Genotypic Characterization of Human Immunodeficiency Virus Type 1 Derived from Antiretroviral Drug-Treated Individuals Residing in Earthquake-Affected Areas in Nepal

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Genotypic characterization of human immunodeficiency virus type 1 derived from antiretroviral drug-treated individuals residing in earthquake-affected areas in Nepal

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Abstract

Molecular epidemiological data on human immunodeficiency virus type 1 (HIV-1) are limited in Nepal, and has not been available in areas affected by the April 2015 earthquake. Therefore, we conducted a genotypic study on HIV-1 genes derived from individuals on antiretroviral therapy residing in 14 districts in Nepal highly affected by the earthquake. HIV-1 genomic fragments were amplified from 40 blood samples of HIV treatment-failure individuals, and a sequencing analysis was performed on these genes. In the 40 samples, 29 protease, 32 reverse transcriptase, 25 *gag* and 21 *env* genes were sequenced. HIV-1 subtyping revealed that subtype C (84.2%, 32/38) was the major subtype prevalent in the region, while CRF01_AE (7.9%, 3/38) and other recombinant forms (7.9%, 3/38) were also detected. In addition, major drug resistance mutations were identified in 21.9% (7/32) of samples, indicating the possible emergence of HIV-1 drug resistance in earthquake-affected areas in Nepal.

Text

A report by the Nepalese Government estimated the number of people living with human immunodeficiency virus (PLHIVs) to be 39,397 in Nepal at the end of 2015[1]. In addition, the prevalence of PLHIVs decreased from 0.35% in 2005 to 0.20% in 2015 among adults in Nepal[1]. Approximately 2,263 deaths were due to acquired immune deficiency syndrome (AIDS)-related complications in 2015, representing a slightly decrease from the 2,558 deaths recorded in 2010. Nepal has recently adopted the National HIV Strategic Plan 2016-2021, which focuses on the "seek, test, treat and retain" strategy to achieve the UNAIDS 90-90-90 targets by 2020: 90% of people living with HIV know their status, 90% of diagnosed people receive sustained antiretroviral therapy (ART) and 90% of people in treatment achieve viral suppression[2]. The districts in Nepal affected by the April 2015 earthquake are areas inhabited by a large number of PLHIVs. Most of the health facilities in these districts were destroyed by the earthquake, emergency conditions persisted and basic health services were halted for a period of time[3], and, therefore, access to ART services was markedly affected.

HIV-1 is characterized by extensive genetic heterogeneity and has been

classified into four groups: M (major), O (outlying), N (new or non-M, non-O) and P (pending). Group M has been further classified into several subtypes and circulating recombinant forms (CRFs). Of these, subtypes A, B, C, D and G as well as CRF01_AE and CRF02_AG are regarded as the major subtypes and CRFs of HIV-1 and are responsible for the worldwide HIV-1 epidemic. Subtype B is prevalent in America, Europe and Australia, while non-B subtypes and CRFs are circulating in Africa and Asia. In the neighboring countries of Nepal, CRF01_AE is prevalent in China[4], while subtype C is prevalent in India[5]. As a result of open border population migration between India and Nepal, subtype C is accountable for most HIV-1 infections in Nepal[6, 7]. HIV-1 subtypes and CRFs show slightly different rates of disease progression, immune responses and responses to ART[8]; therefore, it is important to monitor the global prevalence of HIV-1 subtypes and CRFs for HIV prevention and control as well as for vaccine development.

Although ART does not completely cure HIV, it has markedly reduced its transmission and HIV-related mortality and morbidity. In Nepal, ART coverage is very low and was limited to 30% of all estimated PLHIVs in 2015[1]. Individuals with a CD4

count less than 500 cells/mm^3 are considered eligible for ART. A combination of two nucleoside reverse-transcriptase inhibitors (NRTIs) and a non-nucleoside reverse-transcriptase inhibitor (NNRTI) comprises the first-line regimen of ART in Nepal[9]. Lamivudine (3TC), zidovudine (AZT), tenofovir (TDF), nevirapine (NVP), and efavirenz (EFV) are the main drugs in this regimen. Two NRTIs plus a ritonavir-boosted protease inhibitor (PI) are adopted for second-line ART[9]. Fixed-dose combinations of ritonavir-boosted atazanavir (ATV/r) and ritonavir-boosted lopinavir (LPV/r) are the preferred boosted PI options for second-line ART. ART was initiated more than a decade ago in Nepal and has been successful. However, a reliable system to monitor the emergence of ART drug resistance mutations (DRM) has not yet been established. In addition, a service for viral load testing is not available throughout the country, and only two national laboratories currently exist in Nepal. Under these conditions, information on HIV drug resistance (HIVDR) is currently inadequate in Nepal[10].

In order to identify viral subtypes of HIV-1 and evaluate the efficacy of ART, viral genomes derived from blood samples of PLHIVs on ART in 14 districts highly affected by the earthquake in Nepal were genotypically characterized. Previous findings

showed that treatment failure was associated with HIVDR[11]. Therefore, 40 adult PLHIVs on ART and those that developed treatment failure were recruited from 10 health centers in the central region of Nepal. Treatment failure was defined by two consecutive viral loads having >1000 copies/mL, a CD4 count less than 350 cells/mm^3 or the presence of persistent opportunistic infections. Two-milliliter ethylenediaminetetraacetic acid (EDTA) anti-coagulated peripheral blood samples were collected with written informed consent from study participants between December 2015 and January 2016. Prior to blood collection, ethical approval was obtained from the Ethical Committees of Kobe University Graduate School of Health Sciences (Ethical approval no. 434) and the Nepal Health Research Council (Ethical approval reg.no. 274/2015). DNA was extracted from whole blood samples using the PureLink Genomic DNA Mini kit (Invitrogen, Carlsbad, CA, USA).

Viral *pol* genes encoding full-length protease (PR gene) and reverse transcriptase (RT gene), and partial fragments of the *gag* and *env* genes were amplified from DNA extracted from blood samples by a nested polymerase chain reaction (PCR) using ExTaq (Takara Bio, Shiga, Japan). In the amplification of the PR gene, primers

for first PCR were DRPR05, 5’-AGACAGGYTAATTTTTTAGGGA-3’ [corresponding to nucleotides (nt) 2074 to 2095 of the HIV-1 reference strain, HXB2 (GenBank accession no. K03455)] and DRPR02L-C 5’-TATGGATTTTCAGGCCCAATTTTTG-3’ (nt 2716 to 2692), while those for nested PCR were DRPR01M, 5’-AGAGCCAACAGCCCCACCAG-3’ (nt 2148 to 2167) and DRPR06, 5’-ACTTTTGGGCCATCCATTCC-3’ (nt 2611 to 2592). In the amplification of the RT gene, primers for first PCR were RT1L, 5’-ATGATAGGGGGAATTGGAGGTTT-3’ (nt 2388 to 2410) and GPR2M, 5’-GGACTACAGTCYACTTGTCCATG-3’ (nt 4402 to 4380), while those for nested PCR were RT7L, 5’-GACCTACACCTGTCAACATAATTGG-3’ (nt 2485 to 2509) and GPR3L, 5’-TTAAAATCACTARCCATTGYTCTCC-3’ (nt 4309 to 4285). In the amplification of the *gag* gene encoding Gag p24, primers for first PCR were H1G777, 5’-TCACCTAGAACTTTGAATGCATGGG-3’ (nt 1231 to 1255) and H1P202-C, 5’-CTAATACTGTATCATCTGCTCCTGTGTC-3’ (nt 2352 to 2325), while those for nested PCR were H1Gag1584, 5’-AAAGATGGATAATCCTGGG-3’ (nt 1577 to 1595) and G17-C, 5’-TCCACATTTCCAACAGCCTTTTTT-3’ (nt 2040 to 2017). In the

amplification of the C2-V3 regions of the *env* gene, primers for first PCR were M5-C, 5'-CCAATTCCCATACATTATTGTGCTCCAGCTGG-3' (nt 6858 to 6889) and M10, 5'-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3' (nt 7661 to 7632), while those for nested PCR were M3, 5'-GTCAGCACAGTACAATGIACACATGG-3' (nt 6948 to 6973) and M8, 5'-TCCTTCCATGGGAGGGGCATACATTGC-3' (nt 7547 to 7521). A sequence analysis was performed by Macrogen Japan (http://www.macrogen-japan.co.jp). Sequencing data were assembled and aligned using Genetyx version 10 software (Genetyx, Tokyo, Japan). The sequencing data of 29 PR genes (303-bp; nt 2253 to 2555), 32 RT genes (1681-bp; nt 2550 to 4230), the partial fragments of 25 *gag* genes encoding Gag p24 (382-bp; nt 1627 to 2008), and the partial fragments of 21 *env* genes spanning the C2-V3 region (404-bp; nt 7020 to 7423) were obtained from 40 blood samples. The nucleotide sequences of these PR, RT, *gag* and *env* genes have been registered in the GenBank database under accession numbers KY449164-KY449170, KY449172-KY449174 and KY449176-KY449272.

The recombinant identification program (RIP) available on the website of the HIV sequence database (www.hiv.lanl.gov/) was used for HIV-1 subtyping. In addition,

neighbor-joining (NJ) trees with a Kimura two-parameter model were constructed using MEGA6.2 software[12, 13] with bootstrap values (1,000 replicates) for relevant nodes being reported on a representative tree. Viral subtyping was performed from successfully sequenced PR, RT, *gag* and *env* genes, and a unique recombinant form (URF) of HIV-1 was labeled if any incompatibility existed in the subtype or CRF among the genes. Breakpoints in the recombinant forms were assessed using jpHMM (http://jphmm.gobics.de/)[14]. Viral subtyping by RIP was consistent with that by phylogenetic trees (data not shown). Viral subtyping revealed that 32 samples (84.2%, 32/38) were classified as subtype C, three (7.9%, 3/38) as CRF01_AE, and three (7.9%, 3/38) as URFs including the viral gene fragments of the CRF01_AE/subtype C and subtype C/subtype D (Fig. 1). These results suggest that the prevalence of subtype C is dominant in Nepal, similar to India. A Blast search (https://blast.ncbi.nlm.nih.gov/) revealed that subtype C from Nepal was similar to strains from China and India, while CRF01_AE was similar to those from Thailand (data not shown). Regarding sample NP10, the *gag* and RT genes were classified as subtype C, while the *env* gene was CRF01_AE. In sample NP31, the PR gene and former part of the RT gene were

classified as CRF01_AE, while the latter part of the RT gene was subtype C (breakpoint 3492-3524). The viral *gag* gene of NP38 showed a mixed pattern of subtypes C and D (the breakpoint was not identified). These recombination patterns were not registered as CRF in the HIV sequence database, indicating that these strains were URFs.

The appearance of DRMs in the 29 PR and 32 RT genes was studied according to the IAS-USA panel[15]. A drug resistance-associated major mutation to PR inhibitors, I50V [amino acid substitution from isoleucine (I) to valine (V) at position 50] was detected in the PR gene. This mutation is associated with drug resistance to darunavir/ritonavir or fosamprenavir (FPV)/ritonavir. In addition, several drug resistance-associated minor mutations were detected in PR genes: L10I/V (12.2%), G16E (13.8%), K20R/I (20.7%), L33I (3.4%), M36I/L/V (89%), I64M/L (6.9%), A71T/V (6.9%) and V82I (6.9%) (Table 1). These mutations in the PR gene potentially affect viral susceptibility to ATV, FPV, LPV, tipranavir (TPV), indinavir (IDV), nelfinavir (NFV) and saquinavir (SQV).

Moreover, several DRM appeared in the RT genes derived from 7 PLHIVs (Table 2). The demographic information of the 7 PLHIVs is shown in Table 3. Major

mutations were M41L (3.1%), D67N (3.1%), E138K (6.3%), Y181C (3.1%), M184V (12.5%), Y188L (6.3%), L210W (3.1%) and T215Y (3.1%), while minor mutations were V90I (3.1%) and A98G (3.1%) (Table 2). Major mutations were associated with drug resistance to stavudine (d4T), AZT, rilpivirine (RPV), EFV, etravirine (ETR), NVP, abacavir (ABC), emtricitabine (FTC) and 3TC. The appearance frequency of drug resistance-associated major mutations to RT inhibitors was higher than that in a previous report[7].

In summary, the results of the genotypic study showed that subtype C was the dominantly prevalent HIV-1 subtype in earthquake-affected areas in Nepal. In addition, CRF01_AE and URFs of HIV-1 containing the CRF01_AE/subtype C and subtype D/subtype C gene fragments were also detected in the study. Similarities in HIV-1 subtype C to those in China and India indicate the possible transmission of HIV-1 subtype C to Nepal from those countries. Furthermore, HIV1 CRF01_AE was similar to strains from Thailand. Among treatment failure participants, 21.9% (7/32) had at least one drug resistance-associated major mutation for RT inhibitors, suggesting the possible emergence of HIV-1 drug resistance in earthquake-affected regions in Nepal. Further

studies on drug resistance and the continuous monitoring of treatment failure are needed in order to address drug resistance issues among PLHIVs in Nepal.

Sequence Data

Nucleoside sequences are available under GenBank Accession Numbers KY449164-KY449170, KY449172-KY449174 and KY449176-KY449272.

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Author Disclosure Statement

No competing financial interests exist.

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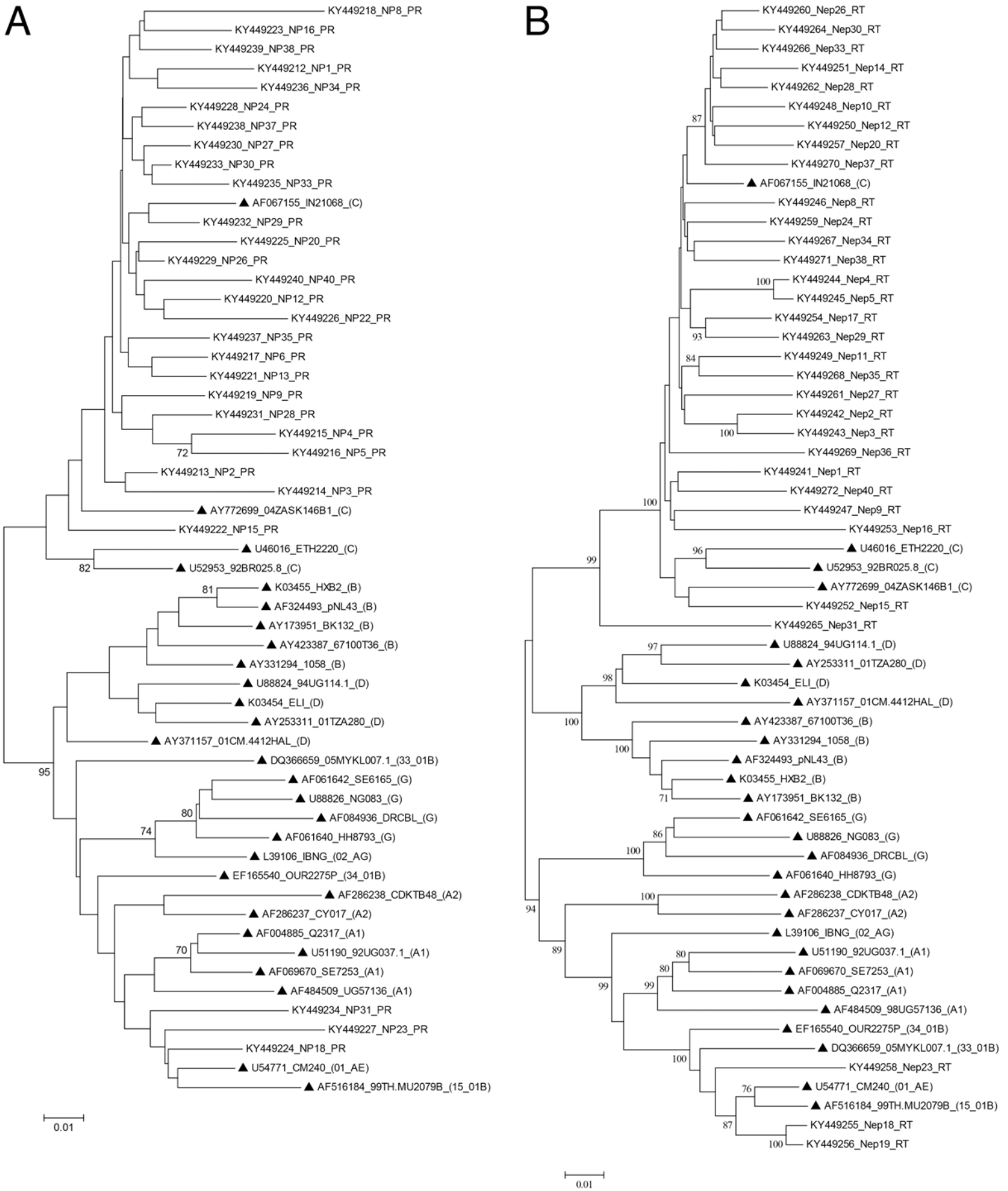
Figure legends

FIG. 1. Phylogenic analysis of HIV-1 PR, RT, *env* and *gag* gene sequences. Phylogenic trees were generated for the PR (A), RT (B), *gag* (C) and *env* genes (D) together with the corresponding viral gene of reference HIV-1 strains (shown with ▲) representing subtype A1 (A1), subtype A2 (A2), subtype B (B), subtype C (C), subtype D (D), subtype G (G), CRF01_AE (01_AE), CRF02_AG (02_AG), CRF15_01B (15_01B), CRF33_01B (33_01B) and CRF34_01B (34_01B). Sequence codes are presented as the GenBank accession number, patient ID, or name of the reference strain and subtype or CRF of the reference strain (shown in parentheses) in that order. Bootstrap values were shown when values were >70. CRF, circulating recombinant form.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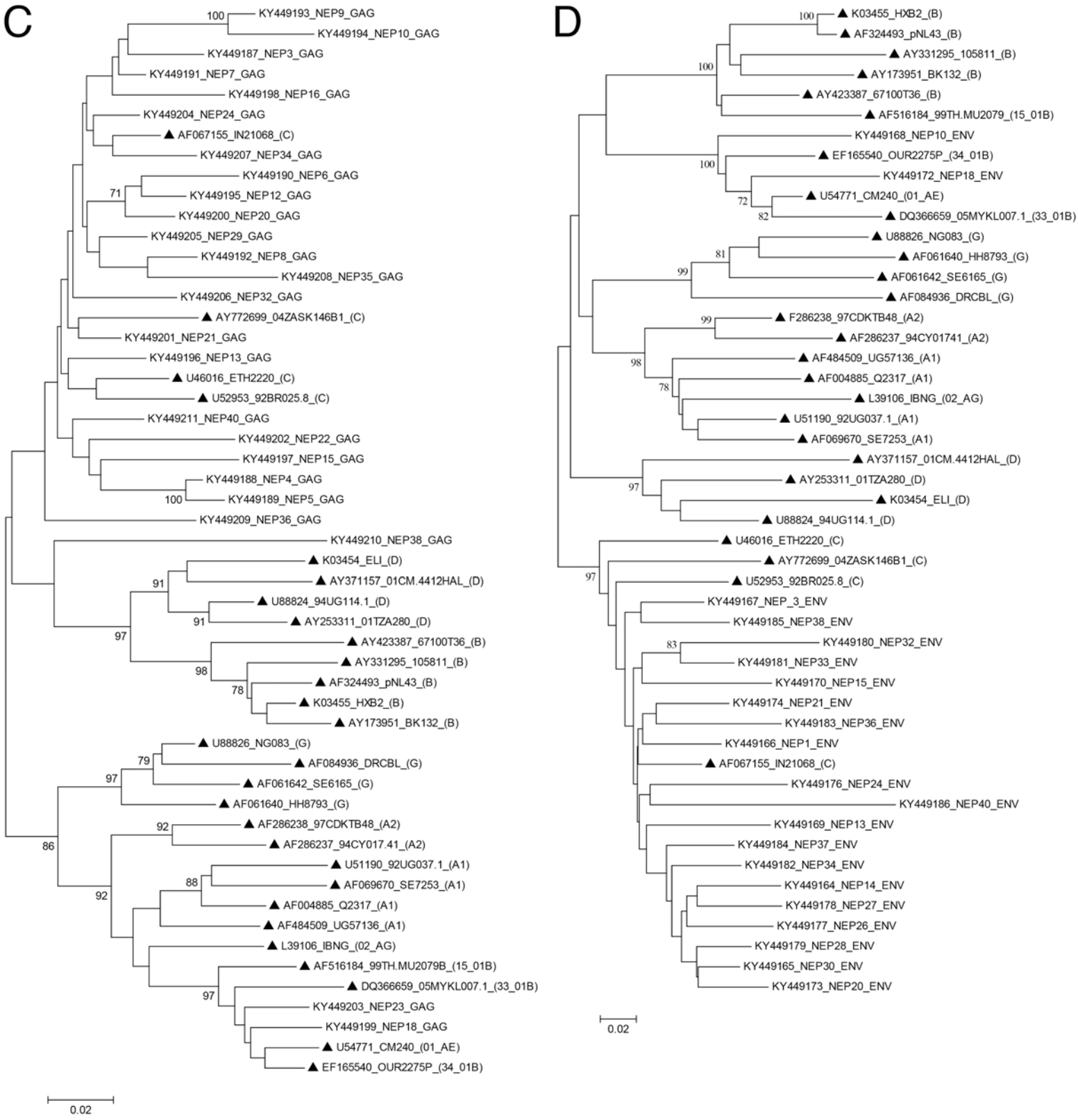
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KY449169_NEP13_ENV
KY449184_NEP37_ENV
KY449182_NEP34_ENV
KY449164_NEP14_ENV
KY449178_NEP27_ENV
KY449177_NEP26_ENV
KY449179_NEP28_ENV
KY449165_NEP30_ENV
KY449173_NEP20_ENV
0.02

TABLE 1. DRUG RESISTANCE-ASSOCIATED MAJOR AND MINOR MUTATIONS DETECTED IN THE PR GENE DERIVED FROM PLHIVS ON ART IN EARTHQUAKE-AFFECTED AREAS IN NEPAL

	Frequency (%)		
Mutations[a]	All (n=29)	CRF01_AE[b] (n=2)	Subtype C (n=27)
Major mutation			
I50V	1(3.4)	0(0)	1(3.7)
Minor Mutations			
G16E	4(13.8)	0(0)	4(14.8)
K20R/I	6(20.7)	2(100)	4(14.8)
L33I	1(3.4)	1(50)	0(0)
M36I/L/V	26(89.7)	2(100)	24(88.9)
I64L/M	2(6.9)	1(50)	1(3.7)
A71T/V	2(6.9)	1(50)	1(3.7)
V82I	2(6.9)	1(50)	1(3.7)

[a]Drug resistance mutations were based on guidelines published by the International AIDS Society United States (IAS-USA).

[b]Subtype of the PR gene assigned based on RIP and phylogenic analyses.

PR, protease; RIP, Recombinant identification program.

TABLE 2. DRUG RESISTANCE-ASSOCIATED MAJOR AND MINOR MUTATIONS DETECTED IN THE RT GENE DERIVED FROM PLHIVS ON ART IN EARTHQUAKE-AFFECTED AREAS IN NEPAL

	Frequency (%)		
Mutations[a]	All (n=32)	CRF01_AE[b] (n=3)	Subtype C (n=29)
Major mutations			
M41L	1(3.1)	0(0)	1(3.4)
M184V	4(12.5)	2(66.7)	2(6.9)
D67N	1(3.1)	1(33.3)	0(0)
E138K	2(6.3)	0(0)	2(6.9)
Y181C	1(3.1)	0(0)	1(3.4)
Y188L	2(6.3)	2(66.7)	0(0)
L210W	1(3.1)	1(33.3)	0(0)
T215 Y	1(3.1)	1(33.3)	0(0)
Minor Mutations			
V90I	1(3.1)	0(0)	1(3.4)
A98G	1(3.1)	0(0)	1(3.4)

[a]Drug resistance mutations were based on guidelines published by the International AIDS Society United States (IAS-USA).

[b]Subtype of the PR gene assigned based on RIP and phylogenic analyses.

PR, protease; RIP, Recombinant identification program.

TABLE 3. DEMOGRAPHIC CHARACTERISTICS AND DRUG RESISTANCE-ASSOCIATED MAJOR MUTATIONS DETECTED IN PR AND RT GENES DERIVED FROM PLHIVS ON ART IN EARTHQUAKE-AFFECTED AREAS IN NEPAL

				Drug Resistance Mutations[a]			
ID	Age	Gender	Subtype[b]	PI	nNRTI	NRTI	Resistance
NP_31	32	Male	Subtype C	I50V		M41L	Darunavir, Fosamprenavir, Stavudine, Zidovudine
NP_9	39	Female	Subtype C			M184V	Abacavir, Emtricitabine, Lamivudine
NP_18	49	Female	CRF01_AE		Y188L,	M184V, D67N, L210W, T215 Y	Abacavir, Emtricitabine, Lamivudine, Stavudine, Zidovudine, Efavirenz, Nevirapine, Rilpivirine
NP_19	32	Female	CRF01_AE		Y188L	M184V	Abacavir, Emtricitabine, Lamivudine, Efavirenz, Nevirapine, Rilpivirine
NP_35	36	Male	Subtype C		Y181C	M184V,	Abacavir, Emtricitabine, Lamivudine, Efavirenz, Nevirapine, Rilpivirine, Etravirine
NP_16	63	Male	Subtype C		E138K		Rilpivirine
NP_27	37	Male	Subtype C		E138A		Rilpivirine

[a]Drug resistance-associated major mutations were based on guidelines published by the International AIDS Society United States (IAS-USA).

[b]Subtype of genes assigned based on RIP and phylogenic analyses.

RIP, Recombinant identification program.