RESEARCH ARTICLE

Molecular Antiretroviral Resistance Markers of Human Immunodeficiency Virus-1 of CRF01_AE Subtype in Bali, Indonesia

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Abstract: **Background:** Molecular epidemiological study of human immunodeficiency virus drug-resistant (HIVDR) markers is challenging in areas where the dominant subtype is non-B.

Objective: Here we provide molecular data for HIVDR in the CRF01_AE subtype in Bali, Indonesia.

Method: Seventy patients were enrolled in this study and grouped into treatment failure and treatment naïve groups. The full-length pol gene was amplified using nested reverse transcriptase polymerase chain reaction and the product was then sequenced. The readable sequence was then subjected to Stanford HIV Drug Resistance Database genotyping.

Results: We found that clinical classification was in accordance with the presence of HIVDR markers in the pol gene. Independent of therapy history, the treatment failure group showed resistance markers against nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), ranging from 72%–100% of patients. Only a small proportion of naïve patients harbored HIV with drug resistance markers to NNRTI. No protease inhibitor-resistant marker was found in either patient group. Molecular marker mutations, which were found in more than 50% of treatment failure patients, were M184V (100%), T215A/Y/F (88.2%), D67N/G (76.5%), and M41L (58.8%).

Conclusion: The protocol used in this study to determine genetic markers of HIVDR based on subtype B can be applied for the rapid determination of resistance of the CRF01_AE subtype. All patients with progressive clinical signs and increased viral load should be recommended to undergo second-line treatment of the ARV regimen.

1. INTRODUCTION

Global surveillance of antiretroviral (ARV) drug-resistant human immunodeficiency virus (HIVDR) needs to be conducted. Effective ARV therapy is essential for HIV infected patients to reduce acquired immunodeficiency syndrome (AIDS) progression and prolong patient life expectancy. The pandemic emergence of HIVDR throughout the world has affected all age groups, including children [1, 2]. Drug resistance leads to treatment failure, resulting in patient death. The epidemiological impact can be disastrous as HIVDR may be transmitted to other patients and transmitted drug-resistant (TDR) strains have been reported in both developed and developing countries [3-5].

Mutations in the reverse transcriptase (RT) gene complex have been used to understand the genetic mechanism of HIVDR. This is particularly valid for the HIV-B subtype that predominantly circulates in America, Europe, and Australia [6]. However, the most globally prevalent subtype is HIV-1 C [7, 8], and the most widespread circulating subtype in Indonesia is CRF01_AE [9-11]. Molecular epidemiological study of HIV resistance is likely to be problematic in areas where the dominant subtype is non-B, as various reports have shown that molecular markers related to drug resistance are less valid for non-B subtypes [12].

Bali is a unique hotspot in understanding molecular markers of HIVDR subtype CRF01_AE. This major tourist

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destination is facing a rapidly growing HIV epidemic [13-15]. The island receives up to five million international visitors annually. Unfortunately, in Indonesia, knowledge of the disease is low and social stigma is high, meaning that only 5–10% of HIV/AIDS sufferers actually get diagnosed and treated [16, 17]. Additionally, HIVDR patients often have a tendency to maintain a high-risk lifestyle [18, 19], further contributing to TDR.

2. MATERIAL AND METHOD

2.1. Ethical Clearance

All subjects received a complete explanation of this study and agreed to sign an accompanying informed consent. Written informed consent was obtained from the patients or their families (in cases of patients under the age of 18 years) before the samples and patient data were collected. Ethical clearance for this study was granted by the Research Ethics Committee of the Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia. The inclusion criteria were patients who (1) underwent ARV therapy and showed increased viral load, or (2) were about to commence the ARV regimen, (3) the HIV-1 CRF01_AE subtype was confirmed, and (4) agreed to participate and signed the informed consent.

2.2. Study Design

This study was an observational cross-sectional analysis performed in 2008–2010. The target populations were HIV-AIDS patients undergoing ARV therapy who showed an increased viral load of >750 copies/ml (treatment failure group) and HIV patients who were about to start the ARV regimen (naïve group) at the Voluntary Consulting and Testing (VCT) clinic in Sanglah Hospital, Denpasar. Viral load was determined by reverse-transcriptase and polymerase chain reaction (RT-PCR) and DNA probe quantification of the product using the Amplicor HIV-1 Monitor kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.3. Sample Collection

Five milliliters of venous blood were collected from all subjects who met the inclusion criteria, using Ethylene Diamine Tetra Acetic Acid as the anti-coagulant. Plasma was separated following clarification using centrifugation at 3000 rpm for 10 minutes.

2.4. RNA Isolation and Pol Gene Amplification

Total RNA was purified from plasma using a QIAamp Viral RNA Mini Kit (Qiagen Corporation, Germany) according to the manufacturer's instructions. The whole RT gene in plasma was amplified using RT-PCR and nested PCR. In the first round, RT-PCR was conducted using Reverse Transcriptase-PCR Superscript[TM] III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase (Invitrogen[®] Carlsbad, CA) with an HIV-1F and HIV-1R primer set [20]. Nested PCR was conducted using Platinum[®] Taq DNA Polymerase High Fidelity (Invitrogen[®]) with an HIVGRT-2F2 and HIVGRT-2R primer set [20].

2.5. Sequencing

Sequencing of the nested PCR product was conducted at the Virology and Cancer Pathobiology Research Center (VCPRC) Faculty of Medicine Indonesia University, Jakarta, Indonesia. Sequencing was done using the BigDye Terminator (Thermo Fisher, Waltham, MA) following the manufacturer's instructions, along with published primers [20]. Capillary electrophoresis of the reaction was performed in an Applied Biosystems 310 Sequencer (Applied Biosystem, Foster City, CA). Sequencing results were aligned using Viroseq[®] (Illinois, USA) or Mega4 [21] and the final sequence was subjected to Stanford HIV Drug Resistance Database genotyping (https://hivdb.stanford.edu).

2.6. Statistical Analysis

Variable normality of the subject demographic data was tested using the Shapiro-Wilk normality test [22]. Quantitative statistics were calculated by the Mann-Whitney test. Non-parametric statistics were calculated using the Chi-Square test. All calculations were conducted using the Statistical Package for the Social Sciences 16.0 (SPSS Inc., Chicago, IL).

3. RESULT

Seventy HIV patients were enrolled in this study. Thirty-four (48.6%) subjects were in the treatment failure group and 36 (51.5%) were treatment-naïve patients. The HIV RT gene could be amplified and sequenced in 48 of the total number of patients. From the positive samples, one was confirmed as HIV subtype A and the remaining 47 (97.9%) as HIV-1 CRF01_AE subtype. Ultimately, the numbers of samples belonging to treatment failure and treatment naïve groups were 17 and 30, respectively. All sequences from this study have been deposited in GenBank with accession numbers of KY927941–KY927982. The sequences covered nucleotide no 1252 – 2953 based on reference CRF01_AE/B of CM240, GenBank Acc. No. AF516184 [23].

Demographic data of ARV treatment failure and naïve patients included in this study, incorporating risk factors, viral load, name and duration of the ARV received, and treatment failure discovery, are available in Supplementary Material 1 and 2. A summary of both data sets is presented in Table **1**. The results show that the groups were not statistically different (p>0.05) in sex, age, and viral load parameters. However, the risk factor for the two groups differed significantly (p=0.018). The predominant at-risk group within the treatment-naïve group was heterosexuals (86.7%), while those present in the treatment failure group were heterosexuals (55.6%), intravenous drug users (27.8%), and perinatal infected infants (16.7%)

Percentage of HIVDR genotype of HIV-1 of CRF01_AE subtype to various Nucleoside Reverse Transcriptase Inhibitor (NRTI) and Non-Nucleoside Reverse Transcriptase Inhibitor (NRTI) in treatment failure and naïve patient groups is shown in Fig. (**1**). This result indicated that treatment failure patients harbor HIV with resistance markers to nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), ranging

Table 1. Summary of demographic data of ARV treatment failure and naïve of HIV-1-CRF01_AE positive patients in Bali, Indonesia 2008 – 2010*.

Variable	ARV Treatment Failure Patients	Naïve Patients	P
Number	18	30	
Sex			
Male	14 (77.8%)	19 (63.3%)	0.296
Female	4 (22.2%)	11 (36.7%)	
Age			
5-15 years	3 (16.7%)	-	
16-44 years	15 (83.3%)	26 (86.7%)	
45-65 years	-	4 (13.3%)	0.56
Age average value	31.6	35.2	
Age range	5 – 48	27 – 50	
Duration (days)			
ARV initiation since date of positive detection	1 – 870	1 – 2,370	
Mean	146.6	324.3	0.163
ARV treatment duration to drug failure discovery	420 – 2,130	-	
Mean	1,077.20	-	
Viral Load (copy/ml)			
Range	6,700 – 750,000	2,345 – 750,000	0.354
Risk factor			
Perinatal infection	3 (16.7%)	-	
Tattoo + heterosexual	-	1 (3.3%)	
IVDU**	5 (27.8%)	1 (3.3%)	
IVDU + heterosexual	-	1 (3.3%)	0.018
Heterosexual	10 (55.6%)	26 (86.7%)	
Heterosexual spouse	-	1 (3.3%)	

*The data of patients, whom the sequences could not be obtained were excluded; **IVDU: Intra-venous drug user;

from 72%–100% of patients, while only a small proportion of naïve patients harbor HIV with NNRTI drug-resistant markers. No protease inhibitor (PI) resistant marker was found in either patient group. Percentage of HIV-1 of CRF01_AE subtype in treatment failure and naïve patient groups harboring deduced amino-acid substitutions that generate HIVDR genotype to NRTI and NNRTI is presented in Fig. (**2**). The result shows that molecular marker mutations, which were found in more than 50% of treatment failure patients, were M184V (100%), T215A/Y/F (88.2%), D67N/G (76.5%), and M41L (58.8%).

Comparison of the percentage of amino acid substitutions in CRF01_AE subtype in treatment failure group in Bali – Indonesia and global CRF01, B, and C subtypes n NRTI±NNRTI Treated Patients available in HIV Drug Resistance Database of Stanford University (https://hivdb. stanford.edu/pages/surveillance.html; accessed January 26, 2019) is presented in Table **2**. The result shows that the major substitutions (percentage >50%) of M41, D67, M184, and T215 were higher than all subtype data, while the K103 substitution was less frequent in our study.

DISCUSSION

As expected, almost all of the HIV-1 identified individuals in this study harbored CRF01_AE subtype, which is the most common circulating subtype in Indonesia [9, 11, 24]. Only one was of A subtype. Along the RT gene, we found unambiguous sequence reads in many positions with a very clean background. The primer set used for sequencing allowed us to identify nucleotides with multiple signals. In some cases, we also repeated the sequencing to confirm the signals. This is a hallmark of the viral quasi-species concept
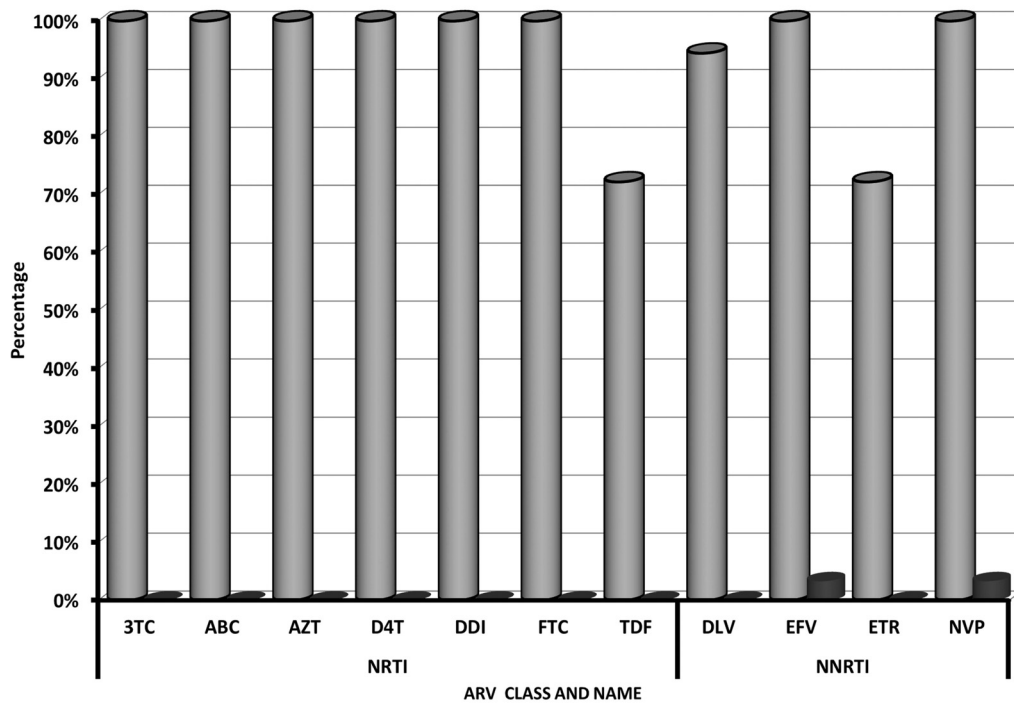
Fig. (1). Percentage of HIVDR genotype of HIV-1 of CRF01_AE subtype to various Nucleoside Reverse Transcriptase Inhibitor (NRTI) and Non-Nucleoside Reverse Transcriptase Inhibitor (NRTI) in treatment failure and naïve patient groups in Bali, Indonesia 2008 – 2010. Grey blocks: treatment failure group; Black blocks: naïve group; NRTI: lamivudine (3TC), Abacavir (ABC), Zidovudine (AZT), Stavudine (D4T), Didanosine (DDI), Emtricitabine (FTC), and Tenofovir (TDF); NNRTI: Delavirdine (DLV), Efavirenz (EFV), Etravirine (ETR), Nevirapine (NVP).
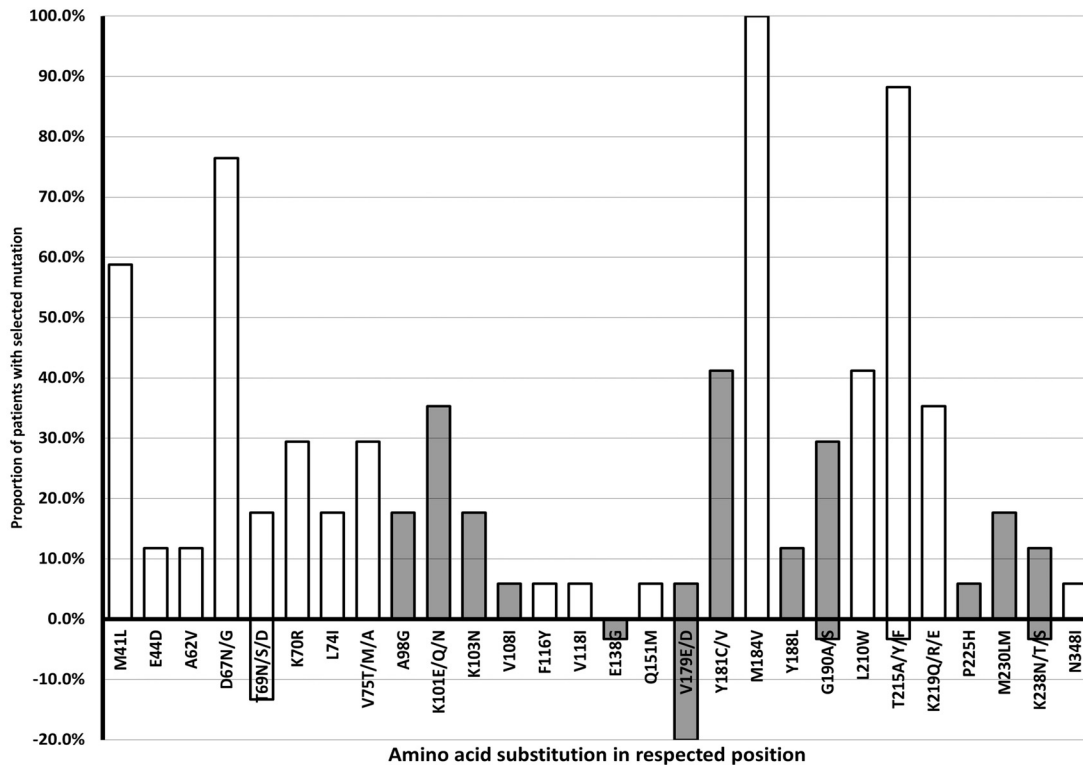

Fig. (2). Percentage of HIV-1 of CRF01_AE subtype in treatment failure and naïve patient groups in Bali, Indonesia 2008 – 2010, with deduced amino-acid substitutions that generate HIVDR genotype to NRTI and NNRTI. Treatment failure: above the axis; naïve: below the axis; HIVDR genotype to NRTI: unfilled bars; HIVDR genotype to NNRTI: filled bars. The numbering was annotated by The Stanford HIV Drug Resistance Database genotyping (https://hivdb.stanford.edu).

Table 2.   Percentage of amino acid substitutions in CRF01_AE subtype in the treatment failure group in Bali – Indonesia and Global CRF01, B, and C subtypes available in HIV Drug Resistance Database of Stanford University.*

Pos.	CR	Subtype			
		CRF01_AE (This Study)	Global CRF01	B	C
41	M	58.8	19.0	40.0	9.2
44	E	11.8	NA	NA	NA
62	A	11.8	NA	NA	NA
67	D	76.5	33.0	37.1	18.8
69	T	17.6	3.3	10.2	3.1
70	K	29.4	21.7	22.6	13.7
74	L	17.6	6.8	16.6	6.4
75	V	29.4	17.2	8.4	5.7
98	A	17.6	10.1	6.3	5.9
101	K	35.3	18.6	9.5	10.5
103	K	17.6	42.6	51.0	76.1
108	V	5.9	NA	NA	NA
116	F	5.9	4.4	2.2	1.4
118	V	5.9	NA	NA	NA
138	E	NA	5.4	4.4	9.7
151	Q	5.9	6.8	3.0	2.1
179	V	5.9	4.4	3.6	6.1
181	Y	41.2	41.4	21.0	15.8
184	M	100	78.7	77.1	69.6
188	Y	11.8	5.9	5.5	9.0
190	G	29.4	41.0	25.4	24.0
210	L	41.2	11.9	27.5	3.3
215	T	88.2	32.7	53.6	16.6
219	K	35.3	22.6	27.8	12.3
225	P	5.9	3.9	3.2	7.7
230	M	17.6	3.4	1.1	3.5
238	K	11.8	2.3	2.6	2.3
348	N	5.9	6.5	13.0	15.1

* In NRTI±NNRTI Treated Patients Available in https://hivdb.stanford.edu/pages/surveillance.html; NA: not available; CR: consensus residue

[25-34]. The reads covered nucleotide no 1252 – 2953 based on reference CRF01_AE/B of CM240, GenBank Acc. No. AF516184 [23]. This should include viral protease, reverse transcriptase, and RT-thumb domain of HIV-1 [35], calculated from the ribosomal slippage site of TTTTTTAG [36].

Global genotyping seems valid for the HIV-1 CRF01_AE subtype. There have been very few reports regarding polymorphisms and molecular drug resistance markers in this subtype, although protease polymorphism has been described [37, 38]. Patterns of point mutations associated

[37, 38]. Patterns of point mutations associated with ARV treatment failure in the CRF01_AE subtype likely differ from that of B subtype [39]. Despite this, we found that there was a strong concordance of molecular markers that were initially established for type B with the clinical signs of the patients in our study. All patients exhibiting signs of treatment failure expressed resistance marker mutations following drug resistance genotyping in the Stanford database.

However, some patterns seem unique to our study. Comparison of incidence of amino acid substitutions in our study to global CFR, B, and C subtypes data shows that the major substitutions (percentage of 50% or higher) of M41, D67, M184, and T215 were higher than all subtype data, while the K103 substitution was less frequent in our study. Substitution of 138E was not found in our study, while others of 44E, 62A, 108V, and 118V were not available in the database. Unique host factors might contribute in replication rate of the HIV-1, therefore its mutation pattern varies. There has been yet no clear evidence to prove this possibility. Host factors have been indicated to contribute to HIV-1 replication [40-42]. The influence of host factors in susceptibility to HIV infection and AIDS progression, but not in HIV mutation pattern, has been described elsewhere [43-46].

All treatment failure subjects harbored resistance to NRTI and NNRTI, which are first-line ARV drugs. The pattern is concordance to a recent report from North Sulawesi, Indonesia [47]. The figure is higher than that reported in South Africa [48]. The group included three pediatric patients who underwent 6.5 years of ARV therapy. The presence of HIVDR strains in children has been reported elsewhere, such as Brazil [49, 50] and France [51], with varying degrees of resistance to NRTI and NNRTI, reaching between 30–80% of patients. The low number of children enrolled in this study might cause the observed difference.

Cross-resistance in HIV-1 subtypes is obvious from this study. Independent of therapy history, the pol gene sequences found in all treatment failure subjects contained all NRTI and NNRTI resistant markers (Fig. **1**), with the prevalence of each molecular marker being 72–100%. All subjects within the treatment failure group received two NRTI and one NNRTI drugs, available during the study period. The NRTIs used were lamivudine and zidovudine, although stavudine was given to zidovudine-allergic patients. The NNRTI was either nevirapine or efavirenz. Of note, no patient was given abacavir, didanosine, emtricitabine or tenofovir, yet genotypic testing showed that all treatment failure subjects were 100% resistant to these drugs despite never having received them, with the exception of resistance to tenofovir, which reached 72.2%. These data demonstrate that cross-resistant HIV is not uncommon [52-59].

We found no TDR in our study. Only a small proportion of naïve patients harbored HIV with drug-resistant markers to NNRTI. This may be unique to this study or to the subtype in question. Transmitted drug resistant strains have been observed elsewhere [4, 60-65], including in Indonesia [66, 67]. The TDR prevalence ranges from 9–25% in Europe and the United States [7]. This might be related to the length of implementation of the ARV program [12]. The program was introduced in 2002–2004 in developing countries, including Indonesia. The resistant markers found in the treatment naïve group are most likely not TDR and seem merely due to genetic variation of HIV through the high mutation rate of the virus [68-72]. The three cases of HIVDR in children were not TDR either, as all of them underwent ARV treatment during the previous five years (Table **1**).

Thymidine analogue mutations (TAMs) of cluster 1 were dominant in our study. Codon polymorphisms were found throughout the RT gene in both groups. Significant differences exist in codon number 41, 67, 70, 75, 184, 210, 215 and 219. Except for codon 75, all others belong to TAM codons, while codon 75 is a nucleoside analogue mutation (NAM) [73, 74]. Molecular markers of TAMs, M184V (100%), T215A/Y/F (88.2%), D67N/G (76.5%), and M41L (58.8%), were found in more than 50% of treatment failure patients. These TAMs were found in the treatment failure group but not in any member of the treatment naïve group. The emergence of TAMs correlates with the use of NRTIs. There are two clusters of TAMs; cluster 1 includes substitutions of M41L, L210W and T215Y, while cluster 2 of D67N, K70R, T125F and K219Q/E/N [75]. The phenotypic picture of cluster 2 mutations is less resistant than cluster 1 [76]. Therefore, the treatment failure in this study is mostly due to cluster 1 TAMs.

The pattern of resistance-associated mutations found in this study differs to that found in Maumere, East Nusa Tenggara, except for M184V [11]. This discrepancy might relate to the subject of the genotypic study. We were targeting naïve as well as treatment failure patients, while the other study did not explain if the subjects were naïve or treatment failure patients [11]. The different time frame of sampling might also cause this dissimilar observation.

There are two major drawbacks of this study. Firstly, the success rate of RT-PCR amplification and sequencing is only around 70% of the samples. This might be because of the quality and quantity of RNA as well as the great variation in primer binding sites. A better approach could be the sequencing of pro-viral DNA [77] and application of next-generation sequencing [78]. The second limitation is the lack of facilities to conduct phenotypic testing to provide *in vitro* evidence. Different approaches to HIVDR should be applied and in-country HIVDR phenotyping infrastructure should be established, including in Indonesia.

That the international database applied in this study is in agreement with the clinical manifestations of subjects with treatment failure demonstrates that the protocol can be recommended for immediate determination of resistance genotype, and ideally, the HIVDR genotyping should combine pro-viral DNA and viral RNA amplification. All patients with progressive clinical signs and increased viral load should be recommended to undergo second line ARV therapy, as no protease inhibitor (PI) resistant marker was found in the treatment failure group.

In conclusion, clinical classification was in concordance with the presence of HIVDR markers in the pol gene. Independent of therapy history, the treatment failure group showed resistance markers against nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), ranging from 72%–100% of patients. Only a small proportion of naïve patients harbored HIV with drug resistance markers to NNRTI. Molecular marker mutations in more than 50% of treatment failure patients were M184V, T215A/Y/F, D67N/G, and M41L. The protocol used in this study to determine genetic markers of HIVDR based on subtype B can be applied for the rapid determination of resistance the CRF01_AE subtype. All patients with progressive clinical signs and increased viral load should be recommended to undergo second-line treatment of the ARV regimen.

CONCLUSION

Clinical classification of patients to treatment failure was in concordance with the presence of HIVDR markers in the pol gene. Independent of therapy history, the treatment failure group showed resistance markers against nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), ranging from 72%–100% of patients. Only a small proportion of naïve patients harbored HIV with drug resistance markers to NNRTI. Molecular marker mutations in more than 50% of treatment failure patients were M184V, T215A/Y/F, D67N/G, and M41L.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Research Ethics Committee of the Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia.

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. All humans research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/ 10 ethics/10helsinki/).

CONSENT FOR PUBLICATION

Written informed consent was obtained from the patients or their families (in cases of patients under the age of 18 years) before the samples and patient data were collected.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

1. Demography of ARV treatment failure, including risk factors, viral load, name and duration of the ARV, and treatment failure discovery of HIV-1 of CRF01_AE subtype positive patients in Bali, Indonesia 2008 – 2010.

2. Demography of ARV-naïve, including risk factors and viral load of HIV-1 of CRF01_AE positive patients in Bali, Indonesia 2008 – 2010.

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