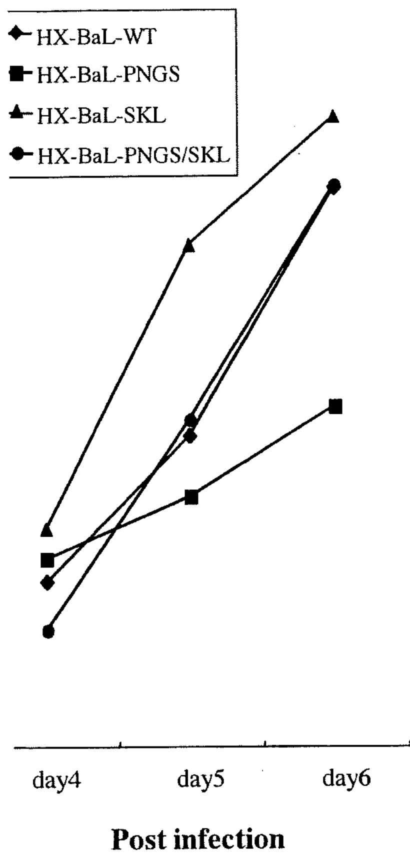
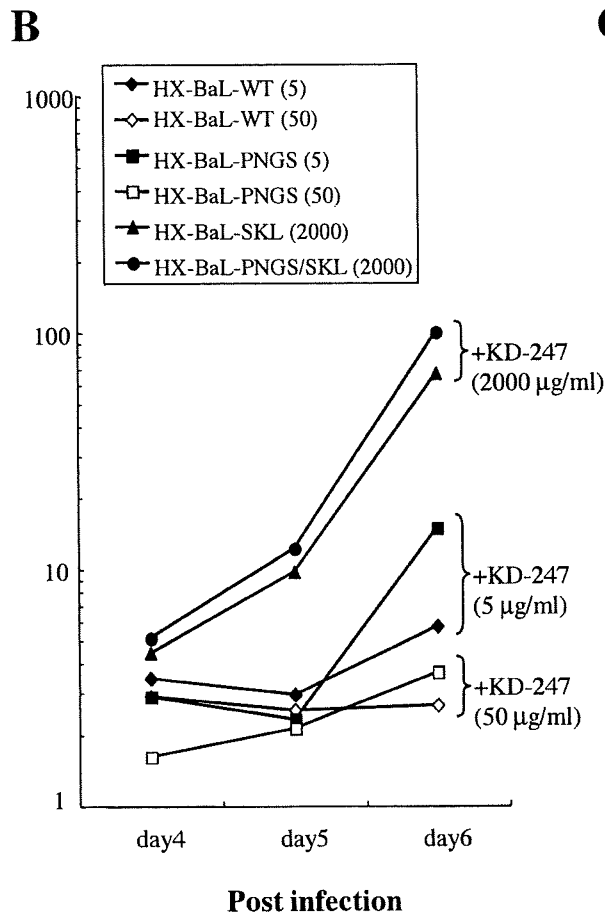
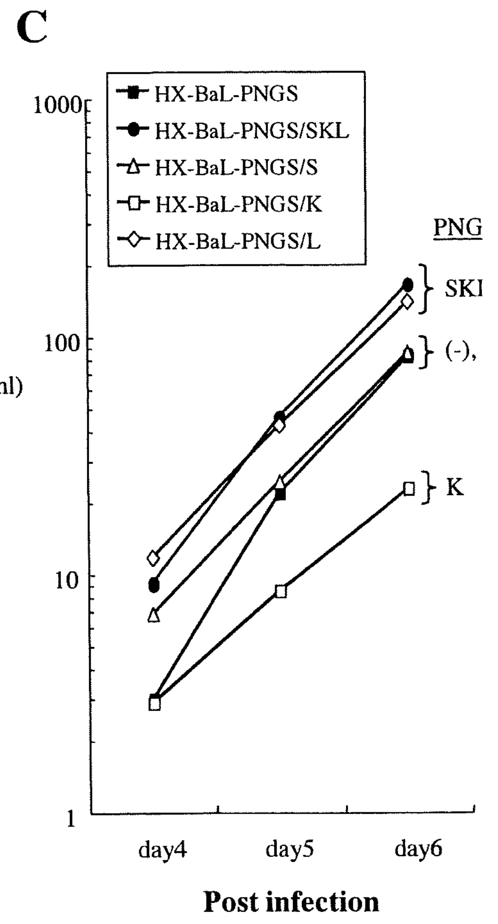
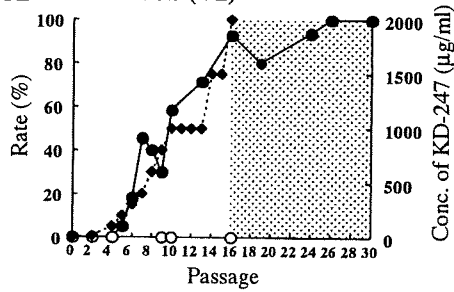


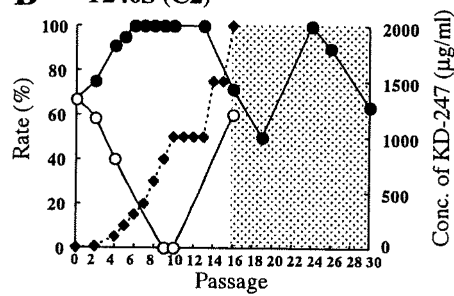
B

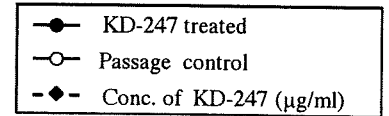


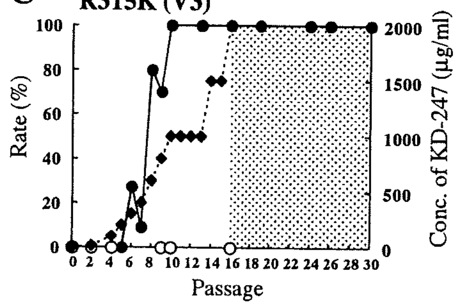
C

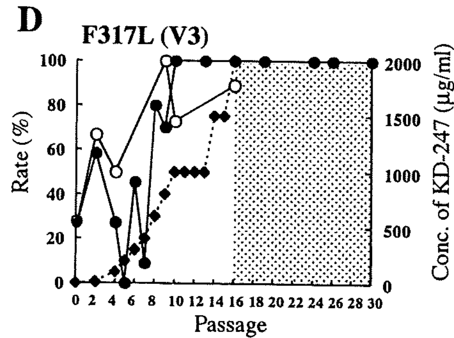


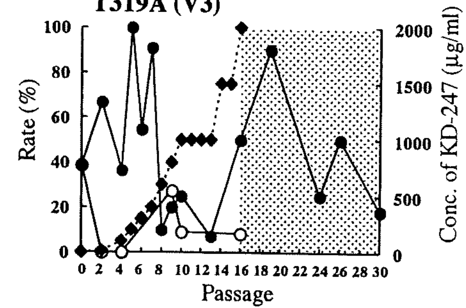

A
186PNGS (V2)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage


B
T240S (C2)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage


KD-247 treated
Passage control
Conc. of KD-247 (μg/ml)


C
R315K (V3)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage


D
F317L (V3)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage


E
T319A (V3)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage

e 1. Neutralization sensitivities of passaged variants to KD-247.

	passage No.	KD-247 conc. ($\mu g\ ml^{-1}$)	IC_{50} ($\mu g\ ml^{-1}$) [a]
Baseline virus	p0	0	0.32 ± 0.20
Passage control	p10	0	0.09 ± 0.04
KD-247 selection	p2	10	0.54 ± 0.19
	p5	200	5.68 ± 1.48
	p6	300	> 100
	p7	400	> 100
	p10	1000	> 100
	p16	2000	> 100

[a] TZM-bl cells (2×10^4 cells $well^{-1}$) were exposed to 300 $TCID_{50}$ of passage control (p10) or KD-247 selected variants (p2, p5, p6, p7, p10, p16) in the presence of various concentrations of KD-247 in 96-well flat-bottom microculture plates and incubated for 48 h. The IC_{50} values were determined using a chemiluminescent assay for β-galactosidase detection. Data shown represents the means ± 1 standard deviation from the results of three independent experiments.

e 2. Anti-HIV1 activities of KD-247 and the CCR5 inhibitor, maraviroc.

	IC_{50} ± SD of maraviroc (nM) [a]	IC_{50} ± SD of KD-247 (μg ml^{-1}) [a]	
HX-BaL-WT	2.0 ± 0.72	0.092 ± 0.028	
HX-BaL-PNGS	1.2 ± 0.28	0.047 ± 0.028	
HX-BaL-Q	1.9 ± 1.3	0.12 ± 0.047	
HX-BaL-S	1.8 ± 0.72	0.087 ± 0.021	
HX-BaL-L	2.6 ± 0.33	0.036 ± 0.012	
HX-BaL-STA	2.5 ± 1.7	4.6 ± 0.71	
HX-BaL-PNGS/SKL	2.4 ± 0.29	214 ± 84	[b]
HX-BaL-K	1.6 ± 0.35	285 ± 76	* (K vs PNGS/K); *** (K vs Q/K)
HX-BaL-PNGS/K	2.7 ± 0.52	582 ± 59	** (PNGS/K vs Q/K)
HX-BaL-Q/K	1.6 ± 0.32	276 ± 31	

[a] TZM-bl cells (2×10^4 cells $well^{-1}$) were exposed to 300 $TCID_{50}$ of the infectious clones with wild-type or mutant Env in the presence of various concentrations of maraviroc or KD-247, and incubated for 48 h. IC_{50} values were determined using a chemiluminescent assay for β-galactosidase detection. All assays were conducted in duplicate or triplicate and the data shown represent means ± 1 standard deviation from the results of three independent experiments.

[b] *P* values < 0.05 were considered statistically significant (Student's t test). *P=0.006, ** P=0.007, *** P=0.89.

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HIV Type 1 Subtype Diversity and Drug Resistance among HIV Type 1-Infected Kenyan Patients Initiating Antiretroviral Therapy

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Abstract

The treatment of HIV-1 infection with antiretroviral drugs has greatly improved the survival of those who are infected. However, HIV-1 diversity and drug resistance are major challenges in patient management, especially in resource-poor countries. To evaluate HIV-1 genetic diversity and drug resistance-associated mutations among drug-naive patients in Kenya prior to antiretroviral therapy (ART), a genetic analysis of HIV-1 *pol*-RT and *env-gp41* was performed on samples collected from 53 (18 males and 35 females) consenting patients between April and June 2005. The average age, baseline $CD4^+$ T cell counts, and viral loads were 38 (range, 24–62) years, 475 (range, 203–799) cells/mm^3, and 4.7 (range, 3.4–5.9) log_{10} copies/ml, respectively. Phylogenetic analysis revealed that 40 samples (75.5%) were concordant subtypes for the two genes and 13 (24.5%) were discordant, suggesting possible recombination and/or dual infections. Prevalent subtypes included 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%). Major reverse transcriptase inhibitor (RTI) resistance-associated mutations were found in four patients (7.5%). Of these patients, three had nucleoside RTI resistance mutations, such as M184V, K65R, D67N, K70R, and K219Q. Nonnucleoside RTI resistance-associated mutations K103N and Y181C were detected in three patients and one patient, respectively. Multiple drug resistance mutations were observed in this drug-naive population. With increasing numbers of patients that require treatment and the rapid upscaling of ART in Kenya, HIV-1 drug resistance testing is recommended before starting treatment in order to achieve better clinical outcomes.

Introduction

Genetic variation is inherent to all RNA viruses, but it has been extensively characterized for human immunodeficiency virus type 1 (HIV-1). The genetic diversity of HIV-1 originates from rapid viral turnover in an infected individual and a high rate of incorrect nucleotide substitutions during HIV reverse transcription in the absence of proofreading mechanisms.[1,2] HIV-1 continuously evolves, overcoming barriers to transmission, avoiding different immune responses, and resisting various antiretroviral regimens.[3–6] Though vaccination is one of the potential options for curtailing the epidemic, the diversity of HIV-1 presents an extraordinary challenge to drug and vaccine development.[7–9]

Antiretroviral therapy (ART) using nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) has sharply reduced HIV transmission, morbidity, and mortality in developed countries, but it has created the long-term specter of drug resistance. Widespread use of ART in these countries has resulted in an increased prevalence of drug-resistant variants, ranging from 10% to 20% among drug-naive patients.[10–12] Intervention through such programs as the World Health Organization's (WHO's) 3 by 5 plan to treat 3 million people by the end of 2005 and the President's Emergency Plan for AIDS Relief has significantly promoted access to ART in low-income and middle-income countries.[13–15]

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As access to ART rapidly increases in these resource-limited countries, the prevalence of HIV-1 drug-resistant strains among drug-naive patients is also expected to increase. Studies conducted among drug-naive individuals in Cameroon found increased drug resistance, from 0% in 2002 before the start of the WHO's 3 by 5 initiative to 9.8% in 2004, when the availability of ART to those in need was 13.5%.[16–18] In Mozambique, where ART was available to 32% of the estimated population in need in 2004, the prevalence of mutations conferring resistance to both NRTIs and NNRTIs among drug-naive patients was reported to be 5.9%.[19] In Botswana, the prevalence was 0% in 2001 before the country's ART program began.[20] Currently, all patients meeting country-based criteria for ART receive treatment and drug resistance among drug-naive patients has been projected to rise to 15% by the end of 2009.[21] In Uganda, Tanzania, and Malawi, major RTI-resistant HIV-1 mutants were rarely found among newly diagnosed patients.[22–24]

In Kenya, the current standard first-line therapy consists of two NRTIs, stavudine (d4T) and lamivudine (3TC), plus one NNRTI, either nevirapine (NVP) or efavirenz (EFV). PI-containing regimens are not yet widely available. The rapid upscaling of ART was accompanied by increased availability to those in need, increasing to 17% by the end of 2005.[25,26] By that time, the WHO's ART guidelines for surveying and monitoring HIV drug resistance in resource-poor countries[27–29] had not been fully implemented. In addition, the use of single dose NVP among HIV-infected antenatal clinic attendees influenced the need for laboratory monitoring.[30–33] Kenyan HIV-1 vertically infected children were reported to have acquired drug resistance mutations.[34] However, the magnitude of drug resistance in adults has not been determined. The current study was conducted to determine HIV-1 diversity and RTI resistance-associated mutations among HIV-1-infected drug-naive adults in Kenya where ART is being rapidly scaled up.

Materials and Methods

Study population and samples

Individuals who were 18 years of age or older and who presented themselves to the clinic for treatment were considered for recruitment after giving informed consent. Demographic data, such as age and gender, together with the ART and/or single dose NVP history for each individual were obtained using a self-reporting questionnaire. Patients who reported prior exposure to ART and/or single dose NVP for prevention of mother-to-child transmission (PMTCT) of HIV, together with those who declined to consent were excluded from the study. A total of 87 patients (50 females and 37 males) from Nairobi were sequentially enrolled between April and June 2005. Five milliliters of blood was collected from each participant and tested for anti-HIV-1 antibodies using Unigold (Trinity Biotech, NY) and Determine (Abbott, IL). HIV-1 antibody positivity was further confirmed by enzyme-linked immunosorbent assay (ELISA) (Enzygnost, Dade-Behring, Marburg, Germany). Ethical clearance was obtained from the National Ethics Committee through the Kenya Medical Research Institute (KEMRI).

$CD4^+$ T cell counts and HIV-1 RNA quantification

Baseline $CD4^+$ T cell counts were performed using a FACSCalibur flow cytometer (Becton-Dickinson, NJ) equipped with automated acquisition and analysis software. Individual test results were reviewed to confirm the accuracy of the automated software analysis. Baseline viral loads were determined using Nuclisens EasyQ (Biomérieux, Marcy l'Etoile, France), with a lower limit of quantitation of 50 (1.69 $\log_{10}$) copies/ml of plasma, according to the manufacturer's instructions.

PCR and sequencing

Peripheral blood mononuclear cells (PBMCs), from confirmed ELISA-positive samples, were obtained by Ficoll-Hypaque density gradient centrifugation. Proviral DNA was extracted from the uncultured PBMCs using DNAzol (GIBCO BRL, Life Technologies) lysis and ethanol precipitation. Nested polymerase chain reaction (PCR) was performed using AmpliTaq Gold (Roche Molecular Systems, Branchburg, NJ). A segment of HIV-1 *env-gp41* corresponding to nucleotides 7850–8310 of HIV-1_{HXB2} was amplified using the primers gp40F1 (5′-TCTTAGGAGCAGCAGGAAGCACTATGGG-3′) and gp41R1 (5′-AACGACAAAGGTGAGTATCCCTGCCTAA-3′) in the first round and gp46F2 (5′-ACAATTATTGTCT GGTATAGTGCAACAGCA-3′) and gp47R2 (5′-TTAAACC TATCAAGCCTCCTACTATCATTA-3′) in the second round (www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1998/III/GP41RENU.pdf). A segment of the HIV-1 RT gene corresponding to nucleotides 2265–3180 of HIV-1_{HXB2} was amplified using the primers RT18 (5′-GGAAACCAAAAAT GATAGGGGGAATTGGAGG-3′) and KS104 (5′- TGACTT GCCCAATTTAGTTTTCCCACTAA-3′) in the first round and KS101 (5′-GTAGGACCTACACCTGTTCAACATAATTG GAAG-3′) and KS102 (5′-CCCATCCAAAGAAATGGAGG AGGTTCTTTCTGATG-3′) in the second round. Amplification was achieved using 1 cycle of 95°C for 10 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The amplicons were sequenced as previously described.[22,34,35]

Table 1. Baseline Characteristics of HIV-1-Infected Kenyan Patients before Initiating Antiretroviral Therapy

	All (n = 53)	Gender: Females (n = 35)	Gender: Males (n = 18)
Age (years)			
Mean (range)	38 (24–62)	37.5[a] (24–57)	39.5[a] (27–62)
Viral load ($\log_{10}$ copies/ml)			
Mean (range)	4.7 (3.4–5.9)	4.7[b] (3.4–5.9)	4.8[b] (3.9–5.8)
$CD4^+$ T cell counts (cells/mm^3)			
Mean (range)	475 (203–799)	449[c] (203–780)	525[c] (233–799)
Range			
<300	6	5	1
301–400	13	10	3
401–500	12	8	4
>500	22	12	10

[a] $p = 0.45$ by paired t-test.
[b] $p = 0.41$ by paired t-test.
[c] $p = 0.28$ by paired t-test.

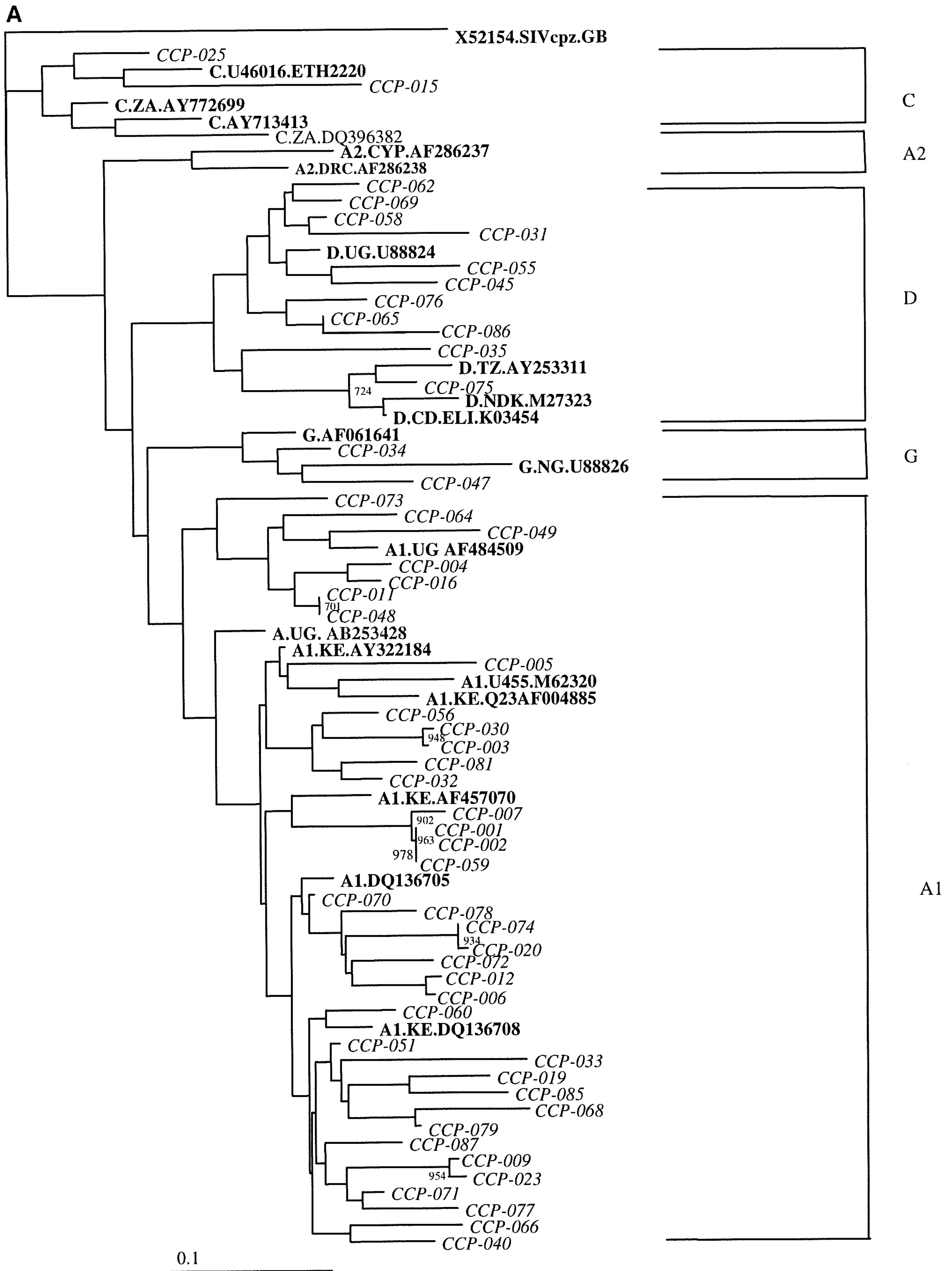

FIG. 1. Phylogenetic tree of the HIV-1 *pol-RT* (**A**) and *env-gp41* (**B**) regions. Patient samples (italics) were aligned and compared with reference sequences from the Los Alamos HIV database (boldface). Phylogenetic relationships were constructed using the neighbor-joining method and rooted with SIVcpzGAB. The bootstrap values of 1000 replicates above 70% are indicated next to the node. Brackets on the right indicate the subtype clusters.

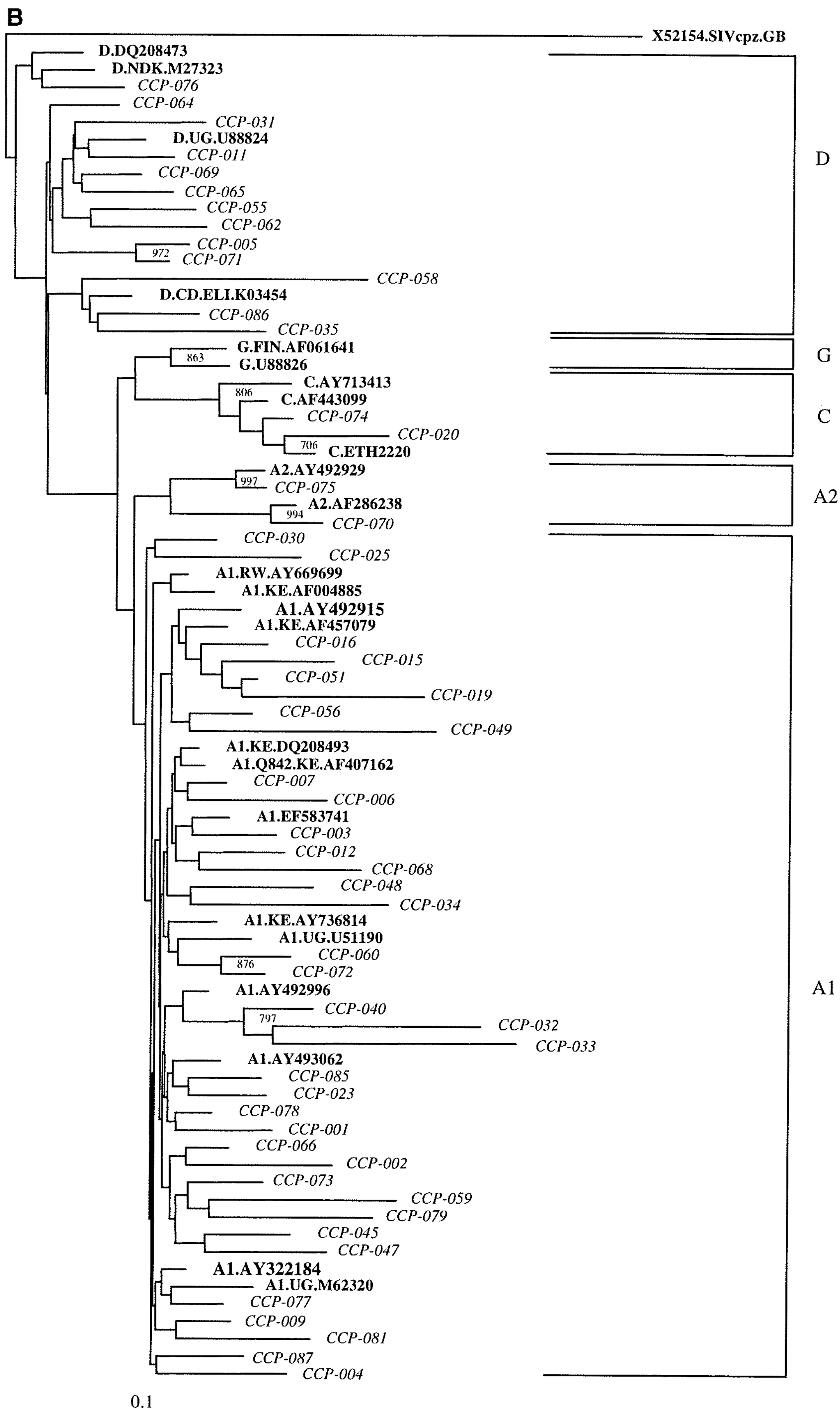

FIG. 1. (Continued).

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*.

Acknowledgments

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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)

Area	Population	The number of HCV isolates categorized into the genotype of						
		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.

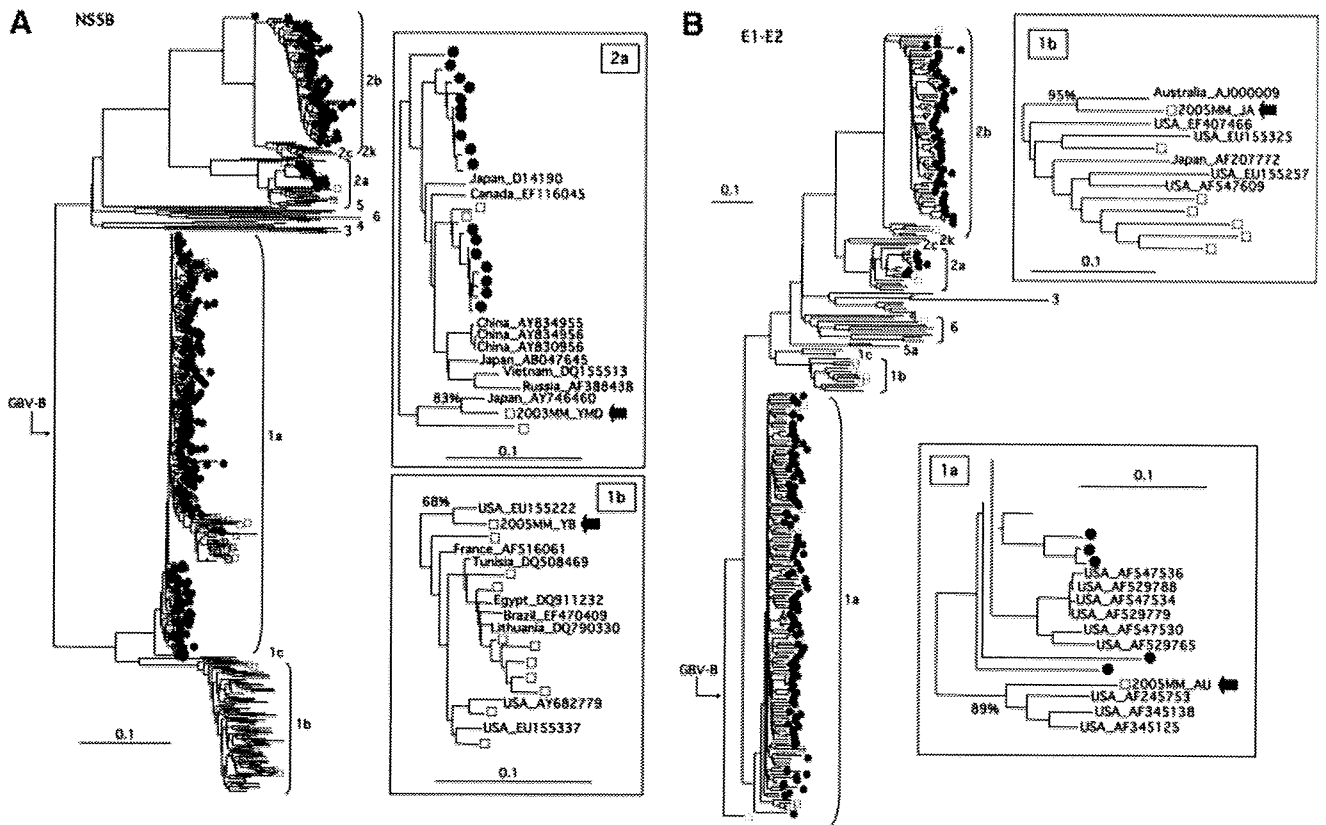

Fig. 1. Phylogenetic trees constructed on the HCV NS5B (**A**) and E1-E2 (**B**) regions The NS5B (328 bases, position 8279-8606 in H77 strain) sequences (Luzon □, Visayas ●, and Mindanao Δ, respectively) were determined and phylogenetic trees were constructed (A). The NS5B-positive samples were further 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 (HB-Pol) 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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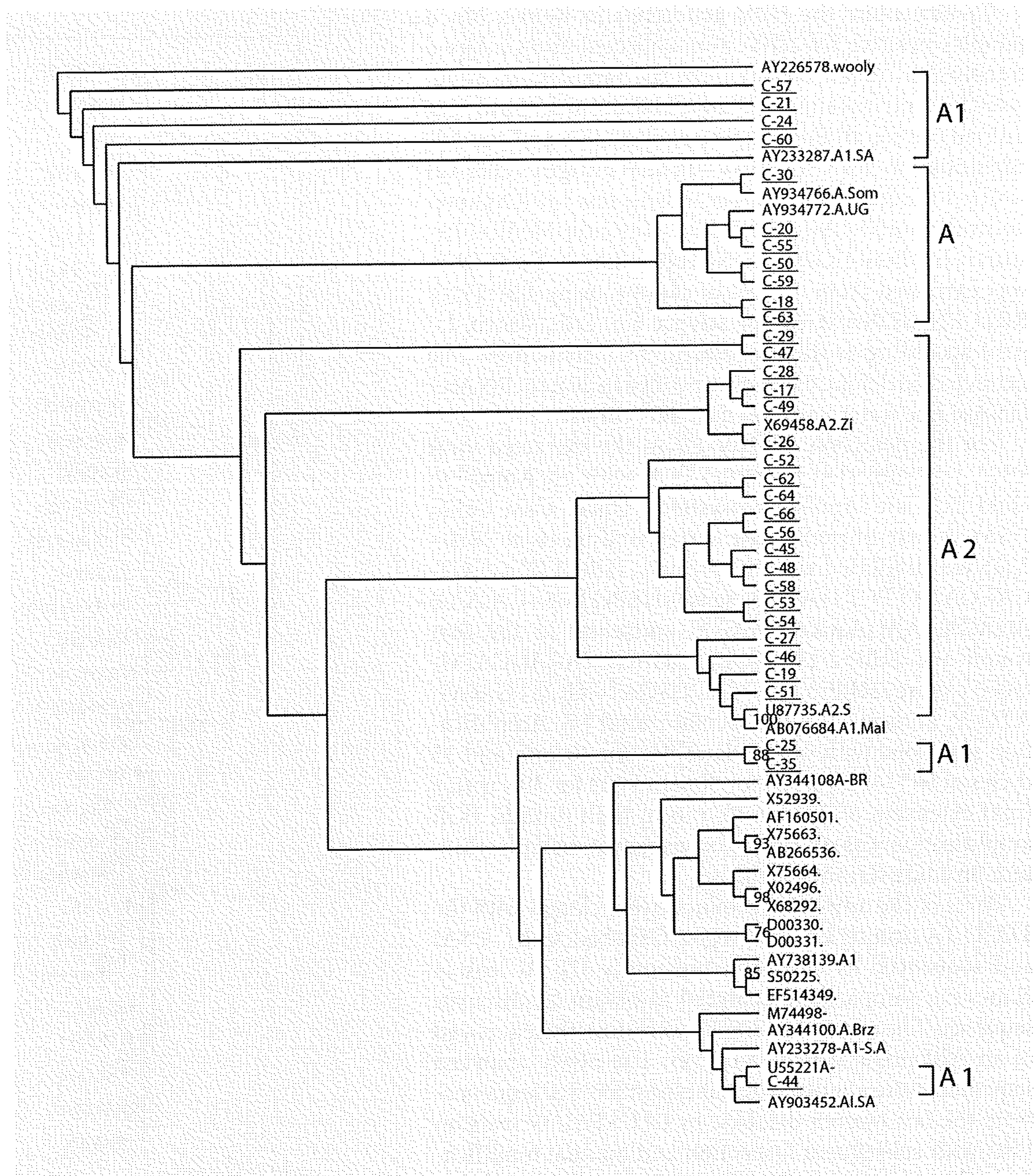

Fig. 1. Phylogenetic analysis of HBV strains in Kenya based on preS1 region. Kenyan isolates underlined.

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.

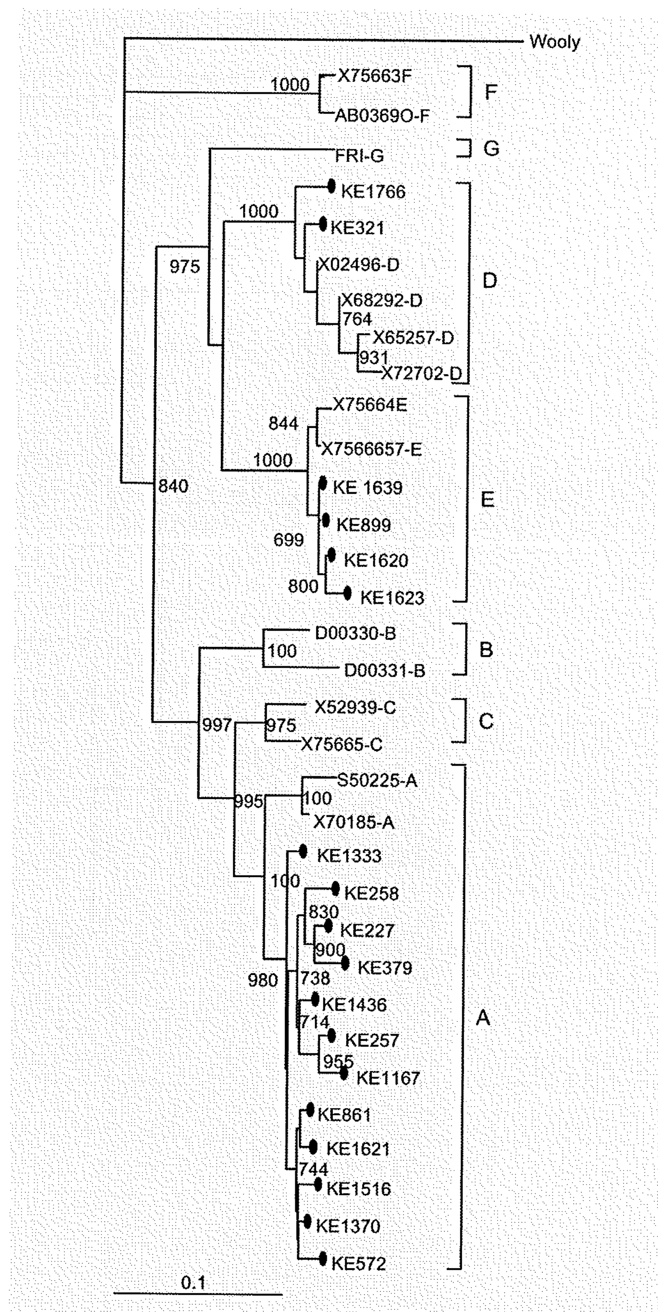

Fig. 2. Phylogenetic analysis of HBV strains in Kenya based on preS1 region. Kenyan isolates bulleted.

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