THE MOLECULAR BASIS OF RESISTANCE ANTIRETROVIRAL MARKERS AND POLYMORPHISMS OF THE HUMAN IMMUNODEFICIENCY VIRUS-1 SUBTYPE CRF01-AE PROTEASE GENE IN NAÏVE AND TREATMENT FAILURE PATIENTS IN BALI

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ABSTRACT

Application of antiretrovirals (ARVs) in patients with Human Immunodeficiency Virus (HIV) infection has proven to reduce mortality rates and prolong life expectancy. On the other hand, the use of antiretroviral drugs has incited the emergence of HIVDR. The resistance is due to mutation at genes associated with drug resistance. Nowadays, the determination of resistance markers mutations are based on HIV-1 subtype B. However, the majority of HIV in Indonesia, particularly in Bali are of subtype CRF01_AE. Genetic variation between HIV viruses has led to variations in subtypes; therefore, resistance markers of subtype B could be polymorphisms of non-B subtypes. This study aims to determine the number and types of the resistance markers mutations and polymorphisms that occur on the PR gene of HIV-1 subtype CRF01_AE of naïve and treatment failure patients in Bali.

This is an observational cross-sectional analytical study, conducted at two VCT clinics in Denpasar, during the period of April 2010 until October 2011. Samples consist of 18 HIV patients with treatment failure and 30 naïve HIV patients. Mutations were evaluated using PCR, sequenced and aligned were carried out using MEGA4. Interpretations of the mutations were made based on the Stanford HIV database. Hypothesis tests used were Mann-Whitney because of abnormal distribution of data. Hypothesis was accepted if the significant level $p<0.05$.

This study found that of the demographic data, only the predisposing factors of the two groups were significantly different ($p<0.05$). Two patients with treatment failure and 5 naïve patients were found to have L10LV/I mutations. Only one patient with treatment failure had the I54FI mutation. No major mutations were found among the two study groups. The number and types of minor mutations were not significantly different ($p>0.05$) between the naïve group and treatment failure group. M36I and H69K polymorphisms of the PR gene were found in all the study samples.

In conclusion of this study, two types of major mutations were found, L10LV/I and I54FI. The number and types of the resistance markers mutations towards the protease inhibitor (PI) group were not significantly different between the two study groups. M36I, H69K mutations of the PR gene are markers of polymorphisms of HIV-1 subtype CRF01_AE.

Keywords: HIV-1 subtype CRF01_AE, HIVDR, protease gene, antiretrovirals, resistance marker, mutation, polymorphism.

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INTRODUCTION

The use of antiretrovirals (ARVs) in patients with Acquired Immune Deficiency Syndrome (AIDS) has proven to reduce mortality rates and prolong life expectancy. On the other hand, the effectiveness of ARVs is limited by the emergence of Human Immunodeficiency Virus drug resistance (HIVDR) strains. The nature of HIVDR strains is that these strains persist for as long as the life of a patient. These resistant strains reside as a minor species in the circulation or are only found in the genome of infected cells in a proviral state.[1,2] HIVDR strains can be grouped into *primary / transmitted HIVDR* found among patients that have never received ARVs (naïve patients) and *acquired/secondary HIVDR*, occurring when patients have received ARVs previously.[1,3] Spread of transmitted drug resistance (TDR) can be influenced by several factors, i.e. the type of drug used, duration of therapy, virus subtype, occurrence of coinfection and persistence of high risk behavior in the patient. Meanwhile, acquired HIVDR can occur because of lack of compliance of the patient in using ARVs and suboptimal therapy because of economic factors.[1,2] Cases of HIVDR have spread to all over the

world, especially in regions where ARVs have been used for more than 4 years.

Antiretrovirals have been used globally as a treatment for AIDS since 1995. In Indonesia, ARV were initially used in 2004. There are 811 HIV patients in Bali that are under treatment with ARVs.[4] Even though many patients with HIV have received ARV therapy in Indonesia, at present there is no data on the prevalence of HIVDR in treatment failure cases. A study in Jakarta in 2009 found a prevalence of transmitted HIVDR among injecting drug users <5%.[5]

HIVDR resistance mechanisms have mostly been acquired through studies of HIV-1 subtype B, which is commonly found in North America, West Europe, and Australia. However, the prevalence of HIV subtype B is only about 12% of all HIV in the world.[6] Meanwhile, in Indonesia, 90.7% of people infected with HIV were infected by subtype CRF01_AE, which for practical reasons is grouped as non-B subtype.[7] A large combination of HIV genome of only 10^4 bp with a large number of mutations and a high rate of replication has led to the emergence of variations in HIV subtypes.[8] Genetic variation between subtypes is 15-20%, whereas variations in nucleotides and amino acids of RT sequences and variations in proteases between subtypes are 10-12% and 5-6%, respectively.[9]

This causes a problem in determining amino acid residues as markers of ARV resistance in non-B HIV subtypes. Several researchers have proven that some mutations that are related to drug resistance occurring in subtype B are normal mutations or normal variations (polymorphisms) in non-B subtypes.[6,10,11] Polymorphisms occur differently in each subtype. A polymorphic position in one subtype is usually nonpolymorphic in another subtype. The opposite applies as well, as a nonpolymorphic position in one subtype is generally a polymorphic position in another subtype.[3] Due to these reasons, studies on HIVDR in non-B HIV subtypes, such as CRF01_AE that is the most commonly found subtype in Indonesia, are still necessary. This study aims to determine the difference in number and types of mutations in markers of ARV resistance and polymorphisms of the protease gene (PR) amino acids in naïve and treatment failure patients with HIV subtype CRF01_AE in Bali. This study will be valuable in choosing therapy regimens in the treatment of patients with HIV.

MATERIALS AND METHODS

This study was performed using an observational cross sectional analytic study design. Samples for this study consisted of blood plasma of people with HIV infection who sought treatment at the HIV clinic of Sanglah Hospital and Kerti Praja HIV clinic in Denpasar, during the period of April 2008 until May 2001, determined through a consecutive sampling technique. Inclusion criteria for the samples were patients with HIV who received antiretroviral therapy for the first time and patients declared as having treatment failure with a viral load of >750 copies/mL. RNA extraction and PCR were performed from April 2010 until October 2011 at the Biomolecular Laboratory of the Faculty of Medicine, Udayana University.

Before the process of RNA extraction, plasma was centrifuged at 21,000 g, 4°C for 90 minutes. As much as 200 µL of the sediment was collected for further processing. RNA of HIV-1 was extracted from the plasma sediment using QIAmp Viral RNA Mini Kit (Qiagen Corporation). Genetic material was amplified by RT-PCR and nested-PCR techniques, using a PCR thermocycle Biorad® machine. RT-PCR reactions used the *Reverse Transcriptase-PCR Superscript™ III One-Step RT-PCR System* with *Platinum® taq DNA Polymerase* (Invitrogen®) reagent. Primers used for the first cycle were HIV-1F and HIV-1R. RT-PCR reaction for a single reaction consisted of RNA mix 12.5 µL, HIV-1F primer 1.5 µL, HIV-1R primer 1.5µL, Taq polymerase 1 µL and template 8.5 µL. The cycle for RT-PCR reactions consisted of 55°C for 60 minutes for the RT reaction, 95°C for 2 minutes, 40x the following cycle: 95°C for 30 seconds 60°C for 30 seconds 68°C for 2.5 minutes, and finally 68°C for 10 minutes. Nested-PCR reactions used the *Platinum® Taq DNA Polymerase High Fidelity* (Invitrogen®) reagent with the primers HIVGRT-2F2 and HIVGRT-2R. Each single nested-PCR reaction consisted of 10x PCR buffer 2.27 µL, dNTP mix (10 mM) 0.45 µL, $MgSO_4$ (50 mM) 0.68 µL, HIV-2F2 primer 0.45 µL, HIV-2R primer 0.45 µL, Taq polymerase 0.23 µL, H_2O 19.56 µL and RT-PCR product 0.91 µL. The cycle for nested-PCR reactions consisted of 94°C for 2 minutes, 30x the following cycle: 94°C for 30 seconds 57°C for 30 seconds 68°C for 2 minutes, and lastly 68°C for 5 minutes. Sequencing was performed at the *Institute of Human Virology and Cancer Biology Universitas Indonesia* (IHBCV-UI) in Jakarta. Sequencing reactions used 7 primers, i.e. primer A, b, C, D as forward primers and F, G, and H primers as reverse primers. Sequencing reactions used *BigDye deoxy Terminator* according to the procedure as instructed by the manufacturer, using the *Applied Biosystems 310 Sequencer* machine. Subtypes and polymorphisms of the protease gene were compared to the standard subtype B based on the *Stanford Drug Resistance Database* (http://hivdb.stanford.edu). Meanwhile, to determine polymorphisms of subtype CRF01_AE, the standard strain CRF01_AE_CM240 was used.

Normal distribution of the demographic variables data was tested using Shapiro-Wilk, because the number of samples was less than 50. For hypothesis testing, Mann-Whitney test was used if abnormal distribution of data was found. Chi square test was used to analyze differences between variables. Statistical analysis was performed using the *Statistical Package for the Social Sciences* (SPSS 16.0, Chicago, Illinois, USA) software.

RESULTS

During this study, number of patients infected with HIV who fulfilled the inclusion criteria was 48 patients. Demographic data showed that more males than females were investigated in both naïve group and treatment failure groups. There were 77.8% of male and 63.3% of female. Based on patients age, young adults (16-44 years old) were the majority in both groups. In the treatment failure group there were 16.7% children (age 5-15 years), while in the group of naïve patients there were no children. The duration from diagnosis of HIV infection until initiation of antiretroviral therapy was shorter among the group with treatment failure (mean 147 ± 216.8 days) compared to the naïve group (mean 324 ± 574 days). Meanwhile, the average duration from starting antiretroviral therapy until treatment failure occurred was 3 years (mean 1077 ± 461.7 days). The most common predisposing factor in both groups was heterosexual. Perinatal infection as a predisposing factor was only identified in the treatment failure group, while tattoos and heterosexual partners were only found in the naïve group (table 1). All patients with treatment failure received first line therapy that did not include antiretrovirals from the protease inhibitor (PI) group. Shapiro-Wilk normality testing of the variables of sex, age, duration and predisposing factors found an abnormal distribution. Mann-Whitney testing of the demographic data of the patients found only the predisposing factors significantly different ($p<0.05$) between the two groups. Demographic analysis results of the patients are presented in Table 1.

The purpose of PCR was to amplify the HIV *pol* gene. The result of nested-PCR consisted of PCR products of 1,800 bp that are detectable through electrophoresis as presented in Figure 1.

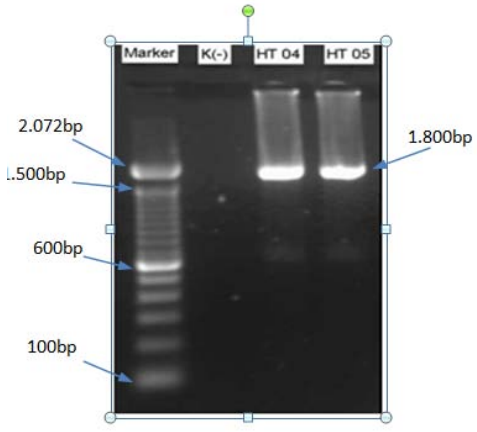

Figure 1
The product of nested PCR of the HIV *pol* gene. Column 1: marker; column 2: negative control; columns 3 and 4: sample.

The PCR sequencing products depend immensely on the nested-PCR quality product. low quality, such as having a noisy background, short peak electrophero grams, or the presence of contaminations, it will be difficult to identify quasi-species in the samples.

Table 1
Demographic data of HIV patients of the naïve and treatment failure groups

Variable	Patients		*p*
	Failure	Naive	
Total	18	30	0.296
• Male	14 (77.8%)	19 (63.3%)	
• Female	4 (22.2%)	11 (36.7%)	
Age (year)			0.56
• 5-15	3 (16.7%)	-	
• 16-44	15 (83.3%)	26 (86.7%)	
• 45-65	-	4 (13.3%)	
• Average	31.6	35.2	
• Range	5 - 48	27 - 50	
Duration (days)			0.163
• Found HIV until starting ARV	1 – 870	1 – 2,370	
• Mean	146.6	324.3	
• Starting ARV until treatment failure	420 – 2,130	-	
• Mean	1,077.2	-	
Viral load (copies/mL)			0.354
• Range	6,700 – 750,000	2,345– 750,000	
• Mean	213,595.72	261,315.83	
Predisposing factors:			0.018
• Perinatal infection	3 (16.7%)	-	
• Tattoo+heterosexual	-	1 (3.3%)	
• IVDU	5 (27.8%)	1 (3.3%)	
• IVDU+heterosexual	-	1 (3.3%)	
• Heterosexual	10 (55.6%)	26 (86.7%)	
• Heterosexual partner	-	1 (3.3%)	

IVDU = intravenous drug user

In this study, to test antiretroviral resistance towards the PI group, all amino acid residues of the PR gene, consisting of 99 amino acid residues, were tested. In both the naïve and treatment failure groups, major mutations as markers of resistance were not found on the PR gene, but several minor mutations were identified. There were 2 amino acid residues as markers of resistant in the treatment failure group and only one amino acid residue in the naïve group. Changes in the amino acid residue type of the PR gene that occurred did not change the characteristic of that

amino acid residue (Table 2). There were 2 patients from the treatment failure group and 5 patients from the naïve group with the L10LV/I mutation. All of the patients were still sensitive to the PI group, as L10LV/I mutations were not found together with major mutations that are markers of PI resistance. Among the patients with treatment failure, one patient (code HNT 09) was found to have a mutation of codon 54. The mutation that occurred was the change of isoleusine (I) to phenylalanine (F) amino acid residue. The I54FI mutation is a polymorphism that is not associated with markers of resistance, therefore patient HNT 09 was still sensitive to all drugs of the PI group.

Table 2
Proportions of the number and types of mutations as markers of PI resistance (minor) among the 99 amino acid residues of the PR gene

No	Mutation	Treatment failure		Naïve		*p*
		n=18	%	n=30	%	
1	L10LV/I	2	11.1	5	16.7	0.696
2	I54FI	1	5.6	0	0	0.375
Number of changed AA		2/99=2.0%		1/99=1.0%		1.000

Notes: 99 = number of amino acids of the PR gene, AA= amino acid, NP.N= non polar neutral, Change in AA type for no.1: NP.N --> NP.N/NP.N, for no.2: NP.N --> NP.N/NP.N

Statistical analysis of minor mutations of the PR gene and markers of NNRTI resistance of the RT gene found p>0.05. Therefore, it can be concluded that there was no significant difference between the numbers of mutations that are markers of resistance among the two groups (Table 3).

Table 3
Comparison of mutations that are markers of resistance of the PR gene

Gene	Mutation	Treatment failure	Naïve	*p*
PR	Major	0	0	-
	Minor	2%	1%	1.00

PR genes of the naïve group had an average of 3.92 polymorphisms, compared to the standard AE_CM240. There was one patient whose PR gene did not have polymorphisms compared to the standard (patient code HT17). Only on position 57 did the amino acids of naïve patients have a 72% difference compared to the standard, i.e. lysine (K) of the standard became arginine (R) in the patient. The other amino acid residues of naïve patients were <25% different from the standard (6 amino acids) (image 3). The average of polymorphisms of the PR gene in the treatment failure group was 3.5. This result was smaller than the result of the naïve group (Figure 3).

Analysis of sequencing results in order to determine resistance patterns towards antiretrovirals used the *Stanford HIV database*. Analysis results identify mutations that are markers of resistance and other mutations with the standard HIV-1 subtype B for comparison.

```
                1111223334 4566777889 99
                0256095791 5723027291 37
#Standard       LTIGKDDNPK KKILKIVVMT IL
_AE_CM240
#HNT_02_n       I.V....... .R........ .. = 3
#HNT_05_n       ?.....?... .R.......? .. = 4
#HR_06_n        .........R .R........ .. = 2
#HT_03_n        ??.?R...S. .R........ .. = 6
#HT_10_n        ...ER..... .?....I... L. = 5
#HT_11n         ...ER....? RRVP?.I... L.= 10
#HT_17_n        .......... .......... .. = 0
#HT_42_n        .......... .R........ .. = 1
#HT_48_n        ....R.E... .R..?..... L. = 5
#HNT_08_n       ...ER.E... .R.?RT..IA .. = 9
```

A. Polymorphisms of the PR gene of 10 study samples in the naïve patient group

Note: amino acids are stated by single letter codes. Dots (.) represent amino acids that at that position are the same as the standard. Question marks (?) represent amino acids that at that position have more than one type of amino acid. n= naïve.

```
                1111111233 5667778999
                0234569057 7130272137
Standard_AE     LTVKIGLKDN KQLKIVVTIL
_CM240
#H01_gt         .......... ..P....... = 1
#HNT_06_gt      .?I?...... R.....I.L. = 6
#HNT_10gt       .......?E. R..R...... = 4
#HR_08_gt       .....A.... R......... = 2
#HR_14_gt       .......R.. R....I.... = 3
#HR_21gt        ?AI.V..... R.PR...... = 7
#HT_02gt        I....E.R.. R........I = 5
#HT_53_gt       ..I....... R.P.....L. = 4
#HT59_gt        ....V..... R........F = 3
#hnt_04_gt      ..I......D R.?.....L. = 5
```

B. Polymorphisms of the PR gene of 10 study samples in the treatment failure group

Note: amino acids are stated by single letter codes. Dots (.) represent amino acids that at that position are the same as the standard. Question marks (?) represent amino acids that at that position have more than one type of amino acid. gt = "*gagal terapi*" (treatment failure).

Figure 4
Polymorphisms of the PR gene of 10 study samples in the treatment failure and naïve groups

Other mutations that are not markers of resistance are polymorphisms of HIV-1 subtype non-B. A mutation is considered a marker of non-B subtypes if polymorphic mutations are found in both patient

groups with a prevalence of >90%. These mutations can be used as markers for HIV-1 subtype CRF01_AE if the prevalence in both study groups reaches 100%. Therefore, amino acid residues that can be used as markers for subtype CRF01_AE in this study are M36I, H69K of the PR gene (Table 4).

DISCUSSION

In this study, two groups of patients were studied, i.e. naïve and treatment failure HIV patients. In the naïve group, the average time from diagnosis of HIV infection until initiation of antiretroviral therapy was 11 weeks. The reason for this is that these patients were diagnosed as infected with HIV for the first time and sought treatment when they were category B or C of the HIV severity classification.

Table 4
Polymorphisms of the PR gene in the treatment failure and naïve groups compared to the standard HIV-1 subtype B

Gene	Mutation	Treatment			
		Failure		Naïve	
PR		n=18	%	n=30	%
	E35D	16	88.9	26	86.7
	M36I	18	100	30	100
	R41K	18	100	28	93.3
	H69K	18	100	30	100
	L89M	18	100	28	93.3

Therefore, these patients are often called chronic naïve HIV patients. In the treatment failure group, the time from diagnosis of HIV infection until receiving antiretrovirals was shorter, with an average of 5 weeks. The majority of patients in the treatment failure group came in the AIDS stage or category C and required immediate antiretroviral therapy. The average duration from the initiation of therapy until treatment failure occurred was 3 years. This result is in accordance with a study in Cameroon, in which mutations that are markers of antiretroviral resistance are found in patients that are exposed to antiretrovirals for more than 14 months. Meanwhile, mutations that are markers of resistance are not found in patients that have used antiretrovirals for 9 months.[12]

Results of statistical testing of the demographic data of both study groups found only the predisposing factors variable to be significantly different ($p<0.05$) between the two groups. In the treatment failure group, more IVDU patients were found, who generally have the tendency of being noncompliant. Kozal et al. (2009) also reported that HIVDR patients have a tendency to maintain their high risk behavior. Compliance of patients in the treatment failure group is expected to be lower than the naïve group.[13]

Mutations that are markers of PI resistance were not found in both of the groups. Minor mutations were found in the treatment failure and naïve groups, 16.6% and 16.7%, respectively. The reason being that none of the patients had used antiretrovirals of the PI group before. Meanwhile, minor mutations can occur as a result of normal mutations during the replication process of HIV. Major mutations (D30N, M64I, G48V, I50N, V82A/F/T, I84V, L90M) and minor mutations (L101I/R/V, K20M/R, L24I, V32I, L33F, M36I, M46I/L, I47V, I54L/M/V, L63P, A71V/T, G73S, V77I, V82I, N88D) associated with the PR gene can be identified based on the *International AIDS Society-USA* (Johnson et al., 2008). In this study, the most common minor mutation was the L10LV/I and only one I54FI mutation was found in the treatment failure group. The L101/F/V/R mutation is a polymorphic mutation, but is associated with resistance to the entire PI group if found together with other mutations.[14-17] Mutations of codon 54 where isoleusine is replaced by Leusine/L or Val/V or Thr/T (I54L/V/T mutation) increases resistance towards the entire PI group if found together with other mutations.[14,17,18] A study in Rio de Janeiro involving 49 naïve HIV patients did not find major mutations in any of the patients. However, minor mutations were found in 85%, particularly L63P mutations that were found in 54%.[20] Natural polymorphisms of non-B subtypes that are commonly found as mutations marking PI resistance in subtype B in Africa are L10I/V, K20I/R, M36I, L63P and V77I.[21]

The result of the PR sequence analysis in this study found several mutations that were always present in both of the patient groups. Mutations that were always found on the PR gene of the treatment failure group and in >90% of the naïve group are M36I, R41K, L89M and H69K, while E35D was only found in >85% of both groups. Santos and Soares (2010) reported several mutations of the non-B subtype that are associated with decreased sensitivity towards the PI group, but are not mutations marking resistance to the PI group in subtype B.[22] These mutations are called *genetic signatures*. Mutations that are always present in one subtype but do not affect sensitivity to drugs are called polymorphisms. These mutations are I13V, K20I, M36I, H69K, V82I and I93L.[22] A study by Ode (2007) found that the samples of subtype CRF01_AE used contained polymorphisms (K20R, E35D, I93L) and genetic signatures (M36I, R41K, H69K, L89M).[23] Sensitivity to atazanavir (ATV), indinavir (IDV), nelfinavir (NFV) and tipranavir (TPV) are associated with the M36I mutation. The H69K mutation is only associated with atazanavir.[22] Other studies have not reported any influence of the R41K and L89M mutations on certain types of PIs.[23] All of the genetic signatures identified by Ode were also found in this study, but only the polymorphism E35D was found in this study.

CONCLUSION

Two types of minor mutations were found, i.e. L10LV/I and I54FI. The I54FI mutation was only found

in one treatment failure patient, and in none of the naïve patients. Major mutations were not found; therefore, no patients were resistant to the PI group. The number and types of mutations between the naïve and treatment failure group were not different significantly (p>0.05). M36I, H69K polymorphisms of the PR gene can be used as a marker of subtype CRF01_AE.

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