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IDENTIFICATION OF ANTIRETROVIRAL MUTATION IN PROTEASE AND REVERSE TRANCRIPTASE INHIBITOR IN HUMAN IMMUNODEFICIENCY VIRUS-1 OF HIV/AIDS PATIENTS IN MIMIKA REGENCY, PAPUA

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ABSTRAK

Terapi dengan pemberian inhibitor RT telah menjadi program lini pertama di Mimika. Penggunaan ARV yang teratur dapat menurunkan jumlah virus. Namun setelah itu HIV dapat menjadi resisten terhadap ARV yang diberikan. Resistensi virus terhadap obat bisa terjadi karena mutasi. Jika mutasi terkait resistensi terjadi pada DNA virus, maka ARV yang diberikan tidak lagi efektif. Penelitian ini bertujuan mengidentifikasi mutasi terkait resistensi pada fragmen DNA pengkode protease dan reverse transcriptase. Penelitian ini dilakukan menggunakan rancangan potong lintang pada 84 subyek yang telah menerima antiretroviral > 6 bulan. Penelitian dilakukan di RS. Mitra Masyarakat Mimika. Proses laboratorium meliputi ekstraksi, RT-PCR, elektroforesis dan sekuensing. Analisis data menggunakan interpretasi algoritma resistensi pada Database HIV. Hasil penelitian mendapatkan 1 subyek yang belum menerima terapi protease diidentifikasi mengalami mutasi minor L10V, 1 subyek yang menerima terapi penghambat nukleotida RT mengalami mutasi dengan motif M184V, 1 subyek dengan motif M41L, dan 1 subyek yang menerima terapi Penghambat Non Nukleotida RT teridentifikasi mengalami mutasi motif Y181C dan V108I. Simpulan, mutan HIV-1 terkait resistensi ARV telah teridentifikasi pada 2 subyek yang telah menerima terapi antiretroviral di RSMM Mimika. ***(FMI 2017;53:56-63)***

Kata kunci: *mutasi, resistensi, HIV-1, Mimika, protease, Rtase*

ABSTRACT

Treatment with RT Inhibitors has been used as first line program in Mimika. Regular use of antiretroviral drugs can lower the amount of the virus, but after that HIV can become resistant to the drugs given. Viral resistance to the drugs can occur because of a mutation. If the resistance-associated mutations occur in the DNA of the virus, then the ARV provided will no longer be effective. The aim of this study was to identify the presence of resistance-associated mutations in DNA fragment that encodes the protease and reverse transcriptase. This study used cross sectional design with 84 subjects who had received antiretroviral for > 6 months. The study was conducted in Mitra Masyarakat Mimika Hospital. Laboratory process included extraction, RT-PCR, electrophoresis and sequencing. Data analysis used resistance interpretation algorithms in HIV Database. Results showed that 1 subject who did not receive protease therapy was identified as having minor mutation L10V, 1 subjects receiving NRTI inhibitors had mutation M184V motive and 1 subjects with M41L motive and 1 subjects who received NNRTI inhibitor therapy identified as having mutated Y181C and V108I motive. In conclusion, mutant HIV-1 related to ARV resistance has been identified in two subjects who had received antiretroviral therapy in Mitra Masyarakat Mimika Hospital. ***(FMI 2017;53:56-63)***

Keywords: *mutation, HIV-1, Mimika, protease, Rtase*

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INTRODUCTION

The number of HIV/AIDS cases has increased. At the end of September 2014 there were 150,285 HIV cases and 55,779 AIDS spread in Indonesia. Papua ranks fifth with 16,051 HIV cases and 10,184 AIDS cases (Directorate General of Communicable Disease Eradication and Environmental Health 2014). ARV treatment in Indonesia began in 2005. The Ministry of Health report shows that in 2005 the number of people living with HIV who received ARVs was 3.904 (82.4%) of the 4,735 people eligible for ART. In 2010, the number of PLWHA eligible for ART was 11,657 people, but those receiving therapy only amounted to 7,755 people and until the end of 2010 that still received ART amounted to 3.509 people (45.2%) of those who had received therapy (Directorate of Disease Control and Environmental Health 2011).

Mimika District, Papua Province, is the district that reports the most HIV/AIDS cases in Papua. Accumulatively, the discovery of HIV cases in Mimika district until the end of 2011 was 2,832 cases with the most contagious pattern of 98% is through sexual intercourse

on heterosexual groups. In 2011 the number of HIV patients eligible for antiretroviral treatment amounted to 282 people, of whom received ARV treatment totaling 127 people (45.4%) (Provincial Health Office of Papua 2011).

The goal of antiretroviral therapy (ART) is to reduce the rate of HIV transmission in the community, reduce morbidity and mortality associated with HIV, improve the quality of life of people living with HIV, restore and maintain immune function, suppress viral replication maximally and continuously (Directorate of Disease Control and Restructuring Environment 2004). Effective antiretroviral treatment is complex, and, if not properly implemented, can adversely affect HIV/AIDS prevention, triggering drug resistance (Directorate of Disease Control and Environmental Health 2006). Resistance to HIV can occur due to several factors. The first factor is the speed of virus replication is very high that is 1010 virus per day. The high rate of replication can lead to gene arrangement and new HIV subtypes that may be resistant to certain antiretrovirals (Santoro & Perno 2013).

The second factor is the error of nucleotide readings by the reverse transcriptase enzyme in the active site polymerase when the conversion of RNA genetic material into cDNA and DNA causes mutation. If the mutation occurs in the part of the gene that produces a functional protein (enzyme) that is used as one of the antiretroviral targets, the antiretroviral can not work on the enzyme because the structure of the enzyme has changed. The occurrence of resistance can be seen from the use of antiretroviral drugs with CD4 cell count and viral load in HIV/AIDS patients. Prolonged use of antiretroviral drugs but a decrease in CD4 cell count or an increase in viral load may indicate resistance (Markowitz et al 2003).

To see the existence of resistance can be done in 2 ways, namely phenotypic assay and genotypic assay. Phenotipic assay aims to determine the ability of antiretrovirals in inhibiting the process of viral replication. Genotypic assay is more advantageous because the process is relatively faster and specific because of which genes that mutate can be known so that the cause of resistance can be recognized (Daniel & Kuritzkes 2007). Several previous studies in some countries in subtype B patients with resistance to the type of antiretroviral drugs in the nucleotide reverse transcriptase inhibitor class resulted in mutations in the reverse transcriptase enzyme thus reducing antiretroviral effectiveness. On administration of d4T (stavudine) and TDF (Tenofovir) have found 4 mutations in the amino acid sequence 65 from lysin to arginine (K65R). Likewise in ddI (didadosine), 3TC (lamivudine), and zidovudine (AZT) have also been found in mutations with 2 to 6 mutation sites in the reverse transcriptase enzyme of each drug (Victoria et al 2008).

In addition, patients with subtype B were also found to have resistance to antiretroviral class of Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs). Mutations are found in the reverse transcriptase enzyme so that the effectiveness of the drug becomes reduced. For example, on the use of EFV (Efavirens) mutations occur in 103 amino acids from lysine to asparagin (K103N) and mutations in 106 amino acids from valine to methionine (V106M) (Victoria et al 2008). In addition the CRF01_AE subtype was also found to be resistant. Mutations that occur in proteases will cause drug resistance that has a working mechanism as a protease inhibitor inhibitor or called protease inhibitor (PI). Mutations in this enzyme are found in the use of TRV (Tipranavir), SQV (Saquinavir), NFV (Nelfinavir), LPV (Lopinavir), IDV (Indinavir), FPV (Fosamprenavir), DRV (Darunavir), and ATV (Atazanavir). The administration of these drugs causes mutations in the amino acid averaging 10-24 mutation sites (Victoria 2006).

Therapy with the provision of RT inhibitors has become a first-line program in Mimika. The inhibitors are nucleotide analogues, namely lamivudine, tenofovir, zidovudine, stavudine and non-nucleotide analogue inhibitors efavirens and nevirapin. Regular use of antiretroviral drugs can reduce the number of viruses, but after that HIV can become resistant to given antiretroviral drugs. Viral resistance to drugs can occur due to mutations. If resistance-related mutations occur in viral DNA, then the given antiretroviral drugs are no longer effective. It is therefore necessary to investigate the occurrence of mutations in HIV reverse transcriptase DNA fragments associated with antiretroviral resistance in HIV patients.

HIV resistance to antivirals can also occur naturally without antiviral administration. In addition, cross-resistance has also been identified in HIV. Cross-resistance is a condition of HIV resistant to other similar drugs even though the drug has never been given to the patient. Giving protease inhibitors has not been done as an alternative in Mimika, but did not rule out mutations that caused resistance to the drug. In Mimika there has been no identification to determine whether there has been a mutation associated with viral resistance to reverse transcriptase and protease antiviral inhibitors. The purpose of this study was to identify mutations related to antiretroviral resistance in Mimika. With the data acquisition, future strategies can be designed more precisely. Identification of mutations may help clinicians in efforts to improve treatment strategies for HIV infection.

MATERIALS AND METHODS

This research is an analytic descriptive research with cross sectional design on HIV/AIDS patient in VCT RS Mitra Masyarakat Mimika. The study was conducted from January to October 2015 at the Virology Laboratory, Indonesian Biomedical Research and Development Center, Ministry of Health

The sample selection is done consequtive sampling of all HIV/AIDS patients who have been getting antiretroviral therapy and met the inclusion criteria. Inclusion criteria include: a data subject has the CD4 and a complete history of treatment, has been undergo-ing therapy > 6 months and sign a consent form (inform-ed consent). The sample size was calculated based on the formula Lameshow (Lameshow et al 1997), with a confidence level of 95%, p = 0.98% and d = 0:03 in order to obtain a sample size of 84 people.

The RNA extraction process uses a manual kit of Qiagen viral RNA minikit with catalog # 52906. The extraction method uses ultrasensitive (Mulder et al 1997), resulting in a high concentration of viral RNA virus. After the extraction process is continued with RT-PCR (Reverse transcriptase-Polymerase chain reaction) amplification process using a specific primer. Primer used were obtained from reference journals HXB2 GenBank access code K03455 composition: Protease: DRPR05: 5'AGACAGGTAATTTTTAGGGA3 ', DRP R02L: 5'TATGGATTTTCAGGCCCAATTTTTG A3', nested PCR: DRPR01M: 5'AGAGCCAACAGCCCC ACCAG3 ', DRPR06: ACTTTTGGGCCATCCATTCC 3', RT-ase: PRA: 5'CCTAGGAAAAAGGGCTGTTGG AAATGTGG3 ', RTA: 5'AACTTCTGTATGTCATTG ACAGTCCA, nested PCR: PRB: 5'ACTGAGAGACA GGCTAATTTTTTAGGGA3', IBR2: 5'CAAAGGAAT GGAGGTTCTTTCTGATG3 '. (Beck et al 2002, Sambrook & Russel 2001).

The next stage after amplification is electrophoresis. This stage aims to see the results of amplification in the previous stage. PCR results were detected by electrophoresis. 5 µl of PCR product plus 1 µl loading buffer on agarose gel 0.8% and 80 V voltage for 40 min. The PCR product is visualized by placing gel on gel-doc. The PCR product appears as a single band with a large base pair as targeted by matching it to the marker. Positive results at this stage are followed by a sequencing process (Lodish et al 2003).

Sequencing results were analyzed using bioedit software. The steps taken are trimming the unreadable sequence results and combining the results of the primary sequences F and R to obtain confirmation. The next step is the BLAST (Basic Local Alignment Search Tool) process at www.ncbi.nlm.nih.gov and the HIV sequence database at www.hiv.lanl.gov.

Demographic characteristics, history of disease and medical history are expressed in percentages. Analysis of the results is descriptive. The sequenced sequence of HIV nucleotides was then compared with HIV virus isolates from the United States, Canada, Thailand, China, Vietnam and Singapore accessed through the GenBank website. Mutations of confirmed rtHIV and protease DNA fragments were identified using the Stanford HIVdb mutation interpretation application on the http://hivdb.stanford.edu website. Each sample identified mutant-related resistance is referred back to the reference sequence to verify the occurrence of the mutation. Ethical Approval is obtained from the Health Research Ethics Commission of the Health Research and Development Agency of the Ministry of Health Jakarta.

RESULTS

Characteristics of HIV/AIDS patient subjects at Mitra Community Hospital include sex, age, pattern of transmission while history history of the disease including CD4 results, opportunistic infections can be seen in Table 1.

Table 1. Characteristics of the subject and history of HIV/AIDS patient illness in RSMM Mimika

No	Variables	Number (n)	Percentage (%)
1	Sex		
	Males	23	27.4
	Females	61	72.6
2	Age		
	0 – 14 years	4	4.8
	≥ 15 years	80	95.2
3	Ethnicity		
	Papua	60	71.4
	Others	24	28.6
4	$CD4^+$ count		
	< 200 cells/mm^3	21	25
	200 - 350 cells / mm^3	28	33.3
	>350 cells /mm^3	35	41.7
5	Opportunisctic Infection		
	TBC	69	82.1
	Others	1	1.2
	No OI	14	16.7
6	Risk Factors		
	Heterosexual	80	95.2
	Others	4	4.8

Table 1 shows that HIV positive respondents in Mimika District, more women (72.6%) and age ≥ 15 years (95.2%). Most patients had CD4 counts> 350 cells/mm 3 of 35 people (41.7%) with TB opportunistic infections of 69 (82.1%) with heterosexual risk factors of 80

(95.2%). In addition to demographic and disease history data, length of therapy data and type of therapy can be seen in table 2.

Of the 84 HIV patients, all (100%) had received anti-retroviral therapy. Patients who have received the most therapy in the time span> 48 months ie 33 people (39.3%). Provision of therapy is entirely first-line therapy with the most antiretroviral combination is Lamivudin + Zidovudin + Nevirapin.

The reverse transcription procedure and the amplification of protease DNA fragments using a one-step Qiagen RT-PCR kit for reverse transcription, and Qiagen hifidelity kit for amplification of HIV protease DNA fragments were performed on 30 samples. The entire sample is purified from the gel to be sequenced. A total of 30 samples were sequenced to confirm mutations and as many as 7 samples could be analyzed and 1 sample confirmed to have minor mutations as shown in Table 3.

Table 2. Duration and type of HIV patient therapy in RSMM Mimika

No	Variables	Number (n)	Percentage (%)
1	Length of therapy		
	6 – 12 months	20	23.8
	13 – 24 months	8	9.5
	25 – 36 months	19	22.6
	37 – 48 months	4	4.8
	>48 months	33	39.3
2	Types of therapy		
	3TC + ZDV + NVP	36	40
	3TC + ZDV + EFV	22	31.2
	3TC + TDF + EFV	12	13.4
	3TC + d4T + EFV	3	3.3
	3TC + d4T + NVP	1	1.1
	D4T + ZDV + 3TC	2	2.2
	D4T + 3TC + NVP	1	1.1
	TDF + 3TC + NVP	5	5.5
	Duvi + EFV	2	2.2

Table 3. Motif of protease mutation in research subjects at RS Mitra Masyarakat Mimika

No	Sample code	Mutation Motives	Mutation status against ARV	
1	RSMM_70	Major Mutation = None Minor Mutation = L10V Other Mutations = I15V, M36I, R41K, L63V, H69K, K70R, L89M, I93L	atazanavir/r (ATV/r) darunavir/r (DRV/r) fosamprenavir/r(FPV/r) indinavir/r (IDV/r) lopinavir/r (LPV/r) nelfinavir (NFV) saquinavir/r (SQV/r) tipranavir/r (TPV/r)	*Susceptible* *Susceptible* *Susceptible* *Susceptible* *Susceptible* *Susceptible* *Susceptible* *Susceptible*

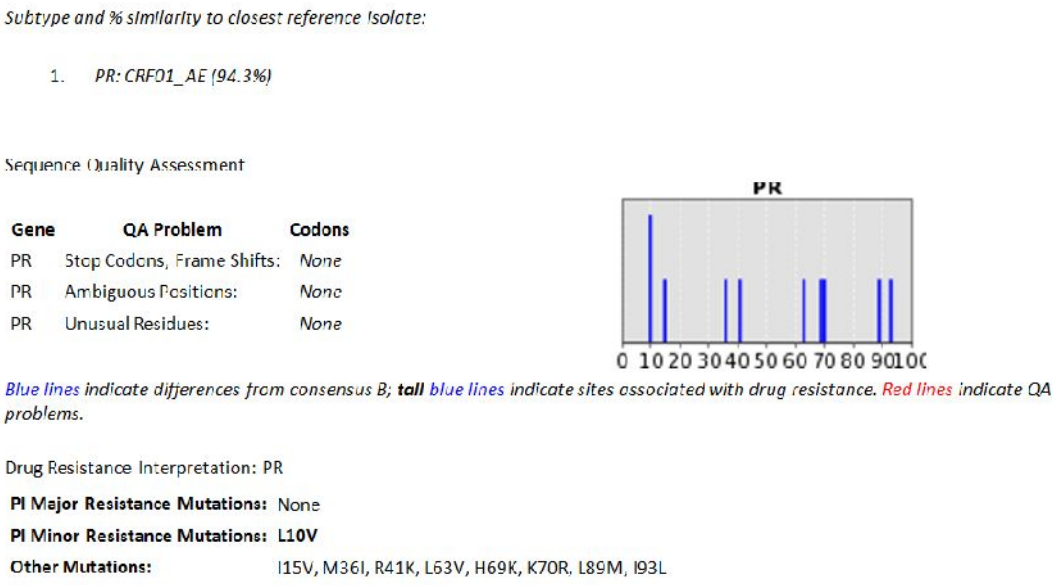

Fig. 1. Interpretation of Protease Genotypic Resistance Algorithms based on Stanford HIV Database

Motif mutation DNA fragment Rtase coding that appears in the study subjects

Mutations appearing in 1 subject with RSMM_58 code on NRTIs are mutations that cause methionine changes in the amino acid 184 to valine which form the M184V motif. M184V mutants cause high levels of resistance to lamivudine and abacavir, and low-level resistance to tenofovir.

Y115F motion mutations appear in 1 sample of subjects simultaneously with the M184V mutant. This combination of mutations results in high resistance to lamivudine, but can still undergo therapy with zidovudine and/or stavudine. Mutations in the NNRTI drug type also occur forming motifs V108I, Y181C, H221Y. This combination results in high resistance to Nevirapin.

Mutations also appear in 1 subjects receiving combination antiviral combination of Lamivudin + Tenofovir + Efavirens in which one carries HIV-1 low-level resistance to several types of NRTIs, lamivudine and tenofovir but still susceptible to NNRTI arv. Combination of antiretroviral drugs containing Efavirens and Nevirapin may be considered for antiretroviral therapy in these subjects. The mutation motif and the status of mutation to antiretroviral can be seen in table 4.

Table 4. Motif Mutation Rtase Appeared in Research Subjects in Mimika

No	Sample code	Mutation Motives	Mutation status against ARV	
1	RSMM_28	NRTI : M41L, D67N, V75M NNRTI : None	Nucleoside RTI	
			lamivudine(3TC)	*Low Level Resistance*
			abacavir (ABC)	*Low Level Resistance*
			zidovudine(AZT)	*Intermediary Resistance*
			stavudine (D4T)	*High Level Resistance*
			didanosine (DDI)	*Intermediary Resistance*
			emtricitabine(FTC)	*Low Level Resistance*
			tenofovir (TDF)	*Low Level Resistance*
			Non-Nucleoside RTI	
			efavirenz (EFV)	*Susceptible*
			etravirine (ETR)	*Susceptible*
			nevirapine(NVP)	*Susceptible*
			rilpivirine (RPV)	*Susceptible*
2	RSMM_58	NRTI : L74V, Y115F, M184V, T215N NNRTI:V108I,Y181C, H221Y	Nucleoside RTI	
			lamivudine(3TC)	*High Level Resistance*
			abacavir (ABC)	*High Level Resistance*
			zidovudine(AZT)	*Low Level Resistance Potential*
			stavudine (D4T)	*Low Level Resistance Potential*
			didanosine (DDI)	*Low Level Resistance*
			emtricitabine(FTC)	*High Level Resistance*
			tenofovir (TDF)	*Low Level Resistance*
			Non-Nucleoside RTI	
			efavirenz (EFV)	*Intermediary Resistance*
			etravirine (ETR)	*Intermediary Resistance*
			nevirapine (NVP)	*High Level Resistance*
			rilpivirine (RPV)	*Intermediary Resistance*

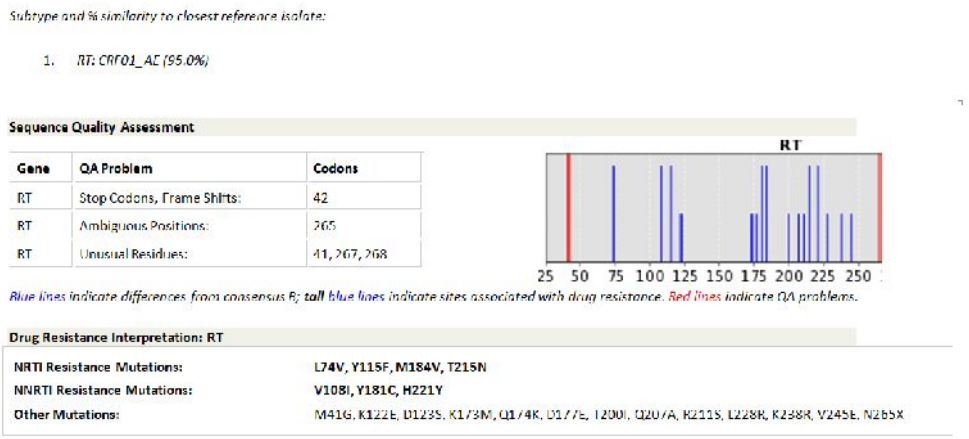

Fig. 2. Interpretation of RTotes Genotipic Resistance Algorithms based on Stanford HIV DataBase

DISCUSSION

This study aims to determine whether or not mutations occur in the sample of the subjects identified. Antiretroviral was given to HIV patients in hospitals. Community Partners Mimika is a class of NRTI (Nucleoside reverse transcriptase inhibitor) and NNRTI (Non-Nucleoside Reverse Transcriptase Inhibitor) inhibitors. The RT inhibitor group is the ideal target of the drug. When the virus does not complete the transcription stage behind the RT enzyme, the preintegration process is not formed so that the virus cannot replicate (Arts & Hasuda 2012).

In this study, mutation detection was performed on 50 samples with reverse transcriptase gene target and 30 samples with target of protease gene. The results of identification showed 2 mutated HIV-related and resistance-related subjects. ARVs with mutations appearing in two sample subjects of M184V and M41L. M184V motion mutations were identified in 1 subject receiving lamivudine + tenofovir + Efavirens therapy with 27 months of therapy duration. M184V is associated with several changes in RT enzyme function in vitro that may affect replication capacity (Petrella et al 2004). M184V mutations reduce viral susceptibility to abacavir two to threefold, and more to lamivudine in invitro (Tisdale et al 1993). M184V motion mutations were also found in HIV patients receiving lamivudine + Zidovudine + Efavirens and lamivudine + Zidovudin + Nevirapin therapy in Surabaya. Likewise, M41 and D67N mutation motifs were also found in HIV patients in Timika who received lamivudine + Zidovudine + Tenofovir (Khairunnisa et al 2014).

Another motive mutation in 1 patient in Timika identified was Y115F. In HIV-1, Y115F is associated with low-level viral resistance to nucleotide analogues. The change from Y to F at position 115 causes the loss of the hydroxyl group. The 115 position on the RT is part of the dNTP binding enclosure. In addition to increasing resistance to abacavir and tenofovir, mutations in this position lead to an increase in RT accuracy (Boyer et al. 2013).

Other mutations also appear in 1 patient receiving combination antiviral lamivudine + Zidovudin + Nevirapin, a mutation with an M41L motif. In addition to M41L, this patient is also identified as having mutations with D67N and V74M motifs. Similar mutations were found in patients in Surabaya, the M41L and D67N motifs in patients receiving lamivudine + Zidovudine + nevirapine therapy. Mutation of these motifs may lead to resistance to the use of stavudine, zidovudine, abacavir and lamivudine (Khairunnisa et al 2014, Johnson et al 2013).

In this study also carried out the detection of mutations in DNA encoding Protease. Patients at RS Mitra Masyarakat have not received second-line therapy using protease inhibitors. The results showed major undetectable mutations in 30 samples examined. Only 1 sample with RSMM_70 code detected minor mutation L10V and other mutations with motive: 115V, M36I, R41K, L63V, H69K, K70R, L89M, I93L. Subjects with different sample codes undetectable have major or minor mutations. The subjects were found to have another mutation with E35D, M36I, I13V, R41K, and K45R motifs. Minor mutations occurring in subjects with L10V motifs are the 10th residue, having mutations from the leucine amino acid to valine (L10V). These mutations are included in minor mutations that can lead to increased drug resistance to the drug when it coincides with other mutations (Johnson et al 2013).

One of the traits of HIV that complicates treatment is mutations. In the protease itself mutations can occur in amino acid residues. Such mutations may lead to secondary structural changes in proteases and inhibitors. Mutations in proteases are divided into major mutations and minor mutations (Victoria et al 2008).

Major mutations make the bonds Between proteases and inhibitors decrease. This is because major mutations alter amino acids that have direct contact with the inhibitor. While minor mutations occur in protease structures that have no direct contact with the drug. If the minor mutation only appears alone, the mutation does not have a significant effect on protease interaction with inhibitors (Victoria 2010).

CONCLUSION

One subject who had not received a protease inhibitor was identified as having a minor L10V mutation; 1 subjects receiving NRTI inhibitor therapy had mutations with M184V motive, 1 subject with M41L motif and 1 subject receiving NNRTI inhibitor therapy identified motif mutations Y181C and V108I. Detection of resistance mutations in patients receiving a line 1 antiretroviral therapy regimen is an early warning for the program to monitor and evaluate the effectiveness of antiretroviral therapy in patients with suspected therapeutic failure.

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