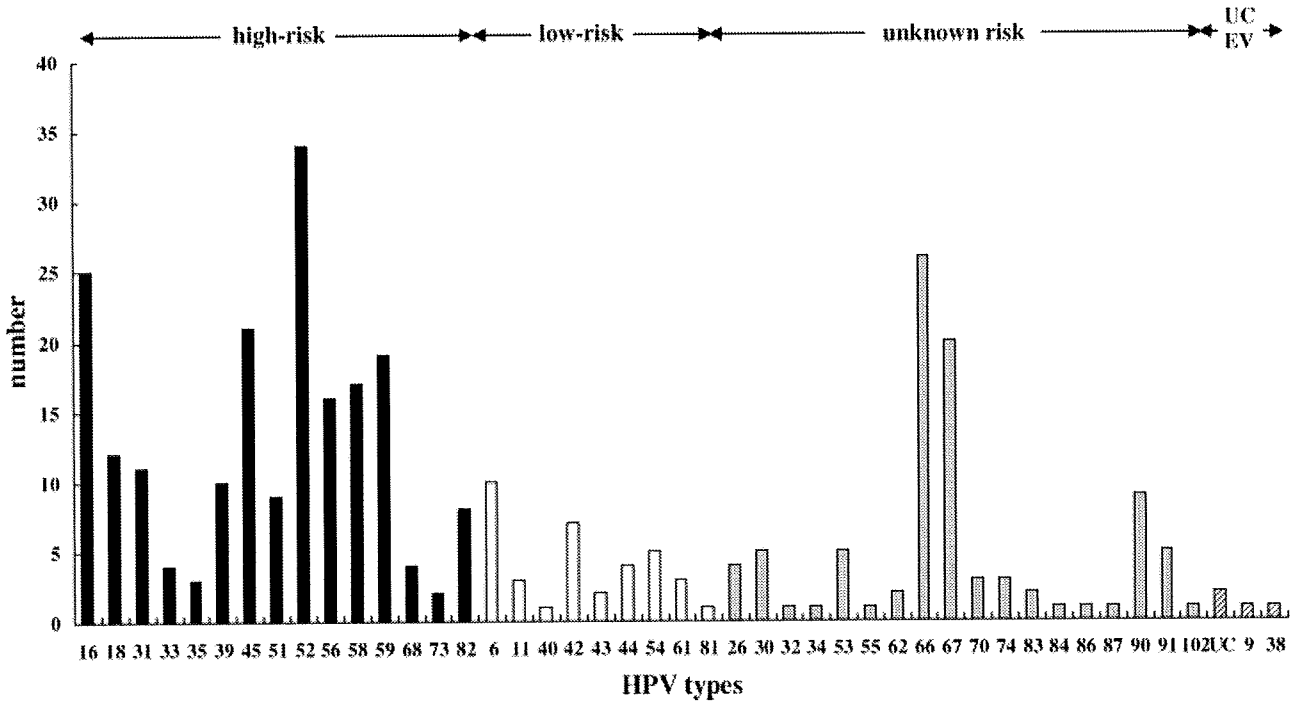

Fig. 1. The prevalence of HPV types among female commercial sex workers (n = 211) in the Philippines. ■, high-risk type; □, low-risk type; ▩, unknown-risk type; ▨, unclassified (UC) and epidermodysplasia verruciformis (EV) types.

TABLE II. HPV Types Associated With Abnormal Cervical Cytology*

HPV type	No. (%) of study participants positive for HPV DNA (n = 295)		Univariate analysis	Multivariate analysis	
	With abnormal cervical cytology (n = 56)	With normal cytology (n = 239)	OR (95% CI)	OR[a] (95% CI)	*P*-value
16	11 (19.6)	10 (4.2)	5.6 (2.2–14)	7.3 (2.7–20)	<0.0001
18	4 (7.1)	6 (2.5)	3.0 (0.8–11)		
26	1 (1.8)	2 (0.8)	2.2 (0.2–24)		
30	2 (3.6)	2 (0.8)	4.4 (0.6–32)		
31	3 (5.4)	5 (2.1)	2.6 (0.6–11)		
34	1 (1.8)	0 (0.0)	—		
35	1 (1.8)	1 (0.4)	4.3 (0.3–70)		
39	5 (8.9)	2 (0.8)	12 (2.2–62)	14 (2.4–83)	0.004
40	1 (1.8)	0 (0.0)	—		
43	1 (1.8)	1 (0.4)	4.3 (0.3–70)		
44	1 (1.8)	2 (0.8)	2.2 (0.2–24)		
45	4 (7.1)	11 (4.6)	1.6 (0.5–5.2)		
51	3 (5.4)	3 (1.3)	4.5 (0.9–23)		
52	13 (23.2)	9 (3.8)	7.7 (3.1–19)	10 (3.9–28)	<0.0001
53	1 (1.8)	4 (1.7)	1.1 (0.1–9.7)		
54	2 (3.6)	0 (0.0)	—		
56	5 (8.9)	9 (3.8)	2.5 (0.8–7.8)		
58	6 (10.7)	8 (3.3)	3.5 (1.2–10)	3.4 (0.96–12)	0.057
59	2 (3.6)	11 (4.6)	0.8 (0.2–4.0)		
66	6 (10.7)	11 (4.6)	2.5 (0.9–7.0)		
67	6 (10.7)	7 (2.9)	4.0 (1.3–12)	4.3 (1.2–15)	0.024
82	3 (5.4)	2 (0.8)	6.7 (1.1–41)	7.6 (1.0–55)	0.046
86	1 (1.8)	0 (0.0)	—		
90	1 (1.8)	6 (2.5)	0.7 (0.1–6.0)		
91	1 (1.8)	3 (1.3)	1.4 (0.1–14)		

OR, odds ratio; CI, confidence interval.
*Abnormal cervical cytology: low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, and adenocarcinoma in situ.
[a]Adjusted for HPV-16, -39, -52, -58, -67, and -82.

population in other countries are 28–39% in Spain [Cañadas et al., 2004; del Amo et al., 2005; Ortiz et al., 2006], 32% in Australia [Tideman et al., 2003], 47% in Korea [Choi et al., 2003], and 55% in Japan [Ishi et al., 2000]. Thus, the prevalence of HPV infection in this group of women in the Philippines is similar to that in Japan and Korea.

For the women in this group, being 25 years old or younger and having worked for a period of 6 months to 2 years were significantly associated with HPV infection, consistent with reports that the prevalence of HPV infection increases with decreasing age [Burk et al., 1996; Hassen et al., 2003; Matos et al., 2003; Baseman and Koutsky, 2005]. As expected, women who had worked longer than 6 months had a higher risk of being infected with HPV than did women who had worked <6 months. However, working longer than 2 years did not increase the risk of HPV infection, suggesting that these women may have acquired some immunity against common HPV types within 2 years of beginning this type of work. The role played by immunity against HPV in decreasing the risk of infection is supported by data from a recent report showing that age-dependent decrease in the prevalence of HPV is attenuated in HIV-infected women in Kenya [Yamada et al., 2008]. In the current study, no information was available for variables such as condom usage by sex partners, smoking, education, area of origin, and annual income,

TABLE III. Modified Versus Original GP5+/6+ PCR for Detecting HPV DNA

HPV infection	No. of positive samples					
	HPV-16 (n = 23)		HPV-52 (n = 34)		HPV-67 (n = 19)	
	Modified[a]	Original[b]	Modified	Original	Modified	Original
PCR positive	23	22	34	23	19	17
Multiple infection	16	15	20	17	14	13
Single infection	7	7	14	6	5	4

[a]Modified: PCR using modified GP5+/6+ primers.
[b]Original: PCR using original GP5+/6+ primers.

which could be factors associated with HPV infection. The absence of these data could limit the types of conclusions that can be drawn from this study.

In the current study, HPV genotyping was performed by sequencing cloned PCR products. In previous studies, direct sequencing or hybridization with HPV type-specific oligo-probes has been used for HPV genotyping. Although these methods are easier and quicker than the method used in this study, their results can sometimes be difficult to interpret [Qu et al., 1997; Coutlée et al., 2002; Perrons et al., 2002; Asato et al., 2004; Gheit et al., 2006]. The direct sequencing method rarely detects multiple-type HPV infection, whereas the hybridization method can detect only HPV types for which probes are available and cross-hybridization of type-specific probe with untargeted HPV types can occur in the dot-blot hybridization method. In contrast, sequencing of cloned PCR products can detect multiple-type HPV infection and identify distinct HPV types. In fact, 46 different HPV types and many cases of multiple-type HPV infections (44.5%) were identified in this study. Therefore, sequencing of cloned PCR products should be considered a preferred method for assessing HPV infection, especially multiple-type infections. However, unless a sufficiently large number of clones are analyzed, a number of types might not be detected, especially in cases of multiple infection with three or more types.

HPV-52 was found to be the most prevalent infecting HPV type in the Philippines; this is not the case in western countries. This difference could be due in part to differences in the methods for detecting HPV DNA. In this study, 57% of the women with single-type HPV-52 infection detected by the modified GP5+/6+ PCR were missed by the original GP5+/6+ PCR, which has been used in previous studies. In contrast, there was no significant difference in the detection of HPV-16 and -67 DNA between modified and original GP5+/6+ PCR, suggesting that the prevalence of HPV-52 may have been underestimated in previous studies.

In the current study, high-risk HPV types were detected in 86% of female commercial sex workers with HPV infection; however, HPV-16 and -18 were detected in only 20% of these women and the most prevalent HPV type was HPV-52. Although HPV-16 is known to be the most prevalent type worldwide [Muñoz et al., 2003; Wheeler, 2007], it has been reported that HPV-52 and -58 are also prevalent in Japan and South Taiwan [Asato et al., 2004; Inoue et al., 2006; Lin et al., 2006]. These results suggest that in addition to HPV-16, HPV-52 may be common in Asian countries in general.

HPV-16, -39, -52, -67, and -82 were found to be significantly associated with abnormal cytology in this study group. HPV-16 and -18 were detected in only 27% of the women with abnormal cervical cytology. Furthermore, HPV-18, -34, -45, and -59 were identified in cases of adenocarcinoma in situ. These results suggest that many high-risk types of HPV other than HPV-16 and -18 might play important roles in cervical carcinogenesis in the Philippines. This is the first study to examine the prevalence of HPV types and their association with abnormal cervical cytology in the Philippines.

The results from clinical trials of first generation vaccines in humans look promising. The data show that an HPV vaccine can prevent HPV infection and precancerous lesions in vaccinated women [Koutsky et al., 2002]. Most HPV vaccines target both HPV-16 and -18 and have been shown highly effective for preventing type-specific HPV infections [Harper et al., 2006; Wheeler, 2007]. However, in the current study, high-risk types of HPV other than HPV-16 and -18, such as HPV-39, -52, -67, and -82, were significantly associated with abnormal cervical cytology in women in the Philippines. In addition, it has been reported that in Japan not only HPV-16 and -18 but also HPV-31, -33, and -58 are significantly associated with cervical cancer (OR > 100) [Asato et al., 2004]. Therefore, the current vaccines might not be sufficient for preventing pre-malignant and malignant lesions of the cervix in women in Asia, although some cross protection of the vaccine has been reported [Harper et al., 2006]. Area-specific vaccines might be needed. Alternatively, the development of type-common HPV vaccines might be more ideal, in light of data showing that a type-common neutralization epitope exists in minor capsid protein *L2* and that a vaccine using the *L2*-epitope is expected to be effective in preventing infection by all high-risk types of HPV [Kondo et al., 2007].

In conclusion, this study determined that HPV-52 is the most prevalent infecting HPV type among female commercial sex workers in the Philippines and that several high-risk HPV types other than HPV-16 and -18 are significantly associated with abnormal cytology. Therefore, the current vaccines may not be sufficient to prevent HPV infection and the subsequent development of pre-malignant lesions of the cervix in women in the Philippines.

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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 ($n = 91$, mean age: 24.8 years old, age range: 17–42); (3) seafarers, who worked for marine companies ($n = 94$, 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′-ATGATAGGGGGAATTG 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.[13] 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 (X52154). 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% (21/91), 0% (0/94), 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]

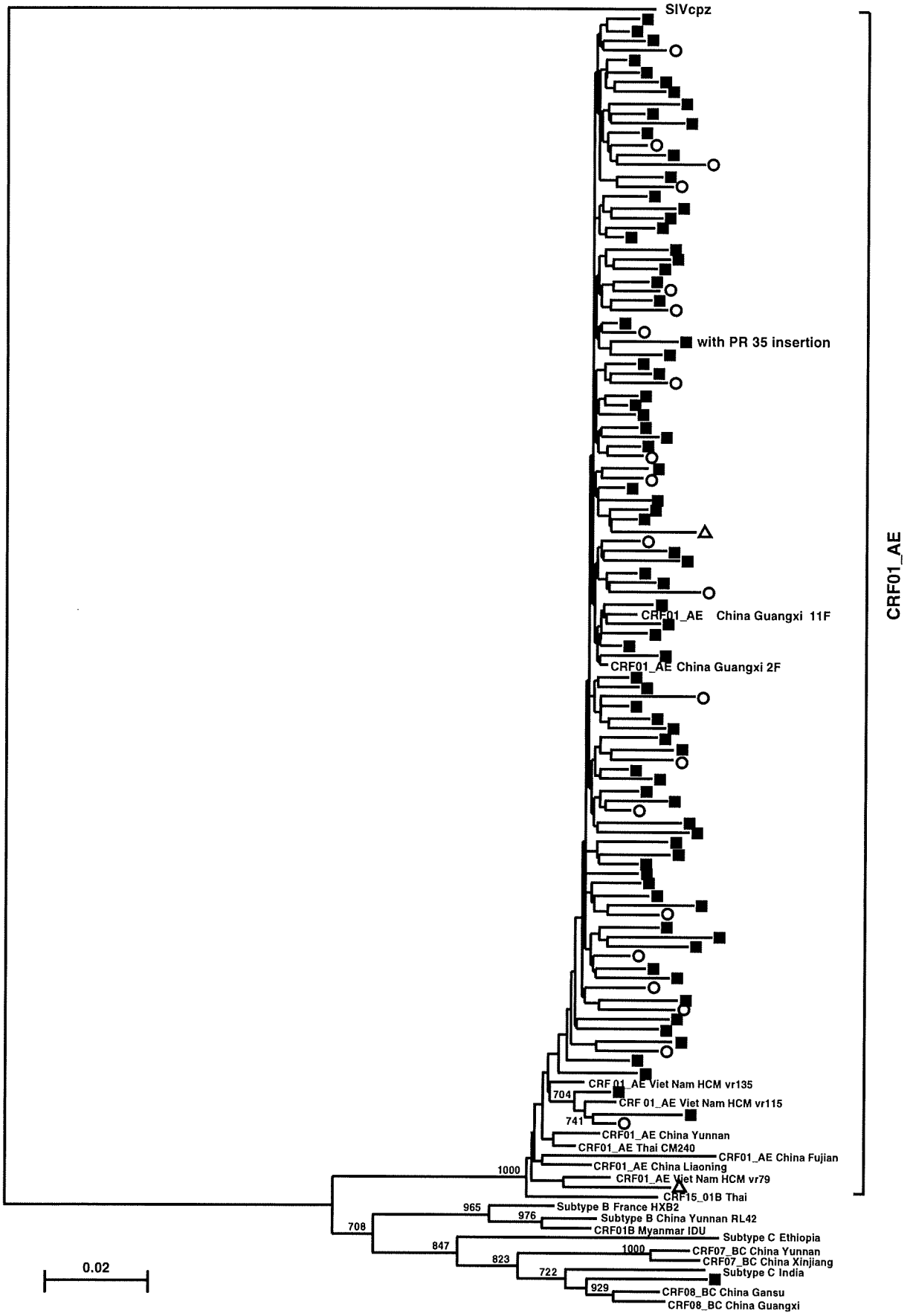

FIG. 1. Phylogenic tree of representative HIV-1 strains from IDU, all 21 strains from FSW, and two strains from blood donors, based on the *pol-PR* and *pol-RT* genes (approximately 957 bases). Filled squares, representative HIV-1 strains from IDU; open circles, HIV-1 strains from FSW; and open triangles, 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		271	T69	L74	V75	V118	**M184**	*L210*	**K219**
01_AE			N (1) S (2)	I (1)	G (1)	I (1)[b]	**I (1)**[c]	*M* (1)	**E (1)**
B+C		1							
B		1							

(C) Position		271	V90	**K103**	V 106	*V 179*	**G190**	*P225*
01_AE			I (1)	**N (3)**	I (4)	*D (4)* *F (1)* *E (1)* *A (1)*	**E (2)**	*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*	*N37*	*P39*	*R41*	*K43*	*K45*	*R57*	*Q61*	*K70*	*I72*	*L89*	*T91*	*Q92*
01_AE	290	R(15)	L (1) V (15)	E (1) M (2)	I (1) M (2) Q (4)	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 (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*	*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)	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