

Nucleic acid amplification of HIV-1 integrase sequence subtypes CRF01_AE and B for development of HIV anti-integrase drug resistance genotyping assay

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Abstract. To anticipate the potential use of anti-integrase drugs in Indonesia for treatment of HIV-1 infection, the development of a drug resistance genotyping assay for anti-integrase is crucial in identifying the genetic drug resistance profile of Indonesian HIV-1 strains. This experiment aimed to amplify a target region in the integrase gene of Indonesian HIV-1 subtypes CRF01_AE and B that contain genetic mutations known to confer resistance to anti-integrase drug. Eleven archived plasma samples from individuals living with HIV-1 were obtained from the Virology and Cancer Pathobiology Research Center for Health Service (VCPRC FKUI-RSCM) laboratory. One of the plasma samples contained HIV-1 subtype B, and the remaining plasma samples contained subtype CRF01_AE. The target regions for all samples were amplified through RT-PCR, with an annealing temperature of 55 °C, using the primer pair AE_POL 4086F and AE_POL 5232R that were designed by VCPRC FKUI-RSCM. The results of this experiment show that 18.2% (2/11) of the samples were successfully amplified using the one-step RT-PCR. While the primer pair was effective in amplifying the target region in the integrase gene sequence for subtype B (100%; 1/1), it had a low efficacy (10%, 1/10) for subtype CRF01_AE. In conclusion, the primer pair can be used to amplify the target region in Indonesian HIV-1 strain subtypes CRF01_AE and B. However, optimization of the PCR condition and an increased number of samples would help to determine an accurate representation of the efficacy of the primer pair.

1. Introduction

The 6th Millennium Development Goal of Indonesia, which is specific for HIV/AIDS, aims to halt and reverse the spread of HIV by 2015. However, evidence indicates that the prevalence of HIV in Indonesia has increased from 0.2% in 2009 to 0.3% of the total population in 2011 [1]. The prevalence is predicted to increase to 0.5% by 2016. The Indonesian Ministry of Health reported that the total number of HIV cases in 2013 was 29,037 cases, which shows an increase from the 21,511 HIV cases recorded in 2012. These data indicate that HIV is still a health concern in Indonesia.

The primary treatment of HIV infection is through antiretroviral therapy (ART). According to the Indonesian Ministry of Health, only ten drugs among the four major classes of antiretroviral (ARV) drugs are currently available in Indonesia. There are six nucleoside reverse transcriptase inhibitors (NRTIs), one nucleotide reverse transcriptase inhibitor (NtRTI), two non-NNRTIs, and one protease inhibitor (PI). The ART requires at least three types of drugs, and the standard regimen involves two NRTI and one NNRTI.



ART requires extremely strong compliance by the individual, especially because it is a lifelong therapy. Nevertheless, a certain degree of HIV drug resistance is expected among individuals with ART [2]. Research conducted by Kusumaningrum (2014) highlighted that, all of the samples (10/10) with virological failure following six months of ART developed a resistance to at least one ARV drug. Based on a systematic review by the WHO, 60% of 573 individuals from low- and medium- income countries in Asia and sub-Saharan Africa that failed first line NNRTI-based therapy developed a resistance to any HIV drug class after 12 months [2]. These data suggest the importance of developing additional drugs to combat the emerging drug resistance.

Currently, PI is the only second-line drug available in Indonesia, even though FDA-approved ARV drug classes, such as HIV anti-integrase, could be an option of the regimen [3]. Several clinical trials of raltegravir, an anti-integrase, have shown that it has potent antiviral properties in regimens for both treatment-naïve and treatment-experienced individuals, especially those with multidrug resistance [4-6]. The capability to incorporate these drugs with the current regimen would be beneficial in the long-term management of HIV infection, especially in HIV drug resistant strains.

Drug resistance is detected primarily using two methods, which are based on either genotyping or phenotyping of the HIV genes responsible for drug resistance [7]. In a resource limited setting such as Indonesia, the WHO recommends using genotyping assay to identify mutations in the HIV genome that confer resistance to ARV drugs [8]. Genotyping assay involves sequencing the target region in the HIV genome. To perform sequencing, the targeted gene needs to be amplified so that it can provide a picture of the potential use and effectiveness of anti-integrase for controlling the HIV infection in Indonesia.

2. Materials and Methods

This descriptive experimental study was conducted in the Virology and Cancer Pathobiology Research Center for Health Service Faculty of Medicine, Universitas Indonesia (VCPRC FKUI-RSCM) on June 2015. The samples were archived HIV-infected plasma samples that were stored in the VCPRC FKUI-RSCM. The number of samples needed to obtain initial data was determined to be at least 10, with a resolution of 10%. Each sample selected has a recorded viral load of more than 5,000 copies per ml, the HIV-1 RNA strain was noted to be either CRF01_AE or subtype B, and there was more than 0.5 ml of available volume to be used.

The RNA from the plasma samples were extracted using a QIAamp Viral RNA Mini Kit. The RNA extracted from each sample was amplified at the annealing temperature of 55 °C, using amplification primers AE_POL 4086F and AE_POL 5232R, which were designed by the staff of VCPRC FKUI-RSCM laboratory. One-step and two-step reverse transcription polymerase chain reactions (RT-PCRs) were attempted. In the two-step RT-PCR, amplification primer, BF_EnvR3, was used for the reverse transcription to produce the cDNA templates. Afterwards, the products of amplification were identified using gel electrophoresis. A good primer performance was noted if the amplification product weighed the desired weight without the presence of additional bands. Each sample was amplified again using primer pair HI857F and HI967C—which have been used regularly by the laboratory—and analyzed using gel electrophoresis to verify the presence of HIV RNA within the samples.

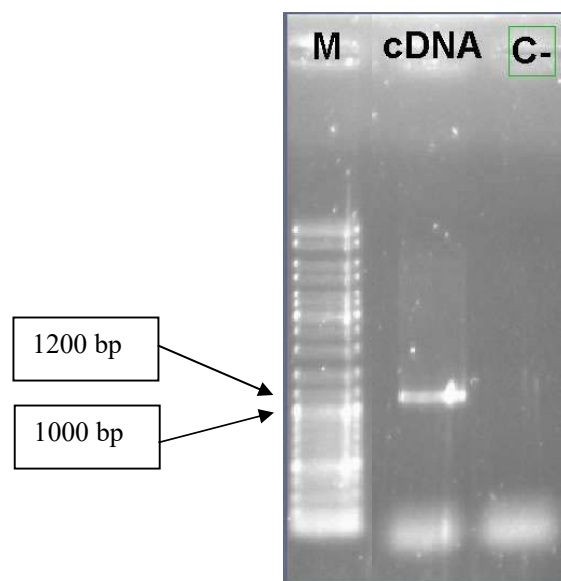
Table 1. List of primers

Primer's name	Sequence (5' → 3')	Tm (°C)
BF_EnvR3	YARRTCTYGAGATRCAGATRCCTGCTCC	61.6
AE_POL 4086F	AGTGAATCAGAGTTAGTCAATCAAATAATAGA	54.5
AE_POL 5232R	CATCCCCTAGTGGGATGTGTACTTCTG	59.9

3. Results and Discussion

3.1 Results

The primer pair AE_POL 4086F and AE_POL 5232R was used with a target product of 1146 base pairs (bp). An initial trial PCR was conducted with HIV cDNA to test the primer pair. The primer pair was noted to amplify the HIV cDNA at 55 °C, as shown by the band appearing between the 1000 and 1200 bp markers in the column labeled “cDNA” in Figure 1.



M: GeneRuler DNA Ladder Mix. cDNA: consists of AE_POL 4086F and AE_POL 5232R primer pair. C-: control negative.

Figure 1. Initial PCR Trial

Eleven archived HIV-infected plasma samples, stored in a -80 °C refrigerator, were selected based on criteria and extracted using the QIAamp Viral RNA Mini Kit (Qiagen). The viral load and characteristic subtype of each sample were assessed beforehand by the VCPRC FKUI-RSCM laboratory. One of the samples contained HIV-1 subtype B, and the remaining 10 samples contained HIV-1 subtype CRF01_AE, as indicated in Table 2. First, a two-step RT-PCR using Thermo Scientific™ RevertAid™ RT Kit for RT and Platinum TaqHiFi (Invitrogen) for PCR was performed for one sample, DRHS48. The primer used for the reverse transcription was BF_EnvR3. However, no band appeared in the first trial. The results of the two-step RT-PCR of DRHS48 for three other instances also failed to show any bands. Thus, an RNA sample that was extracted by the staff of VCPRC FKUI-RSCM laboratory, DRHS62, was amplified using two-step RT-PCR as a control positive for the RNA extraction process. However, the two-step RT-PCR of DRHS62 also failed to show any bands. Figure 2 depict the third trial of two-step RT-PCR of DRHS48. Figure 3 shows the two-step RT-PCR result for DRHS62.



Figure 2. Third trial of two-step RT-PCR of DRHS48.

F-: control negative. F2: A new DRHS48 cDNA template. F+: control positive using HIV-1 vector pNL 4-3. M: GeneRuler DNA Ladder Mix

The RT-PCR in fourth trial of two-step RT-PCR of DRHS48 was performed with a control positive (C+) provided by the RT-PCR kit to evaluate the condition of the reagents. The control positive yielded the desired PCR product, which was 496 bp.

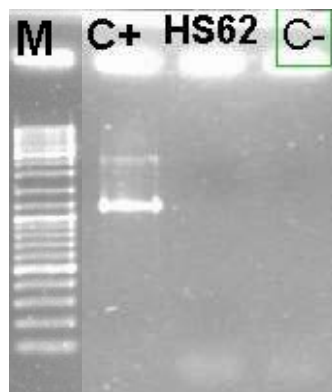


Figure 3. Two-step RT-PCR of DRHS62.

M: GeneRuler DNA Ladder Mix. C+: control positive using pNL 4-3. HS62: DRHS62 sample. C-: control negative.

Based on the results of the two-step RT-PCR for DRHS48 and DRHS62, the RT-PCR protocol was changed to a one-step RT-PCR using SuperScript® III One-Step RT-PCR System with the Platinum® Taq DNA Polymerase kit. One-step RT-PCR was performed on all eleven samples. The desired band appeared between the 1,000 bp and 1,200 bp mark of the GeneRuler DNA Ladder Mix for both DRHS07 and DRSS66, as shown in Figure 6. However, Figures 4, 5, and 6 show that no band of the desired RT-PCR product weight appear for the following nine samples: DRHS09, DRHS32, DRHS48, DRSS20, DRSS22, DRSS24, DRSS45, DRSS57, and DRSS64.

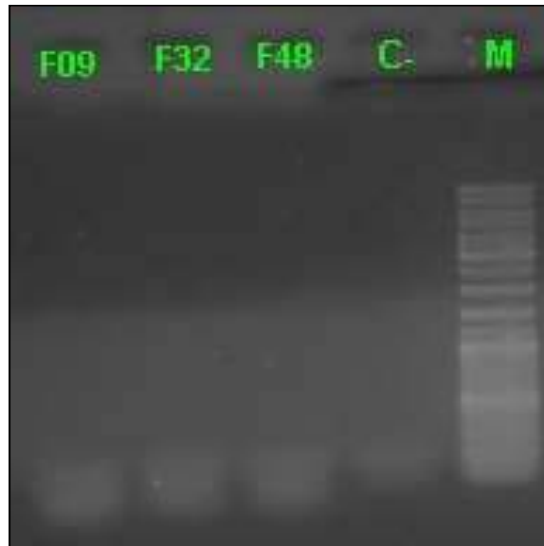


Figure 4. One-step RT-PCR of DRHS09, DRHS32, and DRHS48.

F09: DRHS09 sample. F32: DRHS32 sample. F48: DRHS48 sample. C-: control negative. GR: GeneRuler DNA Ladder Mix.

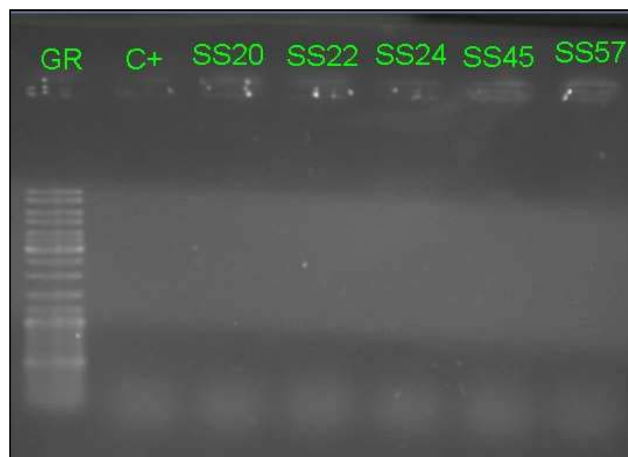


Figure 5. One-step RT-PCR of DRSS20, DRSS22, DRSS24, DRSS45, and DRSS57.

GR: GeneRuler DNA Ladder Mix. C+: control positive. SS20: DRSS20 sample. SS22: DRSS22 sample. SS24: DRSS24 sample. SS45: DRSS45 sample. SS57: DRSS57 sample.



Figure 6. One-step RT-PCR for DRHS07, DRSS64, and DRSS66

HS07: DRHS07 sample. SS64: DRSS64 sample. SS66: DRSS66 sample. C+: control positive. GR: GeneRuler DNA Ladder Mix. C-: control negative

The samples were tested for the presence of HIV-1 RNA using a primer pair with a known performance that was regularly used by VCPRC FKUI-RSCM laboratory. The RNA samples were amplified in a one-step RT-PCR with the primer pair HI857F and HI967C. The presence of the desired product of a 110 bp was found on nine samples, as shown in Figures 7 and 8. No RT-PCR product was detected when the DRHS32 sample was used as the template. The RT-PCR for DRHS48 was not performed because there was no volume of DRHS48 sample left for use in the reaction.



Figure 7. One-step RT-PCR with HI857F and HI967C part 1.

GR: GeneRuler DNA Ladder Mix. C+: control positive. HS09: DRHS09 sample. HS32: DRHS32 sample. SS20: DRSS20 sample. SS22: DRSS22 sample. SS24: DRSS24 sample. C-: control negative.

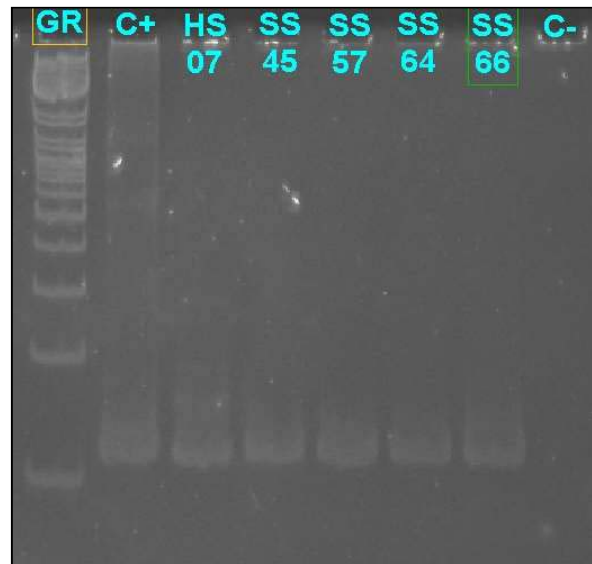


Figure 8. One-step RT-PCR with HI857F and HI967C part 2.

GR: GeneRuler DNA Ladder Mix. C+: control positive. HS07: DRHS07 sample. SS45: DRSS45 sample. SS57: DRSS57 sample. SS64: DRSS64 sample. SS66: DRSS66 sample. C-: control negative.

Table 2. Summary of Samples and RT-PCR Results

Label/ Name	Viral Load (copies/ml)	Subtype	Observed band using primer pair	
			AE_POL 4086F & AE_POL 5232R	HI857F & HI967C
DRHS09	17,296	CRF01_AE	None	Expected
DRHS32	515,966	CRF01_AE	None	-
DRHS48	1,369,136	CRF01_AE	None	Not determined
DRSS20	560,643	CRF01_AE	None	Expected
DRSS22	477,907	CRF01_AE	None	Expected
DRSS24	1,628,913	CRF01_AE	None	Expected
DRSS45	7,813,116	CRF01_AE	None	Expected
DRSS57	1,891,104	CRF01_AE	None	Expected
DRSS64	508,164	CRF01_AE	None	Expected
DRSS66	133,526	CRF01_AE	Expected	Expected
DRHS07	23,999	B	Expected	Expected

3.2 Discussion

The nucleic acid amplification of the target region in the integrase gene of Indonesian HIV-1 subtypes CRF01_AE and B was initially planned to be performed using a two-step RT-PCR. The two-step RT-PCR was preferred because of its advantages over a one-step RT-PCR. While a one-step RT-PCR allows the samples to be stored as cDNA, which is more stable than an RNA, the two-step allows multiple trials to be performed without additional handling of the RNA sample. The two-step RT-PCR also saves the RT reagents for use in future trials [9]. The two-step RT-PCR method was first tested on sample DRHS48, but no band of the desired product was seen on agarose gel electrophoresis, as depicted in

Figure 2. There are several possible reasons for the failure to produce the desired band including low primer quality, low quality of RT-PCR reagents, disrupted integrity and quality of the RNA template, absence of RNA from extraction, absence of cDNA for PCR, potential contamination, and technical errors in the handling process.

To reassess the quality of the primer pair, a different aliquot of the same primer pair was made and used in the PCR reaction. A control positive containing HIV-1 vector pNL 4-3 was also added to verify the capability of the primer pair with the PCR reagents and condition. Nevertheless, the constant appearance of the desired band when pNL 4-3 was used as a template indicates that the primer quality is good (Figure 3). The quality of RT reagents used was verified by including a control positive, which was packed with the kit, to the two-step RT-PCR reaction. The appearance of a band weighing about 496 bp in Figure 5 (labeled C+) indicates that the reagents for RT were in a good condition. Two other separate instances of RT-PCR for DRHS48 were conducted; however, the results showed no presence of the desired PCR product (Figures 2 and 5). The desired band was also not visible when using sample DRHS62, which was extracted by the staff of VCPRC FKUI-RSCM laboratory (Figure 3). This result suggests that inadequate cDNA is synthesized in the reverse transcription reaction, which might be due to the degradation of RNA prior to the RT-PCR. However, it is also possible that the primer is incompatible with the samples (DRHS48 and DRHS62) used.

As a two-step RT-PCR incorporates more procedures in its workflow than a one-step RT-PCR, it is more susceptible to technical errors and other sources that may influence its result [10]. Hence, it is possible that the degradation of the RNA is due to technical errors from the handling process in a two-step RT-PCR. In general, the steps that are most vulnerable to errors in an RT-PCR are the procedures prior to the reaction itself. The storage of the RNA, the process of extracting the RNA, and the quality checks are important in ensuring the quality and integrity of the viral RNA [10]. Contamination of the samples with RNases is also a major cause of disrupting the experiment. Contamination can occur easily because RNases can be found in the hair and skin. During the handling of the specimen, the principal investigator wore gloves and conducted the procedures in a biosafety cabinet to reduce the chances of RNase contamination. The contamination of other samples or PCR products was minimized by performing the steps in pre-PCR and post-PCR at different locations [11]. While the principal investigator adhered carefully to the standard operating procedure provided by VCPRC FKUI-RSCM laboratory, inadequate handling of the samples and technique of the researcher cannot be thoroughly ruled out as a source of errors. Thus, technical errors and possible contamination might be the cause of the RNA degradation. However, the investigator opted to perform a one-step RT-PCR because it is more efficient and less vulnerable to sources of error [9].

One-step RT-PCRs are more commonly used at VCPRC FKUI-RSCM laboratory as part of its health services for HIV infection. A one-step RT-PCR was performed for three samples (DRHS09, DRHS32 and DRHS48); however, no desired bands appeared on the gel electrophoresis (Figure 4). The second batch of one-step RT-PCR consisted of five samples (DRSS20, DRSS22, DRSS24, DRSS45, and DRSS57). A control positive used was based on a sample with known existing RNA since the initial trial run did not result in the appearance of a band. Nevertheless, there was no visible band on the agarose gel for all samples (Figure 5). The final batch of samples was DRHS07, DRSS64, and DRSS66. While a band was found between the 1000 bp and 1200 bp marks for DRHS07 and DRSS66, which corresponds highly to the desired band, none were spotted for DRSS64. A slightly visible band observed in the control positive was most likely caused by some of the PCR products being carried over from DRSS66 by the direction of the pipetting. There was no other contamination, as no bands appeared in the control negative. Overall, the samples that did not produce a band indicate an incompatibility between the primer pair and the samples.

All of the samples are amplified in a one-step RT-PCR using a primer pair that has been routinely used by VCPRC FKUI-RSCM laboratory. The primer pair HI857F and HI967C was designed to produce a 110 bp band. This amplification was conducted to validate the technical aspect of the experiment and to account for a limited degree of RNA viability in the sample. More specific and sensitive methods could be used to check for the quality, purity, and integrity of the viral RNA, such as lab-on-chip

automated capillary electrophoresis; however, these methods are beyond the scope of this research [10]. Figures 7 and 8 showed the result of all samples on the polyacrylamide gel electrophoresis. Aside from DRHS48, which has no available volume left, the 110 bp band was spotted in 90% (9/10) of the samples. The lack of the 110 bp band for DRHS32 might be due to the absence of RNA, which supports the absence of a band when using the primer pair AE_POL 4086F and AE_POL 5232R.

The primer pair AE_POL 4086RF and AE_POL 5232R is capable of amplifying the target region in 18.2% (2/11) of the samples. It can amplify the target region HIV-1 subtype B (100%; 1/1) well but only 10% (1/10) of CRF01_AE is amplified. The success of amplifying the target region has little correlation with the individual viral load of the samples [11]. While DRHS07 and DRSS66 have a relative low viral load compared to the other samples, both manage to produce the desired band. These data demonstrate the compatibility of the primer pair with the samples of Indonesian HIV-1 strains, although the quality and integrity of the RNA in the samples are still in doubt. This research study has some limitations; thus, the principal investigator could not optimize the PCR condition. A limit on time, resources, and budget meant that the samples could only be amplified once, and there were no accurate past records of the use or handling of the samples used in this experiment. Therefore, the small sample size cannot be generalized to represent the population, and the reproducibility is not certain.

4. Conclusion

The primer pair AE_POL 4086F and AE_POL 5232R that was designed by VCPRC FKUI-RSCM can be used to amplify the target region in the integrase gene of Indonesian HIV-1 subtypes CRF01_AE and B strains. The performance of the primer pair for the amplification of HIV-1 subtype B is effective (100%; 1/1); however, its efficacy is low (10%; 1/10) for HIV-1 subtype CRF01_AE. Nevertheless, the PCR condition should be optimized and the experiment should be tested with a larger sample size to determine an accurate representation of the efficacy of the primer pair.

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