one LTR-circular HIV-1 DNA with or without two LTR-circular types in 11 of 15 patients in whom real-time PCR showed that intracellular HIV-1 DNA levels were detectable. However, no circular HIV-1 DNA was detected in the remaining 4 patients who were judged positive by real-time PCR (Table 2). In contrast, as predicted, no circular form HIV-1 DNA was detected among the patients who were judged negative by real-time PCR except patient 17. Circular HIV-1 DNA was detected at a rate of 100% in the group with the highest plasma viral load ($>10^5$ copies/ml), with the lowest $CD4^+$ cell count (<200 cells/μl), and with the highest intracellular HIV-1 DNA levels ($>10^4$ copies/10^6 cells) (Table 4).

Discussion

We measured HIV-1 DNA copy numbers in $CD4^+$ T lymphocytes from 21 therapy-naïve patients using real-time PCR with the Roche LightCycler system. The lower limit of detection was 200 copies/10^6 cells. The intracellular HIV-1 DNA copy numbers ranged from below detectable levels to 98,120 copies/10^6 cells. This distribution was similar to those reported by Désiré et al. (7), Andreoni et al. (1) and Riva et al. (15). Although the number of patients studied might be insufficient to statistically determine an association between plasma HIV-1 viral load and levels of HIV-1 DNA, we identified a weak positive relationship. The key point was the demonstration of the presence of $CD4^+$ T lymphocytes containing a minimal level of HIV-1 DNA in 6 of 21 therapy-naïve patients despite high levels of viral load ranging from 8,800 to150,000 copies/ml. Thereupon, we evaluated the tropism of plasma HIV-1 RNA of these viruses by sequencing the *env V3* region. In all cases, the determined tropism was M-tropic suggesting that viral replication is actively ongoing in target cells of M-tropic HIV-1. In contrast, HIV-1 infection in $CD4^+$ T lymphocytes was not widely established. Current belief is that M-tropic HIV-1 predominantly replicates in patients at the first stage of infection, followed by an increase in T-tropic HIV-1 variants as reported by Schuitemarker et al. (16). According to this model, the very low level of HIV-1 DNA in the $CD4^+$ T lymphocytes found in this study might reflect the infection stage where almost all $CD4^+$ T lymphocytes remain free from HIV-1. Circular HIV-1 DNA was undetectable in most such patients, supporting this notion because this molecular species of HIV-1 DNA reflects active reverse transcription and replication.

From this viewpoint, the HIV-1 DNA copy number in $CD4^+$ T lymphocytes could be a new indicator of the clinical status of HIV-1 infection in therapy-naïve patients. In addition, the delayed HIV-1 infection of $CD4^+$ T lymphocytes could provide new insights into anti-HIV-1 therapy. Selective therapy against M-tropic

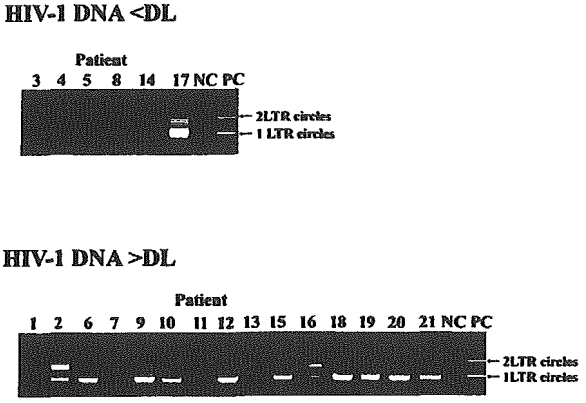

Fig. 2. Detection of unintegrated 1LTR and 2LTR circular HIV-1 DNA in 21 therapy-naïve patients. Molt4 cells were used as negative control (NC) and Molt4-IIIB cells persistently infected with HIV-1 IIIB were used as positive control (PC). Products of PCR electrophoresed in 1% agarose gels were visualized by staining with ethidium bromide.

Table 4. Detection rate of circular HIV-1 DNA in therapy-naïve patients classified as three categories

Categories	Detection rate of circular HIV-1 DNA
$CD4^+$ cell count (cells/μl)	
<200	4/4 (100%)
200–350	6/10 (60%)
>350	2/7 (28.6%)
Plasma HIV-1 RNA (copies/ml)	
<50,000	3/9 (33.3%)
50,000–100,000	2/5 (40.0%)
>100,000	7/7 (100%)
Intracellular HIV-1 DNA (copies/10^6 cells)	
<DL	1/6 (16.7%)
200–10,000	7/11 (63.6%)
>10,000	4/4 (100%)

HIV-1 might retard HIV-1 infection of $CD4^+$ T lymphocytes, delaying or preventing subsequent formation of a lymphocytic HIV-1 reservoir.

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References

1) Andreoni, M., Parisi, S.G., Sarmati, L., Nicastri, E., Ercoli, L., Mancino, G., Sotgiu, G., Mannazzu, M., Trevenzoli, M., Tridente, G., Concia, E., and Aceti, A. 2000. Cellular proviral HIV-DNA decline and viral isolation in naïve subjects with <5000 copies/ml of HIV-RNA and >500×10⁶/l CD4 cells treated with highly active antiretroviral therapy. AIDS **14**: 23–29.
2) Brusswl, A., Mathez, D., Broche-Pierre, S., Lancar, R., Calvez, T., Sonigo, P., and Leibowitch, J. 2003. Longitudinal monitoring of 2-long terminal repeat circles in peripheral blood mononuclear cells from patients with chronic HIV-1 infection. AIDS **17**: 645–652.
3) Chun, T.W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J.A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T.C., Kuo, Y.H., Brookmeyer, R., Zeiger, M.A., Barditch-Crovo, P., and Siliciano, R.F. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **287**: 183–188.
4) Chun, T.W., Stuyver, L., Mizell, S.B., Ehler, L.A., Mican, J.A.M., Baseler, M., Lloyd, A.L., Nowak, M.A., and Fauci, A.S. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. U.S.A. **94**: 13193–13197.
5) Cone, R.W., Gowland, P., Opravil, M., Grob, P., Ledergerber, B., and the Swiss HIV Cohort Study. 1998. Levels of HIV-infected peripheral blood cells remain stable throughout the natural history of HIV-1 infection. AIDS **12**: 2253–2260.
6) Damond, F., Descamps, D., Farfara, I., Telles, J.N., Puyeo, S., Campa, P., Leprêtre, A., Matheron, S., Brun-Vezinet, F., and Simon, F. 2001. Quantification of proviral load of human immunodeficiency virus type 2 subtypes A and B using real-time PCR. J. Clin. Microbiol. **39**: 4264–4268.
7) Désiré, N., Dehée, A., Schneider, V., Jacomet, C., Goujon, C., Girard, P.M., Rozenbaum, W., and Nicolas, J.C. 2001. Quantification of human immunodeficiency virus type 1 proviral load by a TaqMan real-time PCR assay. J. Clin. Microbiol. **39**: 1303–1310.
8) Finzi, D., Blankson, J., Siliciano, J.D., Margolick, J.B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., Quinn, T.C., Chaisson, R.E., Rosenberg, E., Walker, B., Gange, S., Gallant, J., and Siliciano, R.F. 1999. Latent infection of $CD4^+$ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. **5**: 512–517.
9) Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, T.E., Quinn, T.C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D.D., Richman, D.D., and Siliciano, R. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278**: 1295–1300.
10) Leitner, T., Korber, K., Robertson, D., Gao, F., and Hahn, B. 1997. Updated proposal of reference sequences of HIV-1 genetic subtype, p.III-19–24. *In* Korber, B., Hahn, B., Foley, B., Mellors, J.W., Leitner, T., Myers, G., McCutchan, F., and Kuiken, C.L. (eds), Human retroviruses and AIDS 1997, Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N. Mex.
11) Mellors, J.W., Rinaldo, C.R., Jr., Gupta, P., White, R.M., Todd, J.A., and Kingsley, L.A. 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **272**: 1167–1170.
12) Nandi, J.S. 1999. Unintegrated viral DNA as marker for human immunodeficiency virus 1 infection *in vivo* and *in vitro*. Acta Virol. **43**: 367–372.
13) Nicholson, W.J., Shepherd, A.J., and Aw, D.W.J. 1996. Detection of unintegrated HIV type 1 DNA in cell culture and clinical peripheral blood mononuclear cell samples: correlation to diseases stage. AIDS Res. Hum. Retrovir. **12**: 315–323.
14) Piatak, M., Saag, M.S., Yang, L.C., Clark, S.J., Kappes, J.C., Luk, K.C., Hahn, B.H., Shaw, G.M., and Lifson, J.D. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science **259**: 1749–1754.
15) Riva, E., Antonelli, G., Scagnolari, C., Pistello, M., Capobianchi, M.R., Monforte, A.D., Pezzotti, P., and Dianzani, F. 2003. Human immunodeficiency virus (HIV) DNA load and level of immunosuppression in treatment-naïve HIV-1-infected patients. J. Infect. Dis. **187**: 1826–1828.
16) Schuitemaker, H., Koot, M., Kootstra, N.A., Dercksen, M.W., De Goede, R.E.Y., Van Steenwijk, R.P., Lange, J.M.A., Eeftink Schattenkerk, J.K.M., Miedema, F., and Tersmette, M. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus populations. J. Vir. **66**: 1354–1360.
17) Shioda, T., Levy, J.A., and Cheng-Mayer, C. 1992. Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. **89**: 9434–9438.
18) Wong, J.K., Günthard, H.F., Havlir, D.V., Zhang, Z.Q., Haase, A.T., Ignacio, C.C., Kwork, S., Emini, E., and Richman, D.D. 1997. Reduction of HIV-1 in blood and lymph nodes following potent antiretroviral therapy and the virologic correlates of treatment failure. Proc. Natl. Acad. Sci. U.S.A. **94**: 12574–12579.
19) Wong, J.K., Hezareh, M., Günthard, H.F., Havlir, D.V., Ignacio, C.C., Spina, C.A., and Richman, D.D. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278**: 1291–1295.

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Nucleotide and Amino Acid Polymorphisms at Drug Resistance Sites in Non-B-Subtype Variants of Human Immunodeficiency Virus Type 1

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We have compared nucleotide substitutions and polymorphisms at codons known to confer drug resistance in subtype B strains of human immunodeficiency virus type 1 (HIV-1) with similar substitutions in viruses of other subtypes. Genotypic analysis was performed on viruses from untreated individuals. Nucleotide and amino acid diversity at resistance sites was compared with a consensus subtype B reference virus. Among patients with non-subtype B infections, polymorphisms relative to subtype B were observed at codon 10 in protease (PR). These included silent substitutions (CTC→CTT, CTA, TTA) and an amino acid mutation, L10I. Subtype A viruses possessed a V179I substitution in reverse transcriptase (RT). Subtype G viruses were identified by silent substitutions at codon 181 in RT (TAT→TAC). Similarly, subtype A/G viruses were identified by a substitution at position 67 in RT (GAC→GAT). Subtype C was distinguished by silent substitutions at codons 106 (GTA→GTG) and 219 (AAA→AAG) in RT and codon 48 (GGG→GGA) in PR. Variations relative to subtype B were seen at RT position 215 (ACC→ACT) for subtypes A and A/E. These substitutions and polymorphisms reflect different patterns of codon usage among viruses of different subtypes. However, the existence of different subtypes may only rarely affect patterns of drug resistance-associated mutations.

The advent of highly active antiretroviral therapy (HAART) has helped to stabilize the progression of human immunodeficiency virus type 1 (HIV-1) disease in Western countries (19). However, most current knowledge of HIV pathogenesis and responsiveness to antiretroviral therapy is based on work carried out with subtype B viruses, while relatively little information is available with regard to other viral subtypes (13). At the same time, global rates of infection attributable to viruses of other subtypes (A to K) are rapidly increasing (3, 6, 23; K. Fransen, A. Buve, J. N. Nkengasong, M. Laga, and G. van der Groen, Letter, Lancet 347:1403, 1996). There is also evidence for the increased prevalence of non-subtype B infections in Western countries as well as infections caused by recombinant viruses involving subtype B and other viruses (18).

Drug resistance testing has assumed an important role in HIV therapeutics, yet little information is available with regard to the potential effect of subtype diversity on both drug resistance and responsiveness to antiviral therapy (13).

Our goal was to analyze genomic diversity at a number of sites known to be associated with resistance to each of the three major families of antiretroviral drugs (ARVs), i.e., nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). This analysis involved a comparison of known mutational sites among subtype B viruses with the same loci in other viral subtypes. The results illustrate the likelihood that the development of drug resistance among various HIV-1 subtypes may involve different amino acid substitutions and that certain of these changes may result in a variety of phenotypes with regard to the ability of such mutations to confer resistance against individual drugs. This subject is likely to gain in importance as more is learned about HIV genomic diversity at resistance-conferring genomic sites among various subtypes of HIV-1.

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MATERIALS AND METHODS

Patients. The clinical isolates employed in this study included plasma samples from ARV-naive patients provided to our laboratory from sites in Botswana, Kenya, Cote d'Ivoire, Japan, Canada, and Israel (in the final case, the samples evaluated were from patients who had emigrated from Ethiopia). In all instances, samples were obtained with informed consent.

To assist in our analysis of viruses of subtypes A/E, D, G, and A/G, we also studied sequences derived from viruses of untreated patients that are available through the Los Alamos database (http://hiv-web.lanl.gov). We searched only for those mutations relevant to *gag-pol* from among non-subtype B viruses derived from untreated patients. However, some sequences could not be analyzed due to problems of alignment and were excluded. We also analyzed plasma subtype B viral isolates from 50 drug-naive individuals monitored in our clinics. To limit the effect of transmission of drug resistance mutations from treated patients, the presence of more than one major drug resistance-associated mutation in the absence of treatment represented an exclusion criterion. As a result, 12 patients were excluded from the control group. Among them, seven harbored NNRTI-associated mutations, nine harbored thymidine analogue mutations, four possessed the mutation M184V, and six harbored PI-associated mutations. Among the nonexcluded patients, one harbored an L33I mutation, two possessed an A71T mutation, and four harbored an A71V mutation.

Sequencing of the RT and PR genes. To sequence the RT and PR genes, RNA was extracted from plasma viruses using the QIAamp kit and RNA products were amplified by PCR. The sequencing of DNA products was carried out using kits (TruGene) obtained from Bayer Diagnostics Inc. (Toronto, Canada) as described in detail elsewhere (21). Sequencing of both the RT and PR genes also enabled us to determine the subtypes of viral isolates in concert with the Stanford database (http://hivdb.stanford.edu/). Nucleotide and amino acid genomic diversity at resistance sites in the various viral isolates was compared with a consensus subtype B reference virus (http://hiv-web.lanl.gov). The list of resistance sites that were analyzed is as follows: NRTIs: 41, 44, 62, 63, 65, 67, 69, 70, 74, 75, 77, 115, 116, 118, 151, 210, 215, and 219; NNRTIs: 100, 101, 103, 106, 108, 179, 181, 188, 190, 225, 230, and 236; PIs: 10, 20, 24, 30, 32, 33, 36, 46, 47, 48, 53, 54, 71, 73, 77, 82, 84, 88, 89, and 90.

Sequence data. GenBank accession numbers for subtype A/E isolates from Japan are AY211945 to AY211947. Accession numbers for subtype C viruses from Botswana are AF492600 to AF4926004. Accession numbers for subtype C viruses from Ethiopia are AF492618 to AF492622.

GenBank accession numbers from Los Alamos database. (i) **Subtype AE.** GenBank accession numbers for subtype AE were as follows: AB070352, AB097872, AF164485, AF170545, AF170548, AF170549, AF197339, AF197340, AF259954, AF259955, AY008714, AY125894, U51188, U51189 U54771, and AF197338.

(ii) **Subtype D.** GenBank accession numbers for subtype D were as follows: AJ320484, K03454, M22639, M27323, U88824, U88822, AJ519489, AJ519488, AJ488926, and AJ488927.

(iii) **Subtype G.** GenBank accession numbers for subtype G were as follows: AF061642, AF423760, AF450098, and U88826.

(iv) **Subtype AG.** GenBank accession numbers for subtype AG were as follows: AB049811, AB052867, AF063223, AF063224, AF184155, AJ251056, L39106, and AY271690.

Statistical analysis. Fisher's exact test was employed to compare rates of mutational prevalence among the different subtypes.

RESULTS

Analysis of mutational variations among different viral subtypes from different countries. We analyzed 105 HIV-1 sequences of non-subtype B origin from a variety of countries; of these sequences, 38 are present in the Los Alamos database. The distribution of the different subtypes and circulating recombinant forms of these viruses was as follows: subtype A (n = 20) (Canada, Kenya, and Cote d'Ivoire), A/E recombinant viruses (n = 20) (Canada, Japan, Thailand, China, and Central African Republic), subtype C (n = 32) (Canada, Kenya, Botswana, and Ethiopia), subtype D (n = 12) (Canada, Chad, Kenya, and Japan), subtype G (n = 7) (Canada, Sweden, Nigeria, and Spain), and A/G recombinant viruses (n = 14) (Canada, Cameroon, Cote d'Ivoire, Ghana, Senegal, Nigeria, and France).

Prevalence of substitutions and mutations at resistance sites in nontreated patients. This work focused on differences in baseline nucleic acid sequences and amino acids between subtype B isolates and viral isolates from patients infected with viruses of non-B subtypes. The distribution of changes relative to subtype B at resistance sites is shown in Table 1. However, it is important to realize that the substitutions that were commonly observed among non-B subtypes were also present in a reduced proportion of subtype B viruses; this information is also shown in Table 1.

Indeed, only eight polymorphisms with the potential to yield changes in amino acid (Table 1) were seen in non-subtype B viruses relative to subtype B, and only three of these, i.e., V179I in RT and K20I and V82I in PR, yielded a different amino acid in a significant number of cases. Moreover, these eight polymorphisms tended to be restricted to only one or two viral subtypes. This indicates that even at most positions considered in Table 1, polymorphisms unique to non-B viruses that affect the structure of the target enzymes through amino acid differences are rare.

NRTIs. At position D67, a GAT codon was found to encode D in 78% of subtype A/G viruses, whereas GAC was predominant in the other subtypes. Although K70 is encoded by AAA in almost all subtype B isolates (i.e., >92%), a different codon is generally responsible for encoding K in subtype C and D viruses (>80% express AAG). Also, at codon K219, AAA in subtype B is replaced by AAG in 81% of subtype C viruses. Similarly, ACC is the codon that results in T215 in 86% of subtype B viruses, whereas ACT encodes T215 in 75% of subtype A and all recombinant A/E viruses.

NNRTIs. V106 is encoded by GTG in 97% of subtype C and 42% of subtype D viruses. All of these subtype D viruses originated from Chad (Table 2). Three patterns were observed with regard to position V179, with GTG shown to encode this amino acid in >70% of subtype A/G and G viruses and 4% of subtype B viruses. GTC was identified in 89% of subtype C patients from Botswana but in only 41% of all subtype C samples and in only two samples from subtype B patients (Table 2). V179I has specificity for group A and is present in 75% of these samples. A TAC codon that encodes Y (Y181) was observed in all G patients and in 78% of these with A/G recombinants. In contrast, the codon responsible for Y181 in almost all B, C, and A/E viruses was TAT. A silent nucleotide substitution at position P225 (CCT→CCC) was found in 66% of subtype C viruses and in 20% of subtype B viruses. F227 is encoded by TTT in 67% of non-B subtypes and in only 18% of subtype B viruses.

PR. At position L10, a CTC codon was found to encode L in >80% of subtype B viruses, whereas CTT was predominant in non-B subtypes. Similarly, AAA was found to encode K20 in 44% of non-B subtypes while AAG predominated in the case of subtype B. However, among subtype G and A/G viruses, the polymorphism K20I was predominant. At position M36, most non-B subtypes possessed an I36 polymorphism. A nucleotide substitution at position 48 (GGG→GGA) was present in 84% of subtype C viruses and was present in 12% of subtype B viruses. Among subtype C viruses, a dichotomy was observed between viruses of Ethiopian versus non-Ethiopian origin, with ATT being present at position 54 in almost 100% of the former isolates but in relatively few of the latter, in which ATC was predominant (Table 2). Since both ATC and ATT encode I, the significance of this polymorphism is not apparent, although it may suggest a common ancestral variant for Ethiopian isolates, perhaps related to a founder effect. A V82I polymorphism was observed in 86% of subtype G viruses.

Genetic barrier for resistance. To ascertain whether different coding sequences in non-subtype B viruses could influence the genetic barrier for resistance to certain drugs, we analyzed the numbers of nucleotide transitions or transversions needed to obtain a known resistance mutation in viruses of different subtypes (Table 3). In subtype C, the V106M mutation in RT that confers resistance to efavirenz (EFV), nevirapine (NVP), and delevaridine (DLV) is facilitated by a single transition (GTG→ATG), compared to two transitions needed in viruses of other subtypes (GTA→ATG) (transitions are underlined). Two transitions are needed in subtype G viruses to obtain the secondary NNRTI resistance mutation, V108I, compared to only one transition in other subtypes. In subtypes G and AG, a

TABLE 1. Prevalence of nucleotide changes observed between subtype B and other subtypes in nontreated patients

Codon	Subtype B reference sequence	Amino acid	Nucleotide substitution in non-B viruses	Amino acid[a]	Prevalence of nucleotide substitutions (%) in viral subtype[b]:						
					B (n = 50)	A (n = 20)	A/E (n = 20)	C (n = 32)	D (n = 12)	G (n = 7)	A/G (n = 14)
NRTI related											
62	GCC	A	GCT	A	24	20	80**	13	33		
65	AAA	K	AAG	K	4			47**			
67	GAC	D	GAT	D	6					14	78**
69	ACT	T	ACC	T	8		70**		17		
70	AAA	K	AAG	K	12			81**	92**		
74	TTA	L	TTG	L	0					57**	
77	TTC	F	TTT	F	10				33		
115	TAT	Y	TAC	Y	4					42*	
116	TTT	F	TTC	F	4				8		
118	GTT	V	ATA	I	0				8	28	
151	CAG	Q	CAA	Q	24				50		
210	TTG	L	CTG	L	0					14	
210	TTG	L	TTA	L	22				8	28	
215	ACC	T	ACT	T	14	75**	100**				7
219	AAA	K	AAG	K	2			81**			
NNRTI related											
98	GCA	A	GCG	A	0	60**	5	6		42*	43**
98	GCG	A	TCG	S	0					28	
98	GCA	A	TCA	S	26			9			
100	TTA	L	CTA	L	2	55**	25*	3	42*	42*	
100	TTA	L	CTG	L	0				50**		
106	GTA	V	GTG	V	0			97**	42**		
108	GTA	V	GTG	V	2		5			42*	
179	GTT	V	GTC	V	14			41*			
179	GTT	V	GTG	V	4					71*	78**
179	GTT	V	ATT	I	2	75*	15				
181	TAT	Y	TAC	Y	4	5		3		100**	78**
225	CCT	P	CCC	P	20	5		66	25		7
227	TTC	F	TTT	F	18	55*	10	100**	100**	14	93**
PI related											
10	CTC	L	CTT	L	6	70**	65**	97**	66**		14
10	CTC	L	CTA	L	4					71*	7
10	CTC	L	TTA	L	2						64**
10	CTC	L	ATT/A	I	10	5	20			28	
20	AAG	K	AAA	K	2	75**	80**	25	50**	14	
20	AAG	K	ATA	I	0					86**	100**
20	AAG	K	AGA/AGG	R	0	5	5	19			
48	GGG	G	GGA	G	14			84**			7
54	ATC	I	ATT	I	4	5		25			7
71	GCT	A	GCC	A	4						64**
73	GGT	G	GGG	G	0					28	
82	GTC	V	ATC	I	0	5		3		86**	7
88	AAT	N	AAC	N	8	40*	5	9			
90	TTG	L	TTA/CTG	L	4		15				7

[a] Letters in boldface type refer to amino acid changes. Los Alamos database data include information derived from 16, 10, 4, and 8 patients with subtype A/E, D, G, and A/G infections, respectively.
[b] Symbols for statistical significance: *, $P < 0.05$; **, $P < 0.01$.

single transversion can result in a V179E mutation (GTG→GAG) compared to two transversions in viruses of other subtypes (GTT→GAG or GAA). In contrast, viruses of subtype A that possess a V179I polymorphism require both a transition and transversion to yield the V179D resistance mutation (ATT→GAT), compared to only a single transversion in subtype B viruses (GTT→GAT). In PR, the V82S/T resistance mutations may be facilitated by a V82I polymorphism in subtype G viruses compared to viruses of subtype B. In contrast, V82A may be facilitated in subtype B. The prevalence of differences at position 210 in RT and that at positions 73 and 90 in PR were too low for analysis.

At other positions, at which nucleotide substitutions occurred with high prevalence, no differences were observed between subtype B and other viruses with regard to the number of transitions or transversions needed to generate a specific resistance-associated mutation.

As shown in Table 1, the changes at positions 74, 98, 100, and 106 in RT and 20 and 82 in PR were prevalent in non-B subtypes relative to subtype B. Among these substitutions, only those at positions 74 and 106 in RT and 82 in PR are located in proximity to the active sites of these enzymes (http://hiv-web.lanl.gov).

DISCUSSION

HIV-1 group M subtype nucleotide diversity in the Env protein can range between 20 and 30%. With regard to Gag,

TABLE 2. Substitutions in subtype C and D viruses relative to subtype B based on country of origin

Subtype	Origin	No. of patients with indicated nucleotide substitution				
		PR	RT			
		I54I (ATC→ATT)	A62A (GCC→GCT)	F77F (TTC→TTT)	V106V (GTA→GTG)	V179V (GTT→GTC)
C	Botswana (n = 9)	0	0	0	6	8
C	Ethiopia (n = 6)	6	0	0	6	0
C	Other[a] (n = 17)	2	0	0	1	5
D	Chad (n = 4)	0	4	4	4	0
D	Other[b] (n = 8)	0	1	0	1	0

[a] Viruses of subtype C origin from countries other than Botswana or Ethiopia.
[b] Viruses of subtype D origin from sources other than Chad.

variability ranges between 10 and 15% (4) and divergence at the *pol* gene is ~10%. Our group has previously characterized subtype variation at known resistance sites with regard to subtype B and C viruses (16). In the latter study, we analyzed nucleotide substitutions and amino acid diversity at codons known to confer resistance in each of the PR and RT regions, while other studies had only analyzed amino acid variability in this context (12, 14).

Non-B subtypes were defined as a group as having a polymorphism at codon 10 in PR, an M36I substitution in PR, and a polymorphism at codon 227 in RT. Subtype A was identified by a V179I polymorphism in RT. Subtype G and A/G viruses were identified by a silent substitution at codon 181 in RT. Similarly, subtype A/G viruses were identified by a substitution at position 67 in RT. Subtype C was distinguished by silent substitution at codons 106 and 219 in the RT region and codon 48 in PR. Interestingly, two subgroups were recognized within subtype C. The first included viruses from patients of Ethiopian origin with a specific nucleotide substitution at codon 54 in the PR region. The other subgroup included patients from Botswana with a specific nucleotide substitution at codon 179 in RT that is different from previously identified changes at this codon. In addition, three subtype C viruses from Kenyan patients did not harbor these two nucleotide substitutions. Polymorphisms that were specific to a geographic region were also observed in subtype D viruses from Chad, which contained substitutions at positions 62, 77, and 106 (similar to subtype C viruses) in RT. This suggests the possibility of different ancestral variants for viruses of each subgroup.

We previously described a V106M mutation in samples from three patients infected with a subtype C virus who had failed therapy with EFV (5), and others have now shown that this mutation is also seen in subtype C viruses derived from patients who failed therapy with NVP (17). V106M has also been selected in vitro by EFV in subtype C viruses and confers high-level cross-resistance to all three currently approved NNRTIs (5). The selection of this mutation in subtype C viruses results from a single nucleotide change from wild-type in subtype C viruses (*G*TG→*A*TG). In addition, a G→A nucleotide transition is facilitated in this circumstance.

Interestingly variability in codon usage at position V106 between subtype B (GTA) and subtype C (GTG) occurs at the third position in the triplet; third positions rarely have an impact on mutagenesis. The importance of the V106M substitution with regard to subtype C viruses has been confirmed by other groups that also rarely reported the presence of this mutation in subtypes other than C (Z. Grossman, V. Istomin, D. Averbuck, I. Levy, K. Risenberg, M. Chowers, E. Shahar, M. Lorber, E. Mendelson, D. Ram, Z. Kra-Oz, M. Burk, Z. Bentwich, S, Maayan, and J. M. Shapiro, Abstr. 10th Conf. Retroviruses Opportun. Infect., abstr. 624, 2003). As a result, V106M is now reported as an NNRTI resistance-conferring mutation in most algorithms to complement V106A, which is associated with resistance to NVP. Interestingly, variants at position 103 other than K103N (K103S/H/T) may also confer reduced susceptibility to NNRTIs [P. R. Harrigan, B. Wynhoven, J. Montaner, P. McKenna, and L. Bacheler, abstract

TABLE 3. Codons at which differences exist between subtype B and non-B viruses

Region	Amino acid position	Wild-type codon in subtype B[a]	Amino acid	Subtype(s)	Wild-type codon in non-subtype B	Amino acid[b]	Mutated codon[c]	Resistant amino acid	Drugs affected[d]
RT	106	GTA	V	C	GTG	V	ATG[e]	M	EFV, NVP, DLV
	108	GTA	V	G	GTG	V	ATA	I	EFV, NVP
	179	GTT	V	G, A/G	GTG	V	GAG or GAA	E	EFV, NVP, DLV
	179	GTT	V	A	ATT	I	GAT or GAC	D	EFV, DLV
PR	20	AAG	K	A, A/E	AAA	K	ATG	M	IDV, RTV, LPV, ATZ, TPV
	82	GTC	V	G	ATC	I	GCC	A	Multi-PI
	82	GTC	V	G	ATC	I	TCC or AGC	S	Multi-PI
	82	GTC	V	G	ATC	I	ACC	T	Multi-PI

[a] The underlined nucleotides represent transitions or transversions from wild type to nucleotides that code for a resistance-associated amino acid.
[b] Presumed polymorphism.
[c] Nucleotides that encode resistance-associated amino acids.
[d] Abbreviations: DLV, delevaridine; IDV, indinavir; RTV, ritonavir; LPV, lopinavir; ATZ, atazanavir; TPV, tipranavir.
[e] Differences in numbers of nucleotide changes needed to mutate a particular drug resistance-associated amino acid. Only frequently observed mutations are shown.

from the XII International HIV Drug Resistance Workshop 2003, Antivir. Ther. 8(Suppl. 1):S120, 2003].

The present study also suggests several other possible differences between viral subtypes with regard to nucleotide transitions and transversions at known drug resistance sites. A good example is the NNRTI mutation V179D/E, which may be differentially selected in subtypes G, A/G, and A compared to B. In contrast to V106M, however, 179D/E confers only low-level resistance to NNRTIs. The secondary NNRTI mutation, V108I, may be facilitated in subtype B viruses compared to subtype G. In PR, V82A/S/T may be differentially selected compared to subtype B, because of a V82I polymorphism in the former.

Among polymorphisms unique to non-B subtypes, there are only a limited number of codon positions that might affect enzyme structure. For example, position 82 in PR is in close proximity to the active-site Asp 25 in the normally folded PR, and mutations in this region might compromise substrate-enzyme contact sites (24). In contrast, position 20 in PR is located outside the active site. In RT, positions 74 and 106 are close to the active site but positions 98 and 100 are not (http://hiv-web.lanl.gov). Thus, the opportunity for significant differences among viral subtypes with regard to resistance appears to be limited.

In general the effect of subtype diversity on virological response to therapy, as well as the development of both phenotypic resistance and resistance-conferring mutations, is still a topic of conjecture (11). Other groups have shown differences in resistance profiles among subtypes. Among nelfinavir-treated patients, D30N substitutions are apparently present less frequently in non-subtype B viruses than in subtype B [Z. Grossman, E. Paxinos, D. Auerbuch, S. Maayan, N. Parkin, D. Engelhard, M. Lorber, E. Kedem, F. Mileguir, N. Vardinon, Z. Bentwich, C. Petropoulos, and J. M. Schapiro, abstract from the XI International HIV Drug Resistance Workshop 2002, Antivir. Ther. 7(Suppl. 1):S30, 2002]. In addition, a specific mutation at position 88 in A/E circulating recombinant forms has been reported in nelfinavir-treated persons [K. Ariyoshi, M. Matsuda, H. Miura, K. Yamada, N. S. Hellmann, and W. Sugiura, abstract from the XI International HIV Drug Resistance Workshop 2002, Antivir. Ther. 7(Suppl. 1):S150, 2002]. In vitro studies that characterized the enzymatic activity of PR enzymes containing different PI polymorphisms commonly seen in subtypes A and C suggest that the latter may amplify the effect of drug resistance mutations (25). Specific subtype C and intra-C subtype resistance mutations and polymorphisms have also been reported (14, 22), and 55% of group O viruses have a naturally occurring resistance mutation with regard to NNRTIs, i.e., Y181C (7).

Analysis of a large database that included 1,240 non-B-infected persons revealed that virtually all drug resistance mutations known to occur in subtype B can also be found in non-B isolates. However, differences among non-B subtypes were present in untreated individuals, and these included characteristic polymorphisms [R. Kantor, D. Katzenstein, M Gonzales, S. Sirivichayakul, P. Cane, C. Pillay, J. Snoeck, Z. Grossman, A. M. Vandamne, L. Morris, D. Pillay, P. Phanuphak, J. M. Schapiro, and R. W. Shafer, abstract from the XI International HIV Drug Resistance Workshop 2002, Antivir. Ther. 7(Suppl. 1):S142, 2002]. Another study found characteristic minor mutations within the PR region of non-subtype B viruses as well as polymorphisms but no major mutation in either RT or PR (9). A further report from South Africa reported no differences in resistance profiles between subtype B and C viruses (C. Pillay, M. Ntsala, R. Kantor, C. Chezzi, F. Venter, L. Levin, and L. Morris, Abstr. 2nd IAS Conf. HIV Pathog. Treatment, abstr. 775, 2003). Furthermore, several studies of non-B-infected patients have not revealed any association between HIV subtype, adherence to therapy, and virologic response to treatment and disease progression (1, 2, 8, 10, 15, 20; S. De Wit, R. Boulme, B. Poll, J. C. Schmit, and N. Clumeck, Abstr. 2nd IAS Conf. HIV Pathog. Treatment, abstr. 25, 2003). A recent study from Cote d'Ivoire examined 276 patients receiving HAART and found that 50% achieved a viral load of <200 copies/ml, a result similar to that observed in Western countries (15). Among 79 drug-naive African patients who received HAART, 60 had undetectable viral loads after 1 year (10). Another study evaluated ARV therapy in 113 children and did not show evidence of differential in virologic response among viruses of different subtypes (20).

In summary, our analysis of nucleotide substitutions in viruses of various subtypes reveals only limited possibilities for differences with regard to patterns of resistance mutations in comparison to subtype B. This fact as well as the clinical data cited above should be reassuring with regard to the management of HIV disease in populations well represented in non-B subtypes. This is important in the context of efforts to provide ARVs to people in need of treatment in developing countries, while not obviating the need to monitor the emergence of drug resistance-associated mutations in such settings.

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REFERENCES

1. **Alaeus, A., K. Lidman, A. Bjorkman, J. Giesecke, and J. Albert.** 1999. Similar rate of disease progression among individuals infected with HIV-1 genetic subtypes A-D. AIDS 13:901–907.
2. **Alexander, C. S., V. Montessori, B. Wynhoven, W. Dong, K. Chan, M. V. O'Shaughnessy, T. Mo, M. Piaseczny, J. S. Montaner, and P. R. Harrigan.** 2002. Prevalence and response to antiretroviral therapy of non-B subtypes of HIV in antiretroviral-naive individuals in British Columbia. Antivir. Ther. 7:31–35.
3. **Barin, F., A. M. Courouce, J. Pillonel, L. Buzelay, et al.** 1997. Increasing diversity of HIV-1M serotypes in French blood donors over a 10-year period (1985–1995). AIDS 11:1503–1508.
4. **Becker, M. L., G. De Jager, and W. B. Becker.** 1995. Analysis of partial gag and env gene sequences of HIV type 1 strains from southern Africa. AIDS Res. Hum. Retrovir. 11:1265–1267.
5. **Brenner, B., D. Turner, M. Oliveira, D. Moisi, M. Detorio, M. Carobene, R. G. Marlink, J. Schapiro, M. Roger, and M. A. Wainberg.** 2003. A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. AIDS 17:F1–F5.
6. **Couturier, E., F. Damond, P. Roques, H. Fleury, F. Barin, J. B. Brunet, F. Brun-Vezinet, and F. Simon.** 2000. HIV-1 diversity in France, 1996–1998. The AC 11 laboratory network. AIDS 14:289–296.
7. **Descamps, D., G. Collin, F. Letourneur, C. Apetrei, F. Damond, I. Loussert-Ajaka, F. Simon, S. Saragosti, and F. Brun-Vezinet.** 1997. Susceptibility of human immunodeficiency virus type 1 group O isolates to antiretroviral agents: in vitro phenotypic and genotypic analyses. J. Virol. 71:8893–8898.
8. **Djomand, G., T. Roels, T. Ellerbrock, D. Hanson, F. Diomande, B. Monga, C. Maurice, J. Nkengasong, R. Konan-Koko, A. Kadio, S. Wiktor, E. Lackritz, J. Saba, and T. Chorba.** 2003. Virologic and immunologic outcomes and

programmatic challenges of an antiretroviral treatment pilot project in Abidjan, Cote d'Ivoire. AIDS 17(Suppl. 3):S5–S15.
9. **Fleury, H., P. Recordon-Pinson, A. Caumont, M. Faure, P. Roques, J. C. Plantier, E. Couturier, D. Dormont, B. Masquelier, and F. Simon.** 2003. HIV type 1 diversity in France, 1999–2001: molecular characterization of non-B HIV type 1 subtypes and potential impact on susceptibility to antiretroviral drugs. AIDS Res. Hum. Retrovir. **19**:41–47.
10. **Frater, A. J., A. Beardall, K. Ariyoshi, D. Churchill, S. Galpin, J. R. Clarke, J. N. Weber, and M. O. McClure.** 2001. Impact of baseline polymorphisms in RT and protease on outcome of highly active antiretroviral therapy in HIV-1-infected African patients. AIDS **15**:1493–1502.
11. **Harrigan, P. R., J. S. Montaner, S. A. Wegner, W. Verbiest, V. Miller, R. Wood, and B. A. Larder.** 2001. World-wide variation in HIV-1 phenotypic susceptibility in untreated individuals: biologically relevant values for resistance testing. AIDS **15**:1671–1677.
12. **Holguin, A., and V. Soriano.** 2002. Resistance to antiretroviral agents in individuals with HIV-1 non-B subtypes. HIV Clin. Trials **3**:403–411.
13. **Kantor, R., and D. Katzenstein.** 2003. Polymorphism in HIV-1 non-subtype B protease and reverse transcriptase and its potential impact on drug susceptibility and drug resistance evolution. AIDS Rev. **5**:25–35.
14. **Kantor, R., L. S. Zijenah, R. W. Shafer, S. Mutetwa, E. Johnston, R. Lloyd, A. von Lieven, D. Israelski, and D. A. Katzenstein.** 2002. HIV-1 subtype C reverse transcriptase and protease genotypes in Zimbabwean patients failing antiretroviral therapy. AIDS Res. Hum. Retrovir. **18**:1407–1413.
15. **Laniece, I., M. Ciss, A. Desclaux, K. Diop, F. Mbodj, B. Ndiaye, O. Sylla, E. Delaporte, and I. Ndoye.** 2003. Adherence to HAART and its principal determinants in a cohort of Senegalese adults. AIDS 17(Suppl. 3):S103–S108.
16. **Loemba, H., B. Brenner, M. A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, and M. A. Wainberg.** 2002. Genetic divergence of human immunodeficiency virus type 1 Ethiopian clade C reverse transcriptase (RT) and rapid development of resistance against nonnucleoside inhibitors of RT. Antimicrob. Agents Chemother. **46**:2087–2094.
17. **Morris, L., C Pillay, C. Chezzi, P. Lupondwana, M. Ntsala, L. Levin, F. Venter, N. Martinson, G. Gray, and J. McIntyre.** 2003. Low frequency of the V106M mutation among HIV-1 subtype C-infected pregnant women exposed to nevirapine. AIDS **17**:1698–1700.
18. **Osmanov, S., C. Pattou, N. Walker, B. Schwardlander, and J. Esparza.** 2002. Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. J. Acquir. Immune Defic. Syndr. **29**:184–190.
19. **Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, S. D. Holmberg, et al.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N. Engl. J. Med. **338**:853–860.
20. **Pillay, D., A. S. Walker, D. M. Gibb, A. de Rossi, S. Kaye, M. Ait-Khaled, M. Munoz-Fernandez, and A. Babiker.** 2002. Impact of human immunodeficiency virus type 1 subtypes on virologic response and emergence of drug resistance among children in the Paediatric European Network for Treatment of AIDS (PENTA) 5 trial. J. Infect. Dis. **186**:617–625.
21. **Salomon, H., M. A. Wainberg, B. Brenner, Y. Quan, D. Rouleau, P. Cote, R. LeBlanc, E. Lefebvre, B. Spira, C. Tsoukas, R. P. Sekaly, B. Conway, D. Mayers, and J. P. Routy.** 2000. Prevalence of HIV-1 viruses resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or intravenous drug use. AIDS **14**:F17–F23.
22. **Soares, M. A., T. De Oliveira, R. M. Brindeiro, R. S. Diaz, E. C. Sabino, L. Brigido, I. L. Pires, M. G. Morgado, M. C. Dantas, D. Barreira, P. R. Teixeira, S. Cassol, and A. Tanuri.** 2003. A specific subtype C of human immunodeficiency virus type 1 circulates in Brazil. AIDS **17**:11–21.
23. **Thomson, M. M., E. Delgado, N. Manjon, A. Ocampo, M. L. Villahermosa, A. Marino, I. Herrero, M. T. Cuevas, E. Vazquez-de Parga, L. Perez-Alvarez, L. Medrano, J. A. Taboada, and R. Najera.** 2001. HIV-1 genetic diversity in Galicia Spain: BG intersubtype recombinant viruses circulating among injecting drug users. AIDS **15**:509–516.
24. **Todd, M. J., I. Luque, A. Velázquez-Campoy, and E. Freire.** 2000. Thermodynamic basis of resistance to HIV-1 protease inhibition: calorimetric analysis of the V82F/I84V active site resistant mutant. Biochemistry **39**:11876–11883.
25. **Velazquez-Campoy, A., M. J. Todd, S. Vega, and E. Freire.** 2001. Catalytic efficiency and vitality of HIV-1 proteases from African viral subtypes. Proc. Natl. Acad. Sci. USA **98**:6062–6067.

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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

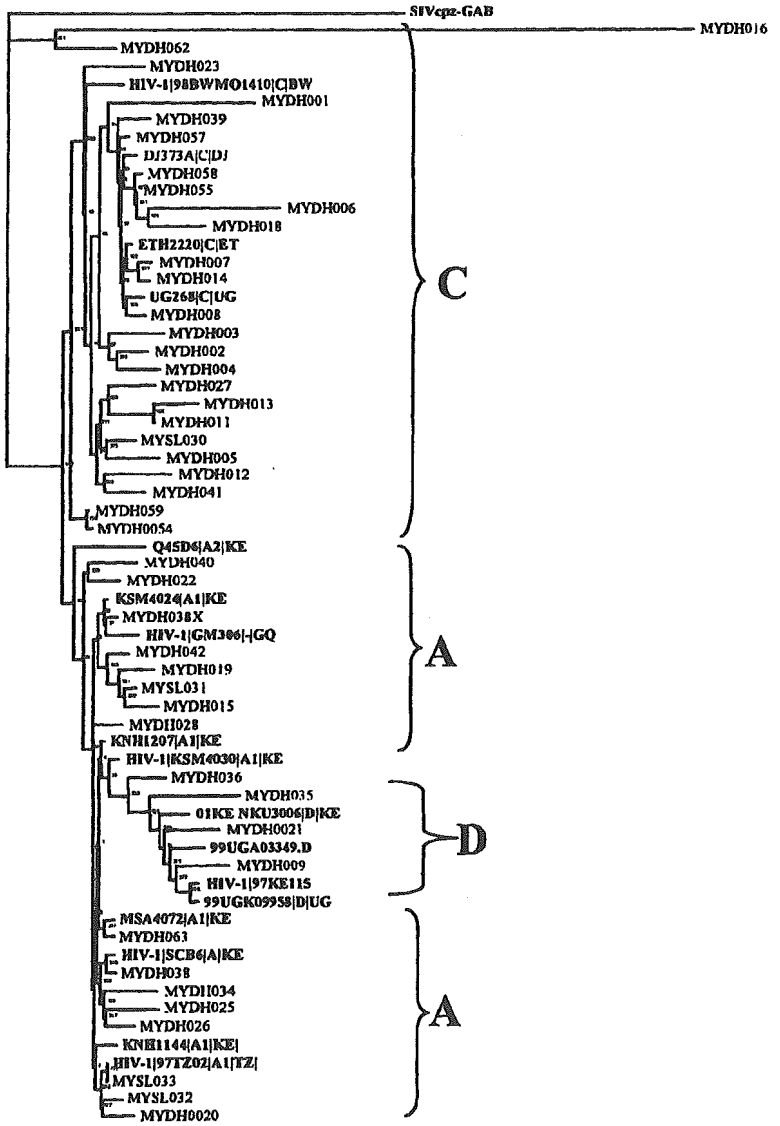

FIG. 1. Phylogenetic analysis of the gp41 *env* region of HIV-1 subtypes from Moyale in northern Kenya. The simian immunodeficiency virus SIV_{cpzgab} was used as the outgroup. The sequences have been indicated by codes MYDH and MYSL denoting Moyale District Hospital and Moyale Sololo, respectively. 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.

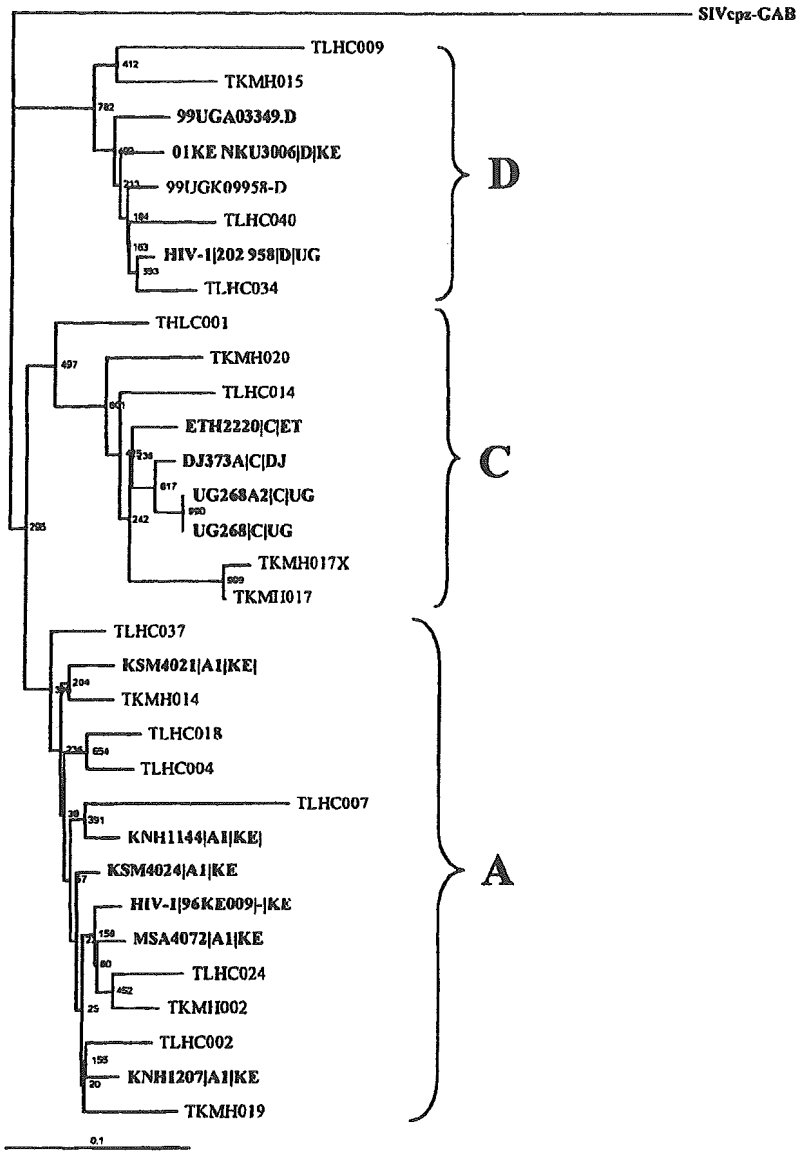

FIG. 2. Phylogenetic analysis of the gp41 *env* region HIV-1 subtypes from Turkana in northern Kenya. The sequences have been indicated by codes TLHC and TKMH denoting Turkana Lobiding Health Centre and Turkana Kakuma Mission Hospital, respectively. The A subtypes in this region clustered with references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

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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

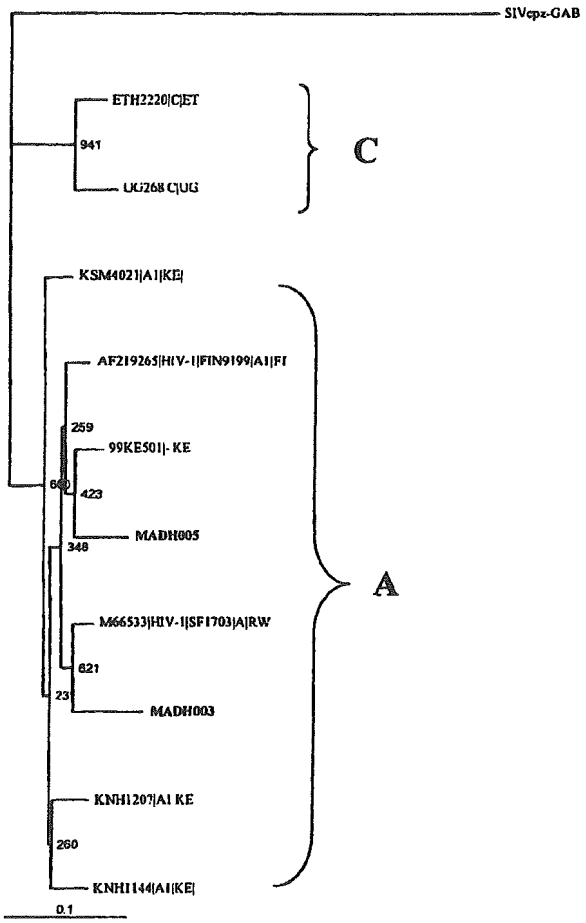

FIG. 3. Phylogenetic analysis of the gp41 *env* region HIV-1 subtypes from Mandera in northern Kenya. The sequences have been indicated by codes MADH denoting Mandera District Hospital. The two samples were all subtype A and clustered with subtypes from Rwanda and Kenya.

REFERENCES

1. Hierholzer M, Graham RR, El Khidir I, Tasker S, Darwish M, Chapman GD, Fagbami AH, Soliman A, Birx DL, McCutchan F, and Carr JK: HIV type 1 strains from East and West Africa are intermixed in Sudan. AIDS Res Human Retroviruses 2002;18(15): 1163–1166.
2. Abebe A, Kuiken CL, Goudsmit J, Valk M, Messele T, Sahlu T, Yeneneh H, Fontanet A, De Wolf F, Rinke and De Wit TF: HIV type 1 subtype C in Addis Ababa, Ethiopia. AIDS Res Human Retroviruses 1997;13(12):1071–1075.
3. Yang C, Dash BC, Simon F, Groen G, Pieniazek D, Gao F, Hahn BH, and Lal RB: Detection of diverse variants of human immunodeficiency virus-1 groups M, N, and O and simian immunodeficiency viruses from chimpanzees by using generic *pol* and *env* primer pairs. J Infect Dis 2000;181:1791–1795.
4. Thompson JD, Higgins DG, and Gibson TJ: CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22: 4673–4680.
5. Kuiken C, Foley B, Hahn B, *et al.*: *HIV Sequence Compendium 2001.* Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.
6. Saitou N and Nei M: The neighbor joining method: A new method for reconstruction of phylogenetic trees. Mol Biol Evol 1987;4: 406–425.
7. Page RDM: TREEVIEW: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 1996; 12:357–358.

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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.

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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.

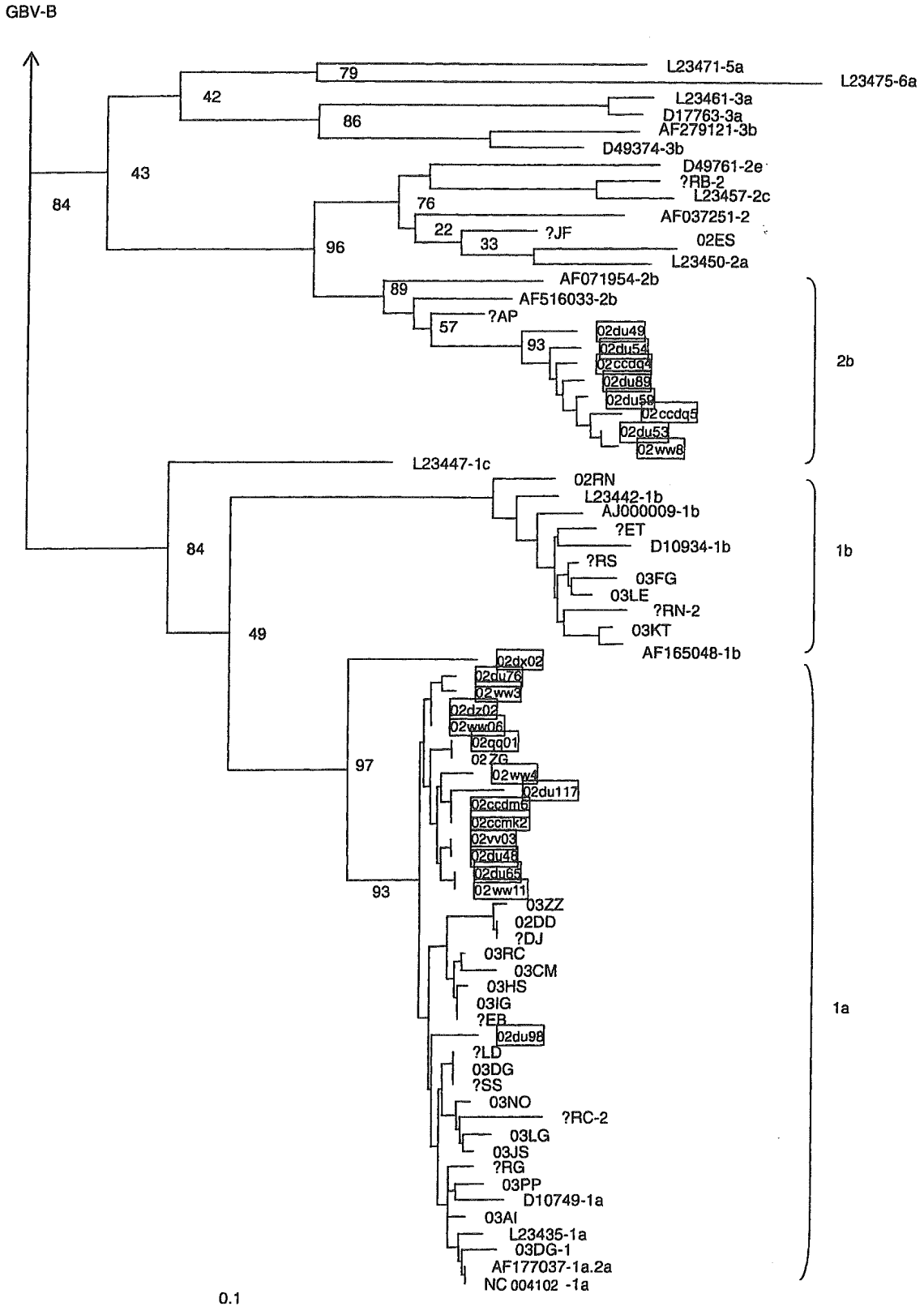

Fig. 1. Phylogenetic trees of 23 HCV strains (highlighted in the boxes) from injecting-drug users in Metro Cebu and 31 HCV strains from other area of the Philippines, performed on 227 nt within the NS5B region by the neighbor-joining method with GBV-B (accession no. NC 001655) as an outgroup. Analyzed 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.

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REFERENCES

Arguillas MO, Domingo EO, Tsuda F, Mayumi M, Suzuki H. 1991. Seroepidemiology of hepatitis C virus infection in the Philippines: A preliminary study and comparison with hepatitis B virus infection among blood donors, medical personnel, and patient groups in Davao, Philippines. Gastroenterol Jpn 26:170–175.

Bukh J, Miller RH, Purcell RH. 1995. Genetic heterogeneity of hepatitis C virus: Quasispecies and genotypes. Semin Liver Dis 15:41–63.

Chamot E, de Saussure P, Hirschel B, Deglon JJ, Perrin LH. 1992. Incidence of hepatitis C, hepatitis B and HIV infections among drug users in a methadone-maintenance programme. AIDS 6:430–431.

Chen PJ, Lin MH, Tai KF, Liu PC, Lin CJ, Chen DS. 1992. The Taiwanese hepatitis C virus genome: Sequence determination and mapping the 5′ termini of viral genomic and antigenomic RNA. Virology 188:102–113.

Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R, Barr PJ, et al. 1991. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA 88:2451–2455.

Crofts N, Hopper JL, Bowden DS, Breschkin AM, Milner R, Locarnini SA. 1993. Hepatitis C virus infection among a cohort of Victorian injecting drug users. Med J Aust 159:237–241.

Espantaleon A, Kageyama S, Bernardo MT, Nakano T, Leano PS, Alban P, Abrenica R, Morimatsu S, Teraoka H, Agdamag DM. 2003. The influence of the expanding HIV genetic diversity on molecular diagnosis in the Philippines. Int J STD AIDS 14:125–131.

Higashi Y, Kakumu S, Yoshioka K, Wakita T, Mizokami M, Ohba K, Ito Y, Ishikawa T, Takayanagi M, Nagai Y. 1993. Dynamics of genome change in the E2/NS1 region of hepatitis C virus in vivo. Virology 197:659–668.

Houghton M, Weiner A, Han J, Kuo G, Choo QL. 1991. Molecular biology of the hepatitis C viruses: Implications for diagnosis, development and control of viral disease. Hepatology 14:381–388.

Katayama Y, Barzaga NG, Alipio A, Soetjipto XX, Doi H, Ishido S, Hotta H. 1996. Genotype analysis of hepatitis C virus among blood donors and inmates in Metro Manila, The Philippines. Microbiol Immunol 40:525–529.

Lauer GM, Walker BD. 2001. Hepatitis C virus infection. N Engl J Med 345:41–52.

Leano PS, Kageyama S, Espantaleon A, Maniar J, Iwasaki M, Saple D, Yoshihara N, Kurimura T, Agdamag DM. 2003. Introduction of human immunodeficiency virus type 2 infection in the Philippines. J Clin Microbiol 41:516–518.

Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Esteban R, Guardia J, Gomez J. 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: Quasispecies nature of HCV genome distribution. J Virol 66:3225–3229.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A, Miyakawa Y, Mayumi M. 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with Reported isolates for conserved and divergent regions. J Gen Virol 72:2697–2704.

Paladin FJ, Monzon OT, Tsuchie H, Aplasca MR, Learn GH Jr., Kurimura T. 1998. Genetic subtypes of HIV-1 in the Philippines. AIDS 12:291–300.

Perriere G, Gouy M. 1996. WWW-query: An on-line retrieval system for biological sequence banks. Biochimie 78:364–369.

Royce RA, Sena A, Cates W Jr., Cohen MS. 1997. Sexual transmission of HIV. N Engl J Med 336:1072–1078.

Ruxrungtham K, Brown T, Phanuphak P. 2004. HIV/AIDS in Asia. Lancet 364:69–82.

Santiago ML, Santiago EG, Hafalla JC, Manalo MA, Orantia L, Cajimat MN, Martin C, Cuaresma C, Dominguez CE, Borromeo ME, De Groot AS, Flanigan TP, Carpenter CC, Mayer KH, Ramirez BL. 1998. Molecular epidemiology of HIV-1 infection in the Philippines, 1985 to 1997: Transmission of subtypes B and E and potential emergence of subtypes C and F. J Acquir Immune Defic Syndr Hum Retrovirol 18:260–269.

Soriano V, Sulkowski M, Bergin C, Hatzakis A, Cacoub P, Katlama C, Cargnel A, Mauss S, Dieterich D, Moreno S, Ferrari C, Poynard T, Rockstroh J. 2002. Care of patients with chronic hepatitis C and HIV co-infection: Recommendations from the HIV-HCV International Panel. AIDS 16:813–828.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Van Ameijden EJ, Van den Hoek JA, Mientjes GH, Coutinho RA. 1993. A longitudinal study on the incidence and transmission patterns of HIV, HBV and HCV infection among drug users in Amsterdam. Eur J Epidemiol 9:255–262.

van Asten L, Verhaest I, Lamzira S, Hernandez-Aguado I, Zangerle R, Boufassa F, Rezza G, Broers B, Robertson JR, Brettle RP, McMenamin J, Prins M, Cochrane A, Simmonds P, Coutinho RA, Bruisten S. 2004. Spread of hepatitis C virus among European injection drug users infected with HIV: A phylogenetic analysis. J Infect Dis 189:292–302. Epub 2004 Jan 2008.

van den Hoek JA, van Haastrecht HJ, Goudsmit J, de Wolf F, Coutinho RA. 1990. Prevalence, incidence, and risk factors of hepatitis C virus infection among drug users in Amsterdam. J Infect Dis 162:823–826.

White PA, Zhai X, Carter I, Zhao Y, Rawlinson WD. 2000. Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. J Clin Microbiol 38:477–482.

Zuckerman AJ, Zuckerman JN. 1995. Prospects for hepatitis C vaccine. J Hepatol 22:97–100.

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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.

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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

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