JOURNAL OF CLINICAL MICROBIOLOGY, July 2005, p. 3484–3486 Vol. 43, No. 7
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.7.3484–3486.2005

Frequent Detection of Epstein-Barr Virus and Cytomegalovirus but Not JC Virus DNA in Cerebrospinal Fluid Samples from Human Immunodeficiency Virus-Infected Patients in Northern Thailand

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Received 18 August 2004/Returned for modification 18 October 2004/Accepted 27 February 2005

Applying nested-PCRs, we frequently detected DNA of Epstein-Barr virus and cytomegalovirus but not JC virus in cerebrospinal fluid samples from 140 human immunodeficiency virus-infected patients with central nervous system symptoms in northern Thailand. Despite the low incidence of primary central nervous system lymphoma or cytomegalovirus encephalitis among Thai AIDS patients, Epstein-Barr virus and cytomegalovirus infections in the central nervous system are common.

According to reports from the Thai Ministry of Public Health, opportunistic infections are common in the central nervous system (CNS) of Thai AIDS patients and have caused a significant portion of mortality. Cryptococcal meningitis was noted as 20.3% of the first AIDS defining illness in northern Thailand; toxoplasma encephalitis was 5.3%; tuberculous meningitis was also seen, though the exact prevalence in the total number of AIDS patients is unknown (2). Virus infections in the CNS such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), and JC virus (JCV) can result in life-threatening consequences as they cause primary CNS lymphoma, cytomegalovirus encephalitis, and progressive multifocal leukoencephalopathy, respectively. In developed countries, PCR tests to detect EBV, CMV and JCV DNA in the cerebrospinal fluid (CSF) have been used as a supplemental diagnostic test (5). However, in developing countries, such a test is not available and very limited data have been reported about the prevalence of virus infections in the CNS. The objective of this study is to investigate the significance of EBV, CMV, and JCV infections in the CNS of human immunodeficiency virus (HIV)-infected Thais in northern Thailand.

From March 2001 to June 2003, CSF samples of 140 HIV-1-infected patients at the day care center clinic or the HIV/AIDS ward in Lampang Hospital, which is a Thai government referral hospital for Lampang province in northern Thailand, were examined as they were clinically suspected of having opportunistic infections in the CNS and did not have any contraindication for lumbar puncture. Consequently, 163 CSF samples including follow-up CSF samples were taken. All CSF samples were initially examined for routine laboratory tests such as cell count, protein concentration, sugar level, bacterial and fungal culture, Indian ink stain, Gram stain, acid-fast bacilli stain, and a latex agglutination test for cryptococcal antigen (PASTOREX, Bio-Rad, France). After the routine laboratory tests, residual CSF samples were stored at −80°C until DNA extraction.

All study patients gave informed consent when they participated in the Lampang HIV cohort study, which was approved by the Thai government ethics committee. DNA was extracted from 200 μl of CSF (QIAGEN blood mini DNA extraction kit, QIAGEN, California), eluted with 50 μl of distilled water, and 10 ul were used as the target for PCR. PCR amplifications were performed using ExTaq DNA polymerase (TaKaRa Biomedical, Osaka, Japan) and nested primer sets targeting specific sequences of virus genes as previously published: the EBNA-1 gene for EBV (PCR product, 209 bp) (3), immediately early protein gene for CMV (146 bp) (1), and regulatory regions for JCV (approximately 396 bp) (7, 10).

Diagnosis of EBV and CMV infection was made on the basis of the size of amplicons, but for the diagnosis of JCV, we further sequenced PCR products. The positive control for EBV PCR was DNA extracted from Namalwa cells as previously described (11). DNA extract from culture supernatant of CMV-producing fibroblast cells was used as a positive control for CMV PCR. DNA extract from the urine of a healthy JCV carrier was used as a positive control for JCV PCR. The detection limit of nested PCR for EBV and CJV was evaluated as previously described (10, 11). The detection limit of CMV PCR was approximately 100 copies/ml of CSF, which was estimated by a limiting dilution method using a DNA sample, of which the number of CMV copies was determined by a quantitative real-time PCR (Mitsubishi-Kagaku BCL, Tokyo, Japan).

The median (interquartile range; range) of age among 140 patients was 33 years (30 to 37 years; 20 to 63 years); 93 patients (66.4%) were male. $CD4^+$ T-cell count data were

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TABLE 1. Clinical characteristics of study patients[a]

Parameter	No. of patients (%)
Symptoms	
Altered mental status	37 (27.0)
Focal sign	18 (13.1)
Chronic headache	121 (88.3)
Fever	104 (75.9)
Diagnosis of CNS infection	
Cryptococcal meningitis[b]	98 (70.0)
Toxoplasmic encephalitis[b]	10 (7.1)
Tuberculous meningitis	6 (4.3)
Aseptic meningitis	3 (2.1)
No diagnosis	24 (17.1)
Antiretroviral drug therapy	
None	131 (93.6)
Two drugs	3 (2.1)
Three drugs	4 (2.9)
Unknown	2 (1.4)
Status at discharge	
Improved	99 (70.7)
Dead	29 (20.7)
Referred to another hospital	7 (5.0)
Unknown	5 (3.6)

[a] Medical records were available for 137 patients.
[b] Includes one case with both cryptococcal meningitis and toxoplasmic encephalitis.

available in 48 patients; the median (IQR; range) was 16 (7 to 42/μl; 0 to 605/μl). Clinical pictures of the patients are summarized in Table 1. Cryptococcal meningitis was by far the most common opportunistic infection in the CNS. There was no case of primary CNS lymphoma, CMV encephalitis, or progressive multifocal leukoencephalopathy. However, one patient developed clinical symptoms of progressive multifocal leukoencephalopathy during the follow-up period.

Thirty-one of 140 patients (22.1%) were positive for EBV PCR and 16 of 140 (11.4%) patients were positive for CMV PCR. Six patients were positive for both EBV and CMV PCR. More than one CSF sample was collected from 20 patients. The results of CMV PCR were concordant in all pairs of samples, but the results of EBV PCR were discordant in five pairs. None of the 140 first CSF samples was positive for JCV PCR. However, JCV was detected in the second CSF sample of one cryptococcal meningitis case. We found that patients with EBV DNA in the CSF tended to be older than the other patients and had a significantly higher protein concentration and a higher number of cells in the CSF (Table 2). We did not find any factor significantly associated with CMV DNA detection in CSF.

We found that EBV infection in the CNS is common in advanced HIV-infected patients in northern Thailand. This frequency was higher than the result of a similar study in Italy (4). The majority of our study patients were suffering from cryptococcal meningitis, but the detection rate of EBV DNA did not significantly differ according to the clinical diagnosis of cryptococcal meningitis. A significant association of EBV detection with a CSF cell count raised the concern that we may have detected EBV in the lymphocytes circulating in the peripheral blood, which invaded the CSF, rather than EBV of the CNS involvement. However, EBV was also often detected in patients without a CSF cell: EBV DNA was detected in CSF from 9 (19.6%) of 46 patients with a CSF cell count of 0.

Several studies from Western countries have shown a high sensitivity and specificity of EBV PCR in CSF for diagnosing primary CNS lymphoma (5). However, we have not seen any primary CNS lymphoma cases in our experience of having seen over 2,400 HIV-1-infected patients at the day care center clinic from its establishment on October 1995 to July 2004. Furthermore, the government report of adult AIDS patients from 1994 to 1998 showed that there were 98 primary CNS lymphoma cases, which represented only 0.1% of all reported first AIDS-defining illness in Thailand (2). According to the Thai national

TABLE 2. Factors associated with EBV or CMV DNA detection in the CSF[a]

Parameter	Median no. of patients (IQR)					
	EBV			CMV		
	PCR positive (n = 31)	PCR negative (n = 109)	P	PCR positive (n = 16)	PCR negative (n = 124)	P
Age (yr)	35 (31–42)	33 (30–36)	0.069	32 (29–38)	33 (30–37)	0.7
No. female	11 (35.5%)	36 (33.1%)	0.79	6 (37.5%)	41 (33.1%)	0.72
CSF cell count (/μl)	8 (0–66)	4 (0–10)	0.045	6 (0–18)	4 (0–16)	0.97
CSF protein concn (mg/dl)	80 (55–160)	53 (34–90)	0.003	75 (30–88)	57.5 (40–100)	0.96
Clinical diagnosis[b]						
Cryptococcal meningitis	21 (70.0%)	76 (69.7%)		12 (75.0%)	85 (69.1%)	
Toxoplasmisc encephalitis	4 (13.3%)	5 (4.6%)	0.40	1 (6.3%)	8 (6.5%)	0.65
Tubercular meningitis	1 (3.3%)	5 (4.6%)		0 (0.0%)	6 (4.9%)	
Aseptic meningitis	0 (0.0%)	3 (2.8)		1 (6.3%)	2 (1.6%)	
No apparent CNS infection	4 (13.3%)	20 (18.3%)		2 (12.5%)	22 (17.9%)	
Symptoms[c]						
Altered mental status	12 (40.0%)	25 (23.4%)	0.07	4 (25.0%)	33 (27.3%)	0.85
Headache	27 (90.0%)	94 (87.9%)	0.75	15 (93.8%)	106 (87.6%)	0.47
Focal sign	4 (13.3%)	14 (13.1%)	0.97	1 (6.3%)	17 (14.0%)	0.34
Fever	22 (73.3%)	82 (76.6%)	0.71	11 (68.8%)	93 (76.9%)	0.48
Death at discharge[d]	5 (17.2%)	24 (24.2%)	0.43	4 (25.0%)	25 (22.3%)	0.81

[a] Data are median (interquartile range) or number of patients (%).
[b] One case with cryptococcal meningitis and toxoplasmic encephalitis was excluded from the analysis.
[c] Medical records were available for 137 patients.
[d] Survival status at discharge was known for 128 patients.

guideline for clinical management of HIV/AIDS patients (8), if patients with a focal sign have poor response to the toxoplasma encephalitis therapy, further investigation with computed tomography scan is recommended to exclude other space-occupying lesions such as primary CNS lymphoma, and the computed tomography scan is available at most government referral hospitals in Thailand. However, this clinical practice may underdiagnose a minimal primary CNS lymphoma, which does not cause CNS symptoms.

Because of a high mortality rate of symptomatic Thai patients (9), patients with a small primary CNS lymphoma might have died due to other opportunistic infections before the primary CNS lymphoma lésion became large and caused CNS symptoms. Recently the Thai government pharmaceutical organization has started mass production of generic antiretroviral drugs. If many insidious primary CNS lymphoma cases exist in Thailand, we expect to see more patients with apparent primary CNS lymphoma lesions as the antiretroviral drug-treated patients survive longer. Alternatively, it is plausible that Thai patients are less susceptible to the development of primary CNS lymphoma and that EBV DNA detection in CSF from AIDS patients does not supplement the diagnosis of primary CNS lymphoma in Thailand.

In our experiences at Lampang Hospital, CMV retinitis is common among our advanced HIV-infected patients, but we have not seen any case with CMV encephalitis. This rarity of CMV encephalitis may be due to the difficulty of making a firm diagnosis in Thailand, since it requires magnetic resonance imaging or biopsy, which is not widely available, and the disease does not induce characteristic clinical symptoms. Our data on CMV PCR warn that we may be overlooking patients with CMV encephalitis.

Progressive multifocal leukoencephalopathy cases have been reported but are not common in Thailand (2, 6). At Lampang Hospital, we had one male patient who presented with hemiparesis and was diagnosed with progressive multifocal leukoencephalopathy on the basis of computed tomography scan findings and clinical course. His CSF was negative for JCV PCR, but this result does not exclude progressive multifocal leukoencephalopathy as the sensitivity of JCV PCR is not high (5). We found one case in which JCV virus was detected in the CSF of the second lumbar puncture. This patient did not have any other CNS symptoms besides headache, but he died shortly after the diagnosis of cryptococcal meningitis. We think that a low prevalence of JCV DNA detection is compatible with our clinical impression, that is, progressive multifocal leukoencephalopathy cases are there but not common, though more patients would be detected if brain magnetic resonance imaging were available.

We thank Noriaki Hosoya, Mieko Goto, and Tadashi Narisawa for their technical assistance and for Suthiraa Kasemsuk, Sriprai Seneewong-na-ayoottaya, Kethkaew Thamachai, Somchai Niyomthai, Anong-nard Ariyakruea, Nutira Boona, and Prapan Wongnamnong for their support.

This study was supported by the Japan International Cooperation Agency (JICA), the Ministry of Health, Labor and Social Welfare of Japan, and the Ministry of Public Health of Thailand.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 21, Number 9, 2005, pp. 810–814

Sequence Note

HIV Type 1 Subtypes in Circulation in Northern Kenya

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ABSTRACT

The genetic subtypes of HIV-1 circulating in northern Kenya have not been characterized. Here we report the partial sequencing and analysis of samples collected in the years 2003 and 2004 from 72 HIV-1-positive patients in northern Kenya, which borders Ethiopia, Somalia, and Sudan. From the analysis of partial *env* sequences, it was determined that 50% were subtype A, 39% subtype C, and 11% subtype D. This shows that in the northern border region of Kenya subtypes A and C are the dominant HIV-1 subtypes in circulation. Ethiopia is dominated mainly by HIV-1 subtype C, which incidentally is the dominant subtype in the town of Moyale, which borders Ethiopia. These results show that cross-border movements play an important role in the circulation of subtypes in Northern Kenya.

KENYA IS BORDERED IN THE NORTH by countries that have had political upheavals in the past leading to a lot of movement of populations across the borders into Kenya. These countries are Ethiopia, Somalia, and Sudan. In this region, not much is known about the circulating subtypes of HIV-1.

Work done between 1998 and 1999 shows that Sudan is dominated mainly by subtypes A, C, and D, with subtype D being the dominant circulating subtype.[1] In Ethiopia, the HIV-1 epidemic is dominated exclusively by HIV-1 C viruses[2] while in Somalia the circulating subtypes have not been clearly defined.

In this study to determine the circulating subtypes of HIV-1 in northern Kenya, HIV-1-positive patients and blood donors attending STD clinics and District hospitals in Mandera, Moyale, and Turkana District between August 2003 and April 2004 were recruited. The study subjects gave written informed consent and 3 ml of blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Peripheral blood mononuclear cells (PBMCs) were extracted and used for polymerase chain reaction (PCR) amplification. A nested strategy was used to amplify about 450 base pairs of the *env* gene (nt 7850–8310) i.e., the gp41 region.[3] The primers used in the PCR were gp40F1 (5′-TCTTAGGAGCAGCAGGAAGCACTATGGG-3′) and gp41R1 (5′-AACGACAAAGGTGAGTATCCCTGCCTAA-3′) for the first round of PCR and primers gp46F2 (5′-ACAATTATTGTCTGGTATAGTGCAACAGCA-3′) and gp47R2 (5′-TTAAACCTATCAAGCCTCCTACTATCATTA-3′ for the nested PCR. The PCR conditions included denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 60 sec, with a final extension at 72°C for 5 min. The resulting products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light to identify the amplified products. The PCR products were sequenced directly using the BigDye Terminator DNA sequencing kit from Applied Biosystems. Electrophoresis and data collection were accomplished with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). The CLUSTAL W method[4] was used to align the resulting 400–450 bp nucleotide sequences to-

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gether with relevant reference sequences from the Los Alamos reference database.[5] Phylogenetic relationships were deduced using the neighbor-joining method.[6] The phylogenetic tree was drawn using the tree view program.[7]

Phylogenetic analysis of the *env* gp41 region of samples from 72 HIV-1-positive patients revealed that 50% (32 samples) of the samples were subtype A, 39% (28 samples) were subtype C, and 11% (8 samples) were subtype D. The results also showed a significant difference in the distribution of the HIV-1 subtypes in Turkana and Moyale. In Moyale a majority of the samples were subtype C (51%); 40% of the samples were subtype A and 9% were subtype D. Moyale contributed 82% of the total subtype C found in this region. This region borders Ethiopia where the dominant HIV-1 subtype is C. In Turkana, the dominant subtype in circulation is A (64%), while the rest is subtype C (20%) and D (16%). The number of samples from Mandera successfully analyzed was too few (2) to draw any significant conclusions. These samples were HIV-1 subtype A. The phylogeny of these viruses

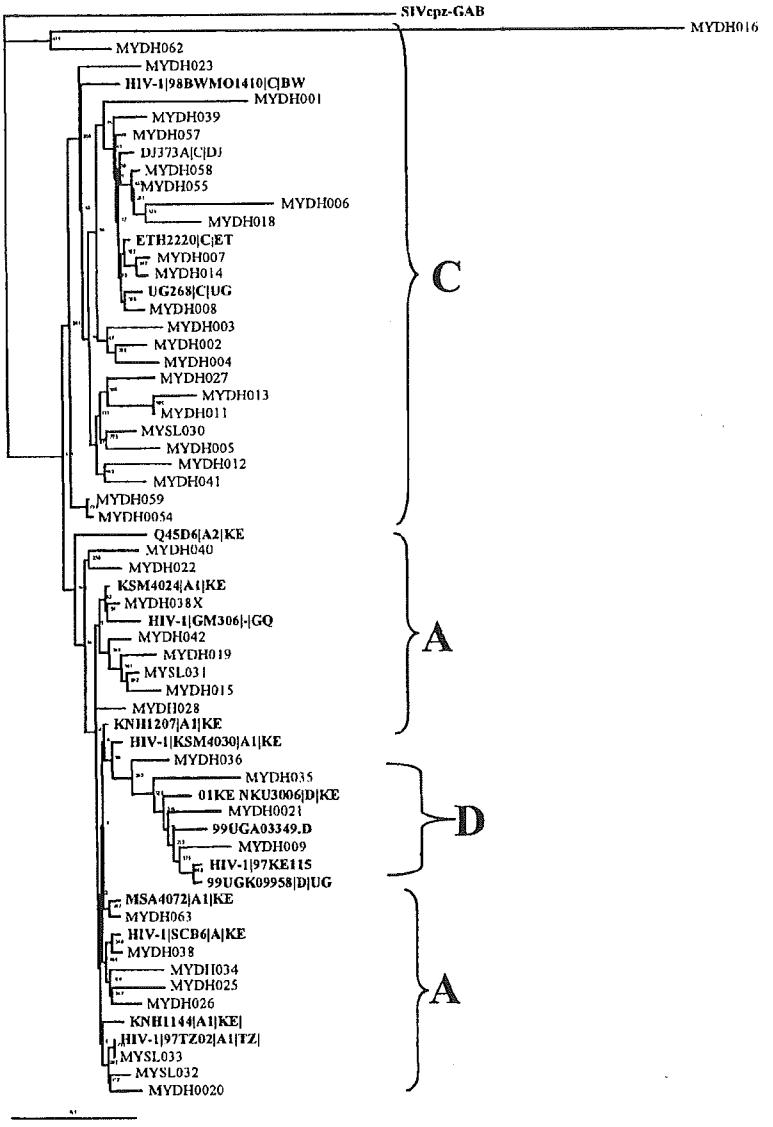

FIG. 1. Phylogenetic analysis of the gp41 *env* region of HIV-1 subtypes from Moyale in northern Kenya. The simian immunodeficiency virus SIV_{cpzgab} was used as the outgroup. The sequences have been indicated by codes MYDH and MYSL denoting Moyale District Hospital and Moyale Sololo, respectively. The A subtypes clustered with references from Kenya, Gambia, and Tanzania; the C subtypes clustered together with references from Ethiopia, Uganda, Djibouti, and Botswana; and the D subtypes clustered with those from Uganda and Kenya.

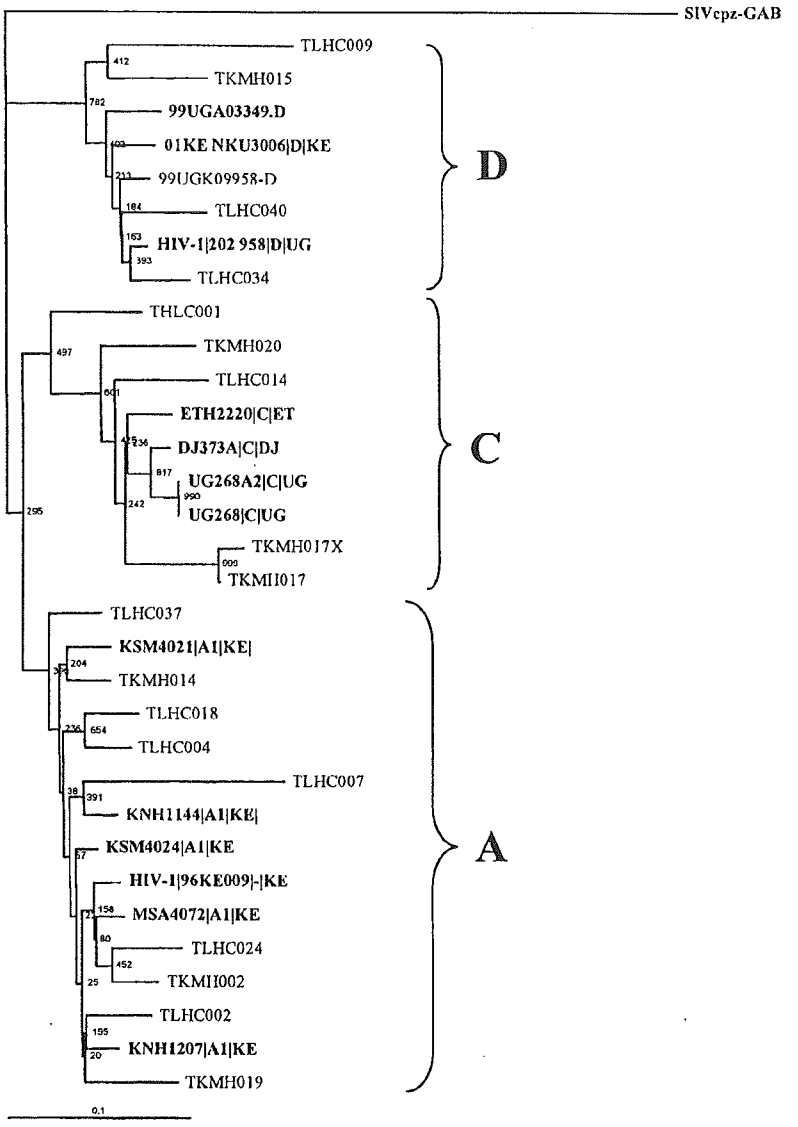

FIG. 2. Phylogenetic analysis of the gp41 *env* region HIV-1 subtypes from Turkana in northern Kenya. The sequences have been indicated by codes TLHC and TKMH denoting Turkana Lobiding Health Centre and Turkana Kakuma Mission Hospital, respectively. The A subtypes in this region clustered with references from Kenya; the C subtypes clustered with references from Ethiopia, Djibouti, and Uganda; and the D subtypes from the region clustered with references from Uganda and Kenya as indicated on the tree.

is displayed in Figs. 1–3. The information available about the study subjects is shown along with the subtype in Table 1. These results indicate a different picture of HIV-1 subtypes in circulation compared to other parts of Kenya where the dominant subtype in circulation is A (70%).

SEQUENCE DATA

GenBank accession numbers (listed in Table 1) for the *env* gp41 sequences are AY697976–AY698021, AY694410–AY694411, AY693585–AY693603, and AY705732–AY705737.

ACKNOWLEDGMENTS

The KEMRI-JICA program funded this project. The work was done in collaboration with the African Medical and Research Foundation. We wish to thank the staff in Northern Kenya, i.e., in Moyale, Mandera, Kakuma, and Lokichogio hospitals, who assisted in the collection and initial processing of the samples.

TABLE 1. INFORMATION ABOUT STUDY SUBJECTS

ID	*Year*	*GenBank accession no.*	*Age (years)*	*Sex*	*Subtype (env)*
MYDH012	2003	AY698019	32	F	C
MYDH013	2003	AY698011	2	F	C
MYSL032	2003	AY697991	50	F	A1
MYDH054	2003	AY698010	34	F	C
MYDH058	2003	AY698002	34	M	C
MYDH057	2003	AY698003	20	F	C
MYDH034	2003	AY698021	23	F	A1
MYDH035	2003	AY698004	25	F	D
MYDH038	2003	AY697981	28	F	A
MYDH055	2003	AY698009	32	M	C
MYDH059	2003	AY698008	34	F	C
MYDH025	2003	AY697978	21	F	A1
MYSL033	2003	AY697984	30	F	A1
MYSL031	2003	AY697986	44	M	A
MYDH023	2003	AY698018	7	M	C
MYDH022	2003	AY697988	50	F	A1
MYDH028	2003	AY698020	26	F	A1
MYDH027	2003	AY697980	32	M	C
MYSL030	2003	AY697985	40	M	C
MYDH021	2003	AY697979	38	M	D
MYDH036	2003	AY697987	32	M	A
MYDH039	2003	AY697976	20	F	C
MYDH020	2003	AY697983	30	F	A1
MYDH038	2003	AY698005	28	F	A1
MYDH026	2003	AY697982	30	M	A1
MYDH003	2003	AY697992	35	M	C
MYDH005	2003	AY697989	40	M	C
MYDH004	2003	AY697990	45	F	C
MYDH002	2003	AY697993	45	M	C
MYDH007	2003	AY697995	20	F	C
MYDH014	2003	AY697996	25	M	C
MYDH001	2003	AY697997	25	F	C
MYDH063	2004	AY698007	35	M	A1
MYDH016	2003	AY697998	46	M	A
MYDH015	2003	AY697994	41	M	A1
MYDH019	2003	AY698017	47	M	A1
MYDH018	2003	AY698016	42	M	C
MYDH006	2003	AY697999	32	M	C
MYDH009	2003	AY698001	32	M	D
MYDH041	2003	AY698014	56	M	C
MYDH011	2003	AY698015	30	F	C
MYDH040	2003	AY698013	27	F	A
MYDH042	2003	AY698012	32	F	A1
MYDH062	2004	AY698005	38	M	D
MYDH008	2003	AY698000	50	M	C
TLHC007	2003	AY693585	28	M	A1
TLHC014	2003	AY693588	33	M	C
TLHC018	2003	AY693587	39	F	A
TLHC024	2003	AY693589	24	M	A1
TLHC034	2003	AY693597	39	F	D
TLHC040	2003	AY693596	34	F	D
TLHC004	2003	AY693591	28	F	A1
TLHC037	2003	AY693595	24	F	A1
TLHC002	2003	AY693586	16	F	A1
TLHC009	2003	AY693590	21	F	D
TLHC001	2003	AY693592	30	F	A
TLHC101	2004	AY705733	24	M	A
TLHC106	2004	AY705737	35	M	A1
TLHC107	2004	AY705732	42	M	A
TLHC109	2004	AY705734	45	F	C
TLHC111	2004	AY705735	46	M	A

TABLE 1. INFORMATION ABOUT STUDY SUBJECTS (CONT'D)

ID	*Year*	*GenBank accession no.*	*Age (years)*	*Sex*	*Subtype (env)*
TLHC112	2004	AY705736	50	M	A1
TKMH017X	2004	AY693601	30	F	C
TKMH017	2003	AY693602	40	M	C
TKMH002	2003	AY693598	20	F	A
TKMH020	2003	AY693599	24	M	C
TKMH014	2003	AY693600	30	F	A1
TKMH019	2003	AY693593	35	M	A
TKMH015	2003	AY693594	40	F	D
MADH005	2003	AY694411	37	F	A1
MADH003	2003	AY694410	20	M	A

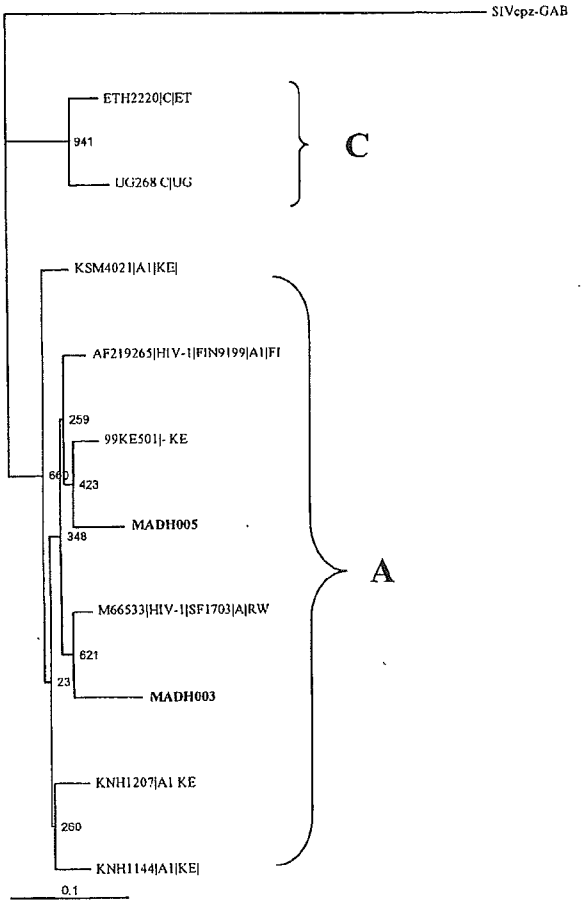

FIG. 3. Phylogenetic analysis of the gp41 *env* region HIV-1 subtypes from Mandera in northern Kenya. The sequences have been indicated by codes MADH denoting Mandera District Hospital. The two samples were all subtype A and clustered with subtypes from Rwanda and Kenya.

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Rapid Spread of Hepatitis C Virus Among Injecting-Drug Users in the Philippines: Implications for HIV Epidemics

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From the trends of human immunodeficiency virus (HIV) epidemics in South and Southeast Asia, it was postulated that an HIV epidemic would start as a blood-borne infection among injecting-drug users in the Philippines. In 2002, 560 individuals were recruited in Metro Cebu, Philippines and tested for HIV, hepatitis C virus (HCV), and hepatitis B virus (HBV) infections. The seroprevalence of anti-HCV among injecting-drug users (70.1%, 61/87) was significantly higher than those among inhalation drug users (16.3%, 7/43; $P=0.00$; OR = 12), sex workers (0%, 0/130; $P=0.00$; OR = ∞), antenatal clinic attendees (0%, 0/100; $P=0.00$; OR = ∞), and students/health care workers (2%, 4/200; $P=0.00$; OR = 115). The seroprevalence of HBsAg among injecting-drug users (10.3%, 9/87) was significantly higher than those among sex workers (2.3%, 3/130; $P=0.01$; OR = 4.9), and antenatal clinic attendees (3%, 3/100; $P=0.04$; OR = 3.7), but was not statistically different from those among inhalation drug users (9.3%, 4/43; $P=0.9$) and students/health care workers (4.5%, 9/200; $P=0.06$). None of the study population was reactive to anti-HIV antibody. The HCV strains obtained from the injecting-drug users belonged to either genotype 1a or 2b and the strains in each genotype clustered closely to each other. There was no dual infection with genotype 1a and 2b. These results suggest that the HCV infection in injecting-drug users may be emanating rapidly from limited number individuals in Metro Cebu, Philippines. ***J. Med. Virol. 77:221–226, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV epidemic; Injecting-drug users; genotype; source; HIV/AIDS outbreak; HIV prevalence

INTRODUCTION

The Philippines is one of the low prevalence countries for human immunodeficiency virus (HIV). Based on the AIDS registry of the Department of Health in the Philippines, the total number of HIV cases has increased but remained at low level at a cumulative total of 2,107 as of June 2004. The main mode of HIV transmission has been reported to be heterosexual contact since 1984. Although HIV-positive cases have appeared sporadically among sexually active populations such as sex workers, no outbreak has occurred among them in this country. However, wide-range HIV strains have been introduced in the country, that is; five HIV-1 subtypes (A, B, C, D, and F), a circulating recombinant form (CRF01_AE) [Paladin et al., 1998; Santiago et al., 1998; Espantaleon et al., 2003], a recombinant strain (*gag*-A/*env*-B) [Espantaleon et al., 2003]. Even HIV-2 [Leano et al., 2003] has been identified. Among these, HIV-1 subtype B was the most predominant, followed by CRF01_AE [Paladin et al., 1998; Santiago et al., 1998; Espantaleon et al., 2003]. The low prevalence and the variety of HIV strains in the Philippines indicate that HIV has been imported mainly from abroad and the gateway of HIV into the Philippines has been quite open.

Grant sponsor: Japan Society for the Promotion of Science; Grant numbers: 16406014, DOST-10417; Grant sponsor: The Nippon Foundation Fellowship for Asian Public Intellectuals.

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Accepted 21 June 2005
DOI 10.1002/jmv.20439
Published online in Wiley InterScience (www.interscience.wiley.com)

Therefore, the migration sites and the subsequent circulation pathways of HIV have become one of the most important concerns for the prevention of an AIDS outbreak in the Philippines.

The past trend of HIV/AIDS outbreak in South and Southeast Asia reported by the World Health Organization (WHO; HIV/AIDS in Asia and the Pacific Region 2003) and others [Ruxrungtham et al., 2004] have implied that the Asian AIDS epidemic may start among injecting-drug users with secondary new infections become evident among sex workers. This is reasonable when considering the fact that the probability of HIV infection is 10-fold higher for transmission through contaminated needle sharing than that through sexual contact [Royce et al., 1997]. Therefore, it could be postulated that an HIV outbreak would start as a blood-borne infection among injecting-drug users in the low HIV-prevalence countries including the Philippines, and that the HIV outbreak could be preceded by other blood-borne infections, such as hepatitis C virus (HCV) and hepatitis B virus (HBV) infections.

HIV, HCV, and HBV are the major blood-borne pathogens, which spread among injecting-drug users via shared syringes and other injection devices [Lauer and Walker, 2001]. The seroprevalence of HCV antibody (anti-HCV) has been reported globally to be 65–90% among injecting-drug users [van den Hoek et al., 1990; Chamot et al., 1992; Crofts et al., 1993; Van Ameijden et al., 1993; Lauer and Walker, 2001; Soriano et al., 2002] and 82.9–100% among HIV-infected injecting-drug users [van Asten et al., 2004]. However, the reports on the prevalence and the characteristics of HCV and HBV have been limited in the Philippines. According to the available data, the positive rate for anti-HCV was 2.2% (9/392 tested) and the same rate was also noted for HBsAg among blood donors in 1990 [Arguillas et al., 1991], and anti-HCV was reported to be 4.6% (23/502 tested) among prison inmates [Katayama et al., 1996].

In this study, an HCV-epidemic site was identified in the Philippines and the genetic links of the HCV strains infecting injecting-drug users were analyzed to determine their migration site, circulation pathways, and the speed of transmission.

MATERIALS AND METHODS

Subjects

From June to August 2002, 560 individuals were recruited in Metro Cebu of the Philippines. Study population was categorized into five groups; injecting-drug users (n = 87), inhalation drug users (n = 43), sex workers (n = 130), antenatal clinic attendees (n = 100), and students and health care workers (n = 200). Characteristics of the study population are shown in Table I. Injecting-drug users were from two areas; an urban area where there was easy access to prohibited drugs and the drug rehabilitation centers. Injecting-drug users were identified by a pre-tested interview questionnaire conducted by trained staff. All of the 560 participants agreed to be part of the study after the researchers explained the objectives and the conduct of the study, and signified their intent to join the study by signing an informed consent form.

Serological Testing

A total of 5-ml whole blood was collected from each participant. Plasma was separated and subjected to each test.

Determine HIV-1/2 (ABBOTT JAPAN, Tokyo, Japan) and Determine HBsAg (ABBOTT JAPAN) were used for the detection of anti-HIV antibody and hepatitis B surface antigen, respectively. HCV PHA (Abbott Laboratories HCV 2nd Generation) was kindly provided by Abbott, Japan, for research purpose and was used for the detection of anti-HCV in this study. All the systems were used 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 (Invitrogen, Carlsbad, CA) with antisense gene-specific primers, hep32 (5′-GCDGARTACCTGGTCATAGC-3′) for NS5B regions of HCV genome. A part of NS5B region of HCV gene was amplified by nested PCR with primers, hep31b (5′-TGGGSTTCTCDTATGAYACC-3′)/hep32 in the first round, and hep33b (5′-AYACCCGMTGYTTTGACTC-3′)/hep34b (5′-CCTCCGTGAAKRCTCKCAG-3′) in the second round. 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 35 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 in the same condition except for the annealing temperature at 60°C. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel [White et al., 2000].

TABLE I. Characteristics of Injecting- and Inhalation-Drug Users and Others

Population	Tested (male/female)	Mean age (range)
Injecting-drug users	87 (80/7)	30 (13–46)
Inhalation-drug users	43 (42/1)	29 (11–53)
Sex workers	130 (2/128)	25 (18–46)
Antenatal clinic attendees	100 (0/100)	26 (17–42)
Students/health care workers	200 (65/135)	31 (6–61)

Genotyping

The PCR product was subjected to nucleotide sequence determination directly with the primers of hep33b and hep34b for NS5B region. Some of the PCR-products were cloned with TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced as described previously [Thompson et al., 1994]. At least 11 clones per sample were analyzed to investigate the possible co-existence of different HCV genotypes.

The sample sequences were aligned with HCV sequences from the database in STD AIDS Cooperative Central Laboratory (Manila, The Philippines) and HCV sequence database (http://gluttony.lanl.gov/content/hcv-db/combined_search/ search) 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 1,000 bootstrap replications. The profile of the tree was visualized with the program of Njplot [Perriere and Gouy, 1996].

Statistical Analysis

Prevalence data of HCV and HBV infection was analyzed by χ^2-test and P-value less than 0.05 was considered to be significant.

RESULTS

Prevalence of HCV, HBV, and HIV Infections

Of the 87 injecting-drug users, 61 (70.1%) were positive for anti-HCV. Twenty-eight of the injecting-drug users were recruited from an area at the downtown of Metro Cebu, and all (100%, 28/28) had anti-HCV. Of the 43 inhalation drug users, only 7 (16.3%) had anti-HCV. No one was positive for anti-HCV in the 130 sex workers and the 100 antenatal clinic patients. Among the students/health care workers (n = 200), only 4 (2%) were positive for anti-HCV (Table II). Thus, the prevalence of anti-HCV was significantly higher among injecting-drug users than inhalation drug users ($P = 0.00$; Odds ratio (OR) = 12, 95% Confidence interval (CI): 5–31), sex workers ($P = 0.00$; OR = ∞), antenatal clinic patients ($P = 0.00$; OR = ∞), and students/health care workers ($P = 0.00$; OR = 115, 95% CI: 38–346), indicating that injecting-drug use is associated significantly with HCV infection.

The seroprevalence of HBsAg among injecting-drug users (10.3%, 9/87) was significantly higher than that among sex workers (2.3%, 3/130; $P = 0.01$; OR = 5, 95% CI: 1–19) and antenatal clinic attendees (3.0%, 3/100; $P = 0.04$; OR = 4, 95% CI: 1–14), but not than that among inhalation drug users (9.3%, 4/43; $P = 0.9$) and students/health care workers (4.5%, 9/200; $P = 0.06$) (Table II).

HIV antibody was not detected in any of these groups (Table II).

Seven (8%) of the 87 injecting-drug users were dually positive for HBsAg and anti-HCV. Among other population groups, there was no dual positive case.

HCV Genotypes

Of the 61 injecting-drug users positive for anti-HCV (Table II), 52 samples were available for further analysis and 38 samples were positive by PCR with NS5B primers. Twenty-three of the PCR-positive samples were selected random and were subjected to nucleotide sequencing. The PCR products were directly sequenced and analyzed phylogenetically. A phylogenetic tree (Fig. 1) based on NS5B sequences (nucleotides, 7,975–8,196 [Choo et al., 1991]) showed two HCV genotypes, 1a and 2b. Of the 23 HCV strains examined, 15 clustered significantly with genotype 1a reference sequences (with bootstrap value 97%), and most of them sub-clustered together, while two strains (02dx02 and 02du98) did not. The remaining eight clustered significantly with genotype 2b reference sequences and formed a significant sub-cluster (with bootstrap value 96%), suggesting that the source of HCV 2b circulation among the injecting-drug users in Metro Cebu is limited and 02du49 could be a founder strain (Fig. 1).

Heterogeneity of HCV Strains in an Injecting-Drug User

To investigate the possible co-existence of different HCV genotypes in injecting-drug users, the PCR products of randomly selected 9 strains (5 genotype 1a

TABLE II. Seroprevalence of Hepatitis B Virus, Hepatitis C Virus, and HIV Infections among Selected Population in Metro Cebu

Population	Tested	Positive cases (%) for: HBsAg	Anti-HCV	Anti-HIV
Injecting-drug users	87	9 (10%)	61 (70)	0
Downtown of Metro Cebu[a]	28	3 (11)	28 (100)	0
Drug rehabilitation centers	59	6 (10)	33 (56)	0
Inhalation drug users[b]	43	4 (9.3)	7 (16)	0
Sex workers	130	3 (2.3)	0	0
Antenatal clinic attendees	100	3 (3.0)	0	0
Students/health care workers	200	9 (4.5)	4 (2.0)	0

[a]Clients from the downtown of Metro Cebu (n = 28) were all injecting-drug users.
[b]All the inhalation-drug users were from drug rehabilitation centers.

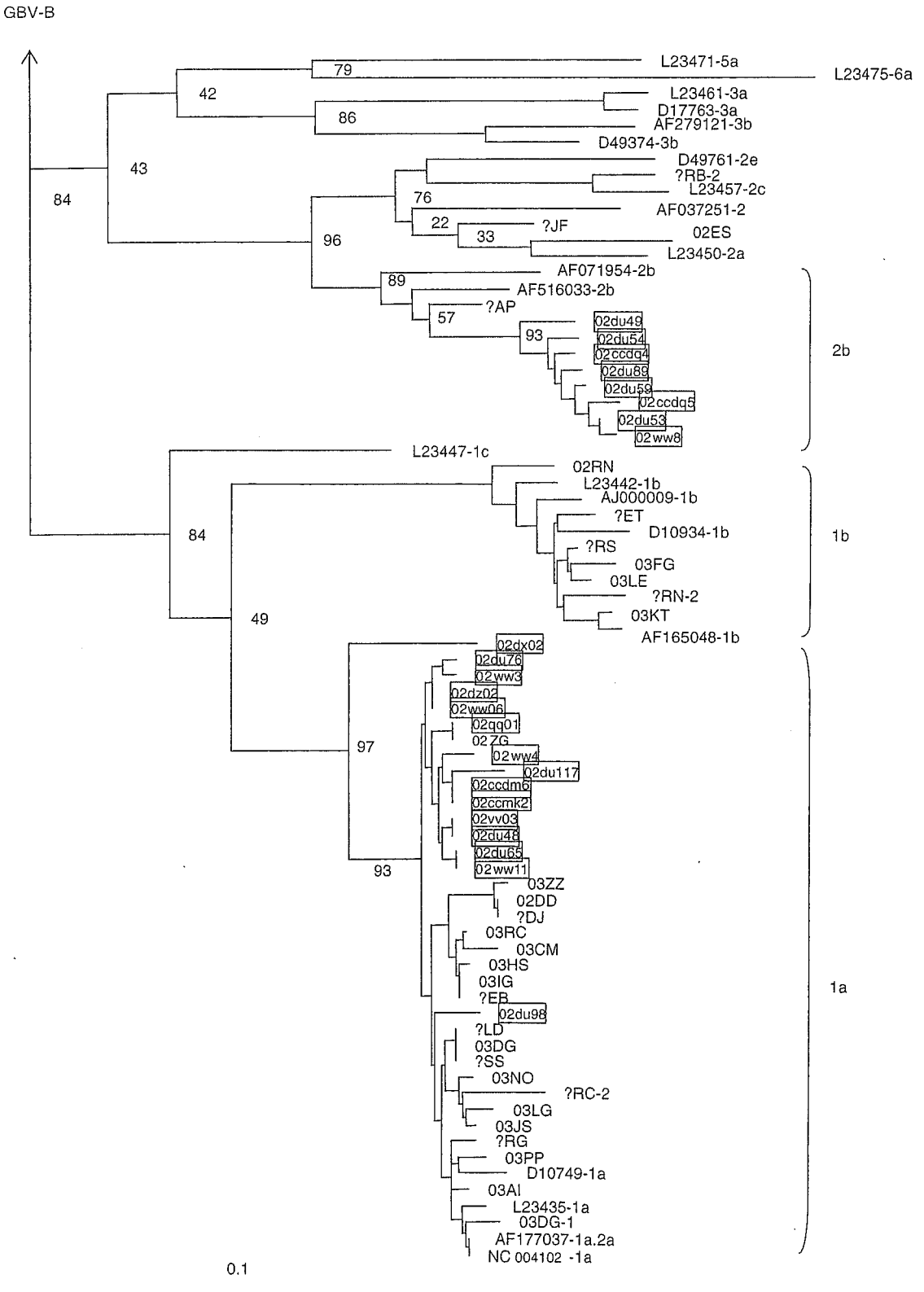

Fig. 1. Phylogenetic trees of 23 HCV strains (highlighted in the boxes) from injecting-drug users in Metro Cebu and 31 HCV strains from other area of the Philippines, performed on 227 nt within the NS5B region by the neighbor-joining method with GBV-B (accession no. NC 001655) as an outgroup. Analyzed samples were indicated with two digits of the collecting year at the head of the ID (e.g., 02ES). If the collecting year is unknown, IDs are shown with the symbol of "?" (e.g.,?JF). Accession numbers were used for the IDs of the genotype-known reference strains with two digits indicating genotypes at the end of the number (e.g., L23471-5a). Bootstrap values are given on the branches as percentage from 1,000 replicates.

strains: 02dz02, 02ccdm6, 02ccmk2, 02du98, and 02qq01; and 4 genotype 2b strains: 02ww8, 02ccdq4, 02ccdq5, and 02du49) were cloned. At least 11 clones per sample were sequenced in the regions of NS5B and analyzed phylogenetically. Phylogenetic trees based on NS5B sequences showed that nucleotide sequences of all the clones in each individual were homogeneous, and co-existence of genotype 1a and 2b were not observed.

DISCUSSION

In the current study, it was found that an HCV infection was epidemic in Metro Cebu of the Philippines, where 70% of injecting-drug users were positive for anti-HCV. The prevalence of anti-HCV among injecting-drug users has been reported to be 65–90% globally [van den Hoek et al., 1990; Chamot et al., 1992; Crofts et al., 1993; Van Ameijden et al., 1993], and that of Metro Cebu in our study was consistent with previous reports. Despite the high prevalence of anti-HCV positive cases among the tested injecting-drug users, HIV infection was not observed.

Like most RNA viruses, HCV exhibits genetic heterogeneity [Bukh et al., 1995; Zuckerman and Zuckerman, 1995], which has been reported even within the same individual [Houghton et al., 1991; Okamoto et al., 1991; Chen et al., 1992; Martell et al., 1992; Higashi et al., 1993]. In our study, two HCV genotypes, 1a and 2b were circulating among injecting-drug users in Metro Cebu, and each injecting-drug user had homogeneous HCV population regardless of the genotypes. These results suggest that these HCV strains have been introduced recently into injecting-drug users in Metro Cebu and spread rapidly among them. However, the origins have not been specified yet and further investigation is required.

The rate of HBsAg was found to be from 2% to 10% among the different population groups in Metro Cebu. However, there was no significant difference in the seroprevalence of HBsAg between injecting-drug users and inhalation drug users ($P = 0.85$). This may be because newly acquired HBV results in acute infection, needle sharing among injecting-drug users may not contribute to the increase in the HBV chronic infection, and HBV antigen carrier state may mainly be induced by vertical infections. For the further discussion, the detection of anti-HBs antibody will be required.

The Philippines and Indonesia are both island countries and have similar distances from Thailand and Cambodia where HIV infection is most prevalent in Asia. By the year 1999, Indonesia had been considered to be one of the low and slow HIV prevalence countries like the Philippines. However, in late 2000, sharp increase in HIV prevalence among injecting-drug users (up to over 35% in Jakarta) was noted (HIV/AIDS in Asia and the Pacific Region 2001, WHO). This increasing trend of HIV prevalence was also noted among blood donors, thereafter, suggesting that the use of contaminated needle sharing (causing HCV infection) triggered an AIDS outbreak before the increase in the number of HIV-infections through sexual transmission. As seen in Indonesia, HIV spreads first among injecting-drug users, followed by sex workers in other Asian countries especially if drug users are the clients of sex workers [Ruxrungtham et al., 2004]. However, it seems that HIV has not yet spread extremely through the blood-borne pathway in the Philippines. As shown in this study, HIV infection was very rare even among HCV-positive injecting-drug users. However, convincing evidence will be required by the further analyses with increasing the number of subjects and in geographically different places in the Philippines. Although HIV is of low prevalence, the rapid spread of HCV infection indicates that the injecting-drug users can be at highest risk in causing an AIDS epidemic in this country.

In this study, it was demonstrated that the HCV infection clustered among injecting-drug users in Metro Cebu of the Philippines. HCV infection seemed to be spreading rapidly among injecting-drug users from limited sources. Further studies must be conducted to identify the migration site(s) and the subsequent circulation mode of HCV infection more precisely, which can serve as a model for probable migration sites of HIV infections at an early phase of a possible AIDS epidemic in the Philippines.

ACKNOWLEDGMENTS

The authors are grateful to Mr. M. Villanobos for the technological assistance. Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research, 16406014 and Ronpaku Program, DOST-10417), Japan and The Nippon Foundation Fellowship for Asian Public Intellectuals, Indonesia, Japan, Malaysia, Philippines, and Thailand, supported this work.

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A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon

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Blood samples ($n=544$) from two different populations (Pygmies and Bantus) in Cameroon, West Africa, were analysed. Serological tests indicated that the anti-hepatitis C virus (HCV) prevalence in Bantus (20·3 %) was higher than that in Pygmies (2·3 %, $P<0·0001$), whereas the distribution of hepatitis B virus (HBV) serological markers was equally high in both populations: in total, 9·4, 17·3 and 86·8 % for HBsAg, anti-HBs and anti-HBc, respectively. HBV genotype A (HBV/A) and HBV/E were predominant (43·5 % each) in both populations, and HBV/D was found in a minority (13 %). The preS/S region was sequenced in nine cases (five HBV/A and four HBV/E) and the complete genome in six cases (four HBV/A and two HBV/E). Subsequent phylogenetic analysis revealed that the HBV/A strains were distinct from the subtypes (subgenotypes) described previously, Ae (A2) and Aa (A1), and in the preS/S region they clustered with previously reported sequences from Cameroon. Based on the nucleotide difference from Aa (A1) and Ae (A2), more than 4 % in the complete genome, the Cameroonian strains were suggested to represent a new subtype (subgenotype), designated HBV/Ac (A3). A high (3·9 %) nucleotide divergence in HBV/Ac (A3) strains suggested that the subtype (subgenotype) has a long natural history in the population of Cameroon. One of the HBV/Ac (A3) strains was found to be a recombinant with an HBV/E-specific sequence in the polymerase reverse transcriptase domain. Further cohort studies will be required to assess detailed epidemiological, virological and clinical characteristics of HBV/Ac (A3), as well as its recombinant form.

Received 26 January 2005
Accepted 12 April 2005

INTRODUCTION

According to the World Health Organization, hepatitis B virus (HBV) infection is one of the major global public health problems. Of the two billion people who have been infected with HBV worldwide, more than 350 million are at risk of developing cirrhosis and hepatocellular carcinoma due to chronic infection (Kane, 1995).

Based on a genomic sequence divergence in the entire genome exceeding 8 %, HBV strains have been classified into seven genotypes, denoted A (HBV/A) to G (HBV/G) (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000). A possible eighth genotype has been proposed with the tentative designation 'H' (Arauz-Ruiz *et al.*, 2002), which is, however, closely related to genotype F phylogenetically, with a complete genome difference of around 8 % (Kato *et al.*, 2005).

Research on HBV genotypes during the last decade has

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences determined in this study are AB194947–AB194955.

demonstrated significant associations between the HBV genotypes and the severity of liver disease, clinical outcomes and the response to antiviral therapies (Kramvis & Kew, 2005). Moreover, it was also demonstrated that the clinical and virological characteristics may also differ among patients infected with the same genotype (Miyakawa & Mizokami, 2003). The existence of different subtypes (subgenotypes) within same genotype helps to explain this for HBV/B, where one of the subtypes (subgenotypes) (widespread in Asia; Ba) possesses a recombination with genotype HBV/C, while another (indigenous to Japan; Bj) does not (Sugauchi *et al.*, 2003). Similarly, two subtypes (subgenotypes) have been reported for HBV/A: one of them, Aa (A′/A1) prevails in sub-Saharan Africa and South Asia, while the other, Ae (A2), is widely distributed in Europe and the USA (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2004). The subtypes (subgenotypes) of HBV/A show no evidence of distinguishing recombination; nevertheless, they are associated with differences in replicative activity, and in the mechanisms of HBeAg seroconversion as a result of specific nucleotide substitutions in the core promoter and precore regions (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004).

The characterization of isolates from indigenous populations, especially in Africa where HBV is hyperendemic, may assist in revealing the origin of HBV and clarify the many questions about its evolutionary history (Kramvis *et al.*, 2005). The genetic diversity and distribution of HBV genotypes in Central West Africa, particularly in Cameroon, are poorly documented. No data were available for the HBV strains from Pygmies in this region. The objectives of the present study were to assess the prevalence of HBV and hepatitis C virus (HCV) markers among Bantus and Pygmies, to compare the distribution of HBV genotypes and to analyse the genomic characteristics of the HBV/A strain in Cameroon. Six full genome sequences, including four representing a new subtype (subgenotype) of HBV/A and two HBV/E strains from the Cameroonian Pygmies, were analysed.

METHODS

Blood serum samples. Blood serum samples were collected in 1994 from 544 voluntary donors, including representatives of two relatively isolated populations (Bantu and Pygmies) in Cameroon, Central West Africa. The Pygmies enrolled were from two forest encampments in the East province, and the Bantu were enrolled from five provinces across the country (Central, South, North, West and East). None of the donors had clinical symptoms of liver disease. Written informed consent was obtained from all subjects enrolled. After isolation of the serum fraction from whole blood, the samples were stored at −40 °C until use. The number of subjects studied in each group, their ages and sexes are summarized in Table 1.

Serological assays for hepatitis virus markers and HBV genotyping. HCV (anti-HCV) and HBV serological markers (HBsAg, HBeAg, anti-HBs and anti-HBc) were examined using a chemiluminescent immunoassay (Ortho Clinical Diagnostics).

HBsAg-positive samples were subjected to HBV genotyping using an enzyme-linked immunoassay (EIA) with monoclonal antibodies to type-specific epitopes of the preS2 region (Usuda *et al.*, 1999), using commercial kits (HBV Genotype EIA; Institute of Immunology Co.).

Table 1. The distribution of HBV and HCV serological markers and HBV genotypes among two populations in Cameroon

Population	Bantu (n=370)	Pygmies (n=174)	P
Male/Female*	177/188	87/82	NS†
Age (years); mean±SD	34·2±14·5	29·9±9·2	<0·05
Anti-HCV	75 (20·3%)	4 (2·3%)	<0·0001
HBsAg	33 (8·9%)	13 (7·5%)	NS
HBeAg	5 (15·2%)	0	NS
Anti-HBs	77 (20·8%)	17 (9·8%)	0·001
Anti-HBc	322 (87·0%)	150 (86·2%)	NS
HBV/A	15 (45·5%)	5 (38·5%)	NS
HBV/E	13 (39·4%)	7 (53·8%)	NS
HBV/D	5 (15·1%)	1 (7·7%)	NS

*Gender and age data were not available for some of the specimens.
†NS, Not significant.

Amplification, quantification of HBV DNA and nucleotide sequencing. DNA was extracted from 27 serum samples, in which HBV/A and HBV/E had been identified by genotyping EIA: 20 (15 from Bantu and five from Pygmies) and seven (only from Pygmies), respectively. Total DNA was extracted from 100 µl serum using a QIAamp DNA mini kit (Qiagen) and suspended in 100 µl storage buffer (supplied by the kit manufacturer). A real-time PCR assay, allowing detection of up to 100 viral DNA copies ml^{-1} (Abe *et al.*, 1999), with slight modifications (Tanaka *et al.*, 2004), was used for HBV DNA screening.

Two overlapping HBV DNA fragments covering the entire genome sequence were amplified using specific primers and PCR conditions that have been described previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly using a Prism Big Dye v3.0 kit (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analysed in both the forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX v11.0 (Software Development). The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers AB194947–AB194955.

Sequence analysis. Sequences were aligned using the CLUSTAL W software program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining (NJ) analysis incorporating the six-parameter distance correction method (Gojobori *et al.*, 1982) with bootstrap test confirmation performed on 1000 resamplings using the Online Hepatitis virus database (http://s2as02.genes.nig.ac.jp/). Preliminary trees were constructed for Cameroonian HBV strains obtained in this study and corresponding data of 632 HBV genome sequences available from the GenBank/DDBJ databases (the trees are available from the authors). The final trees presented herein were constructed for Cameroonian strains together with the selected GenBank/DDBJ references including the HBV/A strains of various geographical origins, and representatives of other known human HBV genotypes.

Nucleotide divergence over complete genomes was calculated using the CLUSTAL method implemented in the MEGALIGN software (Clewley & Arnold, 1997).

Detection of recombination. All Cameroonian strains' complete genome sequences were examined for the presence of recombination with other HBV genotypes, as described previously (Robertson *et al.*, 1995). Bootscan analysis implemented in the SimPlot software program (Lole *et al.*, 1999) was performed for each of the strains.

Statistical analysis. All statistical values were calculated using the Mann–Whitney U test, Fisher's exact test and the χ^2 test with Yate's correction, implemented in the STATA v8.0 software program (Stata). Differences were considered significant for P values less than 0·05.

RESULTS

Hepatitis virus serological markers and HBV genotypes in Cameroon

Table 1 summarizes results of the serological screening and HBV genotyping. The overall anti-HCV seroprevalence was very high (14·5 %), and was significantly higher in Bantus (20·3 %) than in Pygmies (2·3 %, $P<0·0001$), demonstrating that transmission networks of the infection are relatively isolated between two populations (blood transfusion and medical procedures probably contributed to transmission in the Bantus but not in the Pygmies; Kowo *et al.*, 1995). Nevertheless, HBsAg prevalence was equally high in both

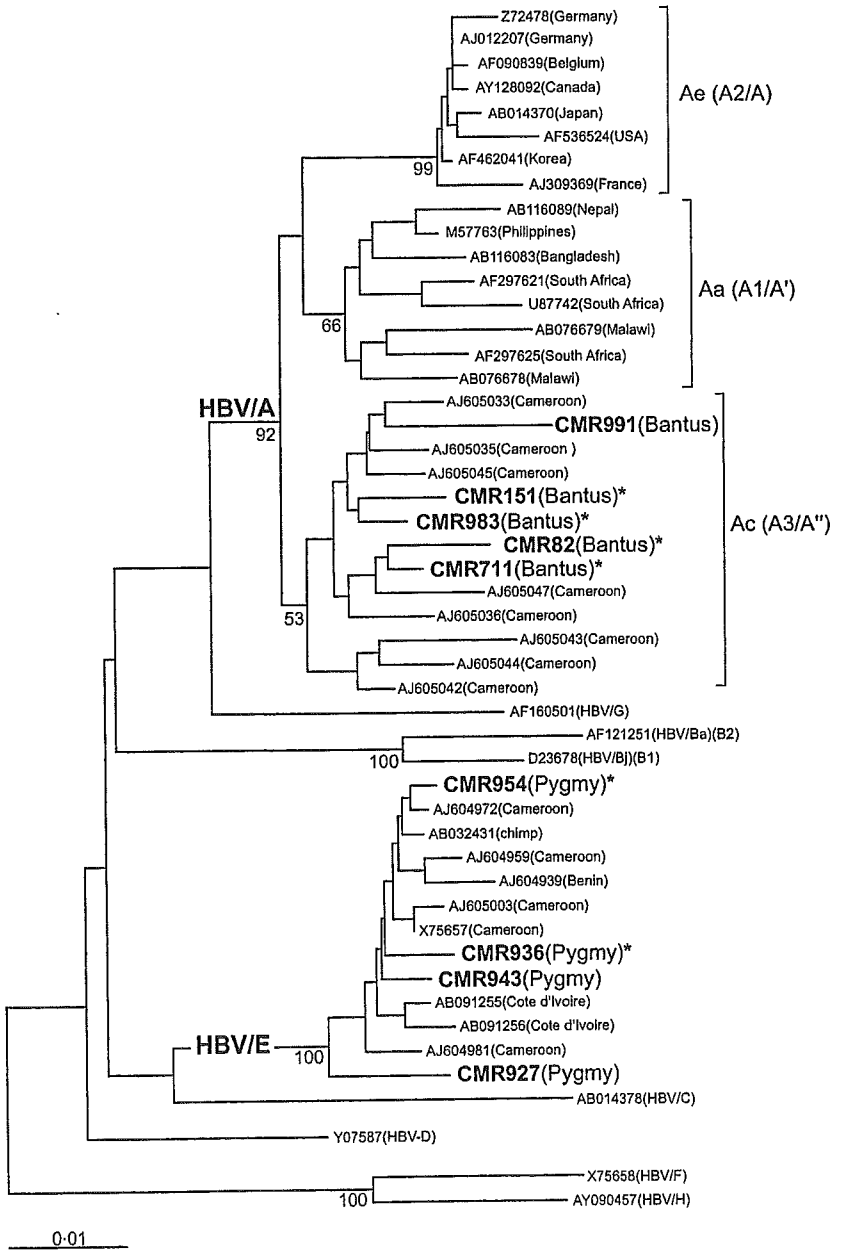


Fig. 1. A phylogenetic NJ tree constructed using the HBV preS2/S nucleotide sequences. Nine strains from Cameroon isolated in this study are indicated in bold. Reference sequences were retrieved from GenBank/EMBL/DDBJ with their accession numbers and origin (in parentheses) indicated. Bootstrap values are indicated in the tree roots. Asterisk (*) marked strains, four HBV/A and two HBV/E, from Cameroon were used for further analyses based on the complete genome sequences.

populations (8·9–7·5 %). HBeAg examined among HBsAg-positive carriers was determined in 10·9 % of cases, all of which were in the Bantus infected with HBV/E (mean age 21·2 years, range 1–30 years). The mean age of HBeAg-negative carriers in the Bantus was 31·6 years, range 17–90 years. All HBsAg-positive carriers among the Pygmies were negative for HBeAg (mean age 29·5 years, range 27–38 years). Thus, the mean age of the HBeAg-negative group was relatively young for HBsAg carriers in both populations in Cameroon, suggesting early HBeAg seroconversion. Anti-HBc seroprevalence was very high in both populations (mean 86·7 %), with no significant difference (86·2 vs 87 %), concordant with a previous report (Ndumbe *et al.*, 1993) and indicating a high incidence of HBV infection in both populations, probably attributable to effective horizontal transmission at a young age, as reported previously in African countries (Kramvis *et al.*, 2005). There was no significant difference in the distribution of the examined viral markers among the Bantu population in different provinces, or among the Pygmies population in the different encampments. A total of 46 serum samples found to be positive for HBsAg were subjected to HBV genotyping using the EIA method. Genotypes A and E identified in 43·5 % of cases were equally predominant in both of the populations, and genotype D was found in a minority (13 %) of cases. No significant difference in distribution of the genotypes was found within the same population in different provinces or between the two populations.

In order to study the molecular genetic characteristics of the prevalent HBV genotypes in Cameroon, 20 HBV/A and seven HBV/E samples, for which sufficient volume was available, were subjected to further investigation. Of the

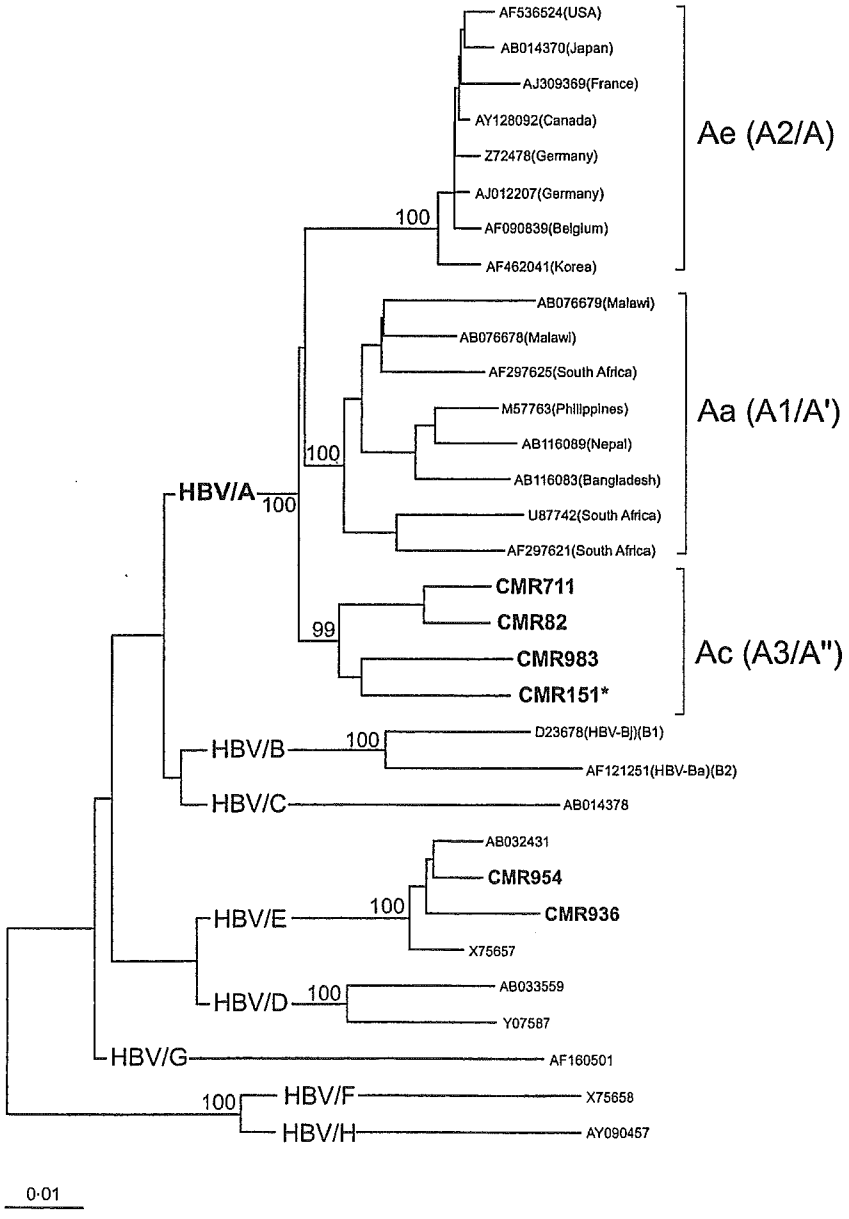
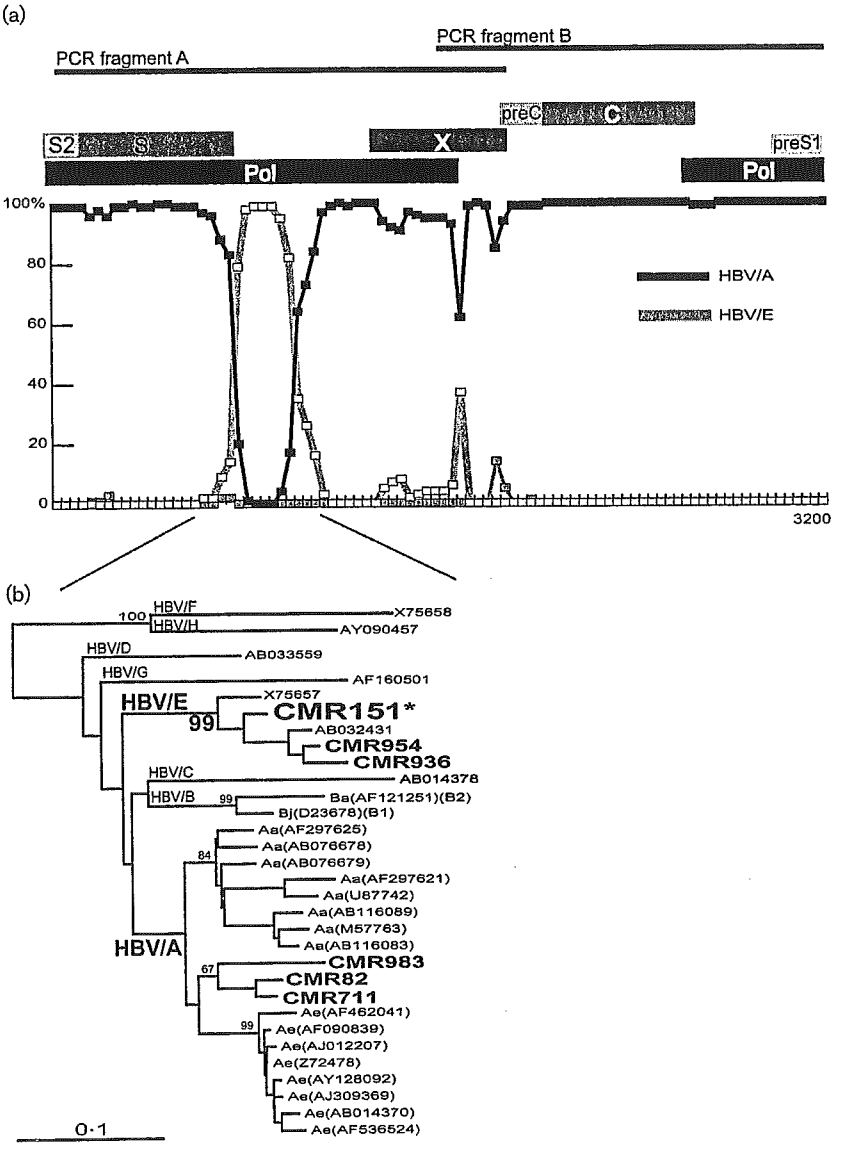


Fig. 2. A phylogenetic NJ tree constructed using the complete HBV genome. Six strains from Cameroon isolated in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. The origins of the previously published HBV/A strains are indicated in parentheses. Bootstrap values are indicated in the tree roots. The strain from Cameroon with the recombination between HBV/A and HBV/E is marked with an asterisk (*).

samples, only 1/27 was HBeAg-positive (HBV/E by EIA), which was obtained from a 1-year-old child, and the rest (26/27) of the HBsAg-positive carriers had undergone HBeAg seroconversion.

HBV DNA quantification, sequencing, phylogenetic relation and genetic diversity of HBV/A subtypes (subgenotypes)

HBV DNA was detected in only 10/27 serum samples: 5/15 Bantus and 5/12 Pygmies. The highest HBV DNA level ($3{\cdot}4 \times 10^{10}$ copies ml^{-1}) was detected in the sample obtained from a 1-year-old child. The rest of the nine positive samples were obtained from (mean) 26-year-old carriers (range 21–30 years), with HBV DNA levels ranging from $1{\cdot}1 \times 10^{3}$ to $7{\cdot}8 \times 10^{5}$ copies ml^{-1}. HBV DNA-negative carriers were (mean) 30·4 years old, range 17–50 years, showing a general tendency of HBV DNA level to decline with age (not statistically significant, probably due to small numbers). HBV large S coding region sequences were successfully amplified from 9/10 samples. The sequences were subjected to a similarity search throughout GenBank/DDBJ using the BLAST search engine, and the most similar strains were used for phylogenetic analysis together with the reference sequences of all known human HBV genotypes. The phylogenetic relationship of the ~800 nt (positions 31–835) preS2/S sequences of the HBV strains is represented in Fig. 1. Within the HBV/A phylogenetic cluster, the HBV/Aa (A1) and HBV/Ae (A2) strains separated out into two clusters and the five Cameroonian strains sequenced in this study together with other Cameroonian strains retrieved from GenBank/DDBJ clustered separately. The Cameroonian strains retrieved from GenBank/DDBJ were previously designated A″ cluster according to partial (Large S) genome sequence (Mulders *et al.*, 2004). The Cameroonian and HBV/Aa (A1) subclusters, however, did not have significant bootstrap indexes.



The complete genome of six strains (four HBV/A and two HBV/E) were sequenced successfully (marked by asterisks in Fig. 1). The lengths of the complete genomes corresponding to HBV/A and HBV/E were 3221 and 3212 nt, respectively. The phylogenetic analysis of the complete genome sequences (Fig. 2) revealed three distinct bootstrap test supported groups within the HBV/A cluster: HBV/Aa (A1), HBV/Ae (A2), and the third group formed by samples from Cameroon. We denoted the third phylogenetic group as 'HBV/Ac', where 'c' stands for Cameroon and Central Africa. The distinctive grouping of HBV/Ac (A3) strains was also confirmed when preS1/S2, preC/C, and complete Pol genes were analysed phylogenetically. S and X genes were phylogenetically related between the HBV/Aa (A1) and HBV/Ac (A3) groups. Estimated inter-group percentage nucleotide divergence over complete genome sequences consisted of [mean ± SD (range)]: 4·9 ± 0·4 (4·2–6·1), Aa (A1) versus Ae (A2); 5·1 ± 0·5 (4·0–6·0), Aa (A1) versus Ac (A3); and 5·2 ± 0·3 (4·7–5·8), Ae (A2) versus Ac (A3). On the other hand, intra-group percentage nucleotide divergence was similar for HBV/Aa (A1) and HBV/Ac (A3) [mean ± SD (range)]: 3·6 ± 0·8 (4·0–4·6) and 3·9 ± 1·1 (1·8–4·8), respectively, and lowest for Ae (A2): 0·9 ± 0·3 (0·4–1·6) ($P<0·0001$).

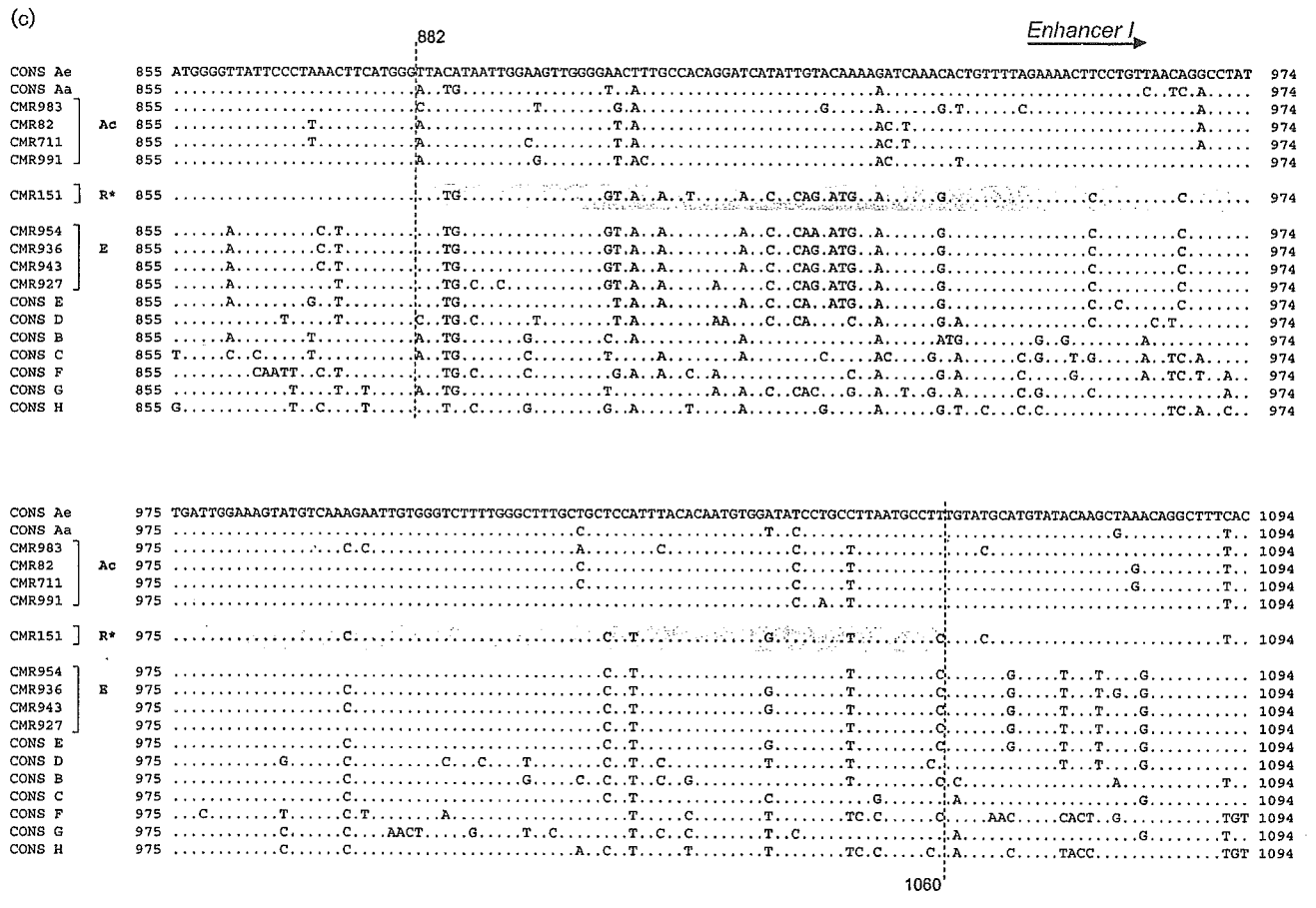


Fig. 3. (a) SimPlot analysis demonstrating the recombination in the non-overlapping part of the polymerase coding region of the CMR151 strain. The strain was subjected to bootscan analysis over the entire genome using the SimPlot program (Lole *et al.*, 1999) with a window size of 300 bp and a step size of 30 bp, under the F84 (ML) model, with bootstrap resampling performed 1000 times. Initially, consensus sequences of each human HBV genotype were used as references; after manual confirmation of the sequence alignment, the final plot was constructed using the consensus of the HBV/A, HBV/E and HBV/D genotypes only. The sequences were obtained from two overlapping PCR fragments, indicated by two lines ('A' and 'B'). HBV genome coding regions are indicated by standard abbreviations (S2, S, Pol, X, preC, C and preS1). (b) The phylogenetic NJ tree constructed using the recombinant segment. Six strains from Cameroon in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. (c) Alignment of all human HBV genotype genome nucleotide sequences in the region corresponding to the recombination in the Cameroonian strain CMR151 (shaded in grey). Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Dashed lines at 882 and 1060 represent the breakpoints.