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Research Report

ANALYSIS OF HIV SUBTYPES AND CLINICAL STAGING OF HIV DISEASE/AIDS IN EAST JAVA

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ABSTRACT

Human Immunodeficiency Virus type 1 (HIV-1) known to cause Acquired Immune Deficiency Syndrome (AIDS) disease are divided into several subtypes (A, B, C, D, F, G, H, J, K) and Circulating Recombinant Form (CRF). Different characteristics of subtype of the virus and its interaction with the host can affect the severity of the disease. This study was to analyze HIV-1 subtypes circulating in HIV/AIDS patients from the East Java region descriptively and to analyze its relationship with clinical stadiums of HIV/AIDS. Information from this research was expected to complement the data of mocular epidemiology of HIV in Indonesia. This study utilited blood plasma from patients who had been tested to be HIV positive who sected treatment to or were reffered to the Intermediate Care Unit of Infectious Disease (UPIPI) Dr. Soetomo Hospital Surabaya from various area representing the East Java regions. Plasma was separated from blood samples by centrifugation for use in the the molecular biology examination including RNA extraction, nested PCR using specific primer for HIV gp120 env gene region, DNA purifying, DNA sequencing, and homology and phylogenetic analysis. Based on the nucleotide sequence of the HIV gp120 env gene, it was found that the most dominant subtypes in East Java were in one group of Circulating Recombinant Form (CRF) that is CRF01_AE, CRF33_01B and CRF34_01B which was also found in Southeast Asia. In the phylogenetic tree, most of HIV samples (30 samples) are in the same branch with CRF01_AE, CRF33_01B and CRF34_01B, except for one sample (HIV40) which is in the same branch with subtype B. HIV subtypes are associated with clinical stadiums (disease severity) since samples from different stages of HIV disease have the same subtype.

Keywords: *HIV, AIDS, molecular biology examination, subtype, clinical stadium*

ABSTRAK

Latar Belakang: *Virus HIV pada manusia (HIV-1) diketahui menyebabkan Acquired Immune Deficiency Syndrome (AIDS) terbagi menjadi beberapa subtipe (A, B, C, D, F, G, H, J, K) dan Circulating Recombinant Form (CRF). Karakteristik yang berbeda dari subtipe virus dan interaksi dengan host dapat mempengaruhi keparahan penyakit.* ***Tujuan:*** *Penelitian ini dilakukan untuk menganalisis HIV-1 subtipe secara deskriptif, yang beredar di kalangan pasien HIV / AIDS dari wilayah Jawa Timur dan untuk untuk menganalisis hubungan dengan stadion klinis HIV / AIDS. Informasi dari penelitian ini diharapkan dapat melengkapi data epidemiologi mocular HIV di Indonesia.* ***Metode:*** *Penelitian ini menggunakan plasma darah dari pasien yang telah diuji untuk menjadi HIV positif yang berobat ke atau dirujuk ke Unit Perawatan Menengah Penyakit Infeksi (UPIPI) Dr Soetomo Surabaya dari berbagai daerah yang mewakili wilayah Jawa Timur. Plasma dipisahkan dari sampel darah melalui sentrifugasi untuk digunakan dalam pemeriksaan biologi molekuler termasuk ekstraksi RNA, proses PCR dengan menggunakan primer spesifik untuk wilayah HIV gp120 gen env, pemurnian DNA, sekuensing DNA, dan analisis homologi dan filogenetik.* ***Hasil:*** *Berdasarkan urutan nukleotida dari gen gp120 HIV env, ditemukan bahwa subtipe yang paling dominan di Jawa Timur berada dalam satu kelompok Circulating Recombinant Form (CRF) yang CRF01_AE, CRF33_01B dan CRF34_01B yang juga ditemukan di Asia Tenggara. Pada pohon filogenetik, sebagian besar sampel HIV (30 sampel) berada di cabang yang sama dengan CRF01_AE, CRF33_01B dan CRF34_01B, kecuali satu sampel (HIV40) adalah di cabang yang sama dengan subtipe B. HIV subtipe dikaitkan dengan stadion klinis (keparahan penyakit) karena sampel dari berbagai tahap penyakit HIV memiliki subtipe yang sama.*

Kata kunci: *HIV, AIDS, pemeriksaan biologi molekuler, subtipe, stadium klinis*

INTRODUCTION

AIDS (Acquired Immune Deficiency Syndrome) is one of the most feared diseases in the world today. A Disease that causes decreased immunity of a person is caused by germs HIV (Human Immunodeficiency Virus). Epidemiological situation of HIV/AIDS in the world is continued to worry about. The prevalence of AIDS cases in East Java is 9.80 per 100,000 populations, by 3540 of the cumulative number of cases in the province. East Java is now ranked as the number of cases and the spread of HIV/AIDS, rising from third to second place in the DKI Jakarta (MOH, 2010). It is known that there are two types of HIV, namely HIV-1 and HIV-2. The main cause of AIDS in the world today is the majority of HIV-1. This species is divided into three groups: group M (main), group O (outlier) and group N (new/non-M, non-O). Group M is widespread and is the most common cause of HIV/AIDS epidemic worldwide. Group M is divided into several subtypes, which until now has been recognized several subtypes, namely A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K.[1] Between a subtype with other subtypes can form the so-called recombinant CRF (circulating Recombinant Form) and, until now, 43 CRF has been found.[2]

Differences in the characteristics of subtypes of the virus and its interactions with the human host may influence the severity of the disease. Some studies proposed that the HIV subtype variation associated with the clinical stage of disease. A research in Senegal, against sex workers who were infected with subtype A, C, D and G, found that the development of AIDS increased in patients infected with non-subtype A.[3] In Thailand, the study of survival of patients infected with CRF01_AE showed shorter time since HIV-1 infection to death compared to Western populations.[4] Several other studies in Africa also state that certain subtypes that increased the severity of disease in patients with HIV/AIDS than other subtypes.

On the basis of the high prevalence of AIDS in the community caused by HIV, the required examination of DNA subtypes can be done in order to make it a more efficient so that AIDS prevention and eradication of disease can be more successful. It is necessary for the proper diagnosis of AIDS patients, and then examined the subtype of virus infected for business/further precautions. Information on the rate of subtype differences in clinical stage of HIV/AIDS is important for proper testing of HIV vaccines aimed at slowing the progression of disease severity and in the management of HIV infected individuals. This genetic information will provide a strong addition to the standard data to determine the epidemiological pattern of the spread of the virus. Molecular epidemiology supports classical epidemiology in terms of import sources to confirm the virus known, a virus subtype that is obtained by a known virus subtypeps circulating in a country.

This research was expected to contribute data about the types of HIV subtypes in East Java, so that the chain of transmission of HIV/AIDS can be controlled both at the national, regional and global levels. Subtypes of HIV virus can also be reported to the WHO to complement the HIV virus molecular epidemiologic data that already exist in the WHO. The data of this study can also be used as a consideration for further research in an attempt to perform virus isolation, and manufacture of candidate HIV vaccine for AIDS is more in line with the subtypes of HIV virus in Indonesia and the clinical stage. HIV genome consisting of genes that encode the viral structural proteins are among the major genes gag, pol and env. Env sequence variation is high enough. Various groups and different HIV subtypes have been characterized genetically by sequences of the env gene. Thus, env is the main target area for studying subtype associated with the epidemiology, as it can provide information on all circulating subtypes in a given geographical area.[5] The present study used the env gene of HIV-1 gp120 as a target due to its high regional variability (V) and constant region (C).

This study aims to determine the descriptive subtypes of HIV-1 circulating in patients with HIV/AIDS from East Java and to study its relationship with clinical stage of HIV disease/AIDS. In particular, the purpose of this study are as follows:

1. Analyzing the most predominant HIV subtype in East Java.
2. Analyzing kinship (phylogenetic analysis) HIV in East Java.
3. Analyzing the relationship between clinical stage of HIV infection/AIDS with HIV subtype in East Java.

MATERIALS AND METHODS

Because the target to be assessed in this study is the type of HIV virus, blood samples were taken only from patients who had been HIV positive for the virus and went to the Intermediate Care Unit of Infectious Diseases (UPIPI) Hospital Dr. Soetomo and had not received antiretroviral therapy. Patients were randomly selected from different regions of origin to represent some of the areas in East Java.

Blood sampling performed by medical personnel Hospital Dr. Soetomo was trained. ± 3 ml of blood sample was collected in EDTA tubes vacutube (to prevent freezing). Then, blood samples were taken into a cool box which had been given the icepack/ice cubes to the laboratory Hepatitis/AIDS Institute for Tropical Diseases (Tropical Disease Center/TDC) within 6 hours maximum after taking them. In the laboratory, the sample tubes were centrifuged to separate the plasma from the blood. Plasma obtained was transferred into a 2 ml microtube and stored at temperatures –80° C until use.

Collection of blood samples was carried on until the minimum amount of sufficient, appropriate, statistical calculations. This ultimately obtained blood sample from 46 patients. In addition to taking blood sample, we also collected other data from the patient such as age, gender, origin, stage of disease, CD4 count, history of other diseases, infection group, and so forth. Furthermore, HIV viral RNA extraction

from blood plasma was collected using a reagent QIAamp Viral Mini Kit from Qiagen. In this study, the gp120 env gene was the target. For the HIV, RNA should be made of DNA by the reverse process trancription. By using the OneStep RT-PCR reagents from QIAGEN, we could the reverse trancription and PCR amplification in one step. The process of the first round of PCR (first round PCR) was used, namely HIV-specific primers ED5 and ED12 forward reverse.[6,7] From the first round, the PCR amplicon size obtained was 1200 bp. To increase the specificity of the gene sought, and also because the size of the amplicon is large enough so that the concern will complicate the process of DNA sequencing, the nested PCR was performed. On the first round of PCR products, PCR was performed with the second round PCR reagents from Promega GoTaq Green PCR using primers as mastermix ES7x forward and reverse E125[6,7] to produce amplicon size 300 bp ~. PCR process was performed many times for the optimization of annealing temperature to find the right. Therefore, the result of the PCR product was good. Sometimes, the process of PCR has also repeated on the samples that gave negative results. To view the results PCR was performed on PCR products electrophoresis sample of 2% agarose gel, and then observed using UV transiluminator (short wave = 254 nm). Picture of DNA bands on the gel was photographed using a digital camera. Before performing the DNA sequencing, the PCR product had to be purified first. The process of purification was performed using reagents QIAquick Purification Kit from Qiagen. If the sample results of the second round PCR gave a positive result, then the PCR product was used for the sequencing process, but when the second round PCR was negative, it was used for sequencing the first round PCR products. Sometimes there were also bands of DNA samples that did not look positive or too thin in the first round PCR, but they would appear after the second round PCR. Before the purified PCR products of positive/ clear tape on-electrophoresis then what? After that, under long-wave UV light (365 nm) the gel containing the target DNA band was cut, then the gel was diluted with buffers contained in the kit, QIAquick DNA Purification obtained was pure. Purified DNA was then at-labeled by using the PCR primer ES7x specifically for labeling. After obtaining a label, we precipitated DNA sequencing according to standard procedures, then inserted into the DNA sequencing machines ready ABI Prism 310 Genetic Analyzer for sequence traced. The result of this nucleotide sequencing of electroferogram was a diagram showing the peaks representing the nucleotide. Sometimes, there was a sample that produced a good picture electroferogram with clear peaks, but there were also samples of which the electroferogram was not good. In some re-sequencing process the samples needed to be treated to get a good electroferogram. Of the 46 samples, 31 samples that produced a pretty good electroferogram were obtained. The other samples have a negative PCR result was that it was impossible to continue the process of sequencing, and some sample results of electroferogram are not good. Samples that had been successful in HIV-sequencing and homology analysis were treated to make the filogenetic tree.

Table 1. Characteristic epidemiology and clinical of the subjects co infected HIV in East Java

Characteristic	Number (people)	Percentage (%)
Gender		
Male (age 19-54 years; mean: 34.39)	30	65.22
Female (age 25-56 years; mean: 34.25)	16	34.78
From		
Surabaya	25	54.35
Madura	4	8.70
Gresik	4	8.70
Sidoarjo	2	4.35
Pasuruan	2	4.35
Madiun	2	4.35
Bojonegoro	2	4.35
Banyuwangi	2	4.35
Kediri	1	2.17
Tuban	1	2.17
Lumajang	1	2.17
Risk Factor		
Penasun	3	6.52
Homoseksual	2	4.35
Heteroseksual	41	89.13
Stage of disease		
Stadium I	7	15.22
Stadium II	0	0
Stadium III	29	63.04
Stadium IV	10	21.74

RESULTS AND DISCUSSION

In this study, several samples of 46 patients with positive Acquired Human Immunodeficiency Virus infection (HIV) were obtained. These patients referred to hospitals for treatment or Dr. Soetomo Surabaya from some areas in East Java. Epidemiological and clinical characteristics of the study subjects can be seen in Table 1.

The 46 patients who were positively infected by HIV in this study, consisted of 30 men (65.22%) and 16 women (34.78%). The ages of the patients ranged from 19 to 56 years old with the average of 34.39 years old. The ages of the male patients range of 19 to 54 years old, with the average of 34.47 years old. Meanwhile, the ages of the female patiens ranged from 25 up to 56 years old, with the average of 34.25 years.

The subjects in the study came from several areas in East Java and were referred to hospitals for treatment or to Dr. Soetomo Hospital Surabaya from some areas in East Java as shown in Table 1. Patients were randomly selected from different regions of origins to represent some of the areas in East Java. Patients from Surabaya, amounted to 25 people (54,35%), dominated the whole subjects of the study. Other subjects came from including that include Madura (4 people), Gresik (4 people), Sidoarjo (2 people), Makati (2 people), Madison (2 people), Bojonegoro (2 people), Banyuwangi (2 people), Karachi (1 person), Tuban (1 person) and Lumajang (1 person). Transmission of HIV/AIDS can occur through various methods of disease transmission, or so-called risk factors, namely injecting drug users (IDUs), heterosexual behavior or illicit sex, homosexual sex, from pregnant mothers to the fetus, blood transfusion, and other unknown causes. Based on the data obtained in this study, the most dominant risk factor was heterosexuality amounted to 41 cases (89.13%), while the other risk factors are injecting drug users (only 3 cases) and homosexuality (only 2 cases) (Table 1). According to the report issued by the National AIDS Commission (NAC) in the international symposium in Padalarang, West Java on October 21, 2011, the behavior or heterosexual sex is now the main culprit in the spread of HIV/AIDS in Indonesia. In 2006, the trend of transmission of HIV/AIDS in Indonesia was dominated by the use of a syringe while a 54.42% of contributor to HIV/AIDS cases were skil unreported, and contributet to 38.5%. Conditions to the contrary were the place in 2011 where injecting drug users risk factor decreased to 16.3%, while the heterosexual risk factors reached 76.3%. This means that the majority of HIV/AIDS in Indonesia is transmitted through casual heterosexual, sex that apparently also happeneds in East Java.

In Table 1, it can also be seen that clinical characteristics of HIV/AIDS are the subjects of this study. Patients with clinical stage I-stage amounted to only 7 people, patients with stage III amounted to 29 people, and stage IV patients amounted to 10 people. In the present study we found no patients with stage II. It appears that HIV/AIDS patients in hospitals Dr. Soetomo Surabaya are still dominated by advanced stage patients rather than early stage patients. This is probably still due the lack of awareness or the courage to see her early on, that makes them go to hospitals when the condition of the disease is severe. The government, the business, and all the parties need to resolve this issue, for example, by doing a counseling and free examinations for the whole society. Because what needs to be assessed in this study are the type of the HIV virus, the blood samples were taken only from patients who had been HIV positive for the virus had gone to the Intermediate Care Unit of Infectious Diseases (UPIPI) Hospital Dr. Soetomo and had not received any antiretroviral therapy. The overall patients who become the subjects of this study were examined for antibodies to HIV using a standard procedure for patients in hospitals UPIPI Dr. Soetomo, namely by using three kinds of antibody rapid test kit: Oncoprobe, SD Triline and HIV 1/2. The use of the three types of inspections was intended to avoid mistakes in making the diagnosis, since the diagnosis of HIV infection is a diagnosis that widely affects not only the patients but also the surrounding environment and also controls efforts undertaken by the government.

HIV DNA detection using PCR technology is one choice of method for diagnosis of HIV infection under situations in which the detection of antibodies gives negative and still questionable results.[8] For the PCR in this study we used pairs of primers that had been used and published in international journals.[6,7] The 46 samples from HIV-positive patients were infected with HIV. The PCR of the env gene coding for HIV gp120 protein obtained positive PCR results amounted to 34 samples. In the examination of the samples with antibodies to HIV positive and HIV PCR positive, the patients' bodies still contained HIV RNA that they still had the potential to transmit the HIV virus. The PCR process was conducted many times to seek optimization annealing temperature (annealing) until the right one, could be found. Therefore, the resulting PCR product appeared to be good. Sometimes, the process of PCR was also repeated on samples that gave negative results. In the sample that was PCR negative results with the use of pairs in this study, the possibility of change / mutation in the nucleotide sequence point of attachment of the primary, so the primary cannot be attached to the result that the negative PCR results. Primer pairs in this study is that when the primer pair used PCR amplification of nucleotides and can provide a positive, after sequencing, the nucleotide sequence obtained will be used to determine the HIV subtype. To find HIV subtypes, the nucleotide sequence obtained in this study was then compared with the nucleotide sequences that had been published.

HIV DNA PCR result was further purified by amplification and sequencing using the ABI-310 sequencer engine. The result of this sequencing of the electroferogram was a diagram showing the peaks representing the nucleotide. In this study, sometimes, there was a sample that produced a good electroferogram with clear peaks, but

there were also samples of electroferogram which appeared not very good. In some re-sequencing, the samples needed to be treated to get a good electroferogram. The 34 samples that produced positive PCR result obtained 31 samples of electroferogram that were pretty good. The other samples had a negative PCR result that it was impossible to continue the process of sequencing, and 3 samples were positive for PCR-sequencing, the electroferograms which were not good or not readable. From the results obtained by sequencing, and by molecular analysis to determine the HIV genotyping and nucleotide, the sequence homology was obtained. To find HIV subtypes, the nucleotide sequence of sequencing results obtained in this study was compared with other HIV subtypes of nucleotide sequences that have been published.[9,10,11] then analyzed to make a phylogenetic tree. Nucleotide sequence of sequencing results obtained from samples of people with HIV/AIDS was used to detect genetic variations or mutations in HIV DNA PCR results in this study.

It has been argued that the identification of HIV-1 that differs in HIV env causes are grouped into: M, N and O. Group M is the most frequently encountered and is divided into nine subtypes based on the whole genome which are geographically distinct[9,10,11] namely subtypes A, B, C, D, F, G, H , J and K. HIV subtype is further subdivided into subtype, which includes A1, A2, F1 and F2.[10] The argument states that the different HIV subtypes may also differ in the effects of transmission (transmission), the emergence of drug resistance and the disease perogresifitas. It has also been argued that subtypes B (found in North America

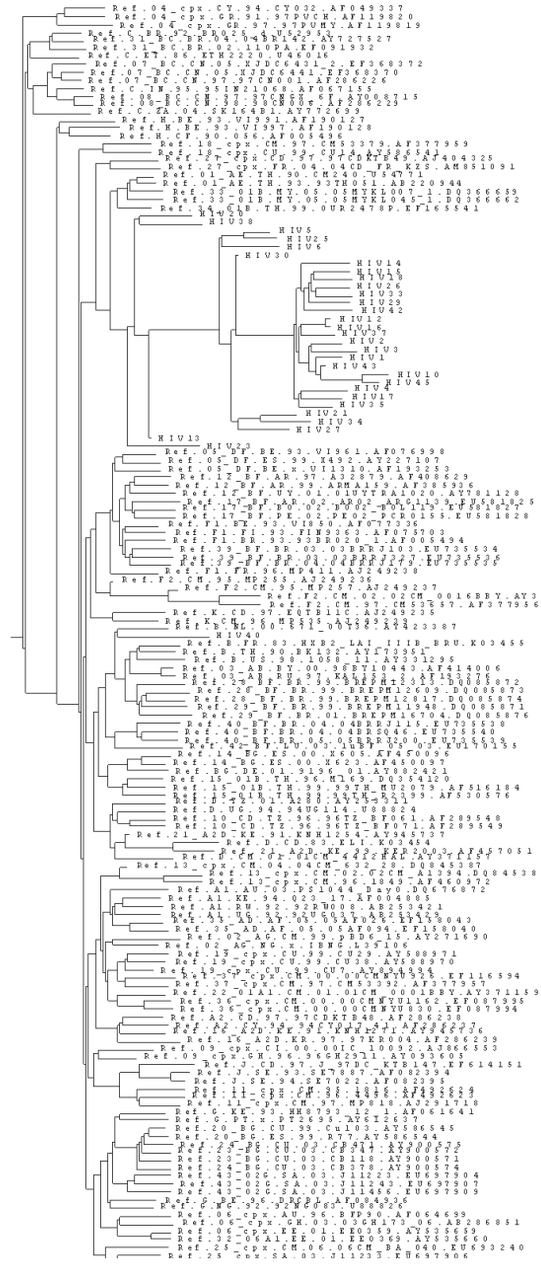

Figure 1. Tree neighbor-joining phylogenetic models of gp120 env sequences of HIV samples and reference

and Europe), A and D (Africa), C (Africa and Asia) are the most prevalent ones. These subtypes form branches in a tree depicting the genetic ancestry of the group M of HIV-1. Co-infection with different subtypes leads to the increased circulating recombinant forms (CRFs). In 2000, a global analysis of prevalent subtypes was made stating that 47.2% of infections worldwide were subtype C, 26.7% were subtype A/CRF02_AG, 12.3% were subtype B, 5.3% were subtype D, 3.2% were CRF_AE, and the remaining 5.3% consisted of other subtypes as well as CRFs.[12] Most research focused on HIV-1 subtype B, while others focud on the few other subtypes.[13]

The HIV nucleotide sequences obtained were analyzed and ready (as many as 31 of the 34 sequences that had been expected). We also conducted a molecular phylogenetic analysis of HIV nucleotide and phylogenetic tree drawn by a computer program Clone Manager Version 6 6:00, with 128 nucleotides with various subtypes of HIV-references that had been published previously (http://www.hiv.lanl. gov). The results obtained, turned out to be HIV from HIV/AIDS patients in this study. As many as 30 samples in one group were circulating Recombinant Forms (CRFs), especially CRF01_AE, CRF33_01B, and CRF34_01B originating from Thailand and Malaysia. For the first sample, the sample was located in one group HIV40 branching with subtype B. The result of the molecular analysis as the result of 31 HIV sequencing of this study homology in the form of multiple nucleotide alignment was 300 nucleotides long. The results of the molecular analysis which were intended to determine the HIV subtype in the form of a phylogenetic tree of nucleotide along with the length of 300 nucleotides (gp120 env gene V3 region) consisted of 31 samples, and the results of this study of HIV subtypes (A, B, C, D, F, G, H, I , J and K) and a variety of CRF have been published. The form of a phylogenetic tree of HIV subtypes are shown in Figure 1.

31 samples were successfully determined. 5 samples were from stage I patients. 19 samples were from stage III patients and 7 samples were from stage IV patients. Almost all the samples had the same subtype and only one sample had a different subtype that it can be connected between the degree or the stage of disease infecting the HIV subtypes. From the results of statistical tests that were obtained between HIV subtype and the clinical stage, there was no significant relationship (Exact p = 1.000 by contingency coefficient = 0.144). Thus, in this study it has not been proven whether the subtypes of HIV influence disease severity. The design of this study was cross sectional so that what the readers can do is analyze the relationship between the subtype of HIV with the degree of disease in a single observation. A longitudinal study in Europe by and another one in Thailand by stated that there was no difference of disease progression among HIV-1 subtypes that were different. However, the study of in his studies in Africa had non-B subtypes that were often found in patients with severe degree of disease (stage III and IV).

CONCLUSION

The results of this study was based on nucleotide sequences of the env gene of HIV gp120 it can also be concluded that the most predominant HIV subtype in East Java in one group circulating Recombinant Forms (CRFs) that CRF01_AE, CRF3x_01B, and CRF34_01B which were also found in various countries of Southeast Asia. In the phylogenetic tree, 30 HIV samples in a branching kinship with the subtype CRF01_AE, CRF34_01B, and CRF33_01B, while the HIV-1 samples are in a branching HIV40 kinship with subtype B. In addition, HIV subtype is not associated with clinical stages (disease severity) while samples from different stages of HIV disease have the same dominant subtype.

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