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Appearance of drug resistance mutations among the dominant HIV-1 subtype, CRF01_AE in Maumere, Indonesia

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Running title: HIV-1 molecular epidemiology in Maumere, Indonesia

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ABSTRACT

Background and Objectives: Human Immunodeficiency Virus (HIV) is still a major health issue in Indonesia. In recent years, the appearance of drug resistance-associated mutations has reduced the effectiveness of antiretroviral therapy (ART). We conducted genotypic studies, including the detection of drug resistance-associated mutations (from first-line regimen drugs), on HIV-1 genes derived from infected individuals in Maumere, West Nusa Tenggara. Maumere, a transit city in West Nusa Tenggara, which has a high HIV-1 transmission rate.

Method: We collected 60 peripheral blood samples from 53 ART-experienced and 7 ART-naive individuals at TC Hillers Hospital, Maumere between 2014 and 2015. The amplification and a sequencing analysis of *pol* genes encoding protease (the PR gene) and reverse transcriptase (the RT gene) as well as the viral *env* and *gag* genes were performed. HIV-1 subtyping and the detection of drug resistance-associated mutations were then conducted.

Results: Among 60 samples, 46 PR, 31 RT, 30 *env*, and 20 *gag* genes were successfully sequenced. The dominant HIV-1 subtype circulating in Maumere was CRF01_AE. Subtype B and recombinant viruses containing gene fragments of CRF01_AE, subtypes A, B, C, and/or G were also identified as minor populations. The major drug resistance-associated mutations, M184V, K103N, Y188L, and M230I, were found in the RT genes. However, no major drug resistance-associated mutations were detected in the PR genes.

Conclusion: CRF01_AE was the major HIV-1 subtype prevalent in Maumere. The appearance of drug resistance-associated mutations found in the present study supports the necessity of monitoring the effectiveness of ART in Maumere.

Keywords: HIV-1, CRF01_AE, antiretroviral therapy (ART), drug resistance-associated mutations, Maumere, Indonesia

1. INTRODUCTION

The prevalence of human immunodeficiency virus type 1 (HIV-1) infection is high in Indonesia. The Ministry of Health reported 232,323 cases of HIV-1 infection and 86,780 cases of acquired immune deficiency syndrome (AIDS) in Indonesia at the end of 2016. Furthermore, there were 2,627 and 1,954 cases of HIV infection and AIDS, respectively, in East Nusa Tenggara in December 2016 [1]. Maumere is located in the Sikka District and has a high number of HIV cases because this city is one of the transit places in Flores Island, East Nusa Tenggara. Commercial sex workers come to Flores Island from Makasar, Manado, and other areas outside Maumere. Therefore, there is an increased transmission rate of HIV among this high-risk population in Maumere.

Heterosexual intercourse and the sharing of needles and syringes among injecting drug users are two transmission types that have predominantly been attributed to the high incidence of HIV-1 infection. The Indonesian Ministry of Health recently reported a high incidence of HIV infection among housewives, who were previously neglected in HIV surveillance because this group was not classified as a high-risk population. In Maumere, 26 housewives were included among the 104 women diagnosed as being HIV-positive between January and December 2014 [2], which represents a higher number than commercial sex workers (3 cases). These women were infected with HIV-1 from their husbands who were returning from work outside of Maumere. The HIV-1 infection of housewives also resulted in a high incidence of the mother-to-child transmission (MTCT) of HIV-1 in Maumere.

Antiretroviral therapy (ART) has greatly reduced HIV-1 mortality. According to previous WHO ART guidelines, first-line regimens contain two nucleoside reverse transcriptase (RT) inhibitors (NRTIs), stavudine (d4T) or zidovudine (AZT), combined with lamivudine (3TC) plus a non-NRTI (NNRTI), efavirenz (EFV) or nevirapine (NVP). HIV-1-infected individuals with a CD4 count lower than 350 cells/mm³ are considered to be eligible for initial ART. In developing countries, a combination of d4T, 3TC, and NVP is more affordable for HIV-1-infected individuals [3,4,5,6,7]. However, the WHO suggested reducing the usage of d4T in 2010 because a single or dual combination of the NRTIs, didanosine (ddI) and d4T, appears to have an impact of the incidence of pancreatitis [8,9,10,11].

Drug resistance is acquired by individuals receiving ART (acquired drug resistance). In addition, drug resistance may be transmitted from ART-experienced individuals to drug-naïve individuals (transmitted drug resistance, TDR). Both acquisition types reduce the effectiveness of first-line ART regimens. However, TDR is considered to have a greater impact, and may rapidly deteriorate the effectiveness of first-line regimens at the

population level. Since individuals with TDR start ART with a lower genetic barrier to resistance, there is a higher risk of virological failure due to the appearance of drug-resistant mutations early after the initiation of first-line therapy [12,13,14,15,16]. Thus, the effectiveness of ART needs to be monitored.

In the present study, we conducted a molecular epidemiological study on HIV-1 at Maumere, for which epidemiological information remains limited. In addition to HIV-1 subtyping, the detection of drug resistance-associated mutations in the RT and PR genes was conducted in order to monitor the effectiveness of ART in Maumere.

2. MATERIALS AND METHODS

2.1, Ethics statement

The present study was conducted with approval from the Ethics Committees of Universitas Airlangga (Ethics Committee approval number: 25-995/UN3.14/PPd/2013) and Kobe University Graduate School of Medicine (Ethics Committee approval number: 784). All patients signed and dated informed consent prior to enrollment in this study. Participants consented to providing blood samples to obtain data on viral subtypes and drug resistance-associated mutations.

2.2, Sample collection and preparation

Sixty HIV-1-infected individuals (35 males and 25 females, mean age of 36 years), comprising 53 ART-experienced and 7 ART-naïve individuals, were recruited from TC Hillers, Maumere, East Nusa Tenggara, Indonesia. Ten milliliters of ethylenediaminetetraacetic acid (EDTA) anticoagulated peripheral blood samples was collected from participants using BD vacutainer®CPT (BD Bioscience, San Jose, USA) between October 2014 and August 2015. Peripheral blood mononuclear cells (PBMC) were then isolated by centrifugation at 2,000 rpm for 10-15 minutes. After centrifugation, a thin layer ring formed and plasma, PBMC, and red blood cells were separated. DNA was extracted from PBMC using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

2.3, Polymerase chain reaction (PCR) and sequencing

Partial fragments of the viral *pol* gene encoding protease (the PR gene) and RT (the RT gene), as well as the *gag* and *env* genes, were amplified from DNA extracted from PBMC samples by nested PCR using Ex Taq (Takara Bio, Shiga, Japan) and the following primers. In the amplification of the PR gene, the primers for first PCR were DRPR05, 5'-AGACAGGYTAATTTTTAGGGA-3' [corresponding to nucleotides (nt) 2074 to 2095 of the HIV-1 reference strain, HXB2 (GenBank accession no. K03455)] and DRPR02L, 5'-TATGGATTTTCAGGCCCAATTTTGA-3' (nt 2716 to 2691), and the primers used 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, the primers for first PCR were RT1L, 5'-ATGATAGGGGGAATTGGAG GTTT-3' (nt 2388 to 2410) and GPR2M, 5'-GGACTACAGTCYACTTGTCCATG-3' (nt 4402 to 4380), and the primers used 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, the primers for first PCR were H1G777, 5'-TCACCTAGA AACTTTGAATGCATGGG-3' (nt 1231 to 1255) and H1P202, 5'-CTAATACTGTATCATCTGCT GCTCCTGT-3' (nt 2352 to 2325), and the primers used for nested PCR were H1Gag1584, 5'-AAAGATGGATAATCCTGGG-3' (nt 1577 to 1595) and G17, 5'-TCCACATTTCCAACAGCCCTTTTT-3' (nt 2040 to 2017). In the amplification of the C2-V3 regions of the env gene, the primers for first PCR were M5, 5'-CCAATTCCCATACATTATTGTGCCCCA GCTGG-3' (nt 6858 to 6889) and M10, 5'-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3' (nt 7661 to 7632), and the primers used for nested PCR were M3, 5'-GTCAGCACAGTACAATGIAC ACATGG-3' (nt 6948 to 6973) and M8, 5'-TCCTTCCATGGGAGGGGCATACATTGC-3' (nt 7547 to 7521).

2.4, Subtyping and drug resistance mutation analysis

A sequencing analysis of amplified viral gene fragments was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 3500 xl genetic analyzer after the purification step using illustra ExoProStar (GE Healthcare Life Science, Little Chalfont, UK). Sequencing data were then assembled and aligned using Genetyx version 10 software (Genetyx, Tokyo, Japan). The sequencing data of the full lengths of 46 PR genes (297-bp), the N termini of 31 RT genes (762-bp), 30 *env* genes spanning the C2-V3 region (389-bp), and the partial fragments of 20 *gag* genes encoding gag p24 (382-bp) were obtained from 60 blood samples. The nucleotide sequences of these PR, RT, *env*, and *gag* genes have been deposited in the GenBank database under accession numbers MG793068 - MG793194.

HIV-1 subtyping was conducted using the recombinant identification program (RIP), available on the website of the HIV sequence database (www.hiv.lanl.gov/). In addition, neighbor-joining (NJ) trees with a Kimura two-parameter model were constructed using MEGA6.2 software with Bootstrap values (1,000 replicates) as a comparison to the results obtained with RIP. Viral subtyping was performed based on the successfully sequenced PR,

RT, *env*, and *gag* genes, and if there was an incompatibility in the subtype or CRF among the PR, RT, *env*, and *gag* genes, the viral gene was considered to be a recombinant form of HIV-1.

Drug resistance-associated mutations were examined according to the International Antiviral Society-United States (IAS-USA) report [17]. We analyzed drug resistance-associated mutations on the RT and PR genes, which were the targets of first- and second-line ART regimens in Indonesia, respectively.

3. RESULTS AND DISCUSSION

3.1, Demographic information of study participants

In the present study, peripheral blood samples derived from 53 HIV-1-infected, ART-experienced individuals and 7 ART-naïve individuals were subjected to HIV-1 subtyping and to an analysis of drug resistance-associated mutations. The demographic characteristics and possible HIV-1 transmission routes of study participants are shown in Table 1. The average age of study participants was 36 ± 11 years old. The percentages of males and females among participants were 58% and 42%, respectively. Approximately 58% were Sikkimese who live in the Sikka district in Maumere.

Sexual transmission was the predominant route of HIV-1 infection in Maumere (90%), which is consistent with previous findings [2]. The sharing of needles and syringes among intravenous drug users (1.7%) also contributed to the incidence of HIV transmission. Since housewives were infected with HIV-1, MTCT cases were also identified (6.7%). According to a previous study in Maumere [2], these mothers were suspected to have been infected with HIV-1 from their husbands. Therefore, we consider an early diagnosis and the education of women living with husbands/partners with high-risk behavior for HIV infection to be a promising strategy to reduce the number of MTCT cases. The education of pregnant women about their HIV status may also prevent their children becoming infected with HIV.

3.2, CRF01_AE is the predominant HIV-1 subtype in Maumere, Indonesia

HIV-1 subtyping by RIP was consistent with that by a phylogenetic tree analysis (data not shown). Viral subtyping revealed that 26 samples (45.5%) classified as CRF01_AE, 11 samples (20%) were subtype B, while 19 (34.5%) were classified as recombinant forms including viral gene fragments of CRF01_AE and subtype B (14 samples), CRF01_AE and subtypes A and B (2 sample), CRF01_AE and subtype G (1 sample), or CRF01_AE and subtypes B and C (1 sample) (Fig. 1). These results suggested that CRF01_AE viruses were the most prevalent, which is consistent with previous findings showing CRF01_AE as the dominant subtype in other regions in Indonesia [18,19,20].

HIV-1 has continuously evolved in order to escape the host immune system. HIV-1 RT lacks proofreading activity, and is considered to be responsible for high HIV-1 diversity. HIV-1 recombination increases viral diversity inside one individual co-infected with two different subtypes of the virus. Other factors, such as the high rates of

viral replication and turnover, also increase HIV-1 diversity [21,22,23,24,25]. The emergence of several circulating recombinant forms (CRFs) shows the complexity of HIV-1 molecular epidemiology. The CRF01_AE subtype was originally identified in Thailand in the late 1980s [26,27,28,29]. This subtype was initially recognized as subtype E of HIV-1, and was subsequently revealed to contain the subtype A genomic fragment. Therefore, it is now registered as CRF01_AE. CRF01_AE was found to dominantly circulate in Southeast Asia [30,31,32]. Previous studies revealed that since the 1990s, CRF01_AE is the dominant HIV-1 subtype in Indonesia [20,33]. The present study revealed that CRF01_AE is the dominant subtype in Maumere; however, subtype B and other recombinant viruses were also detected (Fig. 1). Therefore, the continuous surveillance of circulating viral subtypes is needed.

3.3, Appearance of drug resistance-associated mutations in RT and PR genes

ART regimens and the duration of treatments for study participants are shown in Table 2. ART with a combination of 3TC, AZT, and NVP was the major treatment regimen (45.3%), and 56.6% of study participants had been receiving ART for more than 3 years (Table 2). Drug resistance mutations were analyzed on the viral RT and PR genes. MOF 16 had several major mutations including two mutations that affected multi-nRTI resistance. In addition, MOF30, MOF39, MOF50, and MOF11 had several major mutations that affected multi-NNRTI resistance (Table 3). The 3TC-associated mutations, M184V/I (an amino acid substitution at position 184 from methionine to valine or isoleucine) were the most common among CRF01_AE viruses (4/12, 33.3%), followed by the NNRTI-associated mutations, M230I (3/12, 25%), K103N (3/12, 25%), Y188L (2/12, 16.7%), and E138A (2/12, 16.7%). These results are consistent with our previous findings [34]; CRF01_AE was the dominant HIV-1 subtype, while the major mutations detected were M184V, K103N, and also TAMs among HIV-1-infected individuals on ART in Surabaya, Indonesia.

HIV-1 subtype diversity strongly influences the development of HIV drug resistance [35,36]. HIV-1 diversity may also affect the degree of cross-resistance to antiretroviral drugs of the same class, and may ultimately have an impact on clinical outcomes, the preservation of immunological responsiveness, and virologic failure [37,38,39]. The dominant mutations, M184V/I were identified as major drug resistance mutations in patients receiving 3TC and TDF [40,34]. However, in the present study, the M184V mutation was not observed in subtype B or recombinants containing CRF01_AE and subtype B gene fragments. Among non-CRF01_AE sequences, M230I and E138A were more dominant, suggesting viral resistance to NVP or EFV included in first-line ART regimens.

Drug resistance-associated major mutations were not observed in PR genes (Table 4). This result is reasonable because protease inhibitors were not included in ART regimens for most study participants (Table 2). However, several minor mutations were detected, such as M36I (85%), H69K (71.7%), and L89M/V/I (69.6%) among 46 PR genes (Table 4). Drug resistance-associated minor mutations in PR genes have also been reported in other studies in Indonesia [34]. Minor mutations have been detected in the PR genes of non-B subtypes, including CRF01_AE, as natural polymorphisms [41].

In the present study, we also included 3 samples from infants infected through MTCT. The average age of the 3 infants was 34 months (24-42 months). Our results showed that all infants had drug resistance-associated mutations against NRTI and NNRTIs in the RT genes (MOF 16, MOF 34, and MOF 19 in Table 3), whereas no major mutations were detected in the PR genes (data not shown).

HIV-1 drug resistance occurs rapidly among infants due to the infants' conditions, such as a higher HIV-1 viral load than that in adults, poor tolerance of ART medication, complex adherence issues, and previous exposure to ART in order to prevent MTCT [42,43,44]. In infants with co-infections such as tuberculosis, treatments may increase adverse events, including drug toxicity and the risk of significant inter-drug interactions. Drug resistance-associated mutations in infants and children may occur as a mother-to-child TDR; however, this was not the case in the present study. Further research with a larger sample size of infants and children will provide further insights into how drug resistance mutations occur in the population in Indonesia.

4. CONCLUSION

Among HIV-1-infected individuals in Maumere, CRF01_AE was present as the dominant subtype of HIV-1. Drug resistance-associated major mutations were detected in 28% of the RT genes derived from ART-experienced individuals with the dominant mutations, M184V, Y188L, and K103N. In contrast, no major mutations were identified in the PR genes. These results suggest that it is important to monitor the effectiveness of ART in order to provide better therapy for HIV-1-infected individuals in Indonesia.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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AUTHOR CONTRIBUTIONS

M.K. and N designed the study; D.W.I ,T.K, S.K.Q. A.M.W., and M. Q. Y. performed experiments; A.P and D.K. contributed to sample preparation in Maumere; D.W.I. and S. U. analyzed data; D.W.I and M.K. wrote the manuscript. All authors contributed to the revision of the manuscript and approved the final version.

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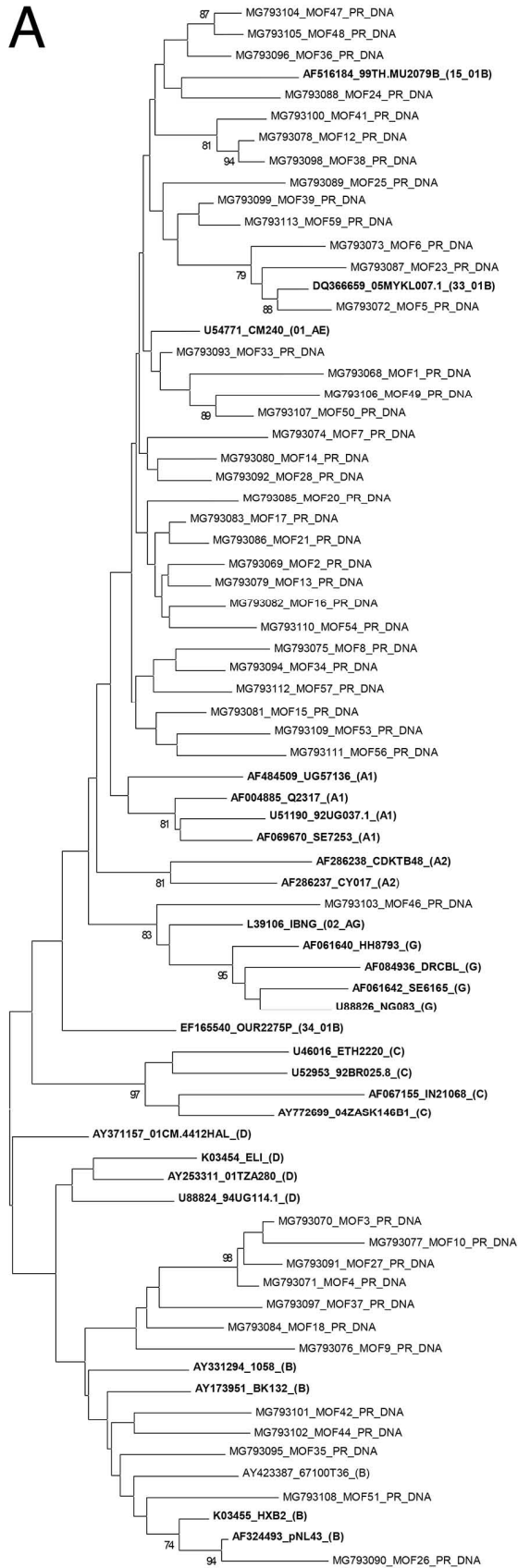
antiretroviral treatment in Uganda. *AIDS Res Hum Retroviruses* 2015; 32(7): 628-635.

FIGURE LEGENDS

Fig. 1. Phylogenetic tree analyses of HIV-1 PR, RT, *gag* and *env* gene fragments obtained in Maumere, Indonesia.

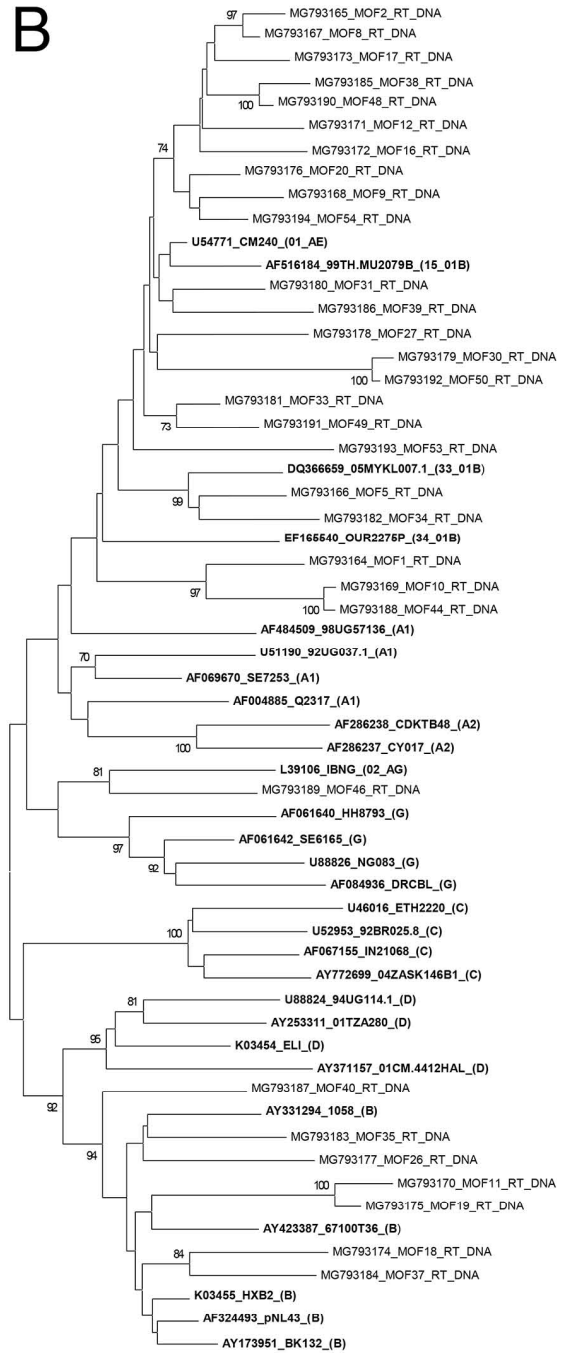
Phylogenetic trees were constructed on HIV-1 PR (A), RT (B), *gag* (C) and *env* gene fragments newly sequenced in this study (D). The corresponding viral gene fragments derived from reference HIV-1 strains classified as subtypes A1, A2, B, C, D and G as well as CRF01_AE, CRF02_AG, CRF15_01B, CRF33_01B and CRF34_01B were included in the analyses. The sequencing data derived from reference HIV-1 subtypes and CRFs were shown in bold letters. The IDs of sequencing samples are shown as an accession number of GenBank, patient ID or the ID of the reference HIV-1 strain, and the assigned subtype or CRF of the reference HIV-1 strain (shown in parentheses) in that order. Bootstrap values were shown if the values were >70.

A



0.01

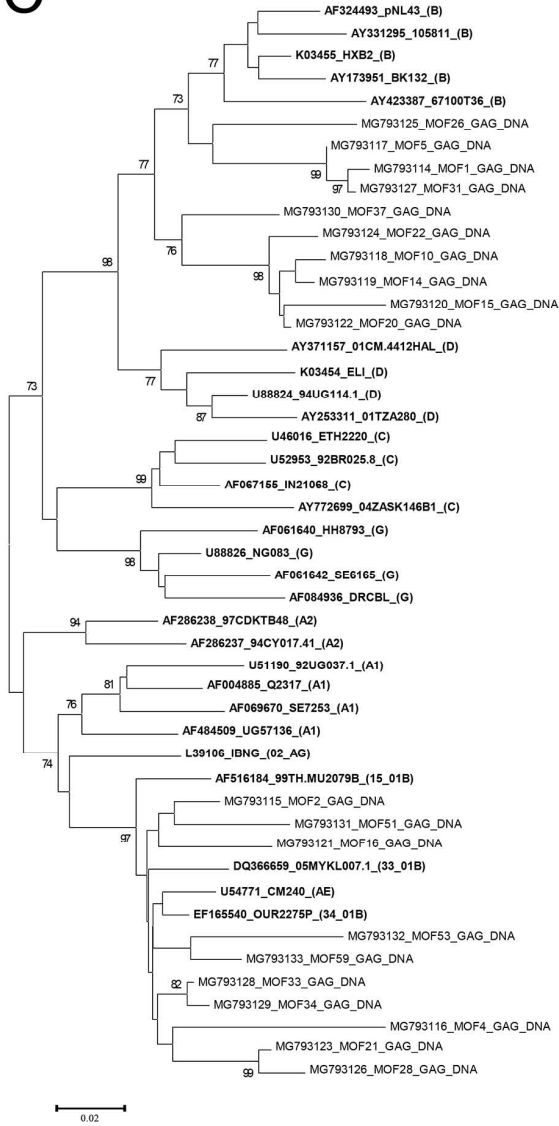
B



0.01

Fig. (1). contd....

C



D

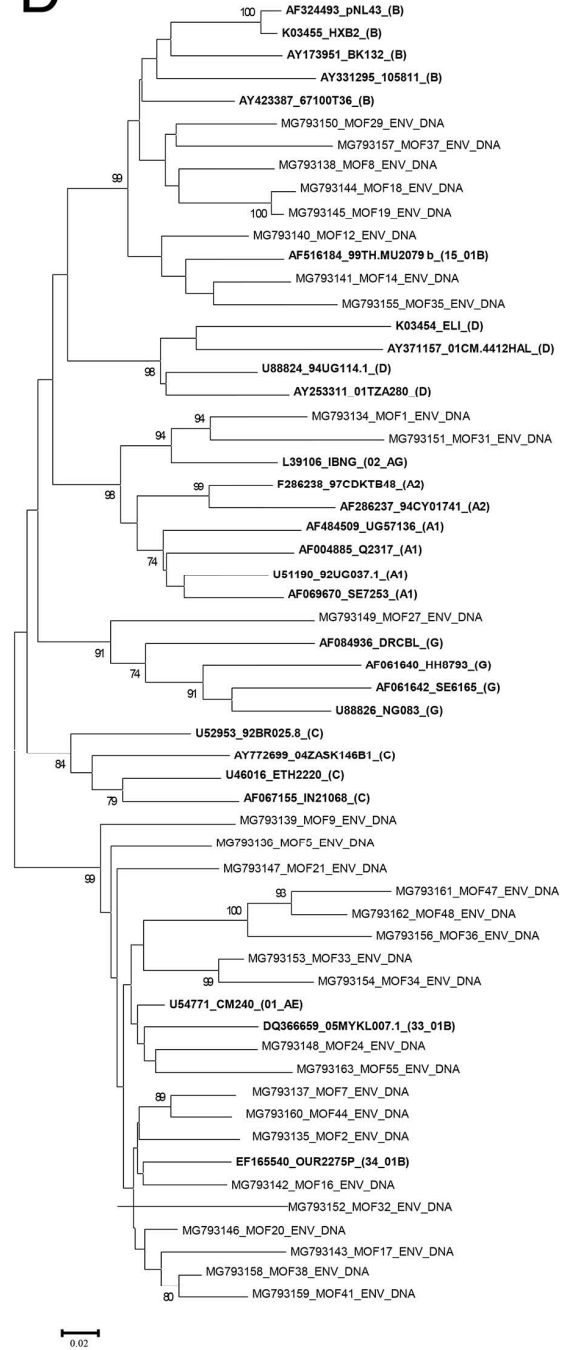


Table 1. Demographic characteristics and HIV-1 transmission routes of HIV-1-infected individuals in

Maumere, Indonesia

Characteristics*	Value (n = 60)
Age, mean years (SD)	36 (11.1)
Gender, n (%)	
Male	35 (58.3%)
Female	25 (41.7%)
Marital Status, n (%)	
Married	49 (81.7%)
Single	9 (15%)
Unidentified	2 (3.3%)
Ethnicity, n (%)	
Sikka	35 (58.3%)
Lio	6 (10%)
Manggarai	6 (10%)
Others (Krowe, Flores Timur, Lembata, Palue, Adonara, Timor Timur, Jawa)	13 (21.7%)
Transmission route of HIV-1, n (%)	
Heterosexual intercourse	54 (90.0%)
Intravenous drug use	1 (1.7%)
Homosexual intercourse	0 (0%)
Mother-to-child transmission (MTCT)	4 (6.7%)
Unidentified	1 (1.7%)

*Demographical information, including age, gender, marital status, ethnicity, and possible transmission

routes of HIV-1, is shown as numbers and percentages.

Table 2. ART regimens and duration of treatments among 53 HIV-1-infected, ART-experienced individuals in Maumere, Indonesia

Types of ART used, n (%)	
3TC+AZT+NVP*	24 (45.3%)
3TC+AZT+EFV*	5 (9.4%)
3TC+TDF+NVP*	11 (20.8%)
3TC+TDF+EFV*	12 (22.6%)
3TC, TDF, Lopinavir/Ritonavir**	1 (1.9%)
Duration of ART, n (%)	
< 1 year	2 (3.8%)
1-3 years	21 (39.6%)
> 3 years	30 (56.6%)

*first-line ART regimen

**second-line ART regimen

Table 3. Genotypic characteristics and drug resistance-associated mutations detected in RT genes derived

from 7 ART-experienced and ART-naive individuals in Maumere

Sample	Subtype*	ART status	Drug Resistance Mutations**		Drug Resistance
			NRTIs	NNRTIs	
MOF 16	CRF01_AE	3TC, TDF, NVP	M41L A98G T215Y M184V	Y181C	3TC, FTC, AZT, EFV, NVP, TMC125, TMC278
MOF 30	CRF01_AE	3TC, TDF, NVP	M184V	K103N Y188L	3TC, FTC, EFV, NVP
MOF 34	CRF01_AE	3TC, TDF, NVP	M184I	M230I	3TC, FTC, TMC278
MOF 39	CRF01_AE	3TC, TDF, EFV	M184V	A98G K103N V108I P225H	3TC, FTC, EFV, NVP
MOF 50	CRF01_AE	3TC, TDF, EFV	M184V	K103N Y188L	3TC, FTC, EFV, NVP
MOF 53	CRF01_AE	ART-Naive		A98G	
MOF 11	B	3TC, TDF, Lopinavir/Ritonavir		E138A E138A M230I	TMC278
MOF 26	B	3TC,TDF,E FV		M230I	TMC278

*The subtype of RT genes was assigned based on RIP and phylogenetic analyses.

****The identification of drug resistance-associated mutations was based on the guidelines published by the International Antiviral Society-United States (IAS-USA). Drug resistance-associated major mutations are shown in bold font.**

Table 4. Drug resistance-associated minor mutations detected in PR genes derived from HIV-infected individuals in Maumere, East Nusa Tenggara

Mutation*	Frequency (%)				
	all (n=46)	CRF01_A E** (n=30)	Subtype B (n=12)	Subtype CRF01_AE/ B (n=3)	Subtype CRF01_ AE/G (n=1)
L10I/V	3 (6.5)	3 (10)	0 (0)	0 (0)	0 (0)
G16E	6 (13)	6 (20)	0 (0)	0 (0)	0 (0)
K20R/I	11 (23.9)	11 (36.7)	0 (0)	0 (0)	0 (0)
L33I/F/V	2 (4.34)	1 (3.3)	1 (8.3)	0 (0)	0 (0)
M36I/K	39 (84.8)	30 (100)	6 (50)	3 (100)	0 (0)
D60E	1 (2.17)	0(0)	0 (0)	1(33.3)	0 (0)
I62V	5 (10.9)	0 (0)	5 (100)	0 (0)	0 (0)
L63P	10 (21.7)	2 (6.7)	6 (50)	1 (33.3)	1 (100)
I64V	3 (6.25)	0 (0)	2 (16.7)	1 (33.3)	0 (0)
H69K	33 (71.7)	29 (96.7)	0 (0)	3 (100)	1 (100)
A71V/T	2 (4.2)	0(0)	1 (8.3)	0 (0)	1 (100)
V77I	2 (4.2)	0(0)	2 (100)	0 (0)	0 (0)
V82I	5(10.9)	4(13.3)	1(8.3)	0 (0)	0(0)
L89M/I/V	32 (69.6)	28 (93.3)	1 (8.3)	2 (66.7)	1 (100)
I93L	13 (28.3)	7 (23.3)	4 (33.3)	1 (33.3)	1 (100)

*The identification of drug resistance-associated mutations was based on the guidelines published by the International Antiviral Society-United States (IAS-USA).

**The subtype of PR genes was assigned based on RIP and phylogenetic analyses.