The Emergence of HIV-1 Transmitted Drug Resistance Mutations Among Antiretroviral Therapy-naive Individuals in Buleleng, Bali, Indonesia

Ni Luh A. Megasari1,2, Devi Oktafiani1, Elsa Fitriana1, Siti Q. Khairunisa2, Shuhei Ueda2,3, Tomohiro Kotaki3, Nasronudin1,2, Soetjipto1,2, Masanori Kameoka3,4

1 Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.
2 Indonesia-Japan Collaborative Research Centre for Emerging and Re-emerging Infectious Diseases, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.
3 Department of Public Health, Kobe University Graduate School of Health Sciences, Hyogo, Japan.
4 Center for Infectious Diseases, Kobe University Graduate School of Medicine, Hyogo, Japan.

Corresponding Author:
Masanori Kameoka, MD. Department of Public Health, Kobe University Graduate School of Health Sciences, 7-10-2 Tomogaoka, Suma-ku, Kobe, Hyogo 654-0142, Japan. email: mkameoka@port.kobe-u.ac.jp.

ABSTRACT

Background: the global scale-up of antiretroviral therapy (ART) is the primary factor contributing to the decline in deaths from acquired immune deficiency syndrome (AIDS)-related illnesses. However, the emergence of transmitted drug resistance (TDR) compromises the effects of ART in treatment-naive individuals, which may hinder treatment success. The present study aimed to identify the presence of TDR among treatment-naive individuals in Buleleng, Bali, which is currently ranked sixth among Indonesian provinces with the highest cumulative human immunodeficiency virus type 1 (HIV-1) infection cases. Methods: thirty-nine ART-naive individuals in Buleleng Regency General Hospital were enrolled in the present study. Blood samples from participants were subjected to a genotypic analysis. Results: 28 protease (PR) and 30 reverse transcriptase (RT) genes were successfully amplified.
and sequenced from 37 samples. HIV-1 subtyping revealed CRF01_AE as the dominant circulating recombinant form in the region. No TDR for PR inhibitors was detected; however, TDR for RT inhibitors was identified in five out of 30 samples (16.7%). **Conclusion:** these results indicate the emergence of TDR among ART-naive individuals in Buleleng, Bali. This issue warrants serious consideration because TDR may hamper treatment success and reduce ART efficacy among newly diagnosed individuals. Continuous surveillance with a larger sample size is necessary to monitor TDR among ART-naive individuals.

**Keywords:** HIV-1, CRF01_AE, Bali, antiretroviral therapy (ART), transmitted drug resistance (TDR).

**INTRODUCTION**

The Joint United Nations Programme on human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (UNAIDS) reported a marked reduction in deaths from AIDS-related illnesses, from a peak of 1.9 million in 2004 to 940,000 in 2017. The global scale-up of antiretroviral therapy (ART) is the primary factor contributing to the decline in deaths. Combination ART decreases the replication of HIV type 1 (HIV-1), thereby improving the survival of infected individuals and lowering the risk of transmission.

UNAIDS estimated a decline in new HIV infections in Indonesia, from 62,000 cases in 2005 to 49,000 cases in 2017. The Indonesian Ministry of Health reported 280,623 cumulative HIV infection cases up to December 2017, with 102,667 cumulative AIDS cases. Provinces with the highest cumulative HIV infection and AIDS cases include DKI Jakarta, East Java, Papua, West Java, Central Java, and Bali.

HIV-1, which is responsible for most of the global HIV pandemic, has been subdivided into group M (major), group O (outlier), group N (non-major, non-outlier), and new group P (pending). Group M, the pandemic group of HIV-1, has been further divided into subtypes A to K. Besides these subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs), as a result of recombination among 2 or more subtypes and/or CRFs, have also been identified in group M. CRF01_AE, the second predominant circulating CRF accounting for 5% of infection cases worldwide in 2004-2007, is responsible for the vast majority of infections in Southeast Asia, including several regions in Indonesia.

In December 2017, 91,369 Indonesians were receiving ART; 88,386 among them received a first-line regimen, and 2,983 a second-line regimen. First-line ART regimens comprise two nucleoside reverse transcriptase (RT) inhibitors (NRTIs) and a non-nucleoside RT inhibitor (nNRTI), while two NRTIs plus a ritonavir-boosted protease (PR) inhibitor (PI) are adopted for second-line regimens.

Although ART is successful in Indonesia in reducing AIDS case fatality rate since 2004, the emergence of drug resistance has been reported not only among ART-experienced individuals, but also among newly diagnosed, ART-naive individuals. The emergence of drug resistance-associated mutations (DRMs) compromises the effectiveness of ART, resulting in lower viral suppression and hinders treatment success. Several DRMs in the HIV-1 pol gene are known to reduce viral susceptibility towards treatment, particularly a first-line regimens combining a NRTI and nNRTI. Thus, the presence of DRMs may be a cause for concern in various countries including Indonesia, where 88,386 out of 91,369 individuals on ART (96.7%) receive a first-line regimen, and thus rely heavily on ART with first-line regimens for the treatment of HIV-1.

The emergence of transmitted drug resistance (TDR) is attributed to the transmission of a drug-resistant virus. TDR compromises the effectiveness of ART for treatment-naive individuals. Our previous studies revealed the appearance of TDR for RT inhibitors among 4.3% (2/47) and 12.9% (4/31) of ART-naive individuals residing in Surabaya and West Papua, respectively. Bali, a popular tourism destination, is now ranked sixth among Indonesian provinces with the highest cumulative HIV cases, and ranks fifth for cumulative AIDS cases. Acquired drug resistance were previously reported to be found in 10% of ART-experienced
individuals in Buleleng, a regency located in the northern of Bali.\textsuperscript{17} The present study aimed to identify the presence of TDR among ART-naive individuals in Buleleng, Bali.

\section*{METHODS}

We determined the necessary sample size by consulting with a statistic lecturer at the Faculty of Medicine, Universitas Airlangga, and recruited 39 individuals recently diagnosed with HIV-1 infection at the Voluntary Counselling and Testing Clinic of Buleleng Regency General Hospital, Bali. Five milliliters of ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood samples were collected from study participants in February 2018. DNA was then extracted from whole blood samples using the Wizard\textsuperscript{\textregistered} Genomic DNA Purification Kit (Promega, Madison, WI, USA). Demographic and clinical data on study participants were retrieved from medical records.

\subsection*{Amplification of HIV-1 Genomic Fragments}

Viral pol gene encoding full-length PR (PR gene) and RT (RT gene) was amplified from DNA extracted from peripheral blood samples by the nested polymerase chain reaction (PCR) using GoTaq Green Master Mix (Promega, Madison, WI, USA) and the following primers. The primers DRPRO5, 5\textsuperscript{\prime} - AGACAGGYTAATTTTTAGGGA-3\textsuperscript{\prime} [corresponding to nucleotides (nt) 2074-2095 of the HIV-1 reference strain, HXB2 (GenBank accession no. K03455)] and DRPRO2L, 5\textsuperscript{\prime} - TATGGATTTTCAGGCCCAATTTTTGA-3\textsuperscript{\prime}, (nt 2716 to 2691) were used in first PCR for the amplification of the PR gene, and the primers DRPRO1M, 5\textsuperscript{\prime} - AGAGCCAACAGCCCCACCAG-3\textsuperscript{\prime} (nt 2148 to 2167) and DRPRO6, 5\textsuperscript{\prime} - ACTTTTG GCCCATCCATCC-3\textsuperscript{\prime} (nt 2611 to 2592) were used for nested PCR. The primers RT1L, 5\textsuperscript{\prime} - ATGATAAGGGGAATTGGAGGTTT-3\textsuperscript{\prime} (nt 2388 to 2410) and GPR2M, 5\textsuperscript{\prime} - GGACTACTGCTTGGCATG-3\textsuperscript{\prime} (nt 4402 to 4380) were used in first PCR for the amplification of the RT gene, while RT7L, 5\textsuperscript{\prime} - GACCTACACCTGTCAACATAATTGGG-3\textsuperscript{\prime} (nt 2485 to 2509) and GPR3L, 5\textsuperscript{\prime} - TTAAAA TCACTARCCATTGYTCTCC-3\textsuperscript{\prime} (nt 4309 to 4285) were used for nested PCR.

\subsection*{Sequencing Analysis, HIV-1 Subtyping, and Detection of Drug Resistance-associated Mutations}

Successfully amplified viral PR and RT genes were subjected to a sequence analysis performed by Macrogen South Korea (http://dna.macrogen.com). Sequencing data were assembled and aligned using Genetyx version 10 software (Genetyx, Tokyo, Japan). HIV-1 subtyping was then conducted using the Recombinant Identification Program (RIP) available on the HIV sequence database website (www.hiv.lanl.gov)\textsuperscript{17} and jumping profile Hidden Markov Model (jpHMM) (http://jpmm.gobics.de/submission_hiv).\textsuperscript{18} In addition, neighbor-joining (NJ) trees with a Kimura two-parameter model were constructed using MEGA 6.2 software with bootstrap values (1,000 replicates) for relevant nodes being reported on a representative tree. The appearance of DRMs in successfully sequenced PR and RT genes was analyzed according to the International Antiviral Society-USA (IAS-USA) panel.\textsuperscript{13} The nucleotide sequences of viral gene fragments have been deposited in the GenBank database under accession numbers MK656030-MK656087.

\subsection*{Ethics Statement}

Ethical approval for this research was obtained from the Ethics and Law Committee of Universitas Airlangga Hospital (Ethical approval no. 033/KEH/2016) and the Institutional Ethics Committee of Kobe University Graduate School of Medicine (approval no.: 784). Written informed consent was obtained from all study participants prior to sample collection.

\section*{RESULTS}

\subsection*{Demographic and Clinical Information on Study Participants}

All participants were confirmed to be ART-naive from medical records. Twenty-four (66.7\%) participants were male. The youngest participants were 16 years old and the oldest was 47 years old, with the most predominant age group being 30-34 years old. Fifteen individuals (38.5\%) were co-infected with tuberculosis (TB). Regarding the clinical stage of HIV
infection, 9 (23.1%), 18 (46.2%), and 12 (30.8%) individuals were classified as stages 2, 3, and 4, respectively. Among participants, 11 (28.2%) were underweight (body mass index <18.5). The demographic and clinical data of 39 study participants are shown in Table 1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>- Male</td>
<td>24 (66.7)</td>
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<tr>
<td>- Female</td>
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</tr>
<tr>
<td>Age (years)</td>
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<tr>
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<td>4 (10.3)</td>
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<tr>
<td>- 20-24</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>- 25-29</td>
<td>8 (20.5)</td>
</tr>
<tr>
<td>- 30-34</td>
<td>9 (23.1)</td>
</tr>
<tr>
<td>- 35-39</td>
<td>8 (20.5)</td>
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<td>- 40-44</td>
<td>3 (7.7)</td>
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<td>- 45-49</td>
<td>1 (2.6)</td>
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<tr>
<td>Clinical stage</td>
<td></td>
</tr>
<tr>
<td>- II</td>
<td>9 (23.1)</td>
</tr>
<tr>
<td>- III</td>
<td>18 (46.2)</td>
</tr>
<tr>
<td>- IV</td>
<td>12 (30.8)</td>
</tr>
<tr>
<td>TB co-infection</td>
<td></td>
</tr>
<tr>
<td>- Yes</td>
<td>15 (38.5)</td>
</tr>
<tr>
<td>- No</td>
<td>24 (61.5)</td>
</tr>
<tr>
<td>Underweight</td>
<td></td>
</tr>
<tr>
<td>- Yes (BMI &lt;18.5)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>- No (BMI ≥18.5)</td>
<td>28 (71.8)</td>
</tr>
</tbody>
</table>

TBD, tuberculosis; BMI, body mass index

### HIV-1 Subtyping

Sequencing data were successfully obtained from 37 out of 39 samples, comprising 28 PR genes (297-bp; nt 2253 to 2549) and 30 RT genes (1680-bp; nt 2550 to 4229). HIV-1 subtyping by RIP and pHMM was consistent with that by NJ trees (data not shown). Thirty-six samples (97.3%) were classified as CRF01_AE, while a sample (2.7%) was classified as CRF02_AG. The NJ trees of the PR and RT genes are shown in Figure 1.

### Appearance of DRMs

TDR was defined as the presence of at least one major DRM listed in the International AIDS Society United States (IAS-USA) panel database.\(^9\) Regarding the results obtained, no TDR was detected in PR genes; however, several minor mutations were detected. Among 28 PR genes, 10 (35.7%) contained L10I/V [amino acid substitution from leucine (L) to isoleucine (I) or valine (V) at position 10 in the PR gene], 12 (42.9%) G16E, 11 (39.3%) K20R/I, 28 (100%) M36I, one (3.6%) D60E, eight (28.6%) L63P, 26 (92.9%) H69K, one (3.6%) A71V, one (3.6%) V77I, six (21.4%) V82I, 28 (100%) L89I, and 10 (35.7%) I93L. M36I, H69K, and L89I, which presented in more than 90% samples, are common polymorphisms in non-B subtypes, including CRF01_AE.\(^9\)

Five out of 30 samples (16.7%) possessed TDR in the RT genes. The demographic characteristics of individuals with major DRMs in RT genes are shown in Table 2. The E138G/A mutation was detected in 2 samples (6.24%), while other major mutations, including K103N, G190A, and K219Q, were each found in one sample (3.12%). Besides the major DRMs, minor mutations were detected in 4 samples (13.3%), including V90I (3.12%), V106I (3.12%), and V179D/F (6.24%).

### DISCUSSION

We herein report the circulating HIV-1 subtype and prevalence of TDR among HIV-1-infected, ART-naive individuals residing in Buleleng, Bali, Indonesia. Among 37 successfully sequenced samples, 36 (97.3%) were classified as CRF01_AE, and the remainder (2.7%) as CRF02_AG. These results are consistent with previous findings for the predominance of CRF01_AE in various regions in Indonesia.\(^6-10\) However, the HIV-1 gene fragments analyzed in the present study were insufficient to identify the actual CRF since recombinant forms of HIV-1 possibly contain various sequences derived from more than 2 different subtypes and/or CRFs. Therefore, full-genomic sequencing analyses of the HIV-1 genome must be carried out in a future study.

A genotypic drug resistance study revealed no evidence of circulating PI-related TDR in Buleleng. This may have been due to the limited usage of PIs in this region. Among ART-experienced individuals in Indonesia, only 3.3% (2,983 of 91,369) were receiving a PI-
Figure 1. Phylogenetic tree analysis of HIV-1 PR and RT gene sequences collected from ART-naive individuals in Buleleng, Bali, Indonesia. Phylogenetic trees were constructed for the HIV-1 PR (A) and RT (B) genes newly sequenced in the present study. The corresponding viral genes of reference HIV-1 strains representing subtypes A1, A2, B, C, D, and G, as well as CRF01_AE (01_AE) and CRF02_AG (02_AG) were included in the analyses (shown in bold). Sequence IDs are presented as a sample ID or the ID of the reference HIV-1 strain, a GenBank accession number, and the subtype or CRF of the reference strain (shown in parentheses) in that order. Bootstrap values were shown if they were >70.
based regimen in 2017. Although no TDR was detected in PR genes, minor mutations, including L10I/V, G16E, K20R/I, M36I, D60E, L63P, H69K, A71V, V77I, V82I, L89I, and I93L, were identified in the present study. These mutations potentially affect viral susceptibility to ritonavir-boosted atazanavir (ATV/r), ritonavir-boosted fosamprenavir (FPV/r), ritonavir-boosted indinavir (IDV/r), ritonavir-boosted lopinavir (LPV/r), nelfinavir (NFV), ritonavir-boosted saquinavir (SQV/r), and ritonavir-boosted tipranavir (TPV/r).\(^{13}\) The presence of these minor mutations needs to be taken into consideration because LVP/r, which is recommended by the Indonesian Ministry of Health as a second-line regimen of ART,\(^{11}\) is potentially affected.

In contrast, the prevalence of TDR against RT inhibitors was 16.7% (5/30), which is higher than that reported in previous studies in Surabaya (4.3%; \(^{2,3}\)) and West Papua (12.9%; \(^{4,5}\)). \(^{10}\) Identified TDR, including E138G/A, K103N, G190A, and K219Q, may affect nNRTIs, such as rilpivirine (RPV), efavirenz (EFV), nevirapine (NPV), and etravirine (ETV), and NRTIs, including zidovudine (AZT) and stavudine (d4T). K219Q, which is also known as a thymidine analogue-associated mutation (TAM), is associated with multi-NRTI resistance, excluding emtricitabine (FTC) and lamivudine (3TC). AZT, EFV, and NPV are included as recommended options for individuals starting first-line ART in Indonesia;\(^{11}\) thus, the presence of TDR affecting these inhibitors need to be considered. Minor mutations, including V90I, V106I, and V179D/F, were also detected. These minor mutations may affect ETV.\(^{13}\)

Based on clinical data, 38.5% ART-naive individuals were co-infected with TB. TB is regarded as the leading opportunistic disease and cause of death in individuals with HIV infection.\(^{20,21}\) Regarding the nutritional status, 28.2% individuals were underweight. Low BMI is correlated with mortality in HIV-infected individuals,\(^{21–23}\) as higher BMI and fat mass among ART-naive individuals were reported to be associated with slower disease progression.\(^{24}\) A previous study reported that women who were underweight prior to ART died from AIDS more than twice as rapidly than normal weight women.\(^{25}\) As for the clinical stage of HIV infection, 46.2 and 30.8% individuals were classified as stages 3 and stage 4, respectively. Higher clinical stages were correlated with higher mortality in HIV-infected individuals.\(^{21,22}\)

The results of the present study indicate the emergence of TDR among ART-naive individuals in Buleleng, Bali. In the WHO TDR surveillance guidelines, the prevalence of TDR is categorized into three groups: low level (<5%), moderate level (5–15%), and high level (>15%).\(^{26}\) According to this guideline, the prevalence of TDR in Buleleng is considered to be high level, indicating inadequate first-line regimens. However, the present results may have overestimated the prevalence of TDR because there were several limitations in the design of the study. The WHO guidelines aim to recruit recently infected individuals (younger than 25 years of age and CD4+ T-cell counts higher than 500 cells/mm\(^3\);\(^{27}\) however, these criteria were not

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Subtype</th>
<th>Drug Resistance Mutations*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nNRTI</td>
<td>NRTI</td>
</tr>
<tr>
<td>BL202</td>
<td>38</td>
<td>Male</td>
<td>CRF01_AE</td>
<td>K103N Efavirenz, nevirapine</td>
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<tr>
<td>BL222</td>
<td>29</td>
<td>Female</td>
<td>CRF01_AE</td>
<td>E138A Etravirine, ritavirine</td>
</tr>
<tr>
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<td>29</td>
<td>Female</td>
<td>CRF01_AE</td>
<td>G190A Etravirine, nevirapine</td>
</tr>
<tr>
<td>BL229</td>
<td>47</td>
<td>Male</td>
<td>CRF01_AE</td>
<td>K209Q Multi-NRTI, stavudine, zidovudine</td>
</tr>
<tr>
<td>BL240</td>
<td>23</td>
<td>Female</td>
<td>CRF01_AE</td>
<td>E138G Etravirine, ritavirine</td>
</tr>
</tbody>
</table>

*a Drug resistance mutations were based on guidelines published by the International Antiviral Society-USA (IAS-USA).

*b The subtype of the RT gene was assigned based on RIP and phylogenetic analyses.

RT, reverse transcriptase; nNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; RIP, recombinant identification program.
applied in the present study for practical reasons. The number of samples collected was also limited. During sample collection in February 2018, less than 50 individuals were newly diagnosed with HIV infection at the Voluntary Counselling and Testing Clinic of Buleleng Regency General Hospital, Bali. Among those individuals, only 39 individuals were agreed to be enrolled in this study. We believe the 39 samples are a minimum necessary number for this study. Therefore, in order to clarify and monitor TDR among ART-naive individuals, continuous surveillance with a larger sample size and compliance with the WHO selection criteria for TDR surveillance are necessary. Besides TDR, the presence of individuals who were co-infected with TB, underweight, and diagnosed with a higher clinical stage also need proper consideration, as these conditions were correlated with HIV-related mortality.20–25

CONCLUSION

There is the emergence of TDR was found among ART-naive individuals in Buleleng, Bali. This issue warrants serious consideration because TDR may hamper treatment success and reduce ART efficacy among newly diagnosed individuals. Continuous surveillance with a larger sample size is necessary to monitor TDR among ART-naive individuals.

ACKNOWLEDGMENTS

This work was supported by Kementerian Riset, Teknologi, dan Pendidikan Tinggi Republik Indonesia through the Pendidikan Magister menuju Doktor untuk Sarjana Unggul (PMDSU) scholarship. This work was also supported by the Japan Initiative for the Global Research Network on Infectious Diseases (J-GRID) from the Ministry of Education, Culture, Sport, Science and Technology in Japan, and the Japan Agency for Medical Research and Development (AMED); and the Institute of Tropical Disease as the Center of Excellence (COE) program by Kementerian Riset, Teknologi, dan Pendidikan Tinggi Republik Indonesia. We sincerely thank the staff at the Voluntary Counselling and Testing Clinic of Buleleng Regency General Hospital, Bali, for their kind cooperation with this study.

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