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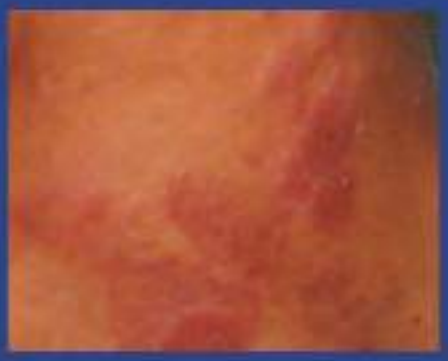

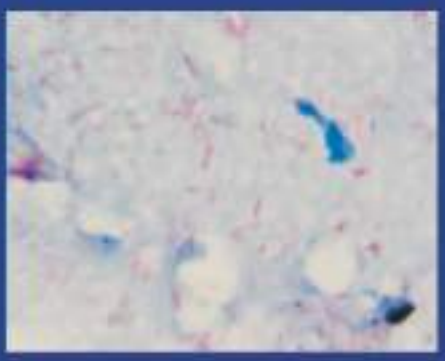

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Research Report

HEPATITIS B SEROLOGY PROFILES ON CHILDREN AGED 1–13 YEARS OLD IN SUMENEP, MADURA

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ABSTRACT

Background: Hepatitis B virus (HBV) which was acquired during perinatal or childhood would promote hepatocellular carcinoma with even higher percentage than that which was acquired during adult age. That is why HBV represents a serious public health threat for children. HBV vaccination has been integrated into national expanded programme on immunization (EPI) since 1997. The aim of they study is to investigate the prevalence of HBV among children who were born after 1997 in Sumenep. Material and Methods: a total of 102 children who were born after 1997 were enrolled in this study. All children were admitted in the Emergency Room and Pediatric Ward of dr. H. Moh Anwar General Hospital for some reasons. Written informed consents were obtained from parents/ guardians of all the children. Study protocol was reviewed and approved by the Ethics Committees. All of these cases were examined for hepatitis B surface antigen (HBsAg), antibody to HBsAg (Anti-HBs), and antibody to hepatitis B core antigen (Anti-HBc). Result and Discussion: Overall, 6 (5.88%) of 102 samples were positive for HBsAg, 51 (50.00%) of 102 samples were positive for anti-HBs, and 49 (48.04%) of 102 samples were positive for anti-HBc. All the children were born after 1997. Conclusion: HBsAg rate is still high even after universal vaccination program, acquired protective antibodies against hepatitis B surface antigen were sufficient, but there is a suspicion for occult hepatitis B infections (OBI). A further study to confirm OBI is needed.

Keywords: *HBV, HBsAg, Anti-HBs, Anti-HBc, immunization*

ABSTRAK

Latar Belakang: *Hepatitis B Virus (HBV) yang diperoleh selama perinatal atau masa kanak-kanak akan menyebabkan karsinoma hepatoseluler dengan persentase lebih tinggi daripada apa yang telah diperoleh selama usia dewasa. Itulah sebabnya HBV merupakan ancaman kesehatan masyarakat yang serius bagi anak-anak. HBV vaksinasi telah diintegrasikan ke dalam program imunisasi nasional yang telah diperluas sejak tahun 1997.* ***Tujuan:*** *untuk menyelidiki prevalensi HBV antara anak-anak yang lahir setelah tahun 1997 di Sumenep. Bahan dan Metode: total 102 anak yang lahir setelah tahun 1997 yang terdaftar dalam penelitian ini. Semua anak-anak dirawat di ruang gawat darurat dan Departemenen Anak dari RSUD dr. H. Moh Anwar untuk beberapa alasan. Informed consent tertulis diperoleh dari orang tua/wali dari semua anak. Studi protokol ditinjau dan disetujui oleh Komite Etika. Semua kasus ini diperiksa untuk antigen permukaan hepatitis B (HBsAg), antibodi terhadap HBsAg (anti-HBs), dan antibodi terhadap antigen inti hepatitis B (Anti-HBc).* ***Hasil:*** *Secara keseluruhan, 6 (5,88%) dari 102 sampel yang positif untuk HBsAg, 51 (50,00%) dari 102 sampel yang positif untuk anti-HBs, dan 49 (48.04%) dari 102 sampel yang positif untuk anti-HBc. Semua anak lahir setelah 1997.* ***Kesimpulan:*** *Tingkat HBsAg masih tinggi bahkan setelah program vaksinasi universal, diperoleh antibodi pelindung terhadap antigen permukaan hepatitis B sudah cukup, tapi ada kecurigaan untuk okultisme infeksi hepatitis B (OBI). Sebuah studi lebih lanjut untuk mengkonfirmasi OBI diperlukan.*

Kata kunci: *HBV, HBsAg, Anti-HBs, Anti-HBc, imunisasi.*

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INTRODUCTION

Hepatitis B is an infectious disease caused by hepatitis B virus (HBV) that affects more than 400 million people worldwide, and 1.3 million die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually.[1] HBV variants are currently classified into the human genotypes A to H.[2]

Up to 90% of infected newborns develop chronic HBV infection, which gives a higher risk of HCC later in their adulthood, while 24% of adults chronically infected during childhood had either HCC or cirrhosis.[2]

A safe and effective vaccine against hepatitis B has been available since 1982. The introduction of a childhood immunization program in many countries has dramatically reduced the carrier rate of HBV and significantly decreased the incidence of HCC. In Indonesia, HBV vaccination had been introduced since 1987, and has been integrated into national expanded programme on immunization (EPI) since 1997. WHO recommends HBV vaccination to all infants, first at birth, then followed by two subsequent vaccinations with each minimum interval of 1 and 2 months respectively.[1]

However, the current serologic status of HBV in children has not been fully investigated in Indonesia. The aim of this study was to investigate the prevalence of HBV among the children who were born after the national immunization program in Sumenep, an area in East Java, Indonesia, which was with medium-to-high endemicity for HBV.

MATERIALS AND METHODS

Study subjects

Three ml of blood samples were taken from all the patients aged 1–13 who admitted in the Emergency Room (*IGD*) and Pediatric Ward (*Zaal Anak*) of dr. H. Moh Anwar General Hospital for some reasons. A hundred and two samples were collected in this study. Serum samples were obtained during January-March 2012 and were stored at -20° C until further usage. Written informed consents were obtained from parents/guardians of all the children. No individual hepatitis B vaccination records remained. The study protocol was reviewed and approved by the Ethics Committees of dr. H. Moh. Anwar General Hospital.

Serological markers of HBV infection

All refrigerated samples were tested for HBsAg with enzyme-linked immunosorbent assay (ELISA) (Hepalisa HBsAg) and for anti-HBs by enzyme-linked immunosorbent assay (ELISA) (Zhongsan Anti-HBS ELISA). In order to differentiate vaccine-induced antibody from naturally acquired antibody (and to identify the suspects of occult HBV infections), the prevalence of antibody to hepatitis B core antigen (anti-HBc) was assessed by enzyme-linked immunosorbent assay (ELISA) (Hepalisa Anti HBc).

RESULTS AND DISCUSSION

A total of 102 children were screened for serological markers of HBV infection. Overall, positivity rates for HBsAg and anti-HBs were 5.88% (6 out of 102) and 50.00% (51 out of 102), respectively, with the mean age of 5.76 years old. All the children (1–13 y.o.) were born after the introduction of the universal vaccination program. Anti-HBc rates were 48.04% (49 out of 102). Of 51 anti-HBs positive children, 23 were negative for anti-HBc. All six HBsAg-positive children were negative for anti-HBs.

Similar study in Borno State, Nigeria showed that overall seroprevalence of HBsAg among primary school pupils was 44.7%,[3] while in those 439 children in Moldova (mean age, 5 years), the prevalence of HBsAg and Anti-HBc were 6.8% and 17.1%, respectively.[4]

Successful vaccination programs had been shown by several countries which previously belonged to high prevalence HBV countries, such as in a study in Karachi, Pakistan, among sixty five (1.8%) out of 3533 children (mean age 10±4 years old) were positive for HBsAg.[5] In Taiwan, after 25 years of nationwide HBV universal vaccination program for infants, HBsAg sero-prevalence sharply declined from 9.8% to 0.6% with HBV vaccination coverage as high as 97%.[6]

This study was unable to assess the actual coverage rate because no individual vaccination records remained. For this reason, efficacy of vaccination was not evaluated in this study. However, this study did show that acquired protective antibody against HBV infection was sufficient among children born after the universal vaccination program.

The HBsAg prevalence of 5.88% in this study was still considered high. A high coverage rate for HBV vacination is crucial for decreasing the prevalence of HBV infection. Program for Appropriate Technology in Health, a non governmental organization in United States of America, (PATH) stated that Birth dose within seven days of birth was 65%, even though HBV 3 coverage was 80-85%.[7] Some of the first dose of HB vacccine in Indonesia has been administered along with the first dose of DPT, which was generally 6 weeks to 2 months of age. Delay in giving the first dose of HB vaccine would not prevent perinatal transmission.[8]

PATH worked with the Indonesian Ministry of Health since the beginning of 1987 to launch a model immunization program on the island of Lombok. The innovative program introduced a comprehensive system for delivering a vital birth dose of the vaccine and established a system for tracking and monitoring pregnancies and births. On October 2002, the Government began an effort to ensure that every newborn is administered Hepatitis B vaccines with prefilled, single use syringe and needle (Unijject®) during the first seven days of life.[7] HBsAg rates on children who were born before 2002 and after 2002 were 0% (0 out of 17) and 7.05% (6 out of 83) respectively. This concludes that even after PATH Unijject® programs in 2002, there had not been any effect in Sumenep.

On the other hand, there was 0% HBsAg rate in children aged 10–13 y.o., means before PATH Uniject® program was promoted? while it was 7.05% in those born after. Anti-HBc rate in previous groups was as high as 47.06%. This means that they had ever been infected before.

Our study showed that HBsAg rate in children Sumenep was still considered high. Hepatitis B immunization coverage of 18.1% in Sumenep, data from National Basic References of Ministry of Health 2007, could be one of the factors which supported this fact.[9] PATH's Uniject® program was one of the solutions to increase the Hepatitis B immunization coverage of birth dose, but it had no significant impact in our study in Sumenep. Some other factors which might have played a role in this result should be searched and overcomed.

The ACIP, the American College of Obstetrics and Gynecology (ACOG), the American Academy of Family Practice (AAFP), and the American Academy of Pediatrics (AAP) recommend that all pregnant women receive prenatal testing for hepatitis B during each pregnancy by screening serum for the presence of HBsAg, regardless of risk factors or immunization history.[10] This routine HBV serological profile screening on pregnant women should be the target of the Ministry of Health of Indonesia in the near future. On pregnant women with positive HBsAg, HBV vaccination and HBIg (0.5 ml) should be administered on their babies within 12 hours after birth.[11] These efforts will lead to a greater control of HBV infection, and furthermore, liver diseases caused by HBV infection would be better controlled.[12] The remaining challenges will be to minimize the rate of vaccine failure and to deal with potential vaccine-related events, such as the emergence of escape surface mutants.[13]

Further studies with larger samples in the future will accomodate better reflections of HBV immunology profile in children. HBV DNA detection among those with HBsAg negative, anti HBc positive, and or anti HBs positive or negative should be tested in order to detect occult HBV infections.

In conclusion, HBsAg rate among children born after the Hepatitis B universal vaccination program is still high in Sumenep, acquired protective antibodies against HBV infection were sufficient, and suspects of occult hepatitis B infections were found. Continuation in PATH's Uniject® program, implementation of immunization programs, and routine HBV serological profile screening on pregnant women should proceed to eradicate HBV infection. Some other aspects which play roles in the high HBsAg rates should be explored, including molecular studies.

Table 1. Seroprevalence of hepatitis B surface antigen (HBsAg), anti-HBs, and anti-HBc among study population

	No.	No. Positive	%
HBsAg	102	6	5.88
Anti-HBs	102	51	50.00
Anti-HBc	102	49	48.04

Table 2. Comparison of prevalence of hepatitis B markers in children born before and after PATH's Uniject® programs.

Age	HBsAg			Anti-HBs			Anti-HBc		
(Years)	No.	Pos.	%	No.	Pos.	%	No.	Pos.	%
1–9	85	6	7.05	85	41	48.23	85	41	48.23
10–13	17	0	0	17	10	58.82	17	8	47.06

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Research Report

ETHNIC AND ATOPIC DERMATITIS: WHAT HAVE WE LEARNED IN ASIAN POPULATION?

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ABSTRACT

Atopic Dermatitis (AD) is a chronic inflammatory skin disease, with relapsing remitting course. Management of AD is challenging due to the complexities of this disease. Two hypotheses concerning the mechanism of AD have been proposed. One holds that the primary defect resides in an immunologic disturbance that causes Ig E-mediated sensitization, with epithelial barrier dysfunction regarded as a consequence of the local inflammation. The others propose that an intrinsic defect in the epithelial cells leads to the barrier dysfunction; the immunologic aspects is considered to be an epiphenomenon. Many studies support that AD is a complex trait which has interactions between genes and environmental factors contributing to disease manifestation, but the result of replicate association between genetic markers and AD is inconsistence. An important factor contributing to this inconsistency is related to population diversity. It is possible that certain genetic markers might contribute to increase the risk in certain ethnic population but not in others, either because of the differences in frequencies of the risk alleles and the specific genes interaction. There is limited information about the role of ethnicity in Asian population. The overall purpose of this review is to present an update on ethnicity approach of AD in Asian population. Research on prevalence, risk factor, innate and adaptive immune response genes, skin barrier dysfunction genes and gene-environment interaction such as epigenetic, is discussed. It is generally approved that the ethnicity of study subject is a key factor in interpreting genetic polymorphism studies. Therefore, discussion of some current areas of research about polymorphism are presented, including filaggrin (FLG) gene and CD14 C-159TSNP. Addressing the issues described above may improve our understanding of AD pathogenesis that has implications for the clinical management of AD.

Keywords: *Atopic Dermatitis, ethnic, Asian, immune system, skin barrier, gene-environment interaction*

ABSTRAK

Latar Belakang: *Dermatitis Atopik (DA) merupakan penyakit keradangan kulit yang bersifat kronis dengan periode remisi dan eksaserbasi. Kompleksitas dari penyakit tersebut menjadikan tantangan bagi Dokter Spesialis Kulit dan Kelamin dalam melakukan penatalaksanaan. Patogenesis DA didasari oleh dua hipotesis. Pertama, gangguan imunologi sebagai penyebab primer, menyebabkan sensitisasi yang diperantarai oleh Ig E, selanjutnya terjadi kerusakan barrier kulit sebagai konsekuensi dari keradangan local yang terjadi. Kedua, gangguan primer pada sel-sel epitel kulit menimbulkan disfungsi kulit sebagai barrier, sehingga memudahkan terjadi keradangan dan diikuti dengan proses imunologi. Berbagai penelitian mendukung kontribusi interaksi antar gen serta lingkungan sebagai factor penting dalam manifestasi klinis DA, tetapi asosiasi antara petanda genetic dan DA masih inkonsisten. Faktor penting yang berkontribusi pada hal tersebut adalah diversitas populasi. Petanda genetic tertentu dapat berperan untuk meningkatkan resiko terjadinya DA pada populasi etnik tertentu karena adanya perbedaan frekuensi alel dan interaksi spesifik berbagai gen. Informasi tentang peran etnisitas pada populasi Asia sangat terbatas.* ***Tujuan:*** *dari penulisan ini adalah untuk menggambarkan pendekatan terkini tentang etnis pada DA di populasi Asia. Diskusi meliputi penelitian yang telah dilakukan tentang prevalensi, factor resiko, gen yang bertanggung jawab pada respon imun alami maupun adaptif, gen yang bertanggung jawab pada disfungsi barrier kulit serta interaksi antara gen dengan lingkungan seperti epigenetic. Secara umum disepakati bahwa etnisitas subyek penelitian merupakan faktor kunci dalam interpretasi penelitian polimorfisme. Oleh karena itu, penelitian polimorfisme termasuk gen penyandi filaggrin*

dan CD 14 C-159T juga dibahas pada tulisan ini. Pemahaman tentang pathogenesis DA diharapkan dapat meningkatkan kualitas penatalaksanaan klinis DA.

Kata kunci: *Dermatitis Atopik, etnik, Asia, sistemimun, barrier kulit, interaksi gen - lingkungan.*

INTRODUCTION

A topic dermatitis (AD) is a common inflammatory skin disease that may affect individuals in any age, race, or ethnicity. It is commonly affected during childhood or infancy with chronic and relapsing course. The etiology of AD is partially understood, genetics and environmental factors are thought to play important roles in the pathogenesis.[1]

Asia is the largest continent in the world and has many different geographic areas. It extends from countries in the sub tropic zones, the Far East (including Japan, Korea, and China) to countries in the tropics, the South and Southeast Asian countries (including Singapore, Malaysia, the Philippines, Indonesia, Thailand, Vietnam, Cambodia, Laos, and Myanmar) and to countries in the subtropics (including the Indian subcontinent and countries in the Middle East). Most of the Asian population has Fitzpatrick skin photo type III, IV, and V, whereas a small proportion has skin photo type II and VI. Wide variety of different ethnicity, culture, hygiene, nutrition, and socioeconomic status also present. The manifestation of dermatologic conditions in one part of Asia is commonly different from those seen in another part.[2]

Racial differences in both the expression of AD and genetics are important areas of research for several reasons. First, there might be different phenotypic expression of the disease in a given racial and or ethnicity group (that might or might not be confounded by environmental differences). Secondly, the frequency of genetic variation varies between races.[3]

Ethnic (racial) differences in AD have been minimally investigated. The current experimental human model for skin is commonly based upon physical and biochemical properties known about Caucasian skin. Thus, anatomical or physiological properties in skin of different races may influence a disease process or a treatment of that disease it self. There is limited information about the role of ethnicity in Asian population.[4]

The overall purpose of this review is to present an update on several approaches to understanding the susceptibility and expression (severity) of AD in Asian population. **Four key questions are addressed in this review: (1) What is the difference between race and ethnic? (2) Do the biophysical properties of Asian skin differ from others? (3) Are there any differences between prevalence and risk factor of AD in Asians and others? (4) What about the research on innate and adaptive immune response genes, skin barrier dysfunction genes and gene-environment interaction such as epigenetic of AD based on ethnicity in Asians?**

QUESTION 1: What is the difference between race and ethnicity?

Definitions of ethnicity and race based on New Oxford American Dictionary are:[5]

Ethnicity is the population subgroup (within a larger dominant national or cultural group) with a common national or cultural tradition.

Race is each of the major divisions of human-kind, having distinct physical characteristic. Race also means a group of people with common features.

QUESTION 2: Do the biophysical properties of Asian skin differ from others?

Racial variability should be considered in terms of different skin responses to topical and environmental agents. Race provides a useful implement to investigate and compare the effects of lifetime sun exposure and ambient relative humidity. Evolution provided over 100,000 years of genetic advantage to survive for those races living in a specific area with specific climatic conditions. To survive in harmful environment requires an optimal adaptation of outermost layers of our body, the skin relates to structural, biochemical, and molecular level.[4]

Stratum Corneum (SC) is equally thick in different races. However, Weigand demonstrated that the SC in blacks contained more cell layers and required more cellophane tape strips to be removed than the SC of Caucasians, while Kampaore and Tsuruta showed that Asian skin was significantly more sensitive tostrip than black skin. No correlation was found between the degree of pigmentation and the number of cell layers. These data clarified that the greater intercellular cohesion in blacks, consequences in an increased number of cell layers and an increased resistance to stripping. This mechanism may involve lipids, because the lipid content of the SC ranges from 8.5% to 14%, with higher values in blacks.[4]

Corcuff investigated the corneocyte surface area and the spontaneous desquamation and found no differences between black, white, and oriental skin. However, an increased desquamation (up to 2.5 fold) was found in blacks. They concluded that the differences might be related to a different composition of the intercellular lipids of the SC.[6] Sugino found significant differences in the amount of ceramides in the SC, with the lowest levels in blacks followed by Caucasians, Hispanics, and Asians. In this experiment, ceramide levels were inversely correlated with transepidermal water loss (TEWL) and directly correlated with water content.[7] Meguro confirmed these correlations.[8] These data may partially explain the controversial findings in the literature on the mechanisms of skin sensitivity. Changes in skin permeability and barrier

function have been reported. Kompaore evaluated TEWL and lag time after application of a vasoactive compound (methyl nicotinate) before and after removal of the SC by tape stripping. Before tape stripping, TEWL was 1.3 times greater in blacks and Asians compared to Caucasians. No difference was found between blacks and Asians, whereas after stripping they found a significantly higher TEWL in blacks and Asians than in Whites. In particular, after stripping Asians showed the highest TEWL (Asians 1.7 times greater than Caucasians).[9,10] They concluded a resemblance with previous studies,[11,12] which concluded that skin permeability measured by TEWL, was higher in blacks than in Caucasians. They also concluded that Asian skin had the highest permeability among the study groups. However, these findings have not yet been confirmed by other groups. Infact, Sugino, that also included Asians in their study found that the baseline TEWL decreased, blacks were greater than Caucasians greater than or equal to Hispanics and greater than or equal to Asians.[7] Another study about Asian skin, has compared the TEWL between Asians and Caucasians and found no statistically significant difference at baseline or after tape stripping without vasoactive substance applied.[13] Reed found differences in the recovery of the barrier between subjects with skin type II/III compared to skin type V/VI, and no differences between general Caucasians and Asians.[14] Darker skin recovered faster after the barrier damage was induced by tape stripping.[4]

Racial differences in skin conductance are difficult to interpret in terms of SC water content, because other physical factors, such as the skin surface or the presence of hair, can modify the quality of the skin electrode contact. Volar and dorsal forearms present a significant differences between all races.[15] These results are apparently contrast with TEWL recordings. Indeed, increased SC water content, correlates with a higher TEWL.[16] These data may be explained on the basis of the differences of intercellular cohesion or lipid composition. The increasing of skin water content could possibly occur due to the greater cell cohesion with a normal TEWL.[4]

There are reasonable evidences that exist to support that black skin has a higher TEWL compared to white skin by means of objective measurements. Although some deductions have been made about Asian skin, the results are contradictive, and further evaluation of Asian skin needs to be performed. Perhaps, more specificity about the origin of their heritage should also be included because "Asian" encompasses a broad spectrum of people.[4]

QUESTION 3: Are there any differences between prevalences and risk factors of AD in Asian and others?

There are still limited studies available pertaining the epidemiologic data of AD in Asian populations. However, several population studies have demonstrated considerable geographic and racial/ethnic variations in the prevalence of AD.[18,19,20] Based on partially understood environmental factors, AD appears to be more common in industrialized nations and urban settings than in developing countries and rural communities.[21] Survey study about population in northern Europe, the United States, and Japan has reported prevalence rates of 15.6%, 17.2%, and 21%, respectively, whereas a prevalence of 8.5% was reported in a recent study from South Eastern Nigeria.[22,23,24,25] However, with expanded urbanization and adoption of Western lifestyle, the prevalence of AD appears to be rising in developing countries, as in more industrialized nations.[25] The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee evaluated the frequency of AD among 463,801 children aged 13–14 years old from 56 countries.[18] In 1999, high prevalence of AD was reported in several regions where non-Caucasian individuals predominated, the centers of Africa (Nigeria, Kenya), Asia (Japan, Pakistan), and of South America (Paraguay, Chile). Several epidemiologic studies have shown increasing prevalence of AD among blacks and Asian/Pacific Islanders when compared with Caucasians. Recently, a 12-month observational prospective study of 182 babies (62 Caucasian, 61 Chinese, and 59 Vietnamese infants) born in Melbourne, Australia, indicated that the incidence rate of AD was varied by ethnicity.[20] In those populations, AD prevalences of the Caucasian, Chinese, and Vietnamese infants were 21%, 44%, and 17% respectively. Because the Caucasian and the Chinese infants were in similar socioeconomical backgrounds, genetic differences likely played a role in the different incidence rates. In contrast, since the Vietnamese infants had a lower socioeconomic background, but were skill in the same racial group as the Chinese infants, environmental factors likely contributed more than genetic factors to these incidence differences.[20] AD was found to be more prevalent among Chinese infants born in San Francisco and Honolulu than among the local Caucasian population.[26] Similar findings were presented in London-born black Caribbean children compared with their white counterparts. Among London-born black Caribbean children, the prevalence of AD was 16.3% compared with 8.7% in white children.[19] However, a study of Indian and Caucasian preschool children in Leicester, United Kingdom, failed to show any ethnic differences in the prevalence of AD.[27] The reasons for the observed differences in prevalence may be based at least in part, on variations of genetic and environmental factors. However, differences of research methodology between epidemiologic studies must be considered when comparing prevalence rates among populations. Further research on the epidemiology of AD among Asians is warranted.[17]

A study about prevalence of AD by ethnic group was conducted by Baker in suburban area of San Diego. Ethnic the data were available for 5912 patients in the study population. Ethnicity was designated as white (not of Hispanic origin), Hispanic, black, Filipino, other Asian, or mixed race. The prevalence of AD was determined to be 3.2% in the overall study population, 3.7% of which were black, 8.5% were Filipino, 2.0% were Hispanic, 2.8% were

white (not of Hispanic origin), 3.2% were mixedrace, and 5.6% were other Asian origins.[28] Because this study was conducted by only one physician in a limited geographic area, it couldn't be assumed that the population study typically represents the overall Filipino population in the United States or in the Philippines. The actual prevalence of AD probably varies considerably by geographic area. Moreover, the population is not ethnically representative of the overall US population. In particular, although he observed a higher prevalence of AD among patients of "other Asian" origin, this group included too many subsets for separate analysis (ie, Japanese, Korean, Chinese, Laotian, Vietnamese, Cambodian, and other ethnic groups were included).[28]

A prospective study was undertaken to investigate several perinatal predictors of AD occurring in the first 6 months of life which were reported from 1005 mothers and their infants who participate in a US cohort study of pregnant women and their offspring. The main outcome measurement was maternal report of an AD diagnosis in the first 6 months of life and analyzed using multiple logistic regression models to assess the association between several simultaneous predictors and incidence of AD. The cumulative incidence of AD in the first 6 months of life was 17.1%. The adjusted odds ratio (OR) for risk of AD among infants born from black mothers was 2.41 (95% confidence interval [CI]: 1.47, 3.94), compared with infants born from white mothers, and was 2.58 (95% CI: 1.27, 5.24), compared with infants born from Asian mothers. Male infants had an OR of 1.76 (95% CI: 1.24, 2.51). Increased gestational age at birth was a predictor (OR: 1.14; 95% CI: 1.02, 1.27, for each 1-week increment), but birth weight for gestational age was not. Infants born by mothers with a history of AD had an OR of 2.67 (95% CI: 1.74, 4.10); paternal history of AD was also predicted, although maternal atopic history was more predictable than paternal history. Several other

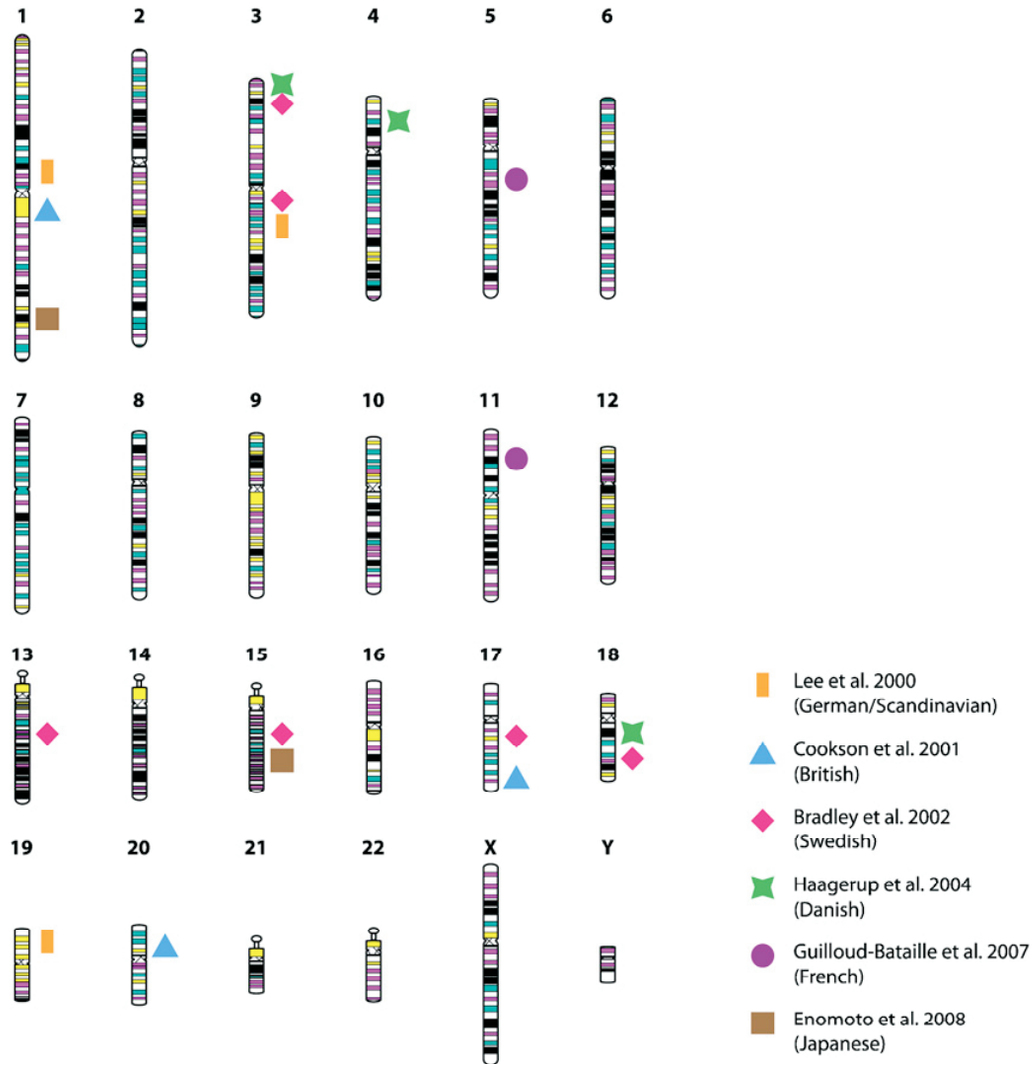

Figure 1. Summary of genome-wide linkage studies of AD: representation of the 23 human chromosomes, highlighting those loci for which genome screens have identified linkage to AD. Loci are mapped to short orlong chromosomal arms and color-coded according to the studies listed in the legend.[30]

perinatal, social, feeding, and environmental variables were not related to the risks of AD. In conclusion, Black and Asian race/ethnicity, male gender, higher gestational age at birth, and family history of atopy, particularly maternal history of AD, were associated with increased risk of AD in the first 6 months of life. These findings suggest that genetic and pre and perinatal influences the essential in the early manifestation of this condition.[29]

QUESTION 4: What about the research on innate and adaptive immune response genes, skin barrier dysfunction genes and gene-environment interaction such as epigenetic of AD based on ethnicity in Asians?

STRATEGIES FOR INVESTIGATING THE GENETIC BASIS OF ATOPIC DERMATITIS

A large amount of research have been undertaken worldwide in the exploration of genetic factors as the etiology of AD. Three main approaches have been used: candidate gene association, selecting genes for study based on a hypothesis of known biological function; genome-wide linkage screens, which are hypothesis free and compare the transmission of genetic information between cases and controls in family pedigrees; and DNA microarray studies, which look at gene expression in selected regions of interest, or across the whole genome. Each of these strategies has been added to the understanding of genetics in AD.[29]

Up to present, there have been 5 genome-wide linkage studies performed on AD, plus a genome-wide linkage screen originally designed for asthma with analyses repeated for the AD outcome (Fig 1). All but one of these screens were performed on families of European ancestry: (1) 199 German and Scandinavian (2) 148 British (3) 109 Swedish (4) 100 Danishand (5) 295 Frenchfamilies, of which 62 affected sib pairs for AD were available for reanalysis. The non-European study was performed on 77 Japanese families selected through 111 sib pairs with AD

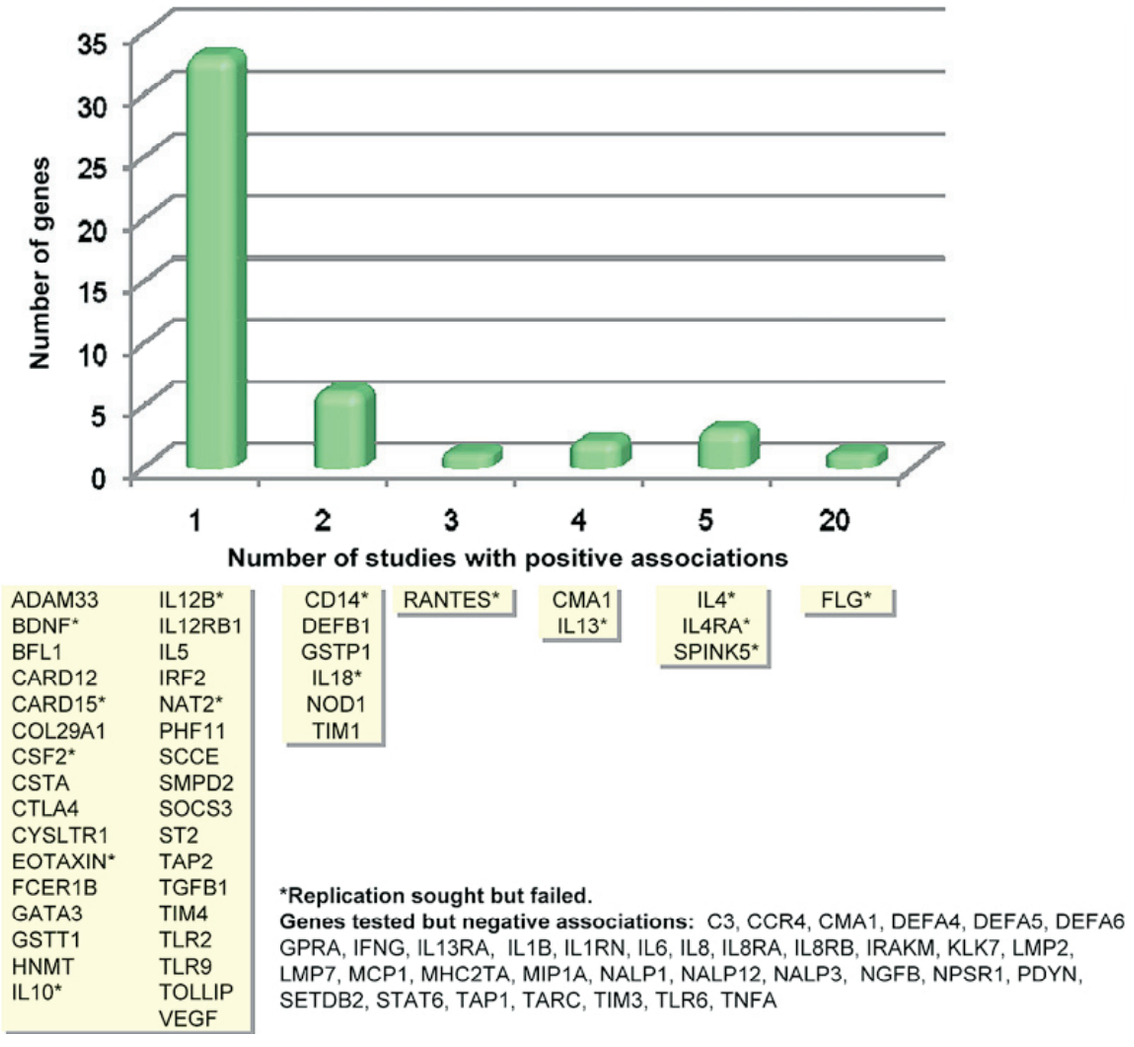

Figure 2. Genes associated with AD in at least 1 published study. Genes are grouped according to how many positive association studies have been reported. The y-axis indicates the number of genes (corresponding to the yellow boxes) for each time that a positive association was reported.[30]

(287 individuals) and relied on a linkage mapping panel of 5,861 single nucleotide polymorphisms (SNPs) rather than the microsatellite panel traditionally used for linkage screens. In the Japanese families, demonstrated linkage at two locus on chromosome 1 and 15 didn't show replication with other studies.[30]

In a search of the public database in June 2009, 111 studies were identified for which results of the tests associated to AD were reported on a candidate gene. The major outcome was limited to AD as a qualitative trait or AD severity. From these 111 published studies, only 42 studies were performed in Asian populations. There are reports on 81 genes, of which more than half (46 genes) had at least 1 positive association study reported (Fig 2). Of these 46 genes, 15 studies failed to replicate associations, and 13 were positively associated in at least 1 other independent study. One of these genes, FLG, has been associated with AD in 20 different reports. There are 35 additional genes studied for which there has been no evidence for a positive association to date.[30]

ADAPTIVE AND INNATE IMMUNE RESPONSE GENES

One approach toward distinguishing potential genes interactions and systematically evaluating the role of candidate genes/polymorphisms in AD susceptibility, for which there is compelling evidence for association, is to implement the program Ingenuity Pathways Analysis (Ingenuity Systems, Inc, Redwood City, Calif; www.analysis.ingenuity.com). As a proof of the concept, Barnes (2010) evaluated the 81 genes summarized in Fig 2 using the Ingenuity Pathway Analysis. There were slightly more than half (n = 48) of the 81 genes studied clustered in 2 major networks, both of which were associated with immune dysregulation, specifically the pathway which is associated with antigen presentation and cell-mediated and humoral immune response, the pathway associated with cell signaling and interaction cellular movement. Six genes (monocyte differentiation antigen CD14 [CD14], GATA-binding protein 3 [GATA3], interleukin 4 [IL4], IL18, nucleotide-binding oligomerization domain 1 [NOD1], and Toll-like receptor 2 [TLR2]), which had previously been significantly associated with AD, were clustered into the antigen presentation and immune response pathway. Meanwhile, 9 previously associated genes (BCL2-related protein A1 [BCL2A1], brain-derived neutrophilic factor [BDNF], Regulated upon Activation,Normally T-Expressed, and presumably Secreted [RANTES], colony stimulating factor 2 [CSF2], glutathione S-transferase P 1 [GSTP1], IL5, interleukin 12 beta [IL12B], interleukin 12 receptor beta 1 [IL12RB1], and suppressor of cytokine signaling 3 [SOCS3]) were clustered into the cell-signaling/movement pathway. Although the studies for which those candidates genes were evaluated did not specifically tested for genes interaction. This investigation potency serves as an example of the power of this approach in selecting optimal candidates for genetic association studies.[30]

In Asian population, there were three genes explored from the antigen presentation and immune response pathway (CD14, IL4 and IL18) and six genes from the cell-signaling/movement pathway (BDNF, RANTES, CSF2, IL5, IL12B, IL12RB1), from total of 42 studies. Barnes presented significant correlation between IL4 and IL18 and AD in Asian population. And also discovered significant correlation between BDNF, RANTES, IL5, IL12B and IL12RB1 in Asian population (Japanese, Chinese and Korean, table 1). Due to the minimal amount of studies study, these results have a limitation to make a conclusion about the role of SNP in there genes of AD from Asian population.[30]

On account of the historical isolation and environmental positive selections, major racial/ethnic groups differ in allele frequencies.[31] Consequently, ethnic differences may contribute to the variations in allele frequencies of phenotype-associated genes in humans. The CD14 protein, which is the receptor for lipopolysaccharides (LPS) and other bacterial wall-derived components, has been suggested to play a critical role in Th1 differentiation of naive T cells, with the likelihood of developing a T helper 1 (Th1)-type response by up-regulating the expression of IL-12, which in turn decreases IgE-mediated immunity.[32]Zhang reviewed the CD14C-159T SNP in the promoter region of CD14 in asthma population. There are significant variations in allele frequencies in different ethnic groups, summarizing that C allele frequencies on the highest in those of African descent, followed by Caucasians and Asians. This indicates that there are significant genetic variations between these ethnic groups in terms of predominant alleles and linkage disequilibrium structure of the SNP CD14C-159T. It is generally approved that the ethnicity of study subjects is a key factor in interpreting genetic polymorphism studies. In AD, there is an inconsistent result of studies of CD14C-159T SNP. There is significant association in American and German population, but not in German and Chinese population. Therefore, we still could not make a conclusion about the role of SNP CD14C-159T in AD from Asian population.[33]

SKIN BARRIER DYSFUNCTION GENES

It is increasingly appreciated that both genetic and environmental factors that affect skin barrier function contribute to AD susceptibility, as well as barrier dysfunction which is an essential feature of AD. A disrupted barrier would allow penetration of microbes and allergens and other environmental insults, such as toxins, irritants, and pollutants, with consequences such as inflammation, allergen sensitization, and bacterial colonization. Although the epidermis functions as the primary defense to the external environment, considerably barrier function is regulated by the SC and by the tight junctions (TJ), which reside at the level of the stratum granulosum. When the SC is compromised, either by the reduced levels of SC lipids, mechanical trauma resulting from extensive scratching that

is precipitated by intensive itch (the hallmark of AD), or as a result of genetic defects in SC proteins (ie, FLG), TJs are the next line of defense. Linkage screens performed on AD to date have not elucidated specific candidate genes per SC, but they have implicated loci harboring clusters of genes associated with skin barrier dysfunction. Specifically, one of the earliest screens indicated linkage at the epidermal differentiation complex (EDC) locus on chromosome 1q21, which contains a very large and diverse family of genes associated with skin barrier dysfunction, including loricrin, involucrin, members of the S100 gene family, and most notably, FLG.[34]Association studies on genes related to the EDC cluster and other barrier dysfunction candidates have been restricted to FLG, also known as filament-aggregating protein, and within FLG, most associations have been limited to 2 null mutations (R501X and 2282del4). In fact, FLG is the most consistently associated gene with risk of AD, as shown in Fig 2, by mid-2009, there were 20 positive reports on genetic associations between FLG mutations and AD.[35] The gene encoding human FLG was firstly cloned in 1989, when it was found to contain numerous tandem FLG repeats localized in chromosome 1q21, and because of its tight regulation at the transcriptional level in terminally differentiating epidermis, it was postulated to be an important candidate for disorders of keratinization.[36]It was subsequently evaluated for its function in the formation of the SC and found to be a critical protein involved in epidermal differentiation and in maintaining barrier function.[37] Full sequencing of the FLG gene has revealed multiple additional polymorphisms with varying frequency across the ethnic groups. However, with a combined allele frequency among patients with AD of 18% and 48% for the R501X and 2282del4 mutations respectively, the 2 null mutations represent the strongest and most compelling genetic risk factors for AD.[38]In the largest meta-analysis performed, that on the R501X and 2282del4 mutations, Rodriguez analyzed data from 24 independent studies, which included 6,448 cases, 26,787 control subjects, and 1,993 families (all selected forAD) and determined that the effect size's risk effect of eczema caused by the 2 FLG null mutations was not dissimilar to previous reports at an OR of just over 3.[39]

Other candidate genes showing somewhat comparable OR include IL-4, OR 1.88, $p = 0.01$[40]; IL-13, OR 1.7, $p = 0.03$[41] and relative risk (RR) 2.5, $p = 0.014$[42]; and mast cell chymaseOR 2.17, $p = 0.009$.[43]

Brown reviewed about genotype-phenotype correlation between FLG and AD, showed the phenotype of early onset (before the age of 2 years), persistent, and severe AD has shown the strongest and the most highly significant statistical association with the combined FLG null genotype, having an OR of up to 7.7 (95% CI 5.3–10.9).[44,45,46]

In Asian population three studies have been conducted to explore the SNP of FLGin AD from Japanese, all of these studies showed positive association (table 1). Further research are required to establish this finding.[30]

EPIGENETIC

Epigenetics is the study of mitotically heritable changes in phenotype (alterations in gene expression) that occur without direct alterations of the DNA sequence. These epigenetic changes include methylation of DNA by the covalent addition of a methyl group to a cytosine residue in a CpG site; posttranslational modification of the amino acid tails of histones by means of acetylation, phosphorylation, methylation and aberrant expression of microRNAs (miRNAs), each of which is capable of posttranscriptionally regulating the expression of a cohort of cognate target genes.[47]

DNA methylation requires the activity of DNA methyltransferases (DNMTs).The mechanism of DNA demethylation is less clear. Loss of binding to methylated DNA-binding proteins might allow the promoter to enter a transcriptional state.[47]

AD is a complex disease for which the risk is convinced to be determined by a complicated interplay of genetics and environmental exposures that have been discussed and debated for many years. The recent understanding of epigenetics as a mechanism mediating gene-environment interaction offers new opportunities to advance novel concepts and re-examine established ones about this disease.[29]

Ho reviewed regarding environmental epigenetics of asthma, including the epigenetic effects of tobacco smoke, microbial allergens, oxidants, airborne particulate matter, diesel exhaust particles, polycyclic aromatic hydrocarbons, dietary methyl donors and other nutritional factors, and dust mites. The discovery and validation of epigenetic biomarkers linked to exposure asthma, or both might lead to better epigenotyping of risk, prognosis, treatment prediction, and development of novel therapies.[47]

In AD, environmental factors, possibly react through epigenetic mechanism, and it may contribute to disease pathogenesis. In support of this, DNA Methyltransferase (Dnmt1) transcripts in peripheral blood mononuclear cells of AD patients with high IgE levels are significantly lower than the control.[48] The effect of reduced Dnmt1 level on IgE may be indirect, with DNA hypomethylation of T cells resulting in their increase production of IL4, which then stimulates IgE production by B cells.[49,50] Enhanced immune response at epidermal surfaces, caused by DNA hypomethylation of methylation-sensitive immune genes, along with the activation of the genes to critical to T/Bcells interactions and inflammation, could initiate a sustained immune response.[51]

Ho also reviewed the trigger factos that influence epigenetics of asthma. That finding in agreement with previous studies reporting that Benzopyrene(BaP)is able to decrease global DNA methylation, inhibit Dnmin vitro, and interfere with recruitment of the methylation machinery. BaP is frequently used as a prototype Polycyclic Aromatic Hydrocarbon (PAH) for many experimental studies. In

Asthma, (PAHs) are one of the most widespread classes of pollutants of the environment and in food. They are present in crude oil, coal, and tar deposits and are derived from incomplete combustion of fossil fuel, oil, garbage, and cigarettes. They are major components of airborne particulate matter (PM) of urban aerosols and widely exist in food products, including grains, vegetables, oils, and fats. PAHs are emitted to the air during the production of coke and aluminum. Cooked meats are contaminated when they are charcoal grilled, roasted, or smoked.[47]

The trigger factor that could reduce Dnmt1 in AD is still unknown, it is possible that BaP has a role in AD as stated above, further studies are required.

SUMMARY

An important factor contributes to the failure of reproducing associations between genetic markers and a complex trait, such as AD, in independent populations is also related to population diversity. It is possible that certain genetic markers might contribute to disease risk in a particular (ie, ethnic or racial) population but not in others, either because of differences in frequencies of the risk alleles or because of specific genes interactions. It is still difficult to evaluate the effect of ethnicity on genetic associations of AD in Asian population, because however, there is relatively little diversity in the populations that have been studied.

FLG is probably the best example of a candidate gene for which ethnicity likely influences the extent to which a polymorphism confers risk. In relevance to clinical practice, individuals with FLG insufficiency are at increased risk of severe and persistent AD, which future therapy may place greater emphasis on barrier repair for this subgroup of patients. AD is a disease in which pharmacogenetics may facilitate the development of primary prevention and treatment regiments tailored according to the individual genetic predisposition in Asian population.

Table 1. Genes associated with atopic dermatitis in at least one study based on ethnicity in Asian population[30]

Gene	Chromosomal location	Variant	Association	Population	No. of subjects*
ADAM33	20p13	rs2853209	Yes	Japanese	140/258
BDNF	11p13	C270T	Yes	Chinese	160/169
CCR4	3p24	C1014 T	No	Japanese	198/183
CD14	5q31.1	C-159T/C-260T	No	Chinese	171/160
			No	Chinese	113/67
CMA1 (MCC)		BstXI	Yes	Japanese	100/100
			Yes	Japanese	145/706
			No	Japanese	100/101
			Yes	Japanese	169!
CSF2	5q31.1	T3606C	No	Japanese	181/100
DEFA4	8p23.1	G-6298C	No	Korean	631/458
DEFA5	8p23.1	G-2819A	No	Korean	631/458
DEFA6	8p23.1	G-4844A	No	Korean	631/458
DEFB1	8p23.1	T-2266C	Yes	Korean	631/458
EOTAXIN(CCL11)	17q21.1-q21.2	C-426 T	No	Japanese	140/140
			No	Japanese	140/140
		A-384 G	No	Japanese	140/140
			No	Japanese	140/140
		G67A	No	Japanese	140/140
			No	Japanese	140/140
FLG	1q21.3	R501X, 3321delA, S1695X, Q1701X, S2554X,			
S2889X, S3296X	Yes	Japanese	118/134		
		S2554X	Yes	Japanese	105 families, 376/923
		S2554X, S2889X, S3296X, and 3321delA	Yes	Japanese	125/133
IFNG	12q14	STR at first intron	No	Chinese	94/186
IL4	5q31.1	C-589T (C-590 T)	Yes	Japanese	88 families
			No	Japanese	302/122
			No	Chinese	94/186

Table 1 Continuation.

Gene	Chromosomal location	Variant	Association	Population	No. of subjects*
		C-3112 T	Yes	Japanese	202/150
		T33C	No	Chinese	94/186
IL4RA	5p13	C-703 T	Yes	Japanese	451/116
		Ile50Val	No	Japanese	27/29
			No	Japanese	302:122
		A184G	No	Japanese	202/150
		G186A	Yes	Japanese	
		A326C	Yes	Japanese	
		C327A	Yes	Japanese	
		Glu375Ala	No	Japanese	27/29
			No	Japanese	302/122
		E375A	No	Chinese	94/186
		L389L	No	Chinese	94/186
		Cys406Arg	No	Japanese	27/29
		C406R	No	Chinese	94/186
		S503P	No	Chinese	94/186
		Glu 551Arg	Yes	Japanese	27/29
			No	Japanese	302/122
		Q576R	No	Chinese	94/186
		T1803C	Yes	Japanese	202/150
		C3112 T	Yes	Japanese	202/150
IL5	5q31.1	24597T/A	Yes	Korean	646/474
IL5R	3p26-p24	28380C/A	No	Korean	646/474
IL8	4q12-q13	2352A/T	No	Korean	646/474
IL8RA (CXCR1)	2q35	3047C/T	No	Korean	646/474
IL8R (CXCR2)	2q35	L262L	No	Korean	646/474
IL10	1q31-q32	A-1082 G	No	Korean	276/140
			No	Chinese	94/186
		T-819C	Yes	Korean	276/140
			No	Chinese	94/186
		A-592C	Yes	Korean	276/140
			No	Chinese	94/186
IL12B	5q31.1-q33.1	A1188C	Yes	Japanese	164/100
		G4237A	No	Chinese	94/186
IL12RB1	19p31.1	A-111 T	Yes	Japanese	382/658
IL13	5q31	C-1112T	No	Chinese	94/186
		A704C	No	Japanese	185/102
			No	Japanese	185/102
		C1103T	No	Japanese	185/102
			No	Japanese	185/102
		G4257A	Yes	Japanese	185/102
			Yes	Japanese	185/102
		G4464A	No	Chinese	94/186
		T7488C	No	Japanese	160/103
IL18 (IGIF)	11q22.2-q22.3	T113G	Yes	Korean	646/474
		G2137C	No	Japanese	160/104
IRF2	4q35.1	C-829T (C-830 T)	No	Japanese	49 families
LMP2	6p21.3	LMP2*R	No	Korean	53/184
LMP7	6p21.3	LMP7*A	No	Korean	53/184
MIP1A (CCL3)	17q12	C954T	No	Japanese	39/65
RANTES (CCL5)	17q11.2-q12	G-401A	Yes	Japanese	62/14
			Yes	Japanese	389/177
			Yes	Japanese	389/177
SMPD2	6q21	Haplotype	Yes	Korean	284/248
SPINK5 (LEKTI)	5q32	G-206A	Yes	Chinese	669/711
		Asp106Asn 41	No	Japanese	families

Table 1 Continuation

Gene	Chromosomal location	Variant	Association	Population	No. of subjects*
			Yes	Japanese	124/110
			Yes	Japanese	41 families
			No	Japanese	124/110
			No	Japanese	41 families
		His396His	Yes	Japanese	124/110
		Glu420Lys	Yes	Japanese	124/110
			Yes	Japanese	118
			Yes	Japanese	41 families
		Glu420Lys	No	Chinese	669/711
		Glu825Asp	No	Japanese	41 families
		A1103G	No	Chinese	669/711
		G1156A	No	Chinese	669/711
		G2475 T	No	Chinese	669/711
		IVS12-26C/T	Yes	Japanese	124/110
		IVS12-10A/G	Yes	Japanese	124/110
		IVS13-50G/A	Yes	Japanese	124/110
		IVS14119G/A	Yes	Japanese	124/110
ST2	11p14.3-p12	A-27639G	No	Japanese	452/636
STAT6	12q13	STR at exon 1	No	Chinese	94/186
TAP1	6p21.3	TAP1*A	No	Korean	53/184
TAP2	6p21.3	TAP2*A	Haplotype	Korean	53/184
TARC (CCL17)	16q13	C-431T	No	Japanese	193/158
		C2134T	No	Japanese	148/158
TIM1	12q12-q13	5383_5397del	Yes	Korean	112/201
TNFA	6p21.3	G-238A	No	Chinese	94/186
		G-308A	No	Chinese	94/186
		C-857 T	No	Chinese	94/186
		C-863A	No	Chinese	94/186
		T-1031C	No	Chinese	94/186

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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

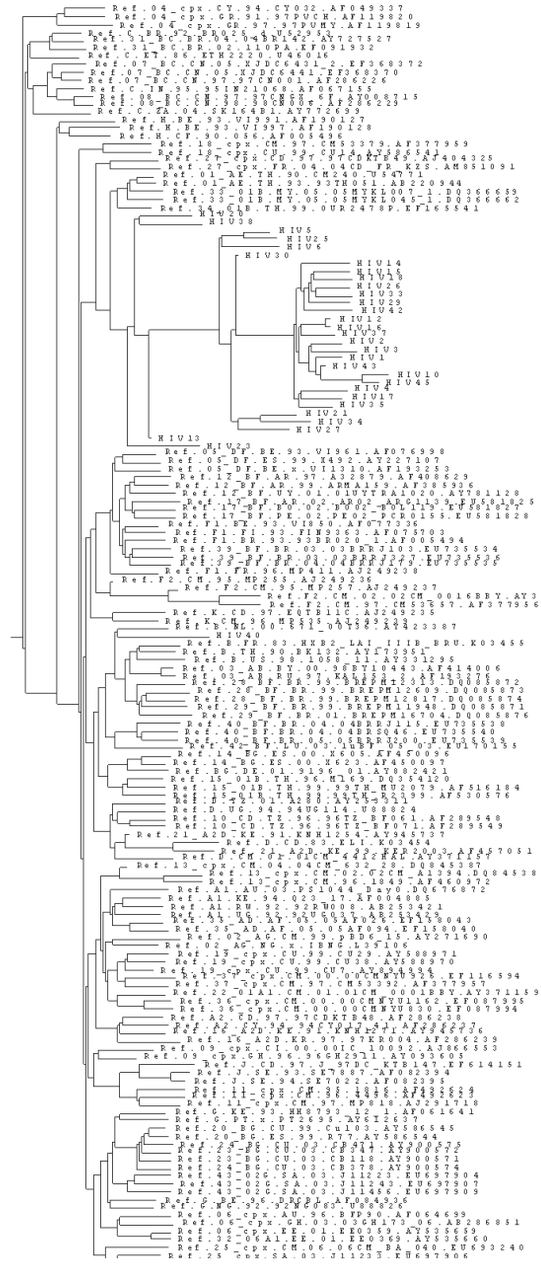

Figure 1. Tree neighbor-joining phylogenetic models of gp120 env sequences of HIV samples and reference

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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Research Report

TUBERCULOSIS COUNTER (TC) AS THE EQUIPMENT TO MEASURE THE LEVEL OF TB IN SPUTUM

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis. This disease is the third killer disease after cardiovascular diseases and respiratory diseases, and is also he number one killer disease in a group of infectious diseases. This is partly due to the late handling and a non real time detection, both of which will inhibit the therapy which yields a large number of microorganisms in the body, and will eventually complicate the recovery. Based on this phenomenon, we offered an alternative solution for detecting the sum of microorganism using Tuberculosis Counter, a tool used to count the number of Tuberculosis bacteria in the patient's sputum. Technically, the patient's sputum preparat was screened using the TCS230 color sensor that was able to filter the color of the preparat. Tuberculosis bacteria in the stained sputum Ziehl-Niellsen preparat was colored red, while the other was colored blue. By utilizing these optical phenomena, the TCS230 color sensor was supposed to filter the red color in the preparat. By using regression equation measurement, we gained the equation which then correlated the bit value as an output of the sensor with the number of Tuberculosis bacteria. Then, the digitalization process yielded the real time and accurate data of Tuberculosis bacteria.

Keywords: *Tuberculosis, Ziehl-Nellsen staining, TCS230 color sensor, counter of bacteria, sputum*

ABSTRAK

Latar Belakang: *Tuberkulosis (TB) adalah penyakit menular yang disebabkan oleh Mycobacterium tuberculosis. Penyakit ini adalah penyakit pembunuh ketiga setelah penyakit kardiovaskuler dan penyakit pernafasan, serta nomor satu penyakit pembunuh dalam kelompok penyakit menular. Hal ini disebabkan sebagian karena penanganan yang terlambat dan tidak mampu mendeteksi masa inkubasi secara tepat Hal tersebut dapat menghambat terapi, sehingga jumlah mikroorganisme dalam tubuh tinggi sehingga mengakibatkan sulitnya pemulihan.* ***Tujuan;*** *Menciptakan alat untuk menghitung jumlah bakteri TBC dalam dahak penderita.* ***Metode:*** *Secara teknis, para preparat dahak penderita akan diputar dengan menggunakan sensor warna TCS230 yang mampu menyaring warna preparat tersebut. Tuberkulosis bakteri dalam preparat Ziehl-Niellsen dahak bernoda akan berwarna merah, sementara yang lain berwarna biru. Dengan memanfaatkan fenomena optik, sensor warna TCS230 akan disaring merah dalam preparat menggunakan gejala optik TCS230 sensor warna akan menyaring warna merah di preparat tersebut. Menggunakan pengukuran persamaan regresi, akan memperoleh nilai persamaan yang akan berkorelasi sebagai output dari sensor dengan jumlah bakteri Tuberkulosis. Kemudian proses digitalisasi akan menghasilkan real time (masa inkubasi) dan data yang akurat dari bakteri Tuberkulosis.* ***Hasil:*** *Prototype alat penghitung jumlah bakteri TBC dalam dahak penderita.*

Kata Kunci: *Tuberkulosis, Ziehl-Nellsen pewarnaan, TCS230 sensor warna, counter bakteri, sputum*

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. *World Health Organization* in 2005 estimated that the number of TB patients was 8,8 million and 1,6 million of them ended in death. TB itself is placed the third among diseases in Indonesia after cardiovascular and airway canal diseases and the first among diseases which cause death in the type of infectious diseases. Indonesia itself is the third country with most TB problems in the worlds, with the death number of one person every 5 minutes. In 2004, 211.753 new cases of TB in Indonesia had been noted and approximately 300 deaths due to TB every day were also noted. New cases of TB in Indonesia always rise to 250 million cases per year.[1]

Based on the TB combat program in Middle Java, it has been shown that the number of TB case findings in 2006 had not reached the target which was 53%.[2] Many kinds of TB therapy have been used including routine standard therapy based on the Ministry of Health's program. But somethimes the therapy do not work well due to a variety of reasons, one of the main reasons is people's poor knowledge of TB. It was considered that even though the therapy has been taken for a long time, the disease has not been cured yet.[11] Long therapy would pase a big physiological pressure to the patients. The patients would feel worried that their disease would never heal or become even worse. This excessive anxiety would also trigger other diseases to occur.

Based on the observation of the case handling which was focused on unspecified physical symptoms in patients, we offer *Tuberculosis Counter* (TC), a diagnostic tool which is capable of counting the number of TB bacteria in sputum in order to provide data of the TB degree of severity so that the clinicians could diagnose the disease in *real-time condition*. By using Ziehl-Nellsen staining in patient sputum, the red one produced by Acid Fast Bacillus (AFB) was filtered and processed digitally then and yielded *real-time* and *up-date*.

MATERIAL AND METHOD

Tuberculosis is on infectious disease which attacks lungs, and caused by *Mycobacterium Tuberculosis*. *Mycobacteria* are aerob bacteria, rod-shaped, and have no capability to produce spores. They are difficult to be colored. If these bacteria have been colored, They will stand to laxative color such as acid or alcohol. There fore they are called acid-resistant bacteria or acid-resistant basil.[3]

TCS230 color sensor is a chip color sensor which works by conversing the acceptance of light emission from certain colors in the form of frequency. TCS230 color sensor is composed of 2 main parts which are acceptance light parts in the form of array photodiode and light converter to frequency. In general, TCS230 color sensor is a light sensor which is complemented with a light filter for basic color RGB (Red-Green-Blue) and a light sensor without a filter in 8 bit scale in each sensor part. Photodiode in TCS230 color sensor extracted the current of which the magnitude of the flow is comparable to basic color of light overwriting. This current then was converted into a pulse shape with a frequency comparable to the magnitude of the current. The output frequency of the TCS230 color sensor could be made as a scale by arranging data configuration S0 and S1 in selector pin S0 and S1 of TCS230 color sensor.[4]

Figure 1. TCS230

Figure 2. IC Microcontroller

Microcontroller is a digital electronic equipment which has on input, on output and a controller with a program which could be written and deleted with special manners. Microcontroller is a computer inside a chip which could be used to control electronic equipment so that it could be efficient and effective in cost. Electronic system usually needs many supporting components such as IC, TTL and CMOS. It could be reduced or minimized. It also has been focused and controlled by this "tiny controller" so that this electronic system could be compendious, faster, easy to modify and the distraction finding could be easier because the system is compact.[5]

RESULT AND DISCUSSION

The making process of the *Tuberculosis Counter* equipment prototype took two months. In the preliminary study, the literature exploration and the experts' statements, indicated that it had been well known that Tuberculosis

bacteria could be detected chemically using Ziehl-Niellsen staining. Acid-resistant bacteria of TB was red in color and the others were be blue in color. From the optical symptoms, then the screening process using TCS230 color sensor had to be done to count and excute a certain calibration.[6,7] Based on the *image*, it would be difficult to differentiate TB bacteria from other artifact that it needs some special ability regarding sputum staining.

From the instrumentation scheme, the sputum of TB patient which had been coloured by Ziehl-Nellsen method was previewed by the camera in the microscope and then previewed on an LCD. TCS230 color sensor was placed above the LCD to gain color bit value on the LCD.[8] The result of output bit value was produced by the bit plot of the number of bacteria in each viewing area. The plot result was analyzed by regression to gain the regression equation which was converter bit formula of the number of bacteria in each viewing area. The formula was inserted into the chip as on analog data processor from the sensor.[9,10,11]

A conversion was performed to compare output bit sensor with the number of bacteria in the preparat. This conversion was performed by making a plot between two variables so that regression equation which would be used in the chip should be obtained. Calibration test result of this equipment was compared the conventional test. The conventional test was performed by paramedical staff by observing the object inb the microscope and counting them per viewing area whereas this equipment technique had the same procedures as those with a conventional manner. The difference between the two methods was only the bacteria counting manner, which sused sensor and the length of detection time which was shorter than that of the conventional way. In conclusions, the Tuberculosis Counter (TC) equipment utilized the optical symptoms of Tuberculosis bacteria by using Ziehl-Nielssen staining, and the processing utilized TCS230 color sensor. The benefit of the utilization of TB Counter was to provide the real time and accurate data of the number of Tuberculosis bacteria.[12]

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Case Report

BRADYCARDIA AND TACHYCARDIA DETECTION SYSTEM WITH ARTIFICIAL NEURAL NETWORK METHOD

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ABSTRACT

Heart disease is one disease with high mortality rate in the world. Based on WHO records from 112 countries at 2004, the rate is 29% of all deaths each year. Medical devices are necessary to diagnose one's health as an indication of a disease. Nowadays, Indonesia still imports medical devices, for the diagnosis of heart failure, from abroad. This research aims to assist the monitoring of cardiac patients with bradycardia and tachycardia appearances of message condition patient's heart rate at the same time. The results were displayed with the output of bradycardia condition of the heart rate (heart rate less than 60 beats per minute) or tachycardia (heart rate over 100 beats per minute). The system displayed the data read from the heart to the PC embedded system to monitor the condition of the patients under decisions based on backpropagation neural network. Classification system could be performed quite well, training data and by testing the 10 pieces, the optimal weight gain was 1727 iteration, the learning rate was 0.1122, and the error was below 0.001 (0.0009997).

Keywords: *Heart Rate, Heart, tachycardia, bradycardia, Backpropagation*

ABSTRAK

Latar Belakang: *Penyakit jantung adalah salah satu penyakit yang memiliki angka kematian tinggi di dunia sebesar 29% kematian global setiap tahun, Perhitungan ini didasarkan pada catatan kematian dari 112 negara pada 2004 dari data WHO (World Health Organization) (Rusciano, 2004). Penggunaan alat medis sangat diperlukan untuk diagnosa kesehatan seseorang sebagai indikasi adanya penyakit. Saat ini Indonesia masih mengimpor alat-alat medis tersebut, termasuk untuk diagnosis gagal jantung.* ***Tujuan:*** *untuk membantu pasien penyakit jantung dalam pemantauan bradikardi dan takikardi dengan tampilan berupa pesan kondisi denyut jantung pasien saat itu.* ***Metode:*** *Hasil yang didapat akan ditampilkan dengan keluaran berupa kondisi denyut jantung yaitu bradikardi (denyut jantung kurang dari 60 kali per menit) atau takikardi (denyut jantung lebih dari 100 kali per menit). Sistem melakukan pembacaan data jantung dari sistem embedded ke PC untuk memonitoring kondisi penderita dengan keputusan berbasis jaringan saraf tiruan backpropagation.* ***Hasil:*** *Sistem dapat melakukan klasifikasi dengan cukup baik, dengan data pelatihan dan pengujian masing-masing 10 buah, memperoleh bobot yang optimal pada iterasi ke 1727, learning rate sebesar 0.1122 dan error di bawah 0.001 (0.0009997)*

Kata Kunci: *Heart Rate, Jantung, Takikardi, Bradikardi, Backpropagation*

INTRODUCTION

Heart is one of the most vital organs. The impaired cardiac function greatly influences other organs, especially kidneys and brains. The main function of heart as a single pump is to pump blood foward the entire body to provide nutrition for the metabolism of the survival cells. Internally, heart is separated into two parts, the right side of the heart functions as a blood pump to the lungs and the left side of the heart pumps blood foward the entire body. At each - there are two halves of the heart chambers of the heart. Blood from each atrium is sent to the ventricles. The blood from the right ventricle flous to the lungs at the pump - lung and blood from the left ventricle flous through the body in the pump. It is still considered normal that heart atrium contracts for approximately six seconds

trillionth preceeding the ventricular contraction, allowing ventricular filling before the ventricles pump blood to the lungs and the entire body. The contraction of the heart works automatically and is generated by electrical currents in the form of action potentials or cardiac conduction and controllable cardiac rhythm. Heart has a special system generated in cardiac conduction to the rhythmic electrical impulses which causes a rhythmic contraction of the heart muscle called the heart rhythm. It sendsaction potentials through the heart muscles toword the heart[1]. As cardiac impulse flows the heart, the electrical current will spread into the tissues surrounding the heart and a small portion of the flow will be spreaded to the body surface. Heart is depicted in Figure 1.

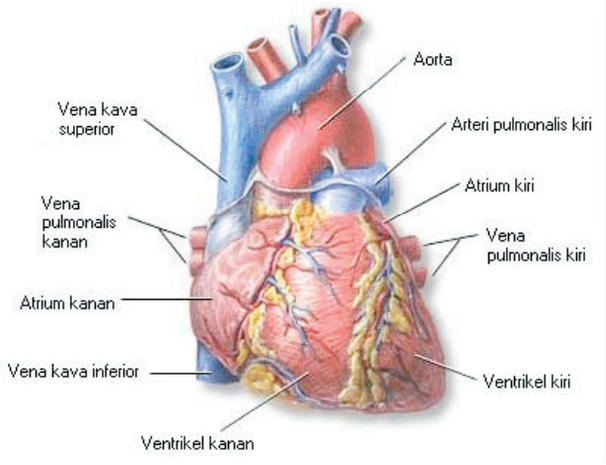

Figure 1. Heart[14]

The direction of the conduction of heart is Sinotrial (SA) node towards Atriventricular (AV) node, then to the bundle of his and branched into the left bundle and the right bundle branches. Left bundle branch's impulses are sent to the left the ventricle, and those of the right bundle branch are sent to the right ventricle. Impulses proceed to the Purkinje fibers and a network of fibers they spread rapidly to the ventricular wall[3]. Cardiac conduction is associated with the amount of heart rate (heart rate) per minute. Heart rate is used as an indication of any abnormalities in the heart. Normal heart rate ranges from 60 to 100 times/min.

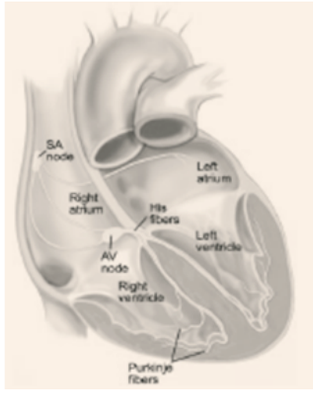

Figure 2. Cardiac Conduction Direction[3]

HEART ABNORMALITIES

Tachycardia

In normal circumstances, the electrical impulses are generated by a pacemaker called SA node. This electrical impulse is passed into the ventricle through an AV node, in which the node will be slowing down the flow of the impulses. The next impulse will spread throughout the ventricles.[2] Fast heart rate, called tachycardia, means that the heart rate exceeds 100 beats per minute. Tachycardia is divided into two main types: supraventricular and ventricular. The emergence of tachycardia is usually indicated by a shortness of breath or wheezing, rapid pulse, chest pain, cold sweat, and uncons ciousness. But in some people, tachycardia does not imply any symptoms.

Bradycardia

Bradycardia or bradyarrhythmias are terms used to indicate the presence of heart rhythm disorders and conduction that causes heart rate to be less than 60 beats/min. The onset of bradycardia is associated with a decrease or failure of impulse formation and of obstacles / interference electrical conductivity. Several causes of bradycardia are as follows:

a. Barriers SA node
This condition is relatively common in the elderly due to the failure of the sinus node impulse to spread to the atrium.

b. Barriers AV node
In this state atrial impulse is blocked in several places on its way into the ventricle.

c. Bundle Branch Block
The disorder is more common nowadays with increasing age in both branches of the left and the right. When encountered individually, these disorders are not dangerous, join what? It could be bad.

DESIGNING THE HARDWARE AND THE SOFTWARE

Designing the Hardware

Hardware design utilizes Arduiono. Arduino is an open-source single-board micro-controller, derived from the wiring platform and designed to facilitate the use of electronics in a variety of fields. The hardware arduino has on Atmel AVR processor while the software arduino has its own programming language

Figure 3. Arduino Board

This study made the hardware detect cardiac abnormalities "Tachycardia" and "bradycardia". These procedures were performed in several stages, namely, preparation of design diagramming tools, hardware design, and software design. The diagram tool is described in Figure 4.

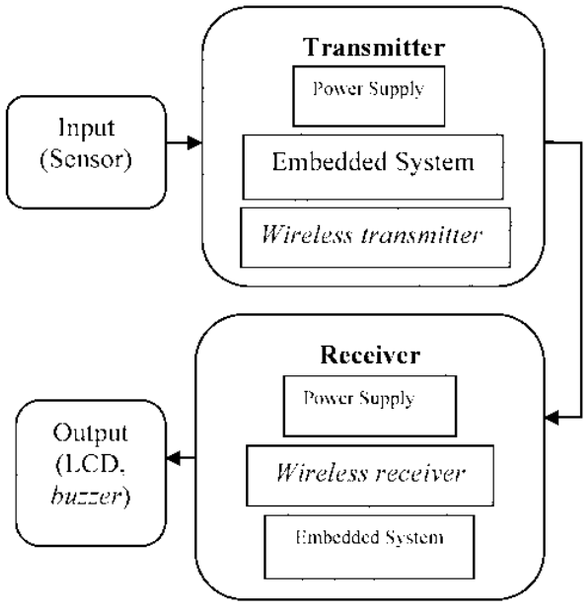

Figure 4. Hardware Diagrams

The explanation to Figure 4: in this study, the design of the sensor used is the Plethysmograph reflection mode as depicted in Figure 5 which depicts the installation of LED and LDR on the finger used as the heart rate detection sensors.

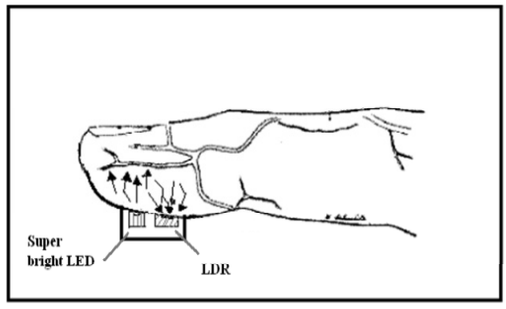

Figure 5. Sensor Plethsymograph.

In this study there are 2 microcontrollers as that functioned for the transmitter and the receiver. The transmitter circuit consisted of a sensor, a power supply, a wireless module and an Arduino Duemilanove. The first work of the transmitter was to detect the heart rate sensors on the fingers and to transmit the data to the wireless module which was enabled as the transmitter controlled by the microcontroller. Receiver circuit consisted of wireless modules that functioned as a receiver that received data from the transmitter and the data of which were processed by a microcontroller to be then displayed on the LCD with an output in the form of heart condition. The software design can be seen in Figure 6.

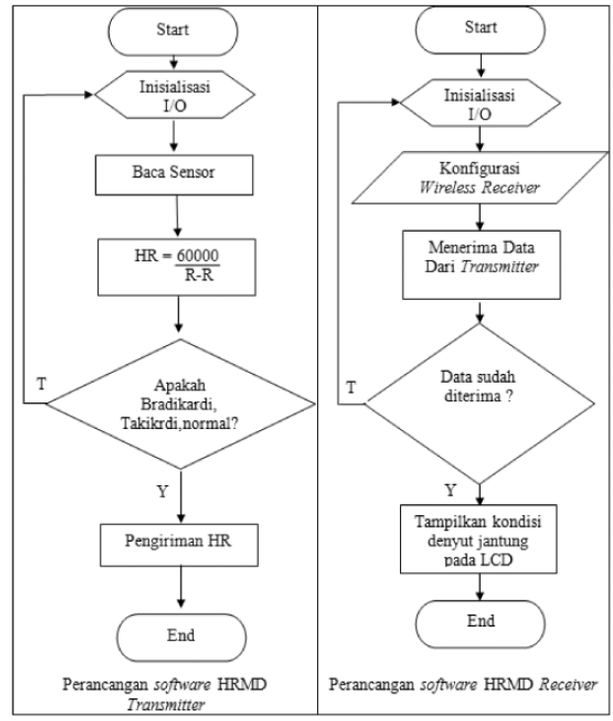

Figure 6. Software Diagrams

Design of Intelligent Network Backpropagation

Artificial neural networks is a system in which the computing architecture and its operation can be inspired from the knowledge of biological nerve cells in the human brain. Neural network is one of the artificial representation of the human brain. To find out more about the origin and how the structure of the neural network is created and can be used as a counter, these will be reviewed briefly by the terms that have generally been used. The structure in Figure 7 is the standard form of the basic units of the human brain tissue that has been simplified. The structure of this standard will change in the future if scientists can find a better standard form or improve the standard form used today. Human brain tissue is composed of 1013 pieces, each neuron is connected by about 1015 pieces of dendrites. The function of dendrites is to transmit signals from neuron to other neurons connected to it. As the output channel, each neuron has axons, while the signal receiver is called synapses.

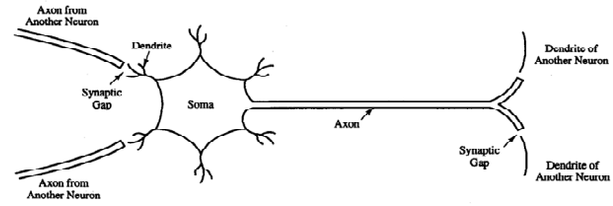

Figure 7. Simple structure a neuron.

In general, neural network is composed of one trillion (even more) neurons that are interconnected and integrated with each other by a trillion synapses so that they can carry out activities to store (memorize) knowledge regularly and continuously as needed.[6] Backpropagation is one of the unsupervised training methods (supervised learning) and is usually designed for operations on multi-layer neural network. According to Rumelhart's backpropagation method that has been applied widely, approximately 90% of backpropagation has been successfully applied in various fields, such as finance, pattern recognition handwriting,

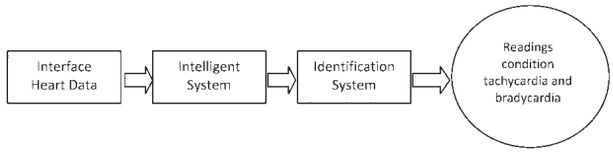

Figure 8. Diagram System

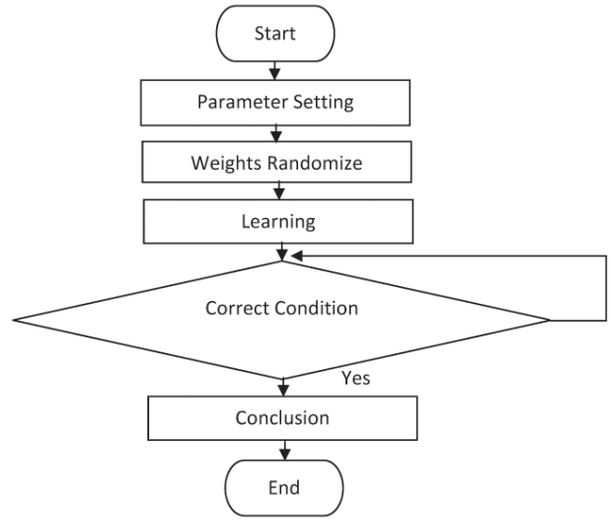

Figure 9. Software Flowchart

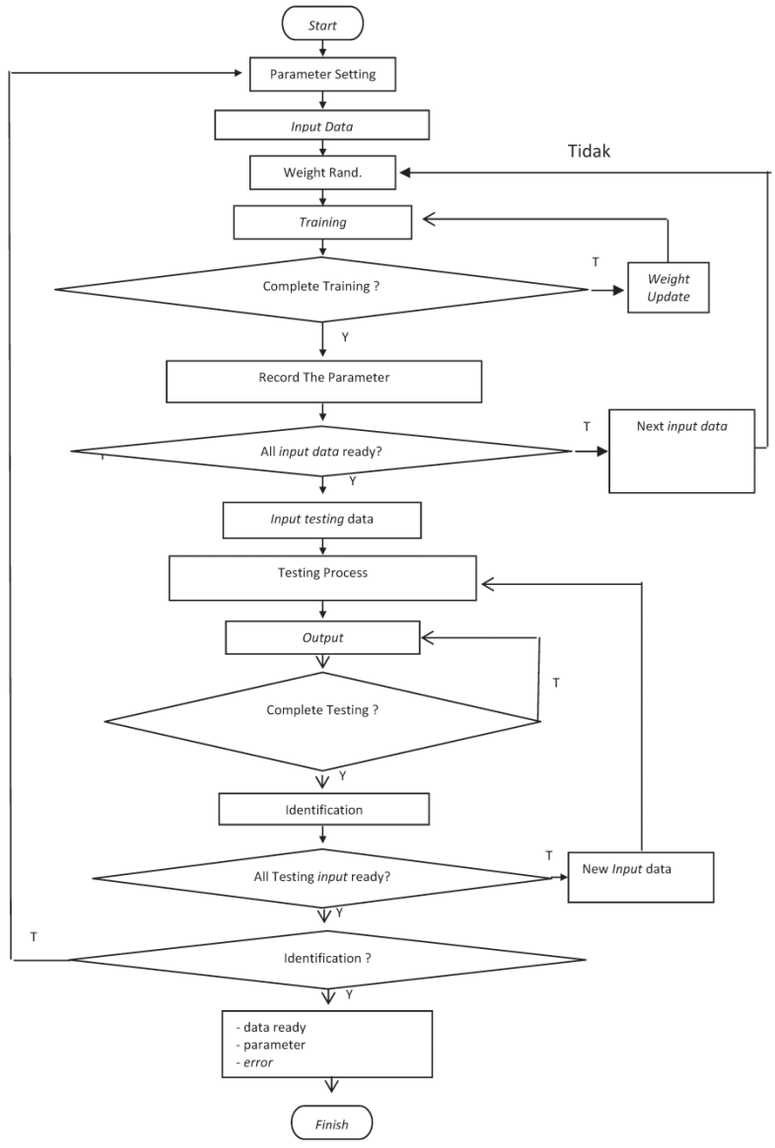

Figure 10. Neural Network Flowchart

voice pattern recognition and medical image processing. This algorithm has a training process that is based on a simple interconnection, for example, if the output gives the wrong result, it will be corrected so that the weight can be reduced and subsequent neural network response could be expected to detect the correct value better. Backpropagation is also capable of transforming and improving the weight of the hidden layer. The system was created as a diagram (Figure 8), the sensor started reading to determine the decision to backpropagation.

Software design cardiac abnormality detection system (bradycardia and tachycardia with a method of Backpropagation Neural Networks). Neural Networks were prepared using 3 pieces of object layers (one input layer, one hidden layer and one output layer). Input layer in a dynamic array was arranged according to the image

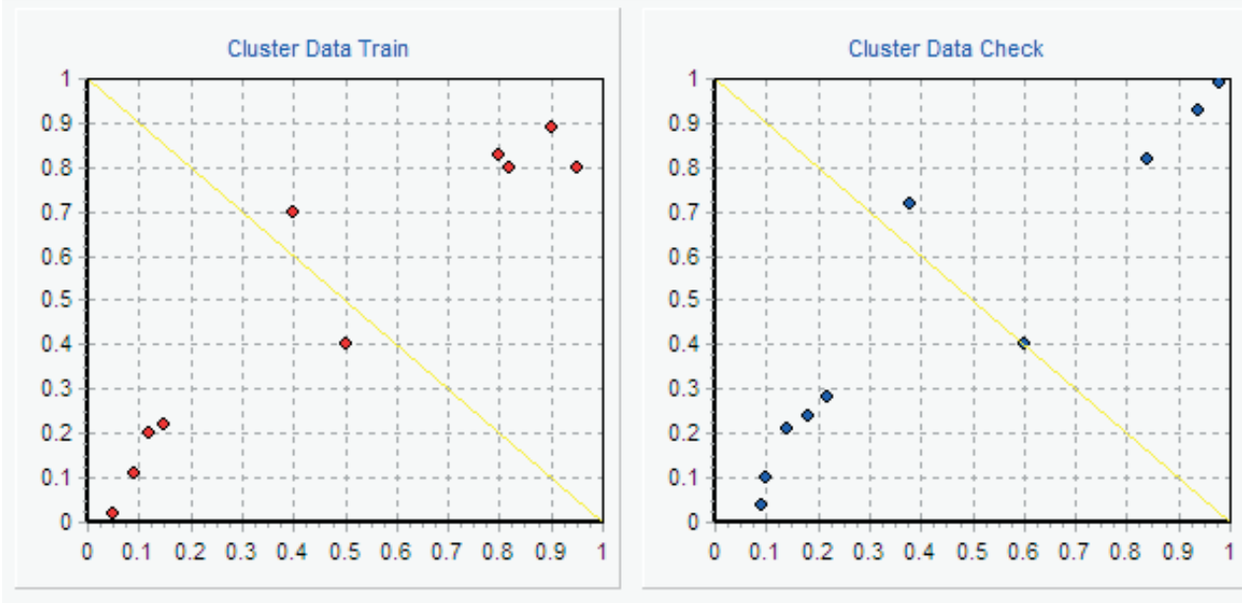

Figure 11. Data Training and Testing
Source: Intelligent System Theory[13]

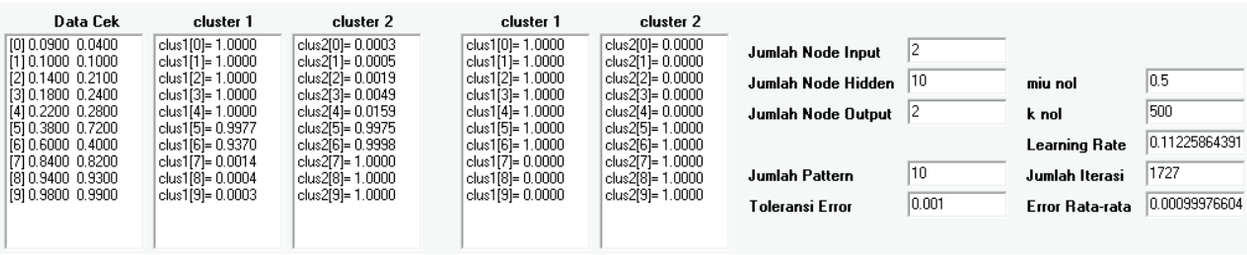

Figure 12. Results Classification of Heart Disease
Source: Intelligent System Theory[13]

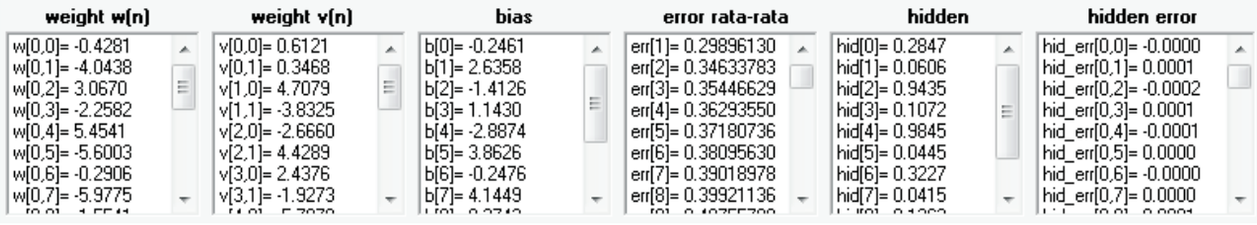

Figure 13. Weight Training Results
Source: Intelligent System Theory[13]

Table 1. Training Data Heart Disease

No	Condition Data		Clasification		Disease
1	0.05	0.02	1	0	Tachycardia
2	0.09	0.11	1	0	Tachycardia
3	0.12	0.2	1	0	Tachycardia
4	0.15	0.22	1	0	Tachycardia
5	0.4	0.7	1	1	Normal
6	0.5	0.4	1	1	Normal
7	0.8	0.83	0	1	Bradycardia
8	0.82	0.8	0	1	Bradycardia
9	0.9	0.89	0	1	Bradycardia
10	0.95	0.8	0	1	Bradycardia

Table 2. Test Data

No	Condition Data		Clasification		Disease
1	0.09	0.04	1	0	Tachycardia
2	0.1	0.1	1	0	Tachycardia
3	0.14	0.21	1	0	Tachycardia
4	0.18	0.24	1	0	Tachycardia
5	0.22	0.28	1	0	Tachycardia
6	0.38	0.72	1	1	Normal
7	0.6	0.4	1	1	Normal
8	0.84	0.82	0	1	Bradycardia
9	0.94	0.93	0	1	Bradycardia
10	0.98	0.99	0	1	Bradycardia

size of the object which roled as an input to the first layer. A number of output nodes were determined based on the number of characters that were supposed to be recognized. The number of nodes in the hidden layer was determined based on the experimental results. The diagram design procedure of Artificial Neural Network method is shown in Figure 9.

Software testing was performed using the type of feedforward Artificial Neural Networks. Artificial Neural Network weights that was going to be used was the value of the weight at the time of learning. Data from the heart to the PC embedded system were compared with the data of the patients who had identified cardiac abnormalities. When the error detection results compared with the data value was less than five percent, the detection result would succeed. The diagram learning procedure and the test methods of Artificial Neural Network are depicted in Figure 10.

RESULTS AND DISCUSSION

Testing of Backpropagation Neural Network

Tests were performed on intelligent systems by observing the system's ability to perform the data clustering. Training data and test data were observed as depicted in Figure 11.

Classification system could perform quite well as depicted in Figure 12, with data such as the training the testing of table 1 and table 2. The system obtained optimal weight by the number of iterations of 1727 patterns 10 pieces of data, learning rate of 0.1122 and the error below 0.001 (0.0009997)

Weight training results were stored in the memory and then were used during testing with different data. Table 1 shows the training data and Table 2 shows the test data.

Discussion

The testing of the data above indicated that the system could work as expected that it could classify patients into normal or diseased conditions by the type of tachycardia or bradycardia.

CONCLUSION

Classification system could be performed quite well as depicted in Figure 13, with data such as the training and the testing of table 1 and table 2. The system obtained optimal weight by the number of iterations of 1727 patterns 10 pieces of data, learning rate of 0.1122 and the error below 0.001 (0.0009997).

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Case Report

EVALUATION ON THE EFFECT OF ANTIRETROVIRAL DRUGS ON CD4 T-CELL AND THE INCREMENT OF BODY WEIGHT AMONG HIV-AIDS PATIENTS IN SURABAYA

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ABSTRACT

Antiretroviral drug discovery has encouraged a revolution in the care of people living with HIV, although it has not been able to cure diseases and to increase the challenge in terms of drug side effects. Side effects of antiretroviral drugs are fairly common occurrences in HIV patients and generally occurr within the first three months after initiation of antiretroviral therapy, although long-term side effects are also often found afterwards. This study aims to evaluate the number of CD4 T-cells in patients with AIDS before and after getting on ARV therapy, the side effects arising during the taking of ARVs are related to the increment of body weight among the HIV-AIDS patients. Subjects were then narrowed down from 25 to 12 due to the incomplete data. The results showed that the top three most side effects which often occur in people with AIDS are appetite loss (20.0%), nausea (17.8%), and diarrhoea (15.6%). Meanwhile, about 58% of the subjects experienced increment of their body weight, and 42% were losing weight due to the side effects of the ARV therapy. Among those who lost their body weight, 50% were in the productive ages between 21–30 years old. The present study shows that combination antiretroviral therapy gives good results to the increased number of CD4 T-cells in patients living with HIV, as shown by the tendency of an increment in the number of CD4 T-cells in patients who received antiretroviral therapy. However, around 42% of those patients were losing weight because of the side effects of the therapy. Therefore, the importance of giving specific nutrient to overcome with the weight loss is needed to be given to the patients HIV instead of only giving the ARV treatment.

Keywords: *antiretroviral drug side effects, CD4 T-cell, weight loss, weight gain, nutrition*

ABSTRAK

Latar Belakang: *Penemuan obat antiretroviral mendorong sebuah revolusi dalam perawatan orang yang hidup dengan HIV, meskipun belum mampu menyembuhkan penyakit dan meningkatkan tantangan dalam hal efek samping obat. Efek samping dari obat antiretroviral adalah kejadian yang cukup umum pada pasien HIV dan umumnya terjadi dalam tiga bulan pertama setelah memulai terapi antiretroviral, meskipun jangka panjang efek samping sering juga ditemukan setelah itu.* ***Tujuan:*** *Untuk mengevaluasi jumlah CD4 T-sel pada pasien dengan AIDS sebelum dan setelah mendapatkan terapi ARV, efek samping yang timbul selama ARV dikonsumsi dan dikaitkan dengan peningkatan berat badan di antara pasien HIV-AIDS tersebut. Subyek kemudian dipersempit dari 25 orang menjadi 12 orang karena data yang kurang lengkap.* ***Hasil:*** *penelitian menunjukkan bahwa terdapat 3 efek samping tertbanyak yang paling yang sering terjadi pada penderita AIDS antara lain kehilangan nafsu makan (20,0%), mual (17,8%), dan diare (15,6%). Sementara itu, sekitar 58% dari subyek memiliki peningkatan berat badan, dan 42% yang kehilangan berat badan karena efek samping dari terapi ARV. Di antara mereka yang kehilangan berat badan, 50% berada di usia produktif antara 21 - 30 tahun. Dalam penelitian ini menunjukkan bahwa kombinasi terapi antiretroviral memberikan hasil yang baik bagi peningkatan jumlah CD4 T-sel pada pasien yang hidup dengan HIV, seperti yang ditunjukkan oleh kecenderungan kenaikan dalam jumlah CD4 T-sel pada pasien yang menerima terapi antiretroviral. Namun, sekitar 42% dari pasien yang kehilangan berat badan karena efek samping dari terapi tersebut. Oleh karena itu, pentingnya memberikan nutrisi yang spesifik untuk mengatasi dengan penurunan berat badan yang perlu diberikan kepada pasien HIV bukan hanya memberikan pengobatan ARV saja.*

Kata kunci: *efek samping obat antiretroviral, CD4 T-sel, penurunan berat badan, berat badan, nutrisi*

INTRODUCTION

The Human Immunodeficiency virus (HIV) is one of the most serious, deadly diseases in human history. HIV causes a condition called Acquired Immunodeficiency Syndrome, commonly known as AIDS. HIV destroys type of defence cell in the body's immune system called CD4 helper lymphocyte.

A healthy body has CD4 helper lymphocytes cells ($CD4^-$ T cells) which helps the immune system to function normally and fight off certain kinds of infections by acting as a messenger to other types of immune system cells, telling them to become active and fight against an invading germ. When HIV destroys these lymphocytes, the immune system becomes weak and the people get more serious infection than that of normal people.

HIV attaches to these $CD4^-$ T cells. The virus then infects the cells and uses them as a place to multiply. While doing so, the virus destroys the ability of the infected cells to do their job in the immune system. The body therefore loses the ability to fight many infections. Once the number of $CD4^-$ T cells per microliter (μL) of blood drops below 200, the cellular immunity is lost. Acute HIV infection usually progress es overtime to clinical latent HIV infection and then early symptomatic HIV infection and later to AIDS which is identified on the basis of the amount of $CD4^-$ T cells remaining in the blood.[1]

Several symptoms of HIV infection and AIDS may not appear for as long as 10 years or more. People with HIV may not notice any signs that they have the virus. However, doctors nowadays can diagnose someone with AIDS when that person's blood lacks a number of $CD4^-$ T cells which are required to fight the infections. Doctors can also diagnose AIDS if the person has signs of specific illness or diseases that may occur to people with HIV infection, such as fatigue or extreme weakness, rapid weight loss, frequent fever, heavy sweating, swollen lymph glands, chronic diarrhoea, coughing, or even minor infections such as skin rashes.[2]

AIMS OF STUDY

The aim of this study is to evaluate the effect of antiretroviral (ARV) drugs that are given to the HIV-AIDS patients. The antiretroviral (ARV) drugs may give effect on the CD4 T-cells and also cause some specific symptoms which lead to neither increment nor deterioration of body weight among the patients.

MATERIALS AND METHOD

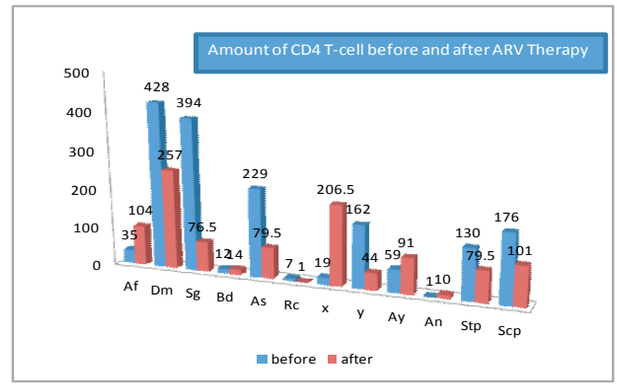
Twenty five patients with HIV positive from a private clinic in Surabaya were involved in the study. The treatment for HIV infection was given with high active antiretroviral (ARV) therapy which slowed down the progression of the diseases, including preventatives and active treatment of opportunistic infections.

Current antiretroviral (ARV) treatment options according to the WHO recommendations are combinations consisting of at least three medications belonging to at least two types of antiretroviral agents: an NNRTI (non-nucleoside reverse transcriptase inhibitor) and two NRTIs (nucleoside analogue reverse transcriptase inhibitors). The typical NRTIs include zidoyudine (AZT) or tenofovir (TDF) and lamiyudine (3TC) or emtricitabine (FTC). The ARV combination may reduce the resistance of HIV replication. Therefore the opportunistic infection could be avoided. However, the side effects of the drug therapy may occur within 3 months after the consumption of ARV. Some medication can upset the stomach which may lead to a weight loss. Nevertheless the ARV therapy is a way to increase the body weight which may be affected due to the infection.[3]

Each patient was given antiretroviral (ARV) drugs with different combinations namely AZT+3TC+NVP, AZT+3TC+EFV, and DAT+3TC+EFV. Conversely, due to the lack of the patient data, the sample of this study reduced to 12 people. Among these patients, we only evaluated the side effects of the drugs without differentiatin the types of drug combination associating with their body weight.

RESULTS AND DISCUSSION

This research shown the evaluation on the effect of ARV drug on CD4 T-cell before and after the therapy, and the increment of body weight of the HIV-AIDS patients, and also shows the side effects that occur during the ARV consumption. There were 25 samples in total; however it was reduced to 12 samples due to the incomplete data. Among 12 samples, the combination of ARV therapy gave a quite good result on the increasing amount of the CD4 T-cell as shown in the graphic below.



Graphic 1. The amount of CD4 T-cell before and after ARV therapy

Based on the graphic, some results show the reduction of the CD4 T-cells. These conditions were caused by the

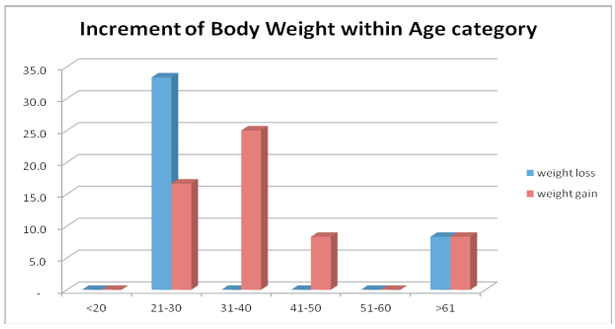
secondary infections which consequenced to the purpose of the therapy. Even though the ARV therapy may give some side effects however it has been proven to improve the amount of the CD4 T-cells continuously.

The 12 samples in this research were vandus age. Most of the patients were in productive ages, between 21 to 30 years old (in category). It can be seen in the table below.

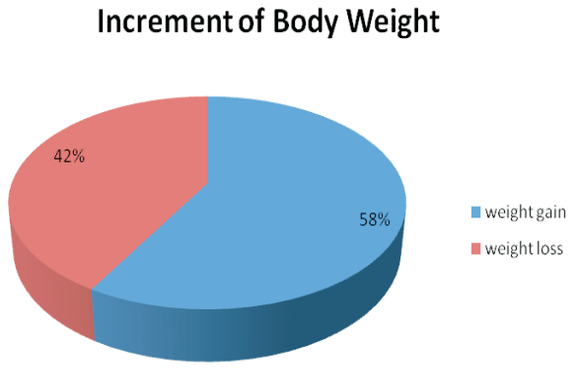
Table 1. Age Category

No	Age	N	Percentage (%)
1	< 20 years old	0	-
2	21 – 30 years old	6	50.0
3	31 – 40 years old	3	25.0
4	41 – 50 years old	1	8.3
5	51 – 60 years old	0	-
6	> 61 years old	2	16.7

Half of the samples were in the ages between 21–30 years old, which were consided productive ages. Comparisons with the increment the of body weight as a result of the ARV therapy are show in the graphic below.



Graphic 2. The increment of body weight within Age category



Graphic 3. Increment of Body Weight

The total samples, approximately 58.3% patients had gained weight. However, around 41.7% lost their weight. This increment of body weight might have associated with the ARV therapy, nevertheless there were still patients whose body weight were deteriorated. The side effects of the ARV therapy may have possibly caused the weight loss.

Based on the information acquired, 10 side effects occurred among the HIV-AIDS patients during the ARV therapy within 1 to 3 years. Each patient had the most frequent side effects that they experienced within the 10 side effects that occured. The side effects are shown in the table below:

Table 2. The most frequent side effect of ARV therapy

No	Side Effect	Freq.	Percentage (%)
1	Nausea	8	17.8
2	Fatigue	3	6.7
3	Sleeping problem	7	15.6
4	Lose appetite	9	20.0
5	Muscle pain	1	2.2
6	Diarrhoea	7	15.6
7	Skin rashes	4	8.9
8	Breathless	1	2.2
9	Fever	2	4.4
10	Headache	3	6.7

The top 3 most common side effects which occur among the HIV-AIDS patients are appetite loss (20.0%), nausea (17.8%), and diarrhoea (15.6%). The appetite loss side effect among the HIV-AIDS patients due to the ARV therapy, may have leaded to the reduction of their body weight, as well as the other side effects such nausea and diarrhoea. Some of ARV medications can upset stomach, this is why the side effects arise and the patients loose their weight.[4]

With respect to the dietary advice for HIV-AIDS patient, some evidence has shown a benefit of micronutrient supplements,[5] Dietary intake of macronutrients at RDA (Recommended Dietary Allowance) levels by HIV-infected adults is recommended by the World Health Organization.[6] Furthermore, WHO states that several studies indicate that supplementation of vitamin A, zinc, and iron can reduce adverse effects in HIV-positive adults to help improve body weight. There are some nutrition guidelines for HIV-AIDS patients. The basic thing is to eat more because extra muscle weight will help to fight HIV.

Balance diet containing macronutrients are essential.[7] Protein helps build and maintain muscle.[8] The good sources of protein came from meat, fish, beans, nuts, and seeds. Carbohydrates are the best macronutrient that give energy, which came from grains, cereals, vegetables, and fruits. Carbohydrates are also a good source of fibre. An other micronutrient is fat, which is needed in an appropriate amount. Monounsaturated fats are considered good fats if they are found in nuts, seeds, olive oil, and fish. To maintain a balance diet, a moderate exercise will help our body to transform the food into muscles. The easiest way is to include exercises into our daily activities (walking or cycling). Moreover, taking supplements can also help to maintain our body weight and get the vitamins and the minerals we need.[9] Last but not least, enough liquid is necessary. Extra water can reduce the side effects of

medications and also helps to concurrence the diarrhoea problem. However, such drinks as tea, coffee, carbonated drinks, chocolate or even alcohol should be avoided since these drinks may actually associate with body liquid loss.

CONCLUSION

Antiretroviral drugs (ARV) have been proven to increase the amount of CD4 T-cells which may help our immune system to function normally and fight off certain kinds of infections. These CD4 T-cells acted as the messenger to other types of immune system cells, telling them to become active and fight against an invading germ. However, the side effects of the ARV therapy among HIV patients are some symptoms related to the appetite which may cause deterioration to body weight. These symptoms are (1) appetite loss, (2) nausea, and (3) diarrhoea, which then lead to a weight loss.

To address the problem, supplementation of vitamin A, zinc, and iron can reduce adverse effects in HIV-positive adults to help them improve their body weight, maintain their body weight and get the vitamins and the minerals they need. Moreover, a balance diet must contain macronutrients that are essential such as high carbohydrates (to give energy), and protein (to help building muscles). Extra water can also help to reduce the side effects of medications and to overcome diarrhoea problems. Furthermore, to maintain a balance diet, it is recommended thats patients keep being active by doing some light exercise such as walking or cycling, in order to retain their body fitness, to gain appetite, as well as to preserve their body muscles.

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Literature Review

EVALUATION ON THE NUMBER OF CD4 T CELLS AND ANTIRETROVIRAL SIDE EFFECTS IN PATIENTS WITH AIDS

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ABSTRACT

Antiretroviral drug discovery has encouraged a revolution in the care of people living with HIV, although it has not been able to cure diseases and to increase the challenge in terms of drug side effects. Side effects of antiretroviral drugs are fairly common occurrences in HIV patients and generally occur within the first three months after initiation of antiretroviral therapy, although long-term side effects are also often found afterwards. This study aims to evaluate the number of CD4 T-cells in patients with AIDS before and after getting on ARV therapy and side effects arising during the taking of ARVs. Samples were collected from 10 patients infected by HIV/AIDS in a clinic in Surabaya. This study is an analytical survey. Data collection was conducted using secondary data obtained from the medical record card status on HIV paients in a clinic in Surabaya. Data results showed that the side effects that often occur in people with AIDS are appetite loss (90%), headache (80%), insomnia (80%) and nausea (70%). While many combinations of antiretroviral drugs have side effects such as a combination of AZT +3 TC + EFV, d4T +3 TC + followed by EFV and AZT +3 TC + NVP. The present study shows that combination antiretroviral therapy gives good results to the increased number of CD4 T-cells in patients living with HIV, as shown by the tendency of an increase in the number of CD4 T-cells in 8 out of 10 AIDS patients who received a antiretroviral therapy.

Keywords: *antiretroviral drug side effects, CD4 T-cell*

ABSTRAK

Latar Belakang: *Penemuan obat antiretroviral mendorong suatu revolusi dalam perawatan ODHA, meskipun hal tersebut belum mampu menyembuhkan penyakit dan menambah tantangan dalam hal efek samping obat. Efek samping obat antiretroviral merupakan kejadian yang cukup sering terjadi pada pasien HIV dan umumnya terjadi dalam tiga bulan pertama setelah inisiasi ARV, walaupun efek samping jangka panjang juga kerap didapati sesudahnya.* ***Tujuan:*** *Untuk mengevaluasi jumlah sel T-CD4 pada penderita AIDS sebelum dan sesudah mendapatkan terapi ARV serta efek samping yang timbul selama mengkonsumsi ARV.* ***Metode:*** *Subyek penelitian adalah sebesar 25 kemudian dipersempit menjadi 10 karena tidak semua data penderita dapat dianalisis (tidak terdapat data CD4 yang lengkap).* ***Hasil:*** *Data hasil penelitian menunjukkan bahwa efek samping yang sering terjadi pada penderita AIDS adalah keluhan nafsu makan yang menurun (90%), sakit kepala (80%), sulit tidur (80%) dan mual (70%). Sedangkan kombinasi ARV yang banyak menimbulkan efek samping adalah kombinasi AZT+3TC+EFV, disusul D4T+3TC+EFV dan AZT+3TC+NVP. Dalam penelitian ini terlihat bahwa kombinasi terapi ARV memberikan hasil yang cukup baik terhadap peningkatan jumlah sel T-CD4 pada pasien ODHA, hal ini ditunjukkan dengan adanya kecenderungan peningkatan jumlah sel T-CD4 pada 8 dari 10 penderita AIDS yang mendapatkan terapi ARV.*

Kata kunci: *antiretroviral, efek samping obat, sel T-CD4*

INTRODUCTION

HIV (Human Immunodeficiency Virus) is a virus that attacks human immune system. If a person is infected by this virus, their immune system decreases, and they become susceptible to diseases. This virus will ultimately lead to a disease called AIDS (Acquired Immune Deficiency Syndrome). Progress from year to year of a person who

is infected by this virus keeps increasing that this case has become a very serious concern for the health sector. Several data indicated that in the end of 2010, nearly 34 million people (31.6 million–35.2 million) people lived with HIV in this world. The increase reached 17% in the year 2001.[1] The results of the development situation of HIV/AIDS in Indonesia in 2009 were as many as 3,863 cases, in 2010 was as many as 4,158 cases, and up to June 2011 the cumulative total was 26,483 cases.[2] The HIV virus transmitted into human body through contact with infected body fluids, will bind to the T cell surface receptor CD4 and replicate in them to produce a new virus and infect other CD4 T cells. The result is a decrease in the number of CD4 T cells that eventually reaches a point that it will significantly decrease the body's immune system, and the body becomes susceptible to opportunistic infections.

Antiretroviral drug discovery has encouraged a revolution in the care of people living with HIV, although it has not been able to cure diseases and to increase the challenge in terms of drug side effects. Many studies have shown that antiretroviral drugs give very good results in various countries. The purpose is to determine the antiretroviral suppresses viral replication rate and the maximum continuity which will result directly or indirectly on the recovery, or maintenance of immune function, improved quality of life of people with HIV, decreased morbidity and mortality associated with HIV, and reduced rate of HIV transmission in the community.[3] Based on the recommendations of WHO and the World Health Organization first-line antiretroviral medicines, what are recommended are two NRTIs (2 types of nucleoside reverse transcriptase inhibitor) and 1 NNRTI (a type of non-nucleoside reverse transcriptase inhibitor).[4] Antiretroviral combination is the basis for the management of the provision of antiretroviral to AIDS patients. Antiretroviral combination can reduce the resistance, effectively suppress HIV replication so that opportunistic infections and other complications can be avoided, and improve the quality and life expectancy of AIDS patients. Side effects of antiretroviral drugs commonly occur in AIDS patients and generally occur in the first three months after initiation of antiretroviral, although long-term side effects are also often found afterwards. This study aims to evaluate the number of CD4 T-cells in patients with AIDS before and after getting an ARV therapy and the side effects arising during the taking of ARVs.

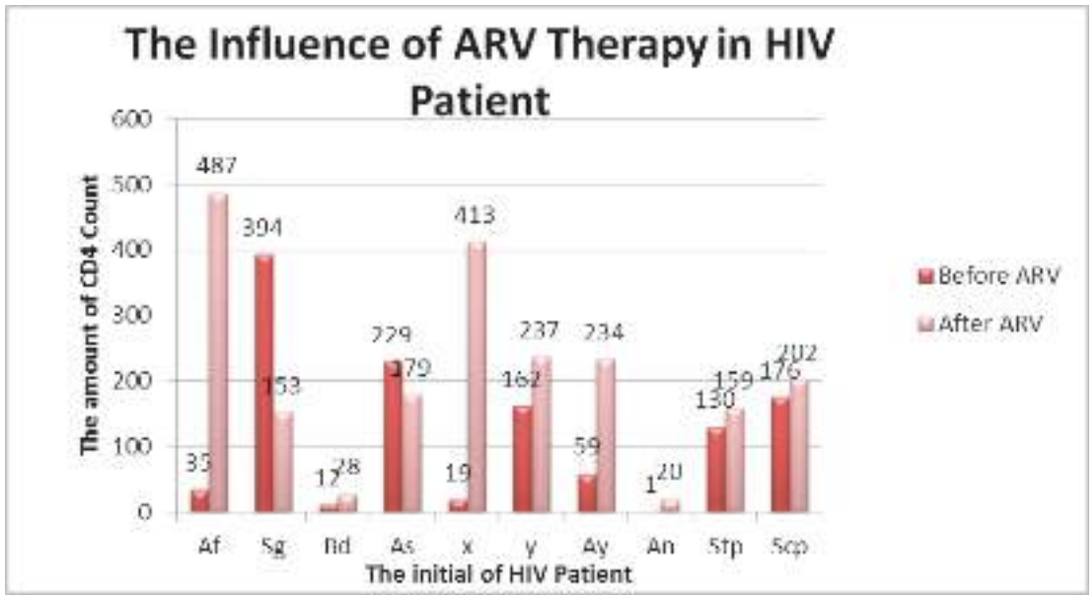
This study is an analytical survey. The data were obtained from medical records in a private clinic in Surabaya. Samples were collected from 10 patients infected by HIV/AIDS in Surabaya.

RESULTS

From the medical records of patients with HIV/AIDS, the data acquired from the disease history of the patients prior to ARV (presence or absence of opportunistic infection) were CD4 cell (counted before and after antiretroviral therapy), patient weight, types of drugs given, and complaints/adverse events after the administration of drugs. Provision of antiretroviral drugs also causes side effects in patients with HIV/AIDS, because each has a toxicity of certain drugs. Side effects of antiretroviral drugs are fairly common occurrences in HIV patients and usually occur within the first three months after initiation of antiretroviral therapy, although long-term side effects are also found often afterwards. According to the indications for changing the ARV regimen due to toxicity of antiretroviral, the side effects of drugs are divided into four degrees, namely (1) degree 1: mild reaction, no need to change on ARV therapy, (2) degree 2: the reaction is thus necessary to continue to give consideration antiretroviral drugs as long as possible, symptomatic treatment may also be given, but if it does not work, replace one kind of anti-HIV drugs that cause side effects, (3) grade 3: severe reaction that requires substitution of ARVs which causes side effects, without stopping antiretroviral, and (4) degree 4 : severe, life-threatening reaction that should be stopped by providing antiretroviral, in which the ARVs were restarted after severe reactions were handled. A research by Kosasih, et al, conducted in Jakarta, found that convenience and drug side effects are major determining factors in people living with HIV antiretroviral adherence. First-line antiretroviral used in Indonesia is the combination with 3TC and NVP

Table 2. ARV Side effects and type of ARV

No	Side Effect	AZT+3TC+NVP	AZT+3TC+EFV	D4T+3TC+EFV
1	Nausea	1	4	2
2	Fatigue	1	1	2
3	Sleeping problem	1	5	2
4	Lose appetite	2	4	3
5	Muscle pain	0	2	0
6	Diarrhea	1	4	2
7	Skin rashes	2	4	2
8	Breathless	0	2	2
9	Fever	0	5	1
10	Headache	1	5	2
	TOTAL	9	36	18



Picture 1. Diagram of the amount of CD4 before and after ARV therapy.

AZT/d4T / EFV. Side effects have been studied including NVP hypersensitivity (27.6%), increased transaminase enzymes (20.8%), and d4T neuropathy (22%).[5] The results showed that the average HIV/AIDS patients have complaints/side effects after receiving ARV therapy. Here are the numbers and the percentages of the side effects complained by the patients of AIDS who underwent antiretroviral therapy for 1 to 3 years.

Table 1. Frequency of the ARV side effects

No	Side Effects	N	Percentage (%)
1	Nausea	7	70
2	Fatigue	5	50
3	Sleeping problem	8	80
4	Lose appetite	9	90
5	Muscle pain	2	20
6	Diarrhea	6	60
7	Skin rashes	6	60
8	Breathless	4	40
9	Fever	6	60
10	Headache	8	80

Side effects had been suffered by HIV patients after receiving ARV therapy according to the result of the medical records of patients. These side effects are shown in Table 1.

According to table 1, it is known that side effects are common in patients with AIDS. The complaints were appetite loss (90%), headache (80%), insomnia (80%) and nausea (70%). Loss of appetite in AIDS patients may be caused by the influence of drugs. Some of these effects such as nausea or difficulty in swallowing food emerge due to mouth sores caused by fungal infections. Side effects such as headaches that often occur in people with HIV/AIDS is caused by the influence of drugs such as AZT. Some interesting findings are that most of ARV drugs cause some types of sleep disorders. Many AIDS patients experience sleep disturbances (difficulty of sleep). Complaints of sleep disorders are also associated with depression and pain, whether it is difficult to fall asleep leading to staying up all night. Nausea often occurs because the path where the ARV drugs are put orally is irritated. It also increases stomach acid. Table 2 is the table of side effects and types of drugs consumed by people with AIDS.

Drugs are given to HIV patients in the form of three drug combinations: Highly Active Anti Retroviral Therapy (HAART) . The recommended combination of two drugs is the group of NRTIs plus one group of NNRTI, such as the combination of AZT +3 TC + EFV, d4T +3 TC + EFV and AZT +3 TC + NVP. There are three (3) combinations of drugs that are usually given to the HIV patients. It depends on the condition of the patients such as changes in weight, CD4 cell count etc. When HIV patients are getting ARV therapy, they should be carried out to clinical or laboratory monitoring which is aimed to findout the ARV toxicity symptoms, to figure out the opportunistic infections/other diseases, to figure out the adherens of the patients, and to see the response of the therapy.[3]

According Table 2, it is noted that the combination of drugs that causes many side effects is a combination of AZT +3 TC + EFV, d4T +3 TC + EFV followed by EFV and AZT +3 TC + NVP. This suggests that any patients get antiretroviral therapy. Failures can vary and depend on the patients condition (opportunistic infection) and the level of the patients compliance, that they need clinical and laboratory monitorings. Therefore, as shown in Table 2, there are various side effects caused by antiretroviral therapy. None of the patients getting ARV therapy got more than one side effect for the combination of the ARV given.

Thus, in Table 2, the total of side effects for one combination of ARV does not always amount to 10. If the antiretroviral therapy given to the patients fails, the combination of ARV will be changed with the next combination of ARV. The present study shows that combination of antiretroviral therapy gives good results to the increased number of CD4 T-cells in patients with AIDS, as seen in picture 1.

The diagram above shows a trend increase in the number of CD4 T-cells in AIDS patients receiving ARV therapy. Some data indicate a decrease in CD4 cell counts. This may be due to secondary infections that affect the success of the therapy and the patients with low levels of compliance to drug therapy. Although the ARV therapy generated a lot of complaints or side effects in people, it could increase the number of CD4 T-cells significantly. It is certainly not independent on the adherence factor which is a dynamic process, but it can change with the subjectivity of individuals who consume drugs. In addition, environmental factors such as support of the family and friends, will also greatly affect the process of clinical improvement (increase in CD4 cell count, weight gain and reduction in viral load) of the AIDS patients.

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Literature Review

THE EXON 5, 6, 7, 8 OF P53 MUTATIONS IN ORAL SQUAMOUS CELLS CARCINOMA

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ABSTRACT

Genetic instability may underlie the etiology of multistep carcinogenesis. The altered p53 gene observed in tumors may represent the expression of such instability and may allow the accumulation of other gene alterations caused by multiple mechanism. p53 gene is the guardian of the genome, that is why we pay more attention to this gene. In this study, we evaluated the significance of p53 mutation in 55 patient with oral squamous carcinoma. Thirty among them underwent well-differentiated carcinoma, while the remaining 25 patients underwent poorly differentiated carcinoma. The mutations were detected by PCR-SSCP (Single strand Conformational Polymorphism) analysis in the region between exon 5 and exon 8. The results indicated that the p53 mutation in exon 5 (40%), exon 6 (28%), exon 7 (24%) and exon 8 (8%) were associated with poorly differentiated carcinoma, whereas mutation in exon 5 (10%), exon 6 (30%), exon 7 (40%) and exon 8 (20%) were associated with well-differentiated carcinoma. These observations suggest that p53 mutation in exon 5, 6, and 7 have strong correlation with poorly differentiated in oral squamous carcinoma while well-differentiated level was related with mutation in exon 6,7 and 8.

Keywords: *p53, oral squamous carcinoma, mutation, PCR-SSCP*

ABSTRAK

Latar Belakang: *Ketidakstabilan genetik mungkin mendasari etiologi multistep karsinogenesis. Perubahan gen p53 yang diamati pada tumor dapat mewakili ekspresi ketidakstabilan tersebut dan memungkinkan akumulasi perubahan gen lain yang disebabkan oleh mekanisme multiple. Gen p53 adalah perwakilan dari genom, sehingga gen ini yang menjadi perhatian.* ***Tujuan:*** *Evaluasi mutasi p53 pada 55 pasien dengan karsinoma skuamosa oral.* ***Metode:*** *Tiga puluh di antaranya mengalami karsinoma diferensiasi baik, sedangkan 25 pasien menjalani karsinoma diferensiasi buruk. Mutasi yang terdeteksi oleh PCR-SSCP (untai tunggal Polimorfisme konformasi) analisis di daerah antara ekson 5 dan ekson 8.* ***Hasil:*** *Hasil menunjukkan bahwa mutasi p53 pada ekson 5 (40%), ekson 6 (28%), ekson 7 (24%) dan ekson 8 (8%) dikaitkan dengan karsinoma diferensiasi buruk sedangkan mutasi pada ekson 5 (10%) , ekson 6 (30%), ekson 7 (40%) dan ekson 8 (20%) dikaitkan dengan karsinoma diferensiasi baik. Pengamatan ini menunjukkan bahwa mutasi p53 pada ekson 5, 6, dan 7 memiliki korelasi yang kuat dengan karsinoma diferensiasi buruk pada skuamosa oral sementara mutasi pada ekson 6,7 dan 8 memiliki korelasi kuat dengan karsinoma diferensiasi baik.*

Kata Kunci: *p53, karsinoma skuamosa oral, mutasi, PCR-SSCP*

INTRODUCTION

Squamous cell carcinoma is the most common intraoral malignancy and is the most frequently occurring malignant tumor of the oral structures. In the malignant oral lesions, the squamous cell carcinoma is about 82–90%, and mainly affects male patients with ages varying between 40–80 years, although recent studies have reported the occurrence of this malignancy in younger patients.[1] Tobacco and betel use, alcohol consumption, viruses, and other occupational and environmental factors have been implicated in the etiology of the disease. Epidemiological studies have shown that the incidence of oral squamous cell carcinoma varies significantly among the continents and within developed and developing countries.[2]

Alterations in tumour suppressor genes like *p53* gene are the most commonly implicated genetic events in a number of human tumors and are frequently found in various types of cancer and have been considered as molecular markers of cancer.[3]

Mutation or increased expression of the p53 protein has also been reported in the head and neck squamous cell carcinoma. However, only few data are available on the frequencies of p53 mutation in oral squamous cell carcinoma correlated with poorly and well differentiated tumor grades. The different exon on *p53* may be mutated in certain stages because it may serve as a regulatory marker in the process of tumorigenesis. Polymorphism in p53 codon 72 may contribute to oral cancer susceptibility.[4] One possibility is that *p53* alteration can enhance genomic instability and thus augment the accumulation of subsequent genetic event necessary for tumor development.[3,5]

Alteration of the specific gene, such as p53, p27, p16 and cyclin D-1, will induce oral cancer development through mutation, amplification, or deactivation mechanism.[4] Therefore, Hsieh and his research team suggest that the pattern of p53 gene mutation in oral squamouse cell carcinoma conduet further study.[5]

Single-Strand Conformation Polymorphism (SSCP) is a screening method for detection of unknowm point mutations. SSCP analysis was orginally described by Orita *et al.* (1989). The general idea is to take a small PCR product, denature it, and electrophorese it through a non-denaturing polyacrylamide gel. Thus, as the PCR product moves into and through the gel (and away from the denaturant), it will regain secondary structure that is sequence dependent.[6] The mobility of the single-stranded PCR products will be different at different sequences. Therefore, PCR-SSCP can be used to detect mutational products. The major advantage of SSCP is that many individual PCR products may be screened for variation simultaneously. Many of PCR products may be analyzed on each full-size sequencing gel (depending upon the comb size used).[7] Most researchers use SSCP to reduce the amount of sequencing necessary to detect new alleles at loci of interest or to estimate allele frequencies of populations better.

The main purpose of the study is to evaluate the significance of *p53* mutation in various stages of patients with oral squamous cell carcinoma. To determine whether mutation of p53 gene was associated with degree of tumorigenesis, we used PCR-SSCP method to detect the mutation in exons 5–8 of *p53* gene.

MATERIALS AND METHODS

Tissues

Mucosal biopsies of 55 patients with oral squamous cell carcinomas (OSCC) from the floor of the mouth were obtained from the surgical pathology files of the Department of Oral Pathology, Faculty of Dentistry, Universitas Airlangga, Surabaya. The 55 cases of squamous cell carcinoma comprised of poorly and well differentiated tumor grades. The a verage of the patient ages was 58.7 (ranging from 21–74) years. Specimens from these patients, from 29 men and 26 women, were screened for adequacy of lesional tissue by staining the tissue sections with hematoxylin and eosin. The histological diagnosis of each specimen was independently reviewed based on the established histological criteria. Sixteen biopsies of normal control epithelium from the floor of the mouth were obtained from non-smoker 6 men and 10 women with the average age of 52.2 years (ranging from 35 to 73).

PCR-SSCP

Fragments of p53 gene exon 5 to 8 were amplified using the following oligonucleotides.

Exon 5,	EX 5/S	5'-TGTTCACTTGTGCCCTGACT-3'
	EX 5/AS	5'-AGTAATCAGTGACGAATCAG-3'
Exon 6,	EX 6/S	5'-TGGTTGCCCAGGGTCCCCAG-3'
	EX 6/AS	5'-GGAGGGCCACTGACAACCA-3'
Exon 7,	EX 7/S	5'-CTTGCCACAGGTCTCCCCAA-3'
	EX 7/AS	5'-AGGGGTCAGCGGCAAGCAAGA-3'
Exon 8,	EX 8/S	5'-ATTTCCTTACTGCCTCTTGC-3'
	EX 8/AS	5'-TCCACCGCCTCTTGTCTTGC-3'

The sequences of all primers were obtained from Laboratoires Eurobio, Paris for the amplification of oral squamous cell carcinoma DNA. The PCR conditions were denaturated at 94° C for 1 min, annealed at 59° C for 1 min and extended at 72° C for 1.5 min, with the total number of cycles of 35. The amplified fragments were analyzed by PCR- SSCP. Single strand of the PCR product was made by snap-freeze technique and loading on 6% polyacrylamide containing 5% glycerol. The gel was running on 0.5×TBE (Tris-borate-EDTA) buffer at 20 W constant power at room temperature for 12–16 h, until the xylene cyanol dye reached 3 cm from the top of the gel. The gel was stained with silver nitrate staining.

RESULTS

In this study, we evaluated the significance of *p53* mutation in 55 patients with oral squamous carcinoma. The male to female distribution in this study was ignored. Thirty among them underwent well-differentiated carcinoma, while the remaining 25 patients underwent poorly differentiated carcinoma. The mutations were detected by PCR-SSCP analysis in the region between exon 5 and exon 8. The results indicated that the p53 mutation in exon 5 (40%), exon 6 (28%), exon 7 (24%) and exon 8 (8%) were associated with poorly differentiated carcinoma whereas mutation in exon 5 (10%), exon 6 (30%), exon 7 (40%) and exon 8 (20%) were associated with well-differentiated carcinoma.

Table 1. Prevalence of mutation in *p53* gene associated with tumor grade in oral squamous cell carcinoma.

Position of mutation	Number of patients with mutation	
	Poorly differentiated (25 patients)	Well differentiated (30 patients)
Exon 5	10 (40%)	3 (10%)
Exon 6	7 (28%)	9 (30%)
Exon 7	6 (24%)	12 (40%)
Exon 8	2 (8%)	6 (20%)

These observations suggest that *p53* mutation in exon 5, 6, and 7 have strong correlation with poorly differentiated in oral squamous carcinoma while the well-differentiated level was related with mutation in exon 6, 7 and 8.

PCR-SSCP analysis showed that mutation on *p53* gene could be detected by extra band eruption (Fig.1). The mobility of the single-stranded PCR products depended upon their secondary structure. Therefore, PCR products that contained change sequence differences as well as insertions, deletions or other mutation, had different mobilities in poly-acrylamide gel electrophoreses.

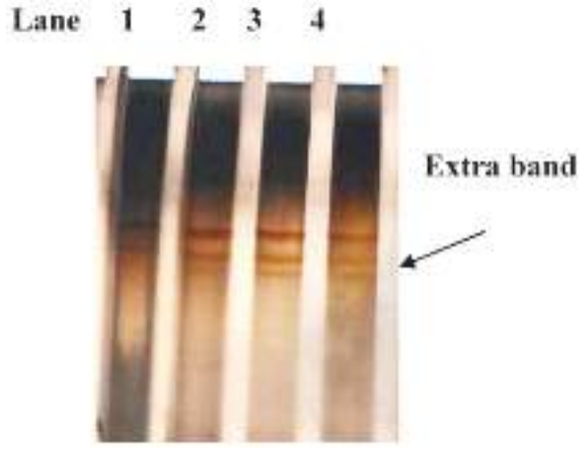

Figure 1. PCR-SSCP analysis of *p53* gene was drawn in lane 1, 2, 3, 4, respectively. Mutations were detected and signed by extra bands.

DISCUSSION

Oral squmous cell carcinoma (OSCC) is the most common cancer found approximately 80% of all cancer in the oral cavity.[8] In the developed country like US, which has managed to eradicate infectious disease, cancer is the second cause of death after cardiovascular diseases. In Indonesia, cancer was recorded as the cause of death besides heart attack, diabetes and hepatitis.

Oral squamous cell carcinoma is one of the most common cancers worldwide, with incidences of more than 30 per 100000 population in India (oral cancer) and in France and Hong Kong (nasopharyngeal cancer). Epidemiological studies have shown that the sites of oral cancer differ widely. Tongue, lip and floor of the mouth are the most frequent sites of lesions of oral squamous cell carcinoma.[2]

Oral cancer constitutes about 4% of all cancers in the United States and 5% in the United Kingdom. A total of 2940 new cases of lip, mouth, and pharyngeal cancer in men were reported in the United Kingdom in 1996: an incidence of 10.2 per 100000 population.[9] Early detection should be a priority, given the excellent prognosis of early stage disease compared with the poor results in advanced stages. In Indian screening programmes, community health workers have been trained in primary prevention and early detection of oral cancer and premalignant lesions, but no evidence indicates that this reduces mortality. Screening is the most cost effective procedure when targeted at high risk groups, for example, heavy drinkers and smokers.[9,10] Oral squamous carcinogenesis is a multistep process in which multiple genetic events occuring, alter the normal functions of oncogenes and tumour suppressor genes. This can result in increased production of growth factors or numbers of cell surface receptors, enhanced intracellular messenger signalling, and/or increased production of transcription factors.[10] Genetic alterations are known to occur during carcinogenesis including point mutations, amplifications, rearrangements, and deletions. Point mutations (single base changes) can lead to overactivity or inactivity of gene products. These are common in genes such as K-ras and p53. Alterations of these genes have frequently been reported in malignant neoplasms.[11]

Oncogenes themselues are not sufficient to cause oral cancer and appear to be initiators of the process. The crucial event in the transformation of a premalignant cell to a malignant cell is the inactivation of cellular negative regulators tumour suppressor genes, and is regarded to be a major event leading to the development of malignancy. Tumour suppressor genes are mostly inactivated by point mutations, deletions, and rearrangements in both gene copies. The p53 gen mutation will not cause cells apoptotic therefore the cancer cells will proliferate.[12] If p53 gene mutated, there was no repair into the apoptosis cell which underwent DNA damage, that mutant cell could enter the cell circulation and it will happen with uncontrolled cell development.[13] One of the important tumor suppressor genes is *p53*, mainly roles as the *guardian of the genome* in apoptotic gene. This protein blocks cell division at the G1 to S boundary, stimulates DNA repair after DNA damage, and also induces apoptosis. These functions are achieved by the ability of p53 to modulate the expression of several genes.[14] The p53 protein transcriptionally activates the production of the p21 protein, encoded by the WAF1/CIP gene, and p21 become an inhibitor of cyclin and cyclin dependant kinase complexes. p21 transcription is activated by wild-type p53, not mutant p53.[15] However, WAF1/CIP expression is also induced by p53 independent pathways such as growth factors, including platelet derived growth factor, fibroblast growth factor, and transforming growth factor β. Wild-type p53 has a very short half life (4–5 minutes) whereas mutant forms of protein are more stable, with a six hour half life.[14,15]

Mutation of *p53* occurs either as a point mutation, which results in a structurally altered protein that sequesters

the wild-type protein, thereby inactivating its suppressor activity, or by deletion, which leads to a reduction or loss of p53 expression and protein function. The tumour suppressor gene p53 is known to be mutated in approximately 70% of adult solid tumors.[15] Mutations of exons 4 to 9 of the *p53* gene were found in 72 of 106 patients with Head and Neck Squamous Cell Carcinoma (HNSCC).[15] *p53* mutations were associated with loss of heterozygosity at chromosome 17p. The prevalence of *p53*-mutated tumors was higher in the group of patients with nonresponse to neoadjuvant chemotherapy than that in the group of responders (81% *v* 61%, respectively).[4]

According to these research the p53 mutation on Poorly differentiated level mostly occurs in exon 5 (40%), whereas the least mutation occurs in exon 8 (8%), followed by exon 6 (28%) and exon 7 (24%). However the least number of p53 mutation in Well differentiated mutation occurs in exon 5 (10%), and most of the mutation occur in exon 7 (40%), followed by exon 6 (30%) and exon 8 (20%). A high prevalence of *p53* mutations on Poorly differentiated level was found in exons 5 to 7. But high prevalence of *p53* mutations on well differentiated level was found in exons 6 to 8. It is relevant to some studies which state that mutation in Oral Squamous Cell Carcinoma occurs beetwen exon 5 and 8. These observation suggest that p53 mutation in exon 5, 6, and 7 have strong correlation with poorly differentiated level in oral squamous carcinoma while well-differentiated level was related with mutation in exon 6,7 and 8.

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Research Report

PLATELET RICH PLASMA PREPARATION PROTOCOLS: A PRELIMINARY STUDY

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ABSTRACT

Currently, therapy with Platelet Rich Plasma (PRP) has been widely used and continues to grow for various clinical applications. Along with its development, there are various options in the method of obtaining PRP, either automatic or manual, while one of the most reliable methods according to the literature is a double centrifugation method. The purpose of this research is to produce an optimization of the double centrifugation method. This study used experimental data obtained by conducting a research at the Clinical Pathology Laboratory of Dr. Soetomo Hospital, Surabaya. Experiments were conducted on stored blood obtained from the blood bag from Indonesian Red Crossand fresh blood from healthy donors with CPD anticoagulant. Results: PRP with optimum platelet count could be made with sufficient personal laboratory skills and amounted to 4.11 times with the platelet count of 1.152 million using 1300 rcf for 5 minutes for the first centrifugation, and 2300 rcf for 7 minutes for the second centrifugation.

Keywords: *Platelet Rich Plasma, Double Centrifugation Method, Stem Cell Therapy*

ABSTRAK

Latar Belakang: *Saat ini terapi dengan menggunakan Platelet Rich Plasma (PRP) telah banyak digunakan dan menjadi pilihan dalam dunia klinis. Adapun dalam perkembangannya terdapat beberapa pilihan metode, baik secara otomatis maupun manual, meskipun metode yang paling dipercaya adalah metode double centrifugation. Tujuan: Untuk menemukan optimalisasi yang paling tepat untuk metode tersebut. Penelitian ini menggunakan data eksperimental yang dilakukan oleh peneliti dari Laboratorium Patologi Klinik RSUD Dr. Soetomo Surabaya.* ***Metode:*** *Penelitian ini menggunakan plasma darah yang diperoleh dari Palang Merah, yang berasal dari donor dengan CPD anticoagulant.* ***Hasil:*** *PRP dengan perhitungan platelet optimum ditunjang dengan kemampuan laboratorium dapat dicapai hingga 4.11 kali dengan perhitungan 1.152 million, menggunakan 1300 rcf selama 5 menit pada putaran pertama dan 2300 rcf selama 7 menit pada putaran kedua.*

Kata kunci: *Platelet Rich Plasma, Double Centrifugation Method, Stem Cell Therapy*

INTRODUCTION

As a relatively new subject, stem cells can be said to have tremendous potential, starting early in life and growing up as a sort of internal improvement system, proliferate and differentiate without definite limits to later form the other cells as long as the relevant person or animal is still alive . As one of the internal improvement system, the proliferation of stem cells can be stimulated in the presence of growth factors. Growth factors are found in PRP (Platelet Rich Plasma) in large numbers.

In Indonesia, the current stem cell therapy is a field that currently emerged, and still not a lot of work of which the method is widely used and applicable. PRP was highly rated in terms of potential for use as a treatment of chronic tendinitis, wound healing, regeneration of cartilage or discs, as well as cardiac applications.

There are various methods used for the manufacture of this PRP, from sophisticated which can only be performed at hospitals using apheresis devices, up to a practical method that can be performed directly in clinics. From variously different methods and protocols, there are still no research in Indonesia on what the most optimal method is. Therefore, it is felt that there is a need to have a research and optimization on double centrifugation method. The method, according to the literature, is the most reliable one and relatively simple. So as to produce a protocol that PRP produces reliable and proven quality.

MATERIALS AND METHODS

This is a laboratory experiment which is intended to get the most reliable PRP making method with platelet count as the indicator. The materials and tools were sterile tubes, syringes, sterile long needle, CPD anticoagulant, centrifuge, and Sysmex automatic hematology analyzer. The experiment was conducted in the laboratory of clinical pathology department of Dr. Soetomo Hospital, Surabaya.

Sample Collection

The blood samples were obtained from a blood bag from a donor, and also from fresh blood from other donors. To get as much as 1 ml of PRP, we took blood samples of 10 ml with 9:1 CPD anticoagulant. For the optimization experiment of centrifugation phase and separation phase, we performed 30 tests, hence the total experiment required a sample size of 300 ml of blood.

PRP Isolation

Platelet count was conducted twice for a sample, one before, and another after the optimization process. The optimization process itself was a series of variation on velocity and time of centrifugation, twice on each sample. It varied from 200 to 2300 rcf, and 5 to 15 minutes on each centrifugation.

Separations were manually done with ± 1 mm of margin after each centrifugation, in order to purify as much as possible, the result was extracted from erythrocytes and Platelet Poor Plasma (PPP). The visible buffy coat from the first separation was essential to be maintained until the end of the optimization process. The first separation was done in order to separate erythrocytes as much as possible, while

Table 1. Platelet count result

Sample No.	1st Centrifugation		2nd Centrifugation		Platelet Count 1 (x 10^3/µl)	Platelet Count 2 (x 10^3/µl)	Rise
	Force (rcf)	Time (min.)	Force (rcf)	Time (min.)			
001	1200	5	2000	6	92	271	2.94 x
002	200	15	1500	15	87	194	2.23 x
003	300	10	1500	15	87	65	0.74 x
004	600	5	1500	15	87	106	1.22 x
005	900	5	1500	15	87	129	1.48 x
006	1200	5	1500	15	87	33	0.38 x
007	1500	5	1500	15	87	41	0.47 x
008	200	15	2000	6	87	90	1.03 x
009	300	10	2000	6	87	47	0.54 x
010	600	5	2000	6	87	23	0.26 x
011	900	5	2000	6	87	31	0.36 x
012	1200	5	2000	6	87	37	0.43 x
013	1500	5	2000	6	87	33	0.38 x
014	200	15	1500	15	170	62	0.37 x
015	200	15	600	15	104	109	1.05 x
016	600	30	1500	10	104	29	0.28 x
017	**1300**	**5**	**2300**	**7**	**280**	**1152**	**4.11 x**
018	900	5	2300	7	104	37	0.36 x
019	1300	5	2300	7	104	22	0.21 x
020	1200	5	2000	6	239	181	0.76 x
021	1300	5	2300	7	239	428	1.79 x
022	600	5	2000	6	234	140	0.59 x
023	1300	5	2300	7	234	186	0.79 x
024	600	5	2000	6	228	288	1.26 x
025	900	5	1500	15	228	292	1.28 x
026	1200	5	2000	6	228	431	1.89 x
027	1300	5	2300	7	228	898	3.94 x
028	600	5	2000	6	183	469	2.56 x
029	1200	5	2000	6	205	269	1.31 x
030	1300	5	2300	7	251	327	1.30 x

the second was to separate PPP leaving the bottom with 1 ml of liquid, which was considered to be the PRP.

Each set of variation was repeated at least 2 times to assure the reliability of the current method. The resulting data were analyzed by using simple analytical statistics. The thrombocyte count of the PRP obtained was measured by automated Sysmex XE-2100 Hematology Analyzer which had a unique fluorescent flow cytometry and hydrodynamic focusing technologies to minimize the review rates and produce accurate results even when interferences or artifacts were present.[1] Hence, the result presented in this study was made to be as accurate as possible, with very less likely to be false positive.

RESULTS AND DISCUSSION

Experiments of optimization of double centrifugation method in platelet rich plasma gave the following result:

Due to the limited budget towards the experiment, a PDGF BB test was performed only as an additional infromation, based on the highest result produced by the protocol, to ensure that it would still be a useful PRP after processed through the protocol. The result proved that it was still a useful PRP with PDGF BB 3401 pg/ml after processing through this protocol. This amount could still increase after activation, which was not included and tested further in this current preliminary study due to some fund limitation. But, as defined by Textor, the conventional human definition of PRP is plasma containing resting platelets at a concentration of at least 106/ul. The PRP may or may not be activated prior to use, hence the term "PRP" does not indicate that activation has occurred.[2] Alone, platelet count itself is considered to be one of the key factors used to standardize research studies for the regenerative capacity of PRP. Maximum platelet concentration of PRP with manual double centrifugation method by Kakudo is 7.9 x.[3] Too, qualitative changes in the platelet may also affect the regenerative potential of PRP. According to Marx, a damaged or nonviable platelet will not release bioactive growth factors, thus the resulting PRP may be disappointing. The PRP for clinical treatment should be sought about 1,000,000 platelets per microliter. Given that whole blood contains approximately 200,000 ± 75 000 platelets per microliter, then the therapeutic PRP must have an average percentage of increase of about 400% in platelet count.[4] Therefore, this experiment indicates that the optimum result from this experiment might be a standard for PRP making.

But as we can see in table 1, there were quite significant inconsistencies in the correlation between force and time of centrifugation to increase the platelet count after the double centrifugation method in PRP making. It seemed that it was very dependent on the fidelity of manual separation processes between the centrifugations. The later samples results were higher than the early ones since the researchers become more accustomed and skilled in the manual separation process. Errors might also be found because of the absence of PRP resuspension before the platelet was counted by the analyst. Resuspension could be important because there were trapped platelets in the buffy coat, so resuspension will show the tangible number of platelets in PRP.[5] Hence, it could be concluded that the factor of trained person in the whole process might be more significantly important rather than the centrifugation process itself. This might also explain the very wide spectrum and range in protocol in centrifugation force and time to this day, which ranges from 72 G for 15 minutes, 160 G for 20 minutes, 250 G for 10 minutes, 900 G for 5 min, 1300 G for 10 minutes, up to 1400 G for 4 minutes, for the first centrifugation. The second centrifugation itself has a variable speed from 400 G to 2000 G, and has a variable period of time from 6 minutes to 15 minutes. Similarly, with temperatures varying from 4° C, 15° C, up to room temperature.[5,6,7,8,9]

Anticoagulant Acid Citrate Dextrose (ACD) type A and low-speed centrifugation can be used to maintain the integrity of the platelet membrane.[5] But according to the study of Shimizu et al, PRP is created with the anticoagulant Citrate Phosphate Dextrose (CPD) had a 4% higher platelet count when using 5 ml of full blood, and 4.5% higher using 200 ml of full blood.[10] Hence, this experiment used CPD anticoagulant instead of ACD.

Overall, the highest increment in platelet count was obtained using 1300 rcf for 5 minutes for the first centrifugation, and 2300 rcf for 7 minutes for the second centrifugation as we can see in samples 017 and 027. This is quite different with the finding of Jo CH and colleagues from orthopedic surgery department of Seoul National University Boramae Hospital, which stated that the platelet count would be optimum with double centrifugation method with the use of 900 G in the first centrifugation for 5 min and 1500 Gin the second centrifugation for 15 minutes.[9]

Besides the double centrifugation method, there are already several automated methods developed independently by many institutions. Among these methods are Curasan PRP Kit by Curasan, Kleinostheim, Germany; method PCCS PRP System by 3i Implant Innovations, Palm Beach Gardens, Florida, United States; and methods SmartPrep by Harvest Technologies, Plymouth, Massachusetts United States. However, research by Castillo showed no significant differences in the platelet concentration, in the number of erythrocytes, the TGFβ1, or in the fibrinogen levels between the various automated methods.[11] And since there was no availability of any automated PRP machines in both the Medical Faculty Universitas Airlangga and Dr. Soetomo Hospital Surabaya, there was no difference in the method used in this experiment. But, based on Kurita et al experiment in 2008, double centrifugation method itself was described as the most accurate and rational choice for manual making experiment of PRP, conformed by Nagata in 2010, since it produced more platelets and more active substances.[7,12] Castillo also suggests that the usage of method and protocol should be based on associated rational choice instead of trying tremendous variability available

by automated machines which are described as "the jungle world of commercial proposals and products".

As the concentrated platelet, PRP rich in basic growth factors and has potential various clinical applications with no virtual risks. To initiate the process of wound healing, there are Platelet Derived Growth Factor (PDGF$\alpha\alpha$, PDGF$\beta\beta$, PDGF$\alpha\beta$), Transforming Growth Factors-β (TGF-β_1 and TGF-β_2), Vascular Endothelial Growth Factor (VEGF), Epithelial Growth Factor (EGF). In addition PRP also contains adhesion molecules necessary for bone grafting and the matrix of bone, such as osteocalcin, osteonectin, and bone morphogenic protein (BMP) -2 and BMP-4.[4,13] Platelets also have a Platelet Factor 4 (PF4), interleukin (IL) -1, Platelet-Derived Angiogenesis Factor (PDAF), Platelet-Derived Endothelial Growth Factor (PDEGF), Epithelial Cell Growth Factor (ECGF), Insulin-Like growth Factor (IGF), vitronectin, fibrinogen, fibronectin, and thrombospondin (TSP) -1.[14,15,16] PRP also shows some potential for treatment of myocardial infarction in experiments using the *musmusculus.*[17] In addition to the usefulness above, PRP is also potentially used in a variety of other clinical applications such as muscle healing,[18] peripheral nerve damages,[19] and maxillofacial surgeries.[20]

Suggestions

This project is a preliminary study about the manual making of for PRP and therefore its goal is to obtain a standard protocol to make PRP according to the present definition. For further study such as therapeutic effect and usage safety, further investigation is encouraged by using both experimental and clinical trials to follow up the therapeutic effects of the PRP in tissues or animal models by investigating the growth factors and the regeneration process from time to time there.

ACKNOWLEDGEMENTS

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Case Report

DAPSONE RESISTANCE IN A *Mycobacterium leprae* ISOLATE WITH TWO POINT MUTATIONS IN *folP* GENE FROM A LEPROSY PATIENT

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ABSTRACT

Drug resistance in leprosy is important for Leprosy Control Program, since the WHO-Multidrug regiment (MDT) has been used for global treatment of leprosy for more than two decades already. A Dapsone resistance case in a Multibacillary (MB) leprosy case is reported. The patient was diagnosed and treated in Tajuddin Chalid Hospital Makassar, South Sulawesi. Previously he was treated in a health center at South Sulawesi and was given a treatment for one year, before referred to the hospital. The leprosy skin lesions are still active with erythematous skin lesions and thickened ear lobe. Bacteriological examination was positive for Acid Fast Bacilli, the Bacterial Index was 3+ and the Morphological Index was 1%. The specimens of M.leprae isolation was sent to the Institute of Tropical Disease Surabaya for drug resistance study. Using the Lp1-2 and Lp3-4 nested primers, PCR test was positive for M.leprae. Sequencing result for folP gene showed a double mutation at codon 53 (ACC / Threonin) which become (AGG / Arginine). Simultaneous mutation at two nucleotides at one codon has never been reported in Indonesia before and this phenomenon is important for leprosy control policy.

Keywords: *leprosy, M.leprae, dapsone resistance, folP gene, mutation*

ABSTRAK

Latar Belakang: *Resistensi obat pada pengobatan kusta adalah faktor penting untuk Program Pengendalian Kusta, Sejak WHO mencanangkan penggunaan Multi Drug Regiment (MDT) untuk pengobatan kusta secara global, dalam kurun waktu lebih dari dua dekade resistensi Dapson dalam kasus (MB) kusta Multibasiler di Indonesia dilaporkan. Pasien didiagnosis dan dirawat di Rumah Sakit Tajuddin Chalid Makassar, Sulawesi Selatan. Sebelumnya ia dirawat di sebuah pusat kesehatan di Sulawesi Selatan dan mendapat perawatan selama satu tahun, sebelum dirujuk ke rumah sakit. Kulit kusta lesi masih aktif dengan kulit eritematosa lesi cuping telinga menebal.* ***Metode:*** *Pemeriksaan Bakteriologis adalah positif untuk Basil Tahan Asam, Indeks bakteri adalah 3 + dan Indeks Morfologis 1%. Spesimen dari pasien dikirim ke Institute of Tropical Disease Surabaya untuk studi resistensi obat. Spesimen dideteksi terlebih dahulu adanya basil M.leprae dengan analisa PCR menggunakan primer LP1-2 dan LP3-4.* ***Hasil:*** *Hasil Sequencing untuk gen folP menunjukkan mutasi pada kodon 53 (ACC / Threonin) menjadi (AGG / Arginine). Mutasi simultan di dua nukleotida pada satu kodon belum pernah dilaporkan di Indonesia sebelumnya dan fenomena ini penting untuk kebijakan pengendalian kusta.*

Kata Kunci: *leprosy, M.leprae, dapsone resistance, folP gene, mutation*

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, primarily attacks peripheral nerves and secondarily affects skin and other organs. The disease can cause disabilities and often creates social problems. Approximately 17.000 new leprosy cases are detected every year in Indonesia, and most of them grow in the Eastern part of the country.[1] The WHO Multi-drug Therapy (MDT) has been implemented for leprosy all over the world since 1980s, using a combination of 3 drugs: Rifampicine, Dapsone and Clofazimine.[2] Dapsone is the

oldest remedy for leprosy which has been used since 1940s as a monotherapy. Dapsone resistance in leprosy was firstly reported from Malaysia in 1964[3] and it was proven by mouse foot pad inoculation technique, which is difficult and time consuming.[4] Due to the difficulties in cultivating *M.leprae*, the molecular biology of the bacilli become very important. Development of molecular biology techniques has made some improvements in *M.leprae* studies,[5,6,7] including the detection of drug resistance study of leprosy, which is more rapid and accurate.[8] DNA sequencing of *folP gene* of the bacilli will give some information if there is a change or mutation which is related to Dapsone resistance. A case of leprosy with Dapsone resistance, proved by molecular biology study is reported. Some aspects related to drug resistance in leprosy are also discussed.

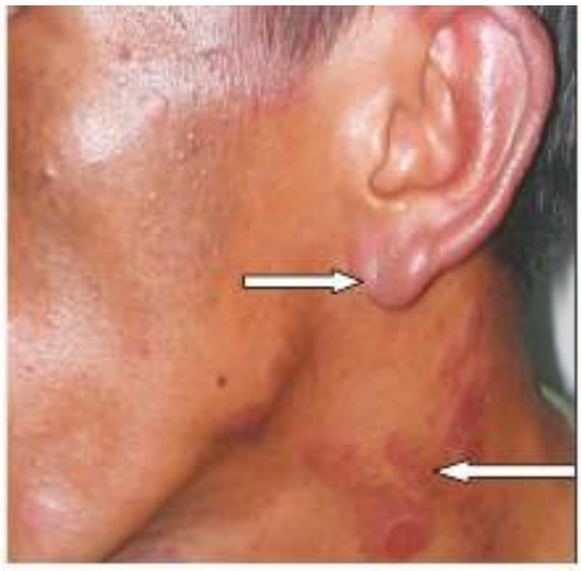

Figure 1. Borderline Lepromatous (BL type) of Leprosy

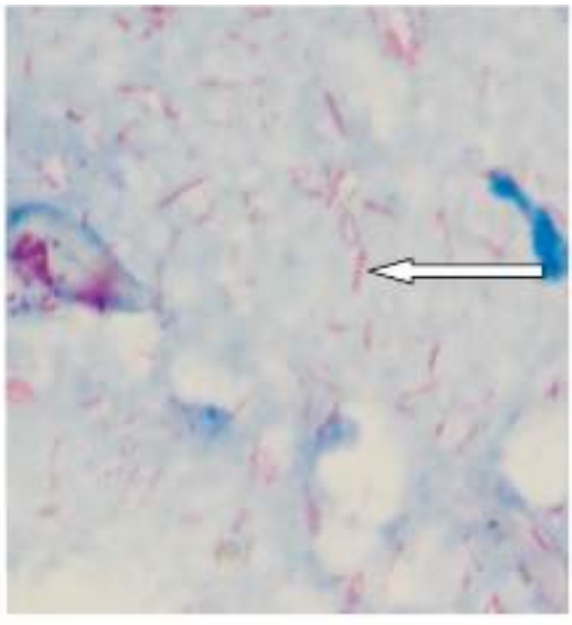

Figure 2. Acid Fast Bacili (AFB) Ziehl Neelsen staining

CASE

A thirty years old man from Makassar, South Sulawesi, was refered to Tajuddin Chalid Hospital Makassar due to a persistent skin lesion after 1 year treated for leprosy in the peripheral health center in South Sulawesi. This patient was given MDT drugs irregularly since he was a sailor. Clinical examination in the hospital revealed a Borderline Lepromatous (BL) Leprosy, with positive bacteriological examination. The Bacteriological Index (BI) was 3+, with the Morphological Index (MI) of 1%.

Skin slit smear specimen from this patient was sent to the Institute of Tropical Disease Universitas Airlangga Surabaya for drug resistance study. The study included Dapsone, Rifampicin and Quinolone resistance.

Poymerase Chain Reaction

Detection of *M.leprae* was perfomed by PCR study. DNA extraction was conducted by mixing the specimen with Qiagen kit. All samples identified the existence of *Mycobacterium leprae* by detection of the 18 kDa antigen *M.leprae* in region RLEP3 *repetitive element* (X17153) using nested PCR. Amplification will produce about a 129 bp for external (*outer*) and a 99 bp for internal (*inner*) product. PCR was carried out using a G mixture of FailSafe PCR System (EPICENTRE, Madison, WI, USA, Cat. No. FSP995G) in a 20 µl volume of reaction mixture containing at least 0.1 pg of genomic DNA in 2 µL of template *Taq* polymerase (Failsafe Cat. No. FS99250) and 2 µL of 5 µM primers. Primers Lp1 5' TGCATGTCATGGCCTTGAGG 3' and Lp2 5' CACCGATACCAGCGGCAGAA 3' and the amplification was conducted in a thermal-cycler-machine (*BioRad i-cycler*) under the conditions of 2 min at 98º C for preheating, 20 sec at 98º C for denaturation, 30 sec at 56º C for annealing and 30 sec at

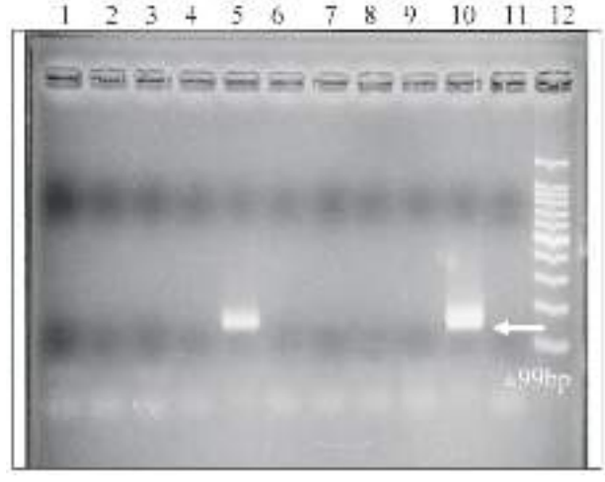

Figure 4. PCR Product of *M.leprae* Detection.
Lane 1-4, 6-9 : negative results
lane 5 : isolate from Makassar: positive result;
lane 10: PC, positive control (*M.leprae* strain *Thai-53*);
lane 11: NC, negative control;
lane 12: DNA size marker of 100bp DNA ladder

72º C for elongation/extension (repeated for 35 cycles) followed by prolonged extension of 5 min at 72º C and then inactivation at 4º C. Amplicon was then nested with primers Lp3 5' TGAGGTGTCGGCGTGGTC 3' and Lp4 5' CAGAAATGGTGCAAGGGA 3' under the conditions of 2 min at 98º C for preheating, 20 sec at 98º C for denaturation, 30 sec at 56º C for annealing and 30 sec at 72º C for elongation/extension repeated for 30 cycles followed by prolonged extension of 5 min at 72º C and then inactivation at 4º C. The full length of this amplicon was separated by electrophoresis in 3% (w/v) HS agarose gel (Cambrex Bioscience, Rockland, ME, USA) using TBE (Tris/Boric/EDTA, pH 8.0) buffer at 100 V. After amplification, the amplicon was distributed in agarose gel for electropheresis process and the PCR results were examined using UV light and recorded. (figure 3).

Amplification of *folP, rpoB* gene of *M.leprae* using their specific primers, all gave positive bands.

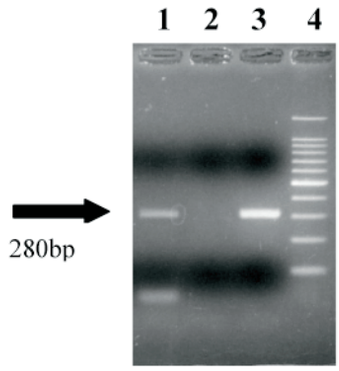

Figure 5. PCR Product of *folP* gene.
lane 1: isolate from Makassar
lane 2: NC, negative control
lane 3: PC, positive control (*M.leprae* strain *Thai*-53)
lane 4: DNA size marker 100bp DNA ladder.

DNA Sequencing Study

Using the Long Reed Tower machine, the results of DNA sequencing study of *rpoB* and *gyrA* gene revealed no mutations, but the sequence of the *folP* showed a mutation at codon 53 (figure 6a). The nucleotides changed from normally *ACC / Threonin* to *(AGG / Arginine* (figure 6b).

The other drug resistance study for Rifampicine (*rpoB gene*) and Quinolone (*gyrA gene*) revealed no mutation regarding this *M.leprae* isolation.

DISCUSSION

Our leprosy case showed a typically Borderline Leprosy (BL), and still no complication or disability. There were some hyperpigmented patches over the patient's body and earlobe thickness which indicated a Multibacillary leprosy.[9] There was peripheral nerve thickening over his both ulnar nerves, but no signs of neuritis. Since there is no data of previous bacteriological examination, it is difficult to categorize the patient as a relapse case or a back-to-

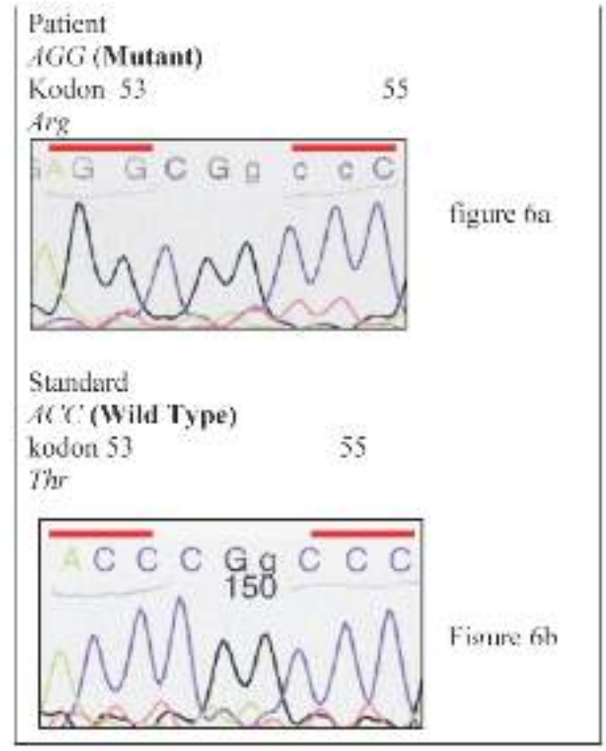

Figure 6. Nucleotides sequencing at codon 53, comparison of mutant type (figure 6a) and normal / wild type (figure 6b).

treatment case. As a sailor who had to sail for weeks or months, the patient could not take the drugs continuously and the MDT treatment became irregular. This situation resulted in persistent skin lesions and also the positive bacteriological examination.

Mycobacterium leprae still can not be cultivated in culture media until today. Detection of Dapsone resistance in the past was performed by injecting the bacilli to the mouse footpad (*in vivo* method), which needed about six months before the result could be established.[10,11] Using the molecular biology technique is possible now to detect the resistance in a few days. The Dihydropteroate synthase enzyme has been known as a target of Dapsone, which is an important enzyme for growth and metabolism of *M.leprae*.[12] The *folP* gene is responsible in the synthesis of this enzyme by coding the formation of amino acids which arrange the protein structure.[13] If a mutation occurs in this gene, the protein arrangement will change and the enzyme will also changed. The end result of this is the failure of Dapsone to inhibit the new enzyme, which means that the bacilli remains active or resistant to this drug.

Using this molecular biological techniques, many Dapsone resistance cases were reported from some Asian countries,[14,15,16] including Indonesia,[17,18] but the incidence was relatively low.

The normal sequence of nucleotides of the *folP* gene has been mapped completely and could be retrieved from GeneBank.[19] The mutation sites usually occurred at codon 53 (normally ACC/Threonine) and only involved one

nucleotide change (i.e. GCC/Alanine or AGA/Arginine). But, in our case we found double mutations, from ACC/ Threonine into AGG/Arginine.

Missense mutations associated with Dapsone resistant in *M.leprae* has been documented and could be outlined as follows.

Table 1. Dapsone resistant in *M.leprae*

drug	*gene*	*Codon no.*	*susceptible*		*resistant*	
Dapsone	folP	53	ACC	(Thr)	GCC	(Ala)
					GTC	(Val)
					ATC	(Ile)
					AGG	(Arg)
					AGA	(Arg)
		55	CCC	(Pro)	TCC	(Ser)
					CGC	(Arg)
					CTC	(Leu)

Adapted from: Leprosy, Science working towards dignity. p 63[21]

From a molecular biology point of view, this type of mutation is relatively rare and should be paid more attention for preventing the spread of primary resistance to Dapsone.[20] Since the WHO-MDT regiment contains Dapsone for daily treatment, an alternative regiment should be anticipated for Dapsone resistant case.

Our case had been treated with Dapsone irregularly, which probably induces the mutation of the bacilli. Although resistance to other drugs (Rifampicine and Quinolone) was not found, the irregular treatment of this patient could induce another drug resistance. This resistance will be solved by changing the Dapsone with other anti leprosy drugs while the patient should take the medicine regularly. It needs patient education and monitoring of regularity of treatment.

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Literature Review

THE PRELIMINARY STUDY OF ANTIOXIDANT ACTIVITY FROM XYLO-OLIGOSACCHARIDE OF CORNCOB (*ZEA MAYS*) HYDROLYSIS PRODUCT WITH ENDO-β-XYLANASE ENZYME

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ABSTRACT

Xylo-oligosaccharide derived from corncob hemicellulose has been reported to possess antioxidant activity. In order to assess the effective scavenging of xylo-oligosaccharide, we conducted in vitro studies based on self-made xylo-oligosaccharide with DPPH (2,2-diphenyl-1-picrilhydrazil) method. Xylo-oligosaccharide was prepared with enzymatic hydrolysis. The enzyme used for hemicellulose hydrolysis was endo-β-xylanase enzyme from PC-01 isolated bactrerium. PC-01 isolated bacterium used in this study was Pacet hot spring which was isolated from East Java. Endo-β-xylanase enzyme is an extracelluler enzyme. There was about 0.199 U/mL after purification and dialysis process. Hydrolisis product of hemicellulose A and B from corncob were analyzed with TLC (Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatography). This analysis showed that hydrolysis product of hemicellulose B had a lot of xylo-oligosaccharide hydrolysis product of hemicellulose than Xylo-oligosaccharide hydrolysis product of hemicelluloses A. Xylo-olygosaccharide was analyzed as on antioxidant activity. Xylo-oligosaccharide hydrolysis product of hemicellulose B (IC_{50} = 48.96) has higher antioxidant activity than Xylo-oligosaccharide hydrolysis product of hemicellulose A (IC_{50} = 92.302). The toxicity of xylo-oligosaccharide can be calculated by the value of LC_{50} (Lethality concentration). LC_{50} of xylo-oligosaccharide derived from corncob hemicellulose was 400 ppm so that xylo-oligosaccharide has anti tumor activity because xylo-oligosaccharide has LC_{50} < 1000 ppm.

Keywords: *Hemicellulose, corncob, endo-β-xylanase, xylo-oligosaccharide, antioxidant activity, toxicity*

ABSTRAK

Latar Belakang*: Xilo-oligosakarida hasil hidrolisis hemiselulosa tongkol jagung dilakukan studi awal uji aktivitas antioksidan.* **Tujuan***: Uji perendaman radikal bebas oleh xilo-oligosakarida, uji antioksidan dari xilo-oligosakarida ini dilakukan secara in-vitro dengan metode DPPH (2,2-diphenyl-1- picrilhydrazil).* **Metode***: Xilo-oligosakarida diperoleh dari hasil hidrolisis secara enzimatis. Enzim yang digunakan untuk proses hidrolisis ini adalah enzim endo-β-xilanase dari isolat bakteri PC-01. Isolat bakteri PC-01 yang digunakan dalam penelitian ini adalah isolat dari sumber air panas Pacet. Enzim Endo-β-xilanase adalah enzim ekstraseluler yang memiliki aktivitas 0,199 U/ml setelah proses pemurnian dan dialisis. Produk hidrolisis hemiselulosa A dan B dari tongkol jagung dianalisis dengan KLT (Kromatografi Lapis Tipis) dan HPLC (High Performance Liquid Chromatography). Analisis tersebut menunjukkan bahwa produk hidrolisis Hemiselulosa B memiliki kandungan xilo-oligosakarida yang lebih banyak dibandingkan dengan produk hidrolisis hemiselulosa A dari tongkol jagung.* **Hasil***: Xilo-oligosakarida hasil hidrolisis hemiselulosa tongkol jagung diuji aktivitas antioksidan. Xilo-oligosakarida hasil hidrolisis Hemi B (IC50 = 48,96) memiliki aktivitas antioksidan yang lebih tinggi dibandingkan xilo-oligosakarida hasil hidrolisis Hemi A dari tongkol jagung (IC50 = 92,302). Toksisitas xilo-oligosakarida dapat dihitung dari harga LC50 (Lethality concentration). Nilai LC50 dari xilo-oligosakarida hasil hidrolisis hemiselulosa B tongkol jagung adalah 400 ppm sehingga xilo-oligosakarida ini memiliki aktivitas antitumor karena nilai LC50 < 1000 ppm.*

Kata kunci: *Hemiselulosa, tongkol jagung, endo-β-xilanase, xilo-oligosakarida, aktivitas antioksidan, toksisitas*

INTRODUCTION

Lately the medical world has been discussing free radicals that give bad effects to human health. These free radicals are physiologically produced by the cells due to the metabolic processes in the body. In addition, free radicals are also produced by other processes outside the body such as ionizing radiation, environmental pollutants (vehicle emission and industrial emissions, asbestos, cigarette smoke, etc.), alcohol, smoke and foods which contain high fat. Free radicals can be easily formed by a compound that is ready to deliver a single electron, such as fatty acids. Free radicals or oxidants in the body can be controlled by the body itself by forming endogenous antioxidants. On the situation of endogenous antioxidants that are not able to suppress free radicals that arise, it needs antioxidants from outside. Antioxidants can be obtained from the synthesis or from natural compounds in plants.[1] Recently, it has been reported that the oligosaccharide compounds also have antioxidant activities.[19] Oligosaccharide is an oligomer of hemicellulose which can be found in many agricultural waste. Oligosaccharide is one example among other xilo-oligosaccharides (XOS), galakto-oligosaccharides, and frukto-oligosaccharides (FOS).Based on this background, the research was conducted as a preliminary study testing for oligosaccharides especially xylooligosaccharides obtained from enzymatic hydrolysis of corn cob as an anti-oxidant with several stages. These stages were hemicellulose isolation of corn cob and enzymatic hydrolisis of hemicellulose into oligimer which was xylooligosaccharides. The enzyme that was used for the hydrolysis of hemicellulose subtrate was endo-β-xylanase from Bacillus subtilis PC-01.[3] Xilo-oligosaccharides derived was used to fix antioxidant tests and toxicity tests using shrimp fry.

METHODS

Xilanolitik enzymes production

Inoculum of *Bacillus subtilis* PC 01 was grown in 1 liter of media production and incubated for 8 hours at 60° C. Cells were harvested after ± 8 hours at 4° C and centrifuged 10000 rpm for 10 minutes. Cell pellet was discarded, while the supernatant (enzyme) was used for the enzyme presipitation process.

Xylanase Enzyme Precipitation Using Amonium Sulfate (enzyme precipitation)

To 100 ml of crude extract enzyme that had been soaked in an ice bath, some ammonium sulfate was added slowly, stirred frequently until the levels of ammonium sulfate saturation percentage reached 60%. Ammonium sulfate saturation percentage was used based on ammonium sulfate saturation table.[4] The enzymes were centrifuged at 6000 rpm for 10 minutes. Precipitated enzyme was again precipitated and dissolved in 100 mM citrate phosphate buffer at pH 5, and then dialyzed. Dialysis was performed, until the ammonium sulfate fraction-free enzyme was marked by the formation of a white precipitate when some buffer was poured into $BaCl_2$ solution.

Xilanolitik Enzyme Assay

The standard reaction mixture, contained 100 µl of substrate and 100 µl of enzyme which was incubated at 70° C for 1 hour and finished by adding 600 µl of DNS, after that, heated for 15 minutes together with the controls, and immediately cooled in ice water for 20 minutes. Absorbance readings were analyzed at a wavelength of λ550 nm. The controls used were 100 µl of enzyme, 100 µl of substrate and 600 of µl DNS. They were treated the same as those, above but without any incubation.

Isolation of Corn cobs Hemicellulose

Agricultural waste of corn cob powder weighed 5 grams. We put the two neck round bottom flask containing 2.0 M NaOH solution up to 100 ml within a magnetic stirrer for heating for 4 hours. After the process was complete, cooled, and then filtered using Buchner funnel, the filtrate was acidified with 4 N acetic acid to a pH of 5.5–6.0 for precipitating hemicellulose A and continued with dicentrifuging (10000 rpm, 20 min) to separate the sediment. The precipitate was freeze-dried to obtain hemicellulose that was of free water. The filtrate obtained was mixed with 96% ethanol to precipitate hemicellulose B. The precipitate obtained was washed with 96% ethanol and then powdered and freeze-dried to obtain a free of water hemicellulose B.[5] The hemicellulose obtained could be used for further testing.

Hemicellulose Enzymatic Hydrolysis

Every 1% of Hemicellulose A and B samples was taken as much as 100 µl and added with 300 µl of xylanase enzyme, incubated at 70° C for 24 hours. After centrifuging, the filtrate obtained was xilo-oligosaccharides and other sugar monomers were dried using a freeze drier.[6]

Analysis of Hydrolysis Products Thin Layer Chromatography (TLC)

A number of oligosaccharide compounds contained in the hydrolysis products were analyzed by TLC with various comparison eluent. The eluent used n-propanol: CH_3CN: water = 5: 3: 2; n-propanol: water: ammonia (70: 29: 1) and n-butanol: acetic acid: water = 2: 1: 1. The three systems of eluent were used to obtain the best separation and as a monitor in the subsequent separation process.[7] The apparition stain used was sulfuric acid in methanol.

High Performance Liquid Chromatography (HPLC)

The HPLC analysis used 2 different columns: the carbohydrates column (mikrobondapak, Waters 2487) and a NH_2Si column, and 2 different detectors refractory index detector and ELSD (Evaporative Light Scaterring Detector), as well as solvent methanol 80% in water and 83% acetonitrile in water, flow rate of 1 µl/min, injection volume of 20 µl.

Table 1. The results of HPLC analysis for the hydrolysis products of corn cobs

Sample	Glucose (%)	Xylose (%)	Arabinose (%)	Xylo-oligosaccaride (%)
CA (xylo-oligosaccharides from Hemi A of corncob)	5,0	0	0	4,6
CB (xylo-oligosaccharides from Hemi A of corncob)	6,0	0	0,1	4,9

Anti - Free Radical Activity Test

The antioxidant activity xylo-oligosaccharide standards and the product of hydrolysis spectrometry were determined by measuring absorbance at a wavelength of 497 nm, 517 nm, and 537 nm. Each sample of xylo-oligosaccharide standards and the product of hydrolysis was dissolved in water with various concentrations: 100, 80, 60, 40 and 20 ppm, taken as much as 1 ml, added with 1 ml of 0.4 M acetic acid buffer at pH 5.5 and 0.5 ml 10–4 M in ethanol and then incubated for 5 min at 20° C. After that, each solution absorbance was measured with UV-VIS spectrophotometer at a wavelength of 497 nm, 517 nm, and 537 nm. Observation of free radical activity of compounds against DPPH reagent absorbance can be calculated as follows.

$$A_{hit} = \frac{A_{517}nm - A_{497}nm\ A_{537}nm}{2}$$

Anti - Free Radical activity as % Scavenging DPPH was as follows.

$$\%\ \text{Scavenging DPPH} = \frac{[1 - (\text{A count of test material})] \times 100\%}{\text{A count DPPH (comparator)}}$$

Determination of the inhibition IC_{50} (Inhibitor Concentration 50%) was based on the linear regression analysis of the concentration of % Scavenging DPPH. If IC_{50} is less than 100 ppm, the compound has the activity as an anti-free radical.[8]

Toxicity Test Using BSLT Method

BSLT test was performed on the isolated pure compound or separation. Sample weighed as much as 10 mg, and then was dissolved in 1 ml of water. After that, it was added with 99 mL of sea water and stirred until homogeneous to obtain a solution with a concentration of 100 ppm. From 100 ppm solution with concentrations of 100, 50, 25, and 12,5 ppm respectively, replication until 3 times was made.

Further into the sample solution and control, each shrimp fry 8–15 was added, thereafter left to stand for 24 hours. The number of dead shrimp fry was counted and recorded for each concentration of the sample solution and the control solution. Good control data were obtamed when there was no dead shrimp fry. Shrimp fry mortality data at each concentration was used for the analysis of LC_{50}.[9] Observations were made after *Artemia salina* contact with the test solution for 24 hours. If the mortality in the control was more than 10 %, the test was canceled and re-tested.

Toxicity of xylo-oligosaccharides was determined by calculating the LC_{50}. To determine LC_{50}, data obtained from the test result bioactivity were processed using SPSS computer program to determine the LC_{50} value. The test result obtained, provided information about the toxicity of the hydrolisis product.

RESULTS

Hemicellulose hydrolysis enzymatically

The enzyme activity of crude endo-β-xylanase was as high as 0.119 U/mL. Crude extract of endo-β-xylanase precipitated by ammonium sulfate showed that the enzyme activity of endo-β-xylanase had on optimum activity at 60% saturation of ammonium sulfate. It was based on the previous research.[10] After the dialysis process was complete, the volume of endo-β-xylanase obtained was 15 mL from 1 liter of media production and the activity of endo-β-xylanase in total after ammonium sulfate precipitation and dialysis was 0.199 U / mL.

In this study, hemicellulose A (Hemi A) and hemicellulose B (Hemi B) were produced. Hemi A was a major hemicellulose whereas Hemi B was hemi residual hemicellulose product. A Hemi obtained was 7.6 grams, while the Hemi B obtained was 6.4 grams. From the TLC results obtained, xylo-oligosaccharide from Hemi B hydrolysis product had a retention factor (Rf) of 0.36, while xylo-oligosaccharide from Hemi A hydrolysis product had a value of Rf as high as 0.41. Based on these two Rf. It was expected that xylo-oligosaccharide from Hemi B hydrolysis product had a Degree of Polymerization (DP)which was higher than that from the Hemi A. This can be seen from

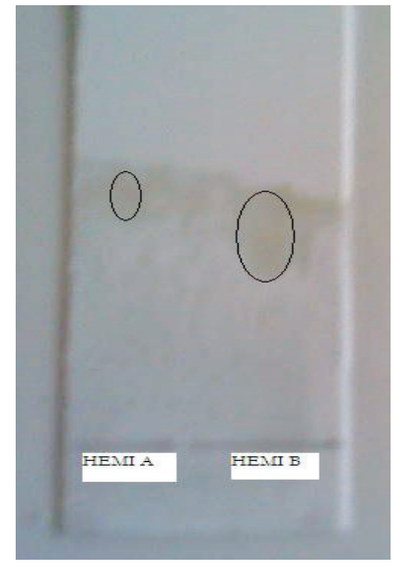

Figure 1. TLC result of xylo-oligosaccharide compounds from Hemi A and B.

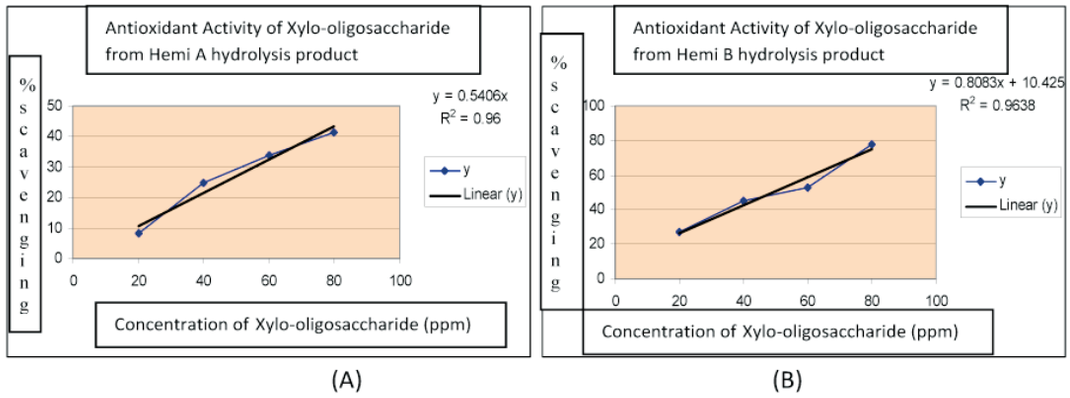

Figure 2. Percentage of Scavenging DPPH curve vs. DPPH concentration xylo-oligosaccharides. (A) HemiA, (B) Hemi B.

the spots on the TLC plates that the highest Rf is the spot for the monomer-monomer sugars/monosaccharides (located around the upper limit of the plate).

From Table 1, we know that Hemi B contains more xylo-oligosaccharides than Hemi A. Xylo-oligosaccharide from Hemi A was 4.6% while from Hemi B was 4.9%.

Anti Free Radical Activity Test

Free radical activity of each xylo-oligosaccharides can be determined based on regression equations derived from these curves of xylo-oligosaccharides (Hemi A), y = 0.5406 x and xylo-oligosaccharides (Hemi B), y = 0.8083x + 10.425. Once the calculation was done by substituting the a value y with 50, it means that the ability was reduced to 50%, and the obtained x as IC_{50} values are as follows.

Based on the data in Figure 2. IC_{50} value of xylo-oligosaccharides (Hemi A) equaled 92.302 ppm, whereas the IC_{50} value of xylo-oligosaccharides (Hemi B) was 48.96 ppm. The antioxidant activity of compounds oligosaccharides could be affected by DP of the compound. In this study, the antioxidant activity in xylo-oligosaccharide hydrolysis results Hemi B with variations hydrolysis time were also tasted.

Table 2. The results of measurements and calculations % DPPH Scavenging by xylo-oligosaccharide from Hemi B hydrolysis product with hydrolysis time variations

Xylo-oligosakarida (Hemi B)	Xylo-oligosakarida concentration (Hemi B)	Absorbance			Antioxidant Activity	
		A_{497}	A_{517}	A_{537}	% Scavenging	IC_{50} (ppm)
Hemi B of corncob	80 ppm	0,081	0,082	0,081	87,5 %	24
Hydrolyzed for 6 hours	60 ppm	0,080	0,085	0,087	81,25 %	
	40 ppm	0,090	0,099	0,104	75 %	
	20 ppm	0,083	0,104	0,115	37,5 %	
	control	0,081	0,100	0,103	-	
Hemi B of corncob	80 ppm	0,269	0,281	0,288	68,75 %	47,61
Hydrolyzed for 12 hours	60 ppm	0,175	0,192	0,203	62,5 %	
	40 ppm	0,148	0,172	0,187	43,75 %	
	20 ppm	0,127	0,152	0,166	31,25 %	
	control	0,081	0,100	0,103	-	
Hemi B of corncob	80 ppm	0,482	0,541	0,565	78,125 %	48,96
Hydrolyzed for 24 hours	60 ppm	0,398	0,474	0,475	53,125 %	
	40 ppm	0,508	0,607	0,619	43,225 %	
	20 ppm	0,477	0,567	0,540	26,875 %	
	control	0,516	0,635	0,594	-	

Table 3. Observational data xylo-oligosaccharide toxicity tests with *Artemia salina* L

Concentration of test solution (ppm)	Number of *Artemia salina* larvae tested		Number of *Artemia salina* larvae dead after treatment		Number of *Artemia salina* larvae dead in control	
	Replication I	Replication II	Replication I	Replication II	Replication I	Replication II
80	10	10	6	8	0	1
60	10	10	4	2	0	0
40	10	10	1	2	0	0
20	10	10	0	0	0	0

From Table 2, the antioxidant activity of xylo-oligosaccharide was affected by the hydrolysis time. Xylo-oligosaccharide from Hemi B hydrolysis product was incubated for 6 hours and had a high antioxidant activity when compared with the incubations for 12 hours and 24 hours, while Hemi B without hydrolysis had the lowest antioxidant activity. The antioxidant activity of xylo-oligosaccharide from Hemi B hydrolysis product incubated for 12 hours and 24 hours were almost the same.

Toxicity test of *Brine Shrimp Lethality Test* (BSLT)

BSLT method was performed by counting the number of dead larvae in each test solution.

Larvae mortality data were obtained and analyzed using a SPSS program to determine the relationship between the number of larvae mortality with the concentration of the test solution. Test result was obtained in LC_{50} value. The calculation of LC_{50} value with SPSS obtained an average LC_{50} for xylo-oligosaccharide of 400 ppm.

DISCUSSION

The development and the advancement of agriculture and agricultural industry in Indonesia have led to an increase in the agricultural waste that are largely a lignocellulosic biomass. Lignocellulosic biomass has not been optimally utilized. Most of biomass will only be destroyed by burning. Continuous combustion process can lead to the accumulation of CO_2 in the air that will give an impact as global warming. When examined more deeply, lignocellulosic biomass is composed of organic materials such as hemicellulose, cellulose and lignin, and has a great potential as raw material for various industries. In addition, fractionation of this waste into its constituent components will increase its utilization in various industries. Among lignocellulosic biomass, corncob is not optimally used. Corncob fibers have a composition comprising starch (10–25% (b/b)), hemicellulose (40–50% (b/b)), cellulose (15–25% (b/b)) and phenolic acid (3–5% (b/b)), while the residual consists of protein and oil.

Natural antioxidant is an antioxidant that comes from nature or synthesized through a chemical reaction, and its structure is derived also from nature. The examples of natural antioxidants are poliphenol, flavonoid (flavonon, flavonol, katekin), vitamin E (tokoferol), vitamin C (asam askorbat), and β-karoten. Synthetic antioxidants are antioxidants that are synthesized through a chemical reaction and their structure is derived from nature such as propyl galat, octyl galat, BHA, BHT and askorbil palmitat.[11] Research have recently observed that oligosaccharide compounds also have antioxidant activity.[2]

Hemicellulose hydrolysis enzymatically has specific properties. The endo-β-xylanase can hydrolyze xylan as a constituent hemicellulose. The endo-β-xylanase (1,4 - β-D-xylanxylanohidrolase, EC.3.2.1.8) can hydrolyze xylan basic structure randomly into xylo-oligosaccharides. The antioxidant activity of a compound can be determined by various methods, such as by measuring the activity of DPPH radical catcher, FTC measurement (Ferry thiocianide), salt reduction method of Fremy, TEAC measurement (Trolox Equivalent Antioxidant capacity), etc. About the Scavenging mechanism of DPPH by the antioxidant, both xylo-oligosaccharide hydrolysis products from Hemicellulose of corn cobs have antioxidant activity because they have IC_{50} values <100 ppm,[8] but these results suggest that xylo-oligosaccharide from Hemi B hydrolysis product have a IC_{50} value greater than the that of Hemi A (Figure 2) that xylo-oligosaccharides from Hemi B is more active as an antioxidant than the Hemi A.

The antioxidant activity of oligosaccharides compounds was affected by Degree of Polymerization (DP) of the compound. Previous studies have succeeded in proving that the existence of the antioxidant activity from galacto-oligosaccharides obtained marine algae by acid hydrolysis, and are influenced by the degree of polymerization of is xylo-oligosaccharides.[12] The higher the degree of the polymerization of xylo-oligosaccharides compound, is the higher the antioxidant activity will be. In this study, xylo-oligosaccharide from Hemi B hydrolysis product based on the TLC has shown that they have a higher degree of polymerization than that from the Hemi A (Figure 1).

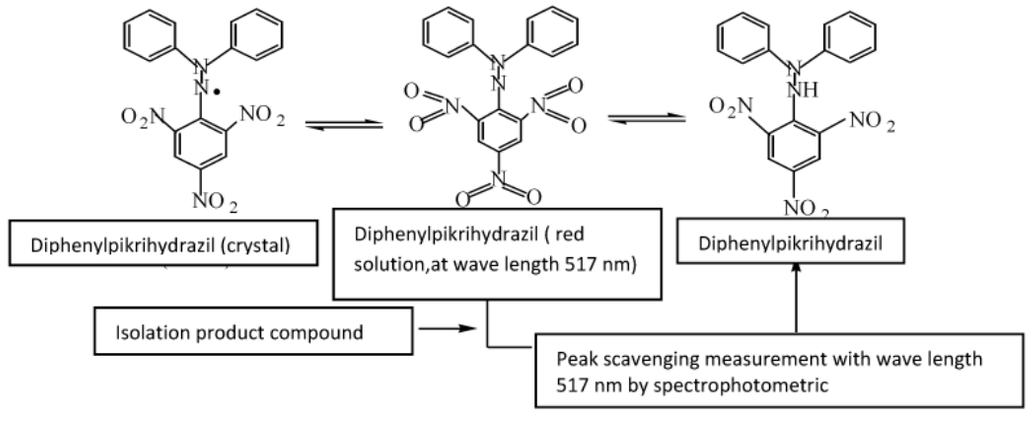

Figure 3. DPPH Scavenging mechanism with antioxidant.

But the spots of both of the xylo-oligosaccharide were tailing although we could distinguish the Rf value of xylo-oligosaccharide. This is because the tailing spots of xylo-oligosaccharides produced a mixture of xylo-oligosaccharides that have degrees of polymerization which are adjacent. We still have not been able to prove the influence of the degree of polymerization toward the antioxidant activity. In this study, the antioxidant activity in xylo-oligosaccharide from Hemi B hydrolysis product with variations hydrolysis time (6 hours, 12 hours, and 24 hours) (Table 2) was also tested. This is due to the very influential hemicellulose hydrolysis products and degree of polymerization of the product of hydrolysis. Hydrolysis time can produce hydrolysis products with low Degree of Polymerization (DP) longer such as sugar monomers. Hemi B was also used without hydrolysis as the polysaccharide production controller with a high molecular weight polysaccharide which was insoluble in water. From the calculation with SPSS, LC_{50} value was obtained, with the average LC_{50} for xylo-oligosaccharide of 400 ppm (Table 3). It shows that xylo-oligosaccharide has antitumor activity since it has LC_{50} less than 1000 ppm.

CONCLUSION

It can be concluded that hemicellulose of corn cobs hydrolysis product has higher antioxidant activity. Hemi B without hydrolysis (Polysaccharides) had no antioxidant activity, and had IC_{50} values > 100 ppm because of its very large molecular weight that the antioxidant activity is influenced by the steric effect of the polysaccharide in reducing free radicals. To figure out the toxicity of xylo-oligosaccharide as an antioxidant compound, BSLT method was performed by counting the number of dead larvae in each test solution.

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