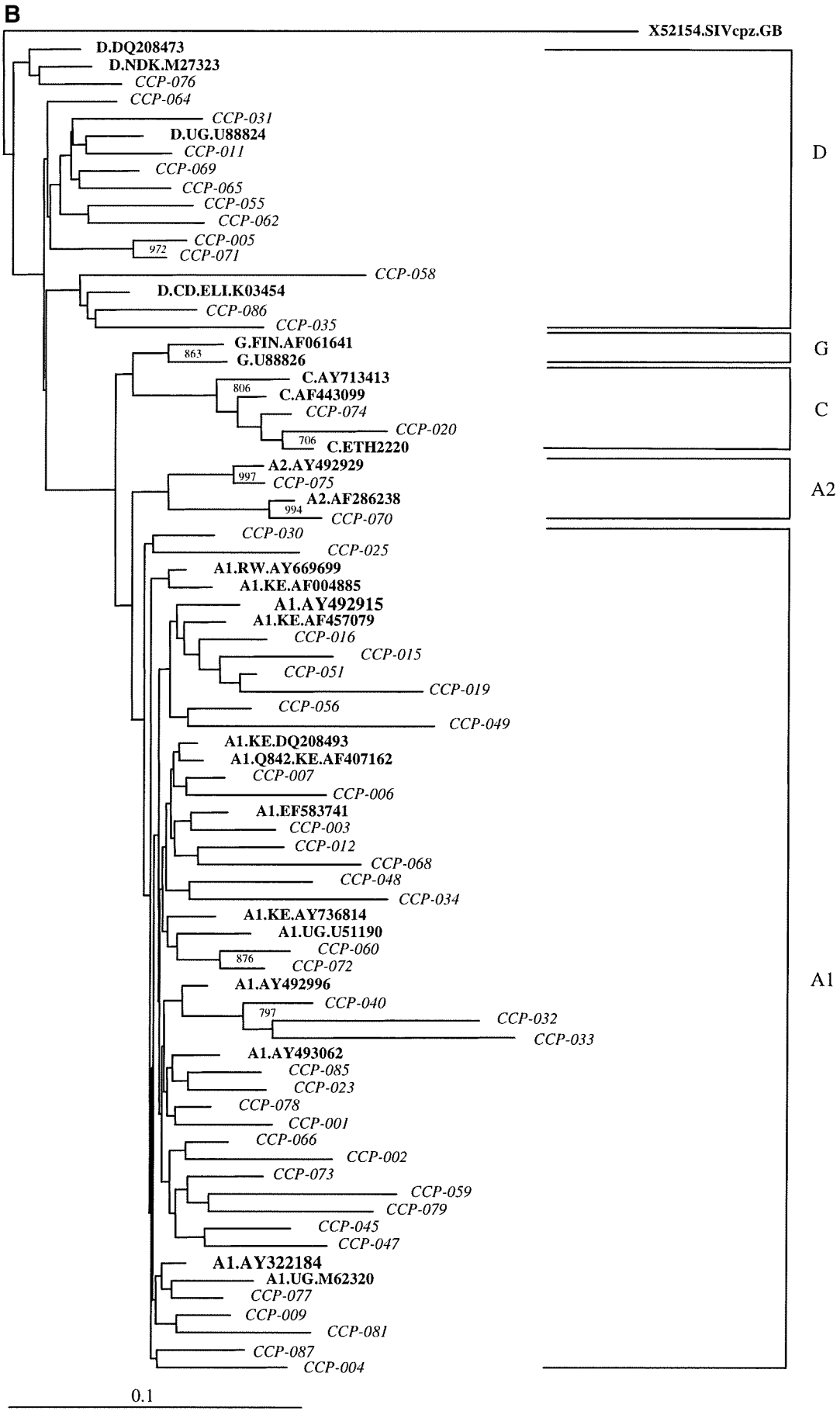

FIG. 1. (Continued).

Phylogenetic analysis

Neighbor-joining phylogenetic trees, including reference sequences from the Los Alamos database, were constructed using Clustal W (version 1.83) and inferred using Tree View (version 1.6.6; Institute of Biochemical and Life Sciences, Scotland, United Kingdom). Bootstrap resampling (1000 data sets) of multiple alignments was performed to test the statistical robustness of the trees.

Genotypic drug resistance analysis

Genotypic drug resistance in the *pol*-RT region was defined as the presence of one or more resistance-related mutations, as specified by the consensus mutation figures of the International AIDS Society-USA.[36]

Results

Study population

None of the patients reported prior exposure to ART and/or single dose NVP for PMTCT. Of the 87 samples obtained, 53 were successfully amplified and analyzed for HIV-1 *pol*-RT and *env-gp41*. The baseline characteristics of the study population are shown in Table 1. There was no significant difference between males and females with regards to the average age ($p = 0.45$), viral load ($p = 0.41$), or $CD4^+$ T cell counts ($p = 0.28$).

HIV-1 subtype distribution

Phylogenetic analyses of the 53 paired sequences revealed that 40 (75.5%) were concordant subtypes in both *pol*-RT and *env-gp41*, whereas 13 (24.5%) were discordant, suggesting possible recombination and/or dual infections (Fig. 1). The prevalent subtypes were A1/A1(*pol*-RT/*env-gp41*), 31 (58.5%); D/D, 9 (16.9%); A1/C, 2 (3.8%); A1/D, 4 (7.5%); G/A1, 2 (3.8%); A1/A2, 1 (1.9%); C/A1, 2 (3.8%); D/A1, 1 (1.9%); and D/A2, 1 (1.9%).

RTI resistance-associated mutations

Major RTI resistance-associated mutations were found in 4 (7.5%) of the 53 sequences (Table 2). Of these sequences, three had NRTI resistance-associated mutations: M184V, K65R, and D67N/K70R/K219Q. The NNRTI resistance-associated mutations K103N and Y181C were detected in three patients and one patient, respectively. Thus, multiple drug resistance mutations were detected in three of these drug-naive individuals. There was no relationship between subtypes and drug resistance mutations. There was no significant difference in the viral load ($p = 0.54$) or $CD4^+$ T cell counts ($p = 0.39$) of patients with ($n = 4$) and without ($n = 49$) drug resistance mutations (data not shown).

Discussion

In the current study, the prevalence of mutations that confer RTI resistance was found to be 7.5% among Kenyan drug-naive individuals sampled in 2005. This finding is consistent with the findings from Cameroon and Mozambique in 2004, which showed a drug resistance prevalence of 9.8% and 5.9%, respectively.[18,19] However, other studies in Africa found the prevalence to be less than 5%.[22–24] The difference may be due to the criteria used in the different studies. The latter studies were based on the WHO's HIV drug resistance threshold survey,[27–29] but the former and our study were hospital based, i.e., among patients seeking treatment. In this study, RTI-resistant mutants were detected in four patients. In particular, one patient had multiple NRTI drug resistance mutations. The NRTI resistance mutations have a higher fitness cost for the virus and would not develop in the absence of drug pressure.[37,38] Although mutations conferring NRTI resistance have previously been reported among drug-naive patients,[17–20,39,40] the possibility that our patients had previous unreported contact with antiretroviral drugs could not be excluded. Therefore, we may have overestimated the number of transmitted HIV drug-resistant strains in our study population. Considering the limitations of a self-reporting system, drug resistance testing would be necessary before initiating ART in order to achieve a better clinical outcome.

In this study, we did bulk sequencing of proviral DNA to investigate drug resistance mutations in the archived viruses, although the use of plasma viral RNA is the gold standard for drug resistance testing.[41] This method is reliable for detecting mutations in replication-competent virions in lymphocytes. However, bulk sequencing compromises the detection of a minor population of HIV-1 drug-resistant variants, which may exist in low copy numbers in drug-naive patients.[42] Under such circumstances, detection of minor viral variants using more sensitive methods, such as allele-specific PCR, single-genome amplification, and clonal or deep sequencing, would be more ideal.

The HIV-1 subtypes among the studied patients were A, C, and D together with their related recombinants, which is in agreement with previous findings in Kenya.[43,44] Although we employed a commonly used subtyping methodology, the absence or presence of recombination in the two gene

TABLE 2. DEMOGRAPHIC, IMMUNOLOGIC, AND VIROLOGIC CHARACTERISTICS OF KENYAN PATIENTS HARBORING MAJOR RTI MUTATIONS[a]

Sample ID	Age (years)/ gender	$CD4^+$ T cell counts (cells/mm^3)	HIV-1 RNA (log_{10} copies/ml)	HIV-1 subtype		RTI resistance mutations	
				pol-RT	env-gp41	NRTI	NNRTI
CCP-049	25/F	500	4.6	A1	A1	M184V	Y181C
CCP-055	37/M	233	5.2	D	D	K65R	K101P, K103N
CCP-068	28/F	288	4.4	A1	A1	D67N, K70R, K219Q	L100I, K103N
CCP-081	40/M	625	3.9	A1	A1	NONE	K103N

[a]RTI, reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; F, female; M, male.

fragments analyzed does not exclude the possibility of recombination elsewhere. There was no statistically significant relationship between subtypes and drug resistance mutations in our patients. This finding is consistent with previous findings that HIV-1 subtypes have common mutational pathways with shared genetic barriers to resistance,[45–47] though differences between subtypes B and C have been reported.[48,49]

The prevalence of HIV among Kenyan adults has remained relatively steady since 2003, after decreasing from a high of 14% in the late 1990s. The Kenya demographic and health survey of 2003 found a prevalence of 6.7% among individuals aged 15–49 years (4.6% in men and 8.7% in women).[26] Access to ART in Kenya has significantly increased since the start of WHO's 3 by 5 initiative. The Kenya AIDS indicator survey of 2007 showed that of the estimated 392,000 Kenyan adults in need of ART, 138,000 (35%) had received the treatment by September 2007, which increased to 212,000 (54%) by June 2008.[26] The increase in ART coverage is expected to lead to an increase in drug-resistant strains among drug-naive patients. In addition, stigma and cultural backgrounds still existing in Kenya may affect ART compliance, resulting in an accelerated appearance of drug-resistant mutants, which are a potential source of transmitted drug resistance.[26]

To our knowledge, this is the first report of HIV drug resistance among drug-naive adults in Kenya, though no PI sequencing was completed to assess for primary resistance. The observed prevalence of mutations conferring drug resistance prompts the establishment of strong intervention strategies to keep the circulation of drug-resistant strains low. Despite the lack of technical capacity[29] and the high cost of resistance testing in resource-poor countries, efforts to mitigate the impact of the pandemic through the surveillance and monitoring of HIV drug resistance have proven viable.[25–27] Continuous countrywide surveillance is required to determine the magnitude of transmitted drug-resistant mutants and viral evolutionary trends in Kenya. This study underscores the need to have readily available, high throughput drug resistance testing for the increasing number of infected individuals in order to effectively manage those initiating ART.

Sequence Data

The sequences discussed in this study were deposited into GenBank under accession numbers FJ599482–FJ599498 and FJ865363–FJ865396 for *pol*-RT and FJ599400–FJ599481 for *env-gp41*.

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Disclosure Statement

No competing financial interests exist.

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Tracking the Entry Routes of Hepatitis C Virus as a Surrogate of HIV in an HIV-Low Prevalence Country, the Philippines

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From 2002 to 2007, 1,590 individuals were enrolled in an active surveillance program conducted in Metro Cebu, Philippines, where the anti-HCV-positive rate was significantly and constantly high among injecting drug users (83%, 793/960; 71–88%), especially among those living in downtown (89%, 683/770; 87–100%), despite the extremely low percentage of anti-HIV-positives (0.34%, 3/874). Sampling areas were then enlarged nationwide and the number of samples increased to 2,645 at the end of 2007. A total of 444 samples were positive for HCV RNA. Phylogenetic analysis based on NS5B and E1–E2 regions revealed that the most dominant HCV subtype was 1a, and followed by 2b, 2a, and 1b, and that the HCV strains had the largest variety in Metro Manila and its vicinity ($P < 0.01$). Interestingly, subtype 1b was detected solely in Metro Manila, and four HCV strains collected in this area showed higher homology to specific foreign strains retrieved from the Genbank/EMBL/DDBJ database with bootstrap values of 68–95% comparing with other strains analyzed in this nationwide study. These data suggest that HCV strains may be introduced occasionally into the Philippines possibly through Metro Manila as a main entry point. Considering the fact that an HIV epidemic started primarily via contaminated needle sharing in Asia, the constantly high rate of HCV infections and the newly introduced foreign HCV strains in the absence of HIV epidemic warrant further investigation on HCV entry and spread for early detection of an HIV epidemic in the Philippines. ***J. Med. Virol. 81:1157–1162, 2009.*** © 2009 Wiley-Liss, Inc.

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KEY WORDS: HIV epidemic; HCV epidemic; injecting drug users; Philippines

INTRODUCTION

It is estimated by World Health Organization that approximately 33 million people were living with HIV and 2.5 million people acquired HIV infections worldwide in 2007. In Asia, approximately 5 million and 440,000 people were and became infected with HIV in 2007, respectively [UNAIDS/WHO, 2007]. Only a few countries still have a low HIV prevalence (<0.1% of HIV positives among population aged 15–49) in Asia, and the Philippines is one of them [UNAIDS/WHO, 2008a,b].

Injecting drug use is the first mode of transmission of HIV in many Asian countries and territories including China, Malaysia, Nepal, Vietnam, and Indonesia. Countries with extensive heterosexual spread of HIV, such as Thailand, Myanmar and India, also have high HIV prevalence rates in groups of injecting drug users. Injecting drug use may drive the growth of HIV epidemics in Asia. Therefore, intensive HIV

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surveillance has been conducted especially focusing on injecting drug users for early detection and prevention of HIV epidemics [Ghys et al., 2001; Aceijas et al., 2004; Ruxrungtham et al., 2004; MAP, 2005]. In addition to HIV surveillance, it may be crucial to simulate the entry routes of HIV into the high-risk population using surrogate markers for early detection and prevention. Hepatitis C virus (HCV) may be one of the most useful surrogate markers of HIV, because HCV transmits mainly by blood-borne pathways such as blood transfusions and sharing of unsterile needles/syringes [Memon and Memon, 2002; Poynard et al., 2003] just like HIV during the early phase of an epidemic.

A primary surveillance on HIV and HCV infections at a model sentinel site in the Philippines showed that HIV infection was very rare but that HCV was prevalent among injecting drug users (70%, 61/87) in 2002 [Agdamag et al., 2005]. Based on this primary surveillance, a chronological study has been conducted to assess the trend of the rates of HIV and HCV infections among those injecting drug users.

MATERIALS AND METHODS

Plasma Samples

Plasma samples were collected from three major islands of the Philippines, that is, Luzon (n = 216), Visayas (n = 1,911), and Mindanao (n = 518). Some samples (n = 49, 7, and 2) had been referred to the national reference laboratory from medical facilities in Luzon, Visayas, and Mindanao, respectively. The others were from dialysis centers in Luzon (n = 167) and collected during active surveillance periods conducted in Visayas (n = 1,904) and Mindanao (n = 516), respectively (Table I).

An active surveillance in Metro Cebu, Visayas, has been conducted since 2002. By the end of 2007, 1,590 individuals were enrolled in the surveillance and 1,904 samples were subjected to laboratory examination. The study population in this surveillance was categorized into six groups; injecting drug users, inhaling drug users, men who have sex with men, sex workers, antenatal clinic attendees, and others. Injecting drug users were subcategorized into three; inhabitants of cities in Metro Cebu, patients in drug rehabilitation centers, and detainees in jails (Table II).

This study was approved by the institutional review board of Kanazawa University, Japan, and also reviewed by the Department of Health, Philippines, and Cebu Medical Society/Cebu Chapter of Philippine Society of Microbiology and Infectious Diseases, Philippines. Active surveillance in Metro Cebu and in dialysis centers in Metro Manila was performed in a linked and anonymous way. An unlinked and anonymous study was conducted in nationwide surveillance. All the procedures were conducted according to the principles of the Declaration of Helsinki.

Serological Tests

Plasma was separated from whole blood with EDTA and subjected to the following tests.

Determine HIV-1/2 (Abbott Japan, Tokyo, Japan), Genscreen Ultra HIV Ag-Ab Assay (Bio-Rad, Marnes La Coquette, France), and HCV-PHA "Abbott" were used for the detection of anti-HIV antibody and anti-HCV antibody, respectively. Assays were performed according to the manufacturer's instructions.

RNA Extraction, Reverse Transcription, and Polymerase Chain Reaction (PCR)

HCV-RNA was extracted from 100 μl of plasma using SMITEST EX-R&D (Genome Science Laboratories, Fukushima, Japan), and reverse-transcribed according to First-Strand cDNA Synthesis protocol with random hexamer (Invitrogen, Carlsbad, CA).

A part of the NS5B region of HCV gene was amplified by nested PCR with primers, hep31b/hep32 in the first round, and hep33b/hep34b in the second round as

TABLE I. HIV and HCV Infections at Sentinel Sites in Luzon, Visayas, and Mindanao (Philippines)

	Anti-HIV-positive samples/tested in		Anti-HCV-positive samples/tested in		
Population	Visayas	Mindanao	Luzon	Visayas	Mindanao
Injecting drug users	3/874	0/499	Not done	793/960	2/516
Inhaling drug users	0/147	Not done	Not done	4/152	Not done
Sex workers	0/130	Not done	Not done	0/170	Not done
Men who have sex with men	Not done	Not done	Not done	5/54	Not done
Antenatal clinic attendees	0/200	Not done	Not done	0/200	Not done
Dialysis patients	Not done	Not done	51/167	Not done	Not done
Others	0/364	Not done	Not done	6/368	Not done
Referral with unknown risks	Not done	Not done	49/49	7/7	2/2
Total	3/1,715	0/499	100/216	815/1,911	4/518
First visit clients	1,401	0	122	1,590	0
Second	54	0	31	54	0
Third	9	0	14	9	0
Fourth	1	0	0	1	0
Unlinked referral	0	0	49	7	2
Unlinked survey	250	499	0	250	516
Total	1,715	499	216	1,911	518

TABLE II. Chronological Observation on the HIV and HCV Infections in a Sentinel Site, Metro Cebu, Visayas (Philippines)

Population collection site	Anti-HIV-positive samples/tested (%)				Anti-HCV-positive samples/tested (%)			
	2002–2003	2004–2005	2006–2007	Total	2002–2003	2004–2005	2006–2007	Total
Injecting drug users	0/120	2/385 (0.52)	1/369 (0.27)	3/874 (0.34)	85/120 (71)	384/471 (82)	324/369 (88)	793/960 (83)
Downtown	0/28	2/357 (0.56)	1/361 (0.28)	3/746 (0.40)	28/28 (100)	333/381 (87)	322/361 (89)	683/770 (89)
Rehabilitation centers	0/92	0/28	ND	0/120	57/92 (62)	34/62 (55)	ND	91/154 (59)
Jails	ND	ND	0/8	0/8	ND	17/28 (61)	2/8 (25)	19/36 (53)
Inhaling drug users	0/56	0/1	0/90	0/147	3/56 (5.4)	1/6 (17)	0/90	4/152 (2.6)
Sex workers	0/130	ND	ND	0/130	0/130	0/40	ND	0/170
MSM	ND	ND	ND	ND	ND	5/54 (9.3)	ND	5/54 (9.3)
Antenatal clinic attendees	0/100	0/100	ND	0/200	0/100	0/100	ND	0/200
Others	0/209	0/138	0/17	0/364	4/209 (1.9)	2/142 (1.4)	0/17	6/368 (1.6)
Total	0/615	2/624	1/476	3/1,715	92/615	392/813	324/476	808/1,904
First visit clients	615	591	195	1,401	615	780	195	1,590
Second	0	33	21	54	0	33	21	54
Third	0	0	9	9	0	0	9	9
Fourth	0	0	1	1	0	0	1	1
Unlinked survey	0	0	250	250	0	0	250	250
Total	615	624	476	1,715	615	813	476	1,904

MSM, men who have sex with men. ND, not done. Clients subjected to the tests at second, third, and fourth visit were all injecting drug users living in downtown.

described previously [White et al., 2000]. Nested PCR was performed with 20 μl reaction mixture containing 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM primers and one unit of Amplitaq Gold® (Applied Biosystems, Foster City, CA). First-round PCR was done with one cycle of 94°C for 10 min, and 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec with a final extension of 72°C for 10 min. Second-round PCR was done under the same conditions except for the annealing temperature at 60°C. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. [White et al., 2000].

A part of the E1–E2 region including hypervariable region 1 was amplified by nested PCR with primers, Lqz188/Lqz187 in the first round, and Lqz189/Lqz187 in the second round as described previously [Zhang et al., 2004]. The thermal cycling program for the E1–E2 region was done under the same conditions as that for the amplification of an NS5B region except for the annealing temperature of 50°C and the extension time of 60 sec.

Determination of Nucleotide Sequences and Phylogenetic Analysis

The PCR products were subjected to the direct sequencing method using BigDye terminator v1.1 Cycle sequencing Kit (Applied Biosystems) with the primers of hep33b/hep34b and Lqz187/Lqz189 for NS5B and E1–E2 regions, respectively. The sequencing results were aligned with the ones retrieved from Genbank/EMBL/DDBJ database by ClustalW with subsequent inspection and manual modification [Thompson et al., 1994]. The frequency of nucleotide substitution in each base of the sequences was estimated by the Kimura two-parameter method. A phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications. The program of Njplot was then used for drawing the trees [Perriere and Gouy, 1996].

Statistical Analysis

The χ^2-test was used to assess the difference between two populations and P-values <0.01 were considered to be significant.

Accession Numbers

The sequences described in this article have been deposited in GenBank/EMBL/DDBJ under accession numbers DQ364460, DQ648505-DQ648517, and EU123078-EU123302.

RESULTS

Prevalence of HIV and HCV Infections in an Active Surveillance Site

Only three anti-HIV-positive samples were observed during the 6-year study period (3/1,715, Table II) in an active surveillance site (Metro Cebu, Visayas). Those three samples were collected from injecting drug users (3/874), and were also positive for anti-HCV. None of the other tested populations (n = 841) including sex workers (n = 130) were reactive for anti-HIV (Table II).

Despite the extremely low percentage of anti-HIV-positive results among injecting drug users (0.34%, 3/874), the anti-HCV-positive rate was significantly high (83%, 793/960 where 86 samples were not tested for anti-HIV; 84%, 734/874) (Tables I and II). In particular, the anti-HCV-positive rate in downtown (89%, 683/770) was higher than that in drug rehabilitation centers or jails (58%, 110/190) ($P < 0.01$), and was constant (87–100%) in the chronological observation (Table II).

Drug users with injecting habits (83%, 793/960) had higher positive rate of anti-HCV than those with inhaling habits (2.6%, 4/152) ($P < 0.01$) (Table II).

The positive rate for anti-HCV among men who have sex with men followed that among injecting drug users, however, it was much lower at 9.3%. No sex workers or antenatal clinic attendees showed positive results for anti-HCV. Six of 368 individuals categorized in others were positive for anti-HCV (Table II). However, the risk of HCV infection among these six individuals has not been clarified yet.

HCV Strains in the Philippines

A total of 444 HCV RNA samples collected in the nationwide surveillance were amplified successfully by RT-PCR and subjected to the determination of NS5B nucleotide sequence. The NS5B (328 bases, position 8279–8606 in H77 strain) sequences of subtype 1a (n = 28, 256, and 30 from Luzon, Visayas, and Mindanao, respectively), subtype 1b (n = 11, 0, and 0), subtype 2a (n = 5, 16, and 0), subtype 2b (n = 2, 94, and 0), genotype 4 (n = 1, 0, and 0), and genotype 6 (n = 1, 0, and 0) were determined (Table III and Fig. 1A). The most dominant HCV genotype (subtype) was 1 (1a) in every area (Luzon, Visayas, and Mindanao), and HCV strains circulating in Luzon had the largest variety among those in the Philippines ($P < 0.01$). Indeed, subtype 1b has only been detected in Luzon (Table III).

The NS5B-positive samples were further subjected to the determination of E1-E2 sequence including hypervariable region 1 (538 bases, position 1322–1859) (Fig. 1B). Four strains showed discordant genotype results between NS5B and E1–E2 regions. One of these strains was from Metro Manila, Luzon, consisting of subtype 2b in E1-E2 and subtype 1b in NS5B (2b-1b, n = 1) [Kageyama et al., 2006], and the other three were from Metro Cebu, Visayas, (1a-2b, n = 2; 2b-1a, n = 1).

The reference strain with the highest homology score to each analyzed HCV strain was retrieved from the GenBank/EMBL/DDBJ database. Four strains had higher homology to specific strains of foreign origin rather than to any other strains collected in the Philippines (Fig. 1, insets).

Viral RNA extracted from three plasma samples positive for both anti-HCV and anti-HIV (Tables I and III) was subjected to HIV RT-PCR with primers for the amplification of HIV-1 *gag* [Heyndrickx et al., 2000] and *pol* [Miura et al., 1990] regions. However, HIV-RNA could not be amplified eventhough HCV-RNAs from the same plasma samples were successfully amplified, presumably because the HIV viral load was low and at the undetectable level for RT-PCR (data not shown).

DISCUSSION

A 6-year chronological study on HIV infections among injecting drug users was carried out based on the theory that injecting drug user-driven growth of the HIV epidemic could be applicable to the future HIV trend in the Philippines like other sites in Asia where the rate of HIV infection had expanded first among injecting drug users [Ghys et al., 2001; Ruxrungtham et al., 2004; MAP, 2005].

The low rate of HIV infections among injecting drug users shown in this study corroborates the trend of low HIV prevalence documented in the government report [UNAIDS/WHO, 2008a]. While HIV has not yet become an epidemic, an HCV epidemic has been established solely among injecting drug users as suggested from the primary study [Agdamag et al., 2005]. This result is also consistent with another report that indicated an extremely low HCV prevalence among blood donors (0.33%) in the Philippines and overseas Filipino worker applicants (0.94%) [Yanase et al., 2007]. HIV infection may preferentially superimpose on such HCV-infected individuals, because HIV and HCV transmit mainly though blood-borne routes during the early phase of an epidemic. In fact, co-infections of HIV/HCV have been popular among injecting drug users (72–95%) in the high HCV-prevalence areas [Alter, 2006; Lee et al., 2008] while the single HIV infection rate is extremely low among injecting drug users populations with high

TABLE III. Genotype Distribution of HCV Strains Isolated in Luzon, Visayas, and Mindanao (Philippines)

		The number of HCV isolates categorized into the genotype of						
Area	Population	1a	1b	2a	2b	4	6	Total
Luzon	Injecting drug users	0	0	0	0	0	0	0
	Dialysis patients	11	1	0	1	1	0	14
	Unknown	17	10	5	1	0	1	34
	Total	28 (58%)	11 (23)[a]	5 (10)	2 (4)	1 (2)[b]	1 (2)[b]	48 (100)
Visayas	Injecting drug users	252	0	13	93	0	0	358
	Dialysis patients	0	0	0	0	0	0	0
	Unknown	4	0	3	1	0	0	8
	Total	256 (70)	0	16 (4)	94 (26)	0	0	366 (100)
Mindanao	Injecting drug users	29	0	0	0	0	0	29
	Dialysis patients	0	0	0	0	0	0	0
	Unknown	1	0	0	0	0	0	1
	Total	30 (100)	0	0	0	0	0	30 (100)

[a]Ten of 11 samples were collected in Metro Manila.
[b]Both samples were collected in Metro Manila.

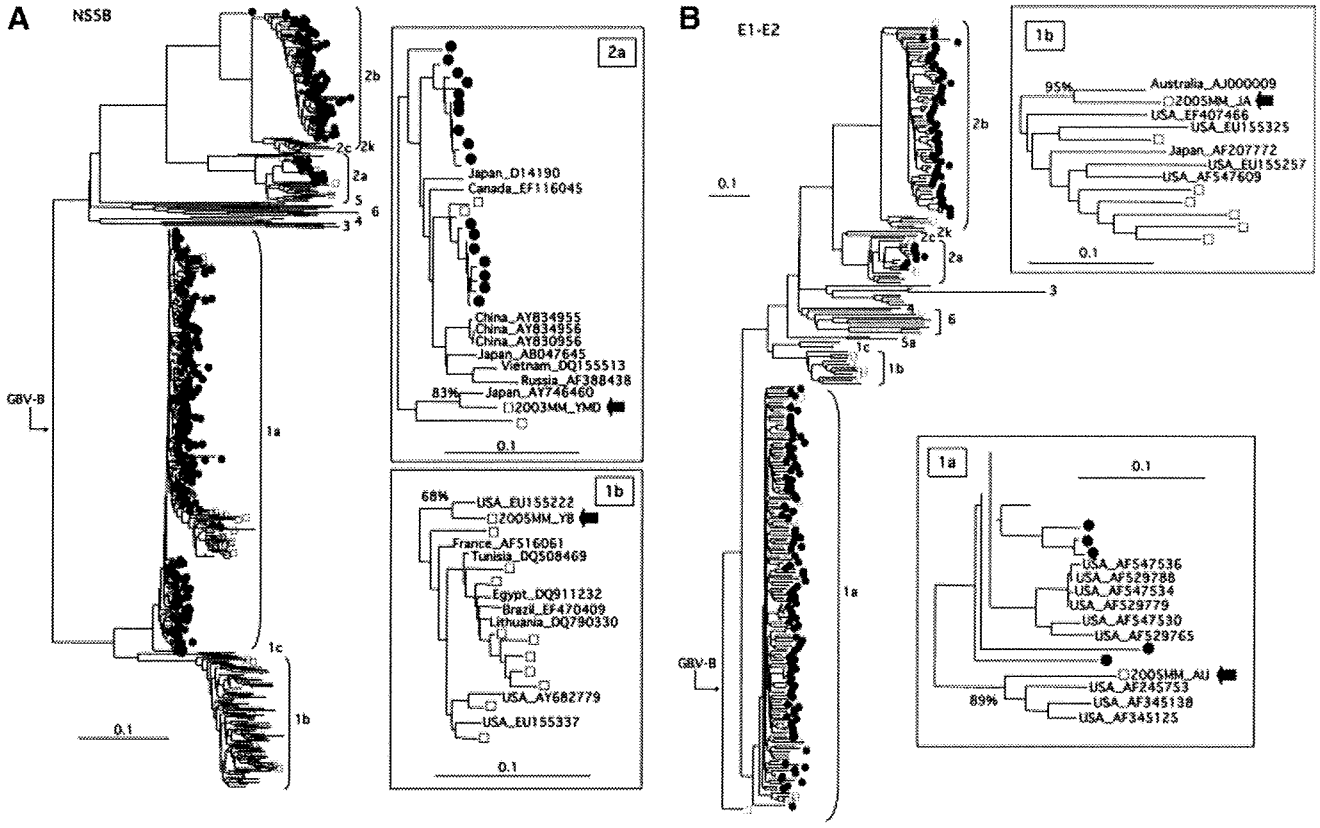

Fig. 1. Phylogenetic trees constructed on the HCV NS5B (**A**) and E1-E2 (**B**) regions The NS5B (328 bases, position 8279-8606 in H77 strain) sequences (Luzon □, Visayas ●, and Mindanao Δ, respectively) were determined and phylogenetic trees were constructed (A). The NS5B-positive samples were further subjected to the determination of E1–E2 sequence including hypervariable region 1 (538 bases, position 1322-1859) (B). The IDs of reference strains were denoted by blanks in the tree, and country of origin was shown only in insets with accession number (e.g., Japan_D14190). GBV-B (accession no. NC_001655) was used as an outgroup [Bukh et al., 1999]. Bootstrap values (≥68%) were given on the branches as percentage from 1,000 replicates (insets). A tested strain was indicated (arrow) when it showed a higher sequence homology to a foreign strain than any other Philippine strain.

HCV infection rates [Patrick et al., 2001; Miller et al., 2004]. These findings warrant further careful observations of the HIV/HCV infection trend among injecting drug users in the Philippine sentinel sites. To date, the HIV/HCV co-infection rate remains low among anti-HCV positive subjects (0.38%) and there was no HIV infection among anti-HCV negatives in the nationwide surveillance in the Philippines.

The nucleotide sequences of the four HCV strains from Metro Manila showed higher homology to foreign strains retrieved from the Genbank/EMBL/DDBJ database with high bootstrap values (68–95%) rather than any other sequences of the strains collected in the Philippines (n = 440). These data suggest that foreign HCV strains are introduced occasionally into the Philippines. However, these four cases were referred to the National Reference Laboratory anonymously with no indicated risk for the HCV transmission such as injecting drug use. Therefore, it will be necessary to carry out further specification of the population bringing such foreign HCV strains into the Philippines and the population susceptible to those strains. Nevertheless, considering the existence of newly introduced HCV strains into Metro Manila, unevenly distributed HCV genotype 1b in Metro Manila, and the main HCV transmission routes confirmed worldwide [Memon and Memon, 2002], not a few HCV strains may be introduced into the Metro Manila occasionally through blood-borne pathways like HIV strains that have caused early phase epidemics in other Asian countries.

As shown above, the constantly high rate of HCV infections and the newly introduced foreign HCV strains in the absence of HIV epidemic warrant further investigation on HCV entry and spread for early detection of an HIV epidemic in the Philippines.

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Molecular Genetic Diversity of Hepatitis B Virus in Kenya

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Key Words
Genotypes · Hepatitis B virus · Kenya · Nuclear acid testing

Abstract
Eight genotypes of hepatitis B virus (A-H) and subgenotypes have been recognized worldwide. However, there is limited information on prevalent genotypes in many countries in Africa. This study was undertaken to determine the hepatitis B virus (HBV) genotypes in Kenya. Seropositive HBV blood samples from a blood donor setting were used in the study. HBV genotypes were determined in 52 nucleic acid-positive samples using specific primer in a nested PCR and sequencing employed in the HBV genotyping. This study shows presence of HBV variants with genotypes A (88%), E (8%) and D (4%). In conclusion, we found that HBV genotype A is the most predominant genotype in Kenya with both subgenotype A1 and A2 present. Genotype D and E are also present in our population. This demonstrates that there could be a high genetic diversity of HBV in Kenya.

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Hepatitis B virus (HBV), a well-known agent of acute and chronic hepatitis, is endemic in many parts of the world, especially Asia and Africa. More than 2 billion people have had contact with the virus and more than 350 million are chronic carriers globally [1, 2], making the infection a public health problem.

HBV is the prototype member of the genus *Orthohepadnaviridae* of the family *Hepadnaviridae* and the viral genome is about 3.2 kb long. It circulates in the serum as a Dane particle which is a round structure consisting of an envelope and an inner core of nucleocapsid protein, enclosing both a polymerase and the partly double-stranded circular viral DNA [3].

The highly compact genome contains the four major open reading frames (ORFs) encoding the envelope (preS1, preS2 and surface antigen HBsAg), core (preCore precursor protein, HBeAg and HBcAg), polymerase (HBPol) and X (HBX) proteins, respectively [4]. Earlier, before the genotype definition, HBV strains were distinguished into 9 hepatitis B surface antigen (HBsAg) subtypes designated *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4q–*, *adrq+*, and *adrq–* by serological analysis [3].

The new classification based on a comparison of the complete genomic sequence classifies HBV into 8 geno-

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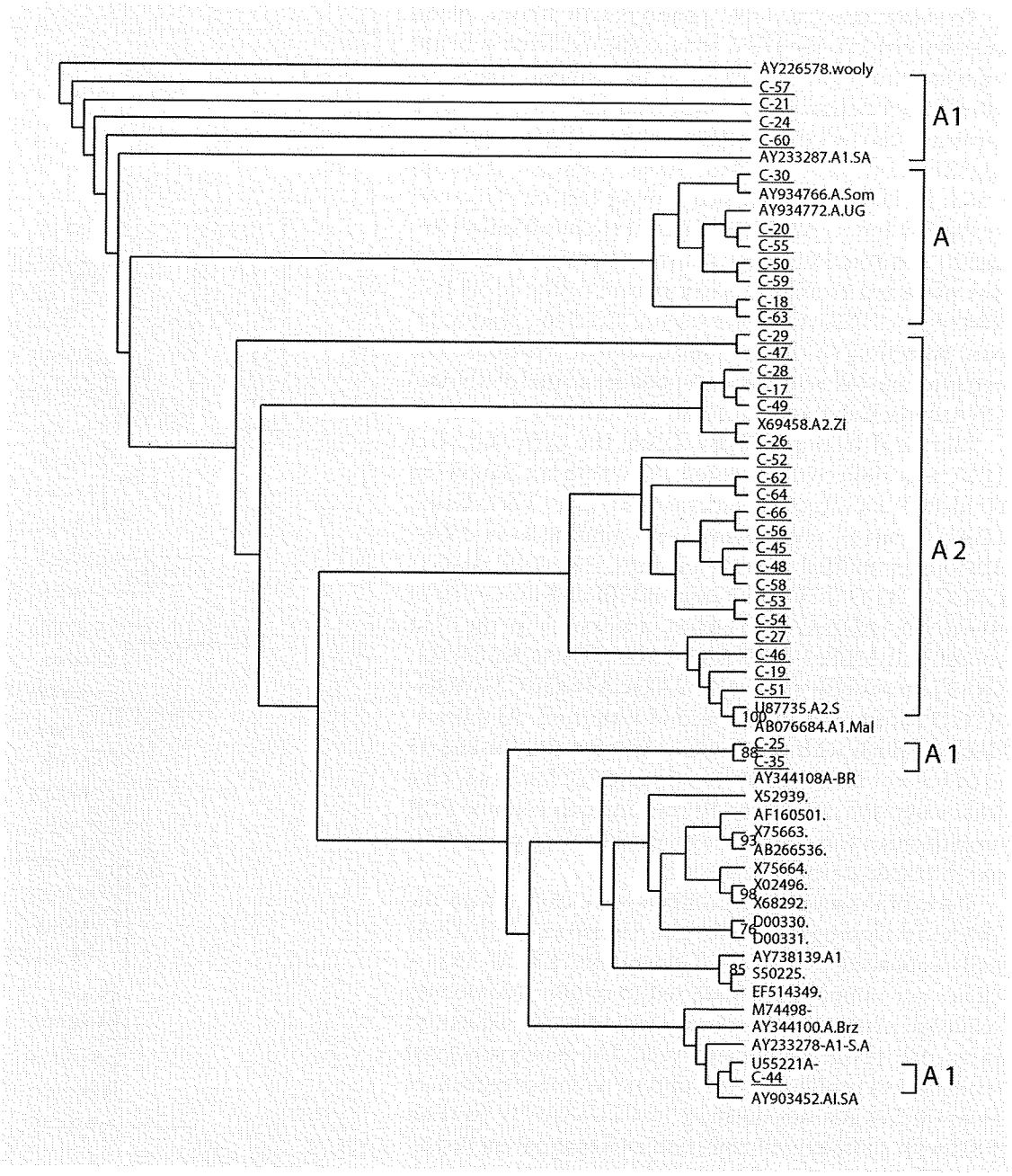

Fig. 1. Phylogenetic analysis of HBV strains in Kenya based on preS1 region. Kenyan isolates underlined.

types, A–H, with each genotype differing by more than 8% at the nucleotide level when compared to each other [1] and less than 4% intragenotype divergence. Some genotypes are further classified into subgenotypes: genotypes A and F into 2 subgenotypes each, and genotypes B, C and D into 4 subgenotypes each [5].

Africa is one of the highly endemic regions of HBV, with 5 genotypes A–E identified. Genotype D in Tunisia, genotype A–D in South Africa and genotype E in Nigeria are the predominantly reported genotypes in these countries [6, 7]. Little information is available on HBV genetic diversity in Kenya, yet the country is considered among the endemic countries for HBV infection.

To determine the HBV genotypes in Kenya, blood samples positive for HBV were collected from a blood transfusion center. Plasma from the samples was separated by centrifugation and unlinked anonymous testing carried out for all the samples using ELISA and particle agglutination tests for HBV surface antigen (HBsAg) which included; Hepanostica HBsAg test kit (Organon Technika) and KEMRI HEPCELL II (Kenya Medical Research Institute (KEMRI), Nairobi, Kenya). Viral DNA was extracted from 100 μl of plasma using SMITEST R&D RNA/DNA (Genome science Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The extracted nucleic acid was resuspended in 20 μl of RNAse/DNAse free water and stored at −80° till use.

For the detection of HBV DNA, the extracted viral DNA was subjected to nested PCR with an AmpliTaq Gold PCR kit (Applied Biosystems, Foster City, Calif., USA). A part of preS1 gene corresponding to nt 2850–3246 was amplified with the primers, HBPr1 (5′-GGGTCACCATATTCTTGGG-3′, sense)/HBPr135 (5′-CA(A/G)AGACAAAAGAAAATTGG-3′, antisense) in the first round and HBPr2 (5′-GAACAAGAGCTACAGCATGGG-3′, sense)/HBPr3 (5′-CCACTGCATGGCCTGAGGATG-3′, antisense) in the second round [4]. Amplification was done with one cycle of 94° for 10 min and 35 cycles of 94° for 30 s, 50° for 30 s and 72° for 30 s with a final extension of 72° for 10 min in both rounds. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel.

Parts of the amplified products were cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif., USA) and sequenced as described previously [7]. At least 4 clones per sample were analyzed to obtain a consensus sequence. The rest of the samples were directly sequenced in an ABI prism genetic analyzer. The newly analyzed sequences were aligned with subtype reference sequences from the Los Alamos database by CLUSTAL W (version 1.81) with subsequent inspection and manual modification. The frequency of nucleotide substitution in each base of the sequences was estimated by the Kimura two-parameter method [8]. A phylogenetic tree was constructed by the neighbor-joining method [9], and its reliability was estimated by 1,000 bootstrap replications. The profile of the tree was visualized with Tree View PPC version 1.6.5.

In this study, of 80 HBsAg seropositive samples, 52 were positive for nuclear acid testing (NAT). The rest of the samples could not amplify specifically; these were considered to be either false-positives or had very low DNA undetectable by PCR. To determine the genotypes, the 52 NAT-positive samples were then sequenced and analyzed phylogenetically. Reference sequences from Gene Bank including all genotypes reported and especially those from Africa were included in the analysis.

Upon sequencing, the 52 preS1 sequences obtained segregate HBV strains in Kenya into 3 genotypes, A, D

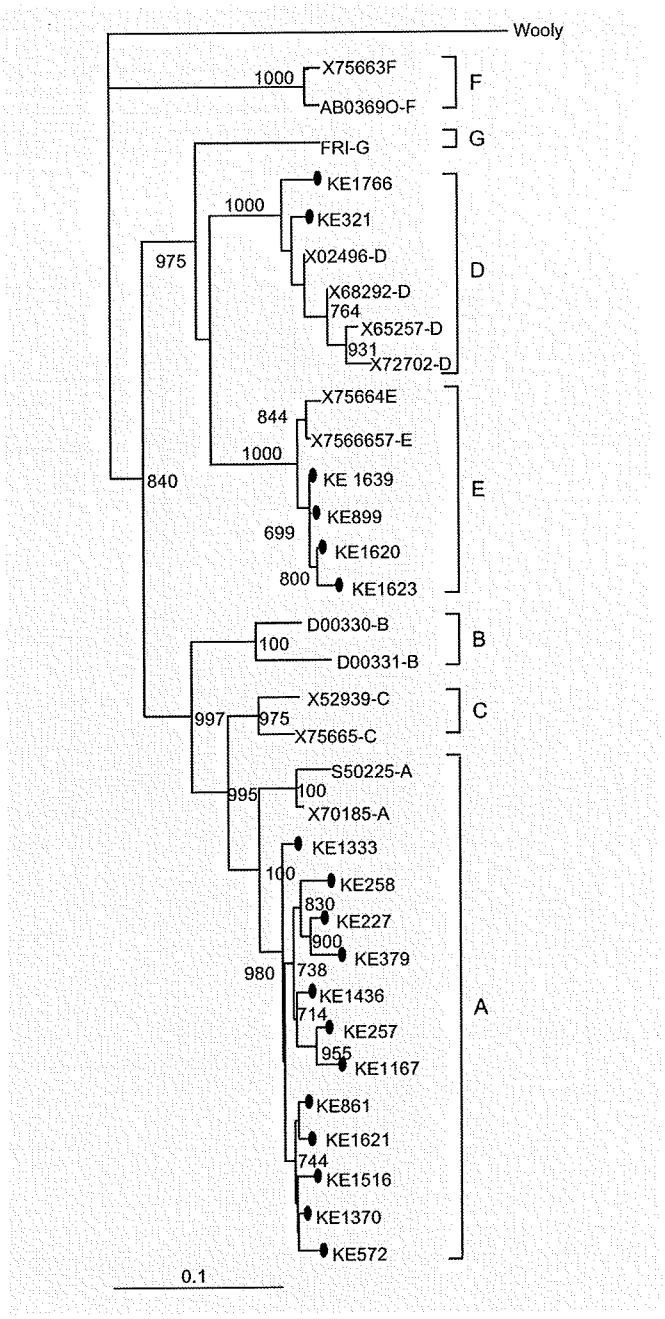

Fig. 2. Phylogenetic analysis of HBV strains in Kenya based on preS1 region. Kenyan isolates bulleted.

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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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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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}.

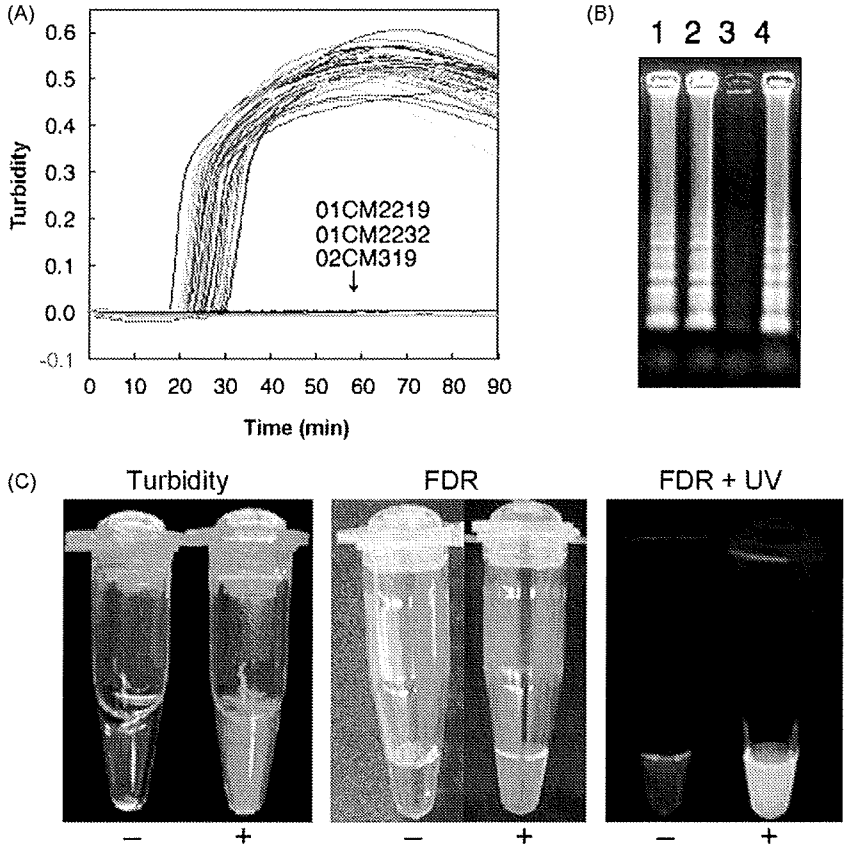

Fig. 1. Real-time detection of HIV-1 RT-LAMP products of 57 HIV-1-positive samples from Cameroon by turbidimeter (LA-200). (A) Agarose gel electrophoresis of HIV-1 RT-LAMP products that were undetectable by LA-200. A turbidity value of more than 0.1 was considered positive. Turbidity of three samples (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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