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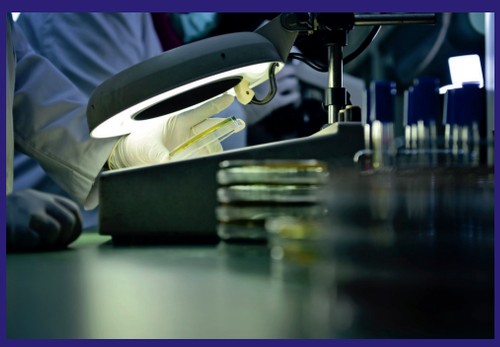

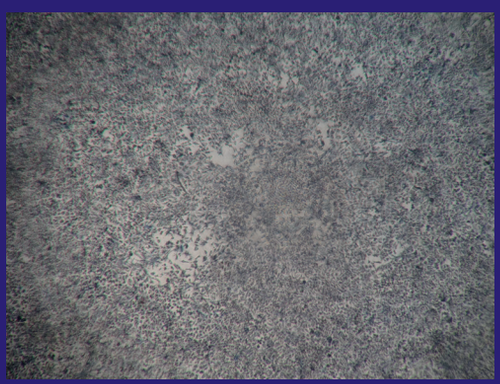

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Research Report

COMBINED TARGET SITE VGSC MUTATIONS PLAY A PRIMARY ROLE IN PYRETHROID RESISTANT PHENOTYPES OF *AEDES AEGYPTI* AS DENGUE VECTOR FROM PALU CITY, CENTRAL SULAWESI

Purwaningsih[1], Sitti Rahmah Umniyati[2a], Budi Mulyaningsih[2]
[1] Post Graduate Student of Tropical Medicine Program, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.
[2] Department of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.
[a] Coresponding author: sitti-rahmah@ugm.ac.id

ABSTRACT

It has been reported that Aedes aegypti mosquitoes in Palu City had been resistant to cypermethrin insecticide but the resistance mechanism is not well known. This study aimed to determine the resistance status of Ae. aegypti to cypermethrin and whether the mutation of voltage-gated sodium channel (VGSC) was associated with pyretroid resistance in high and low dengue endemic areas in Palu City. Aedes aegypti collected from each village was reared to adult and assayed for susceptibility test to cypermethrin using the CDC bottle bioassay method. PCR primers of AaSCF1 and AaSCR4 were used for screening of IIS6 VGSC gene mutation. PCR primers of AaSCF7 and AaSCR7 were used for screening of IIIS6 VGSC gene mutation. For an identification of mutation sites were sequenced and aligned to Gene bank (access No. AB914689 and AB914690) for IIS6 VGSC and Gene bank (access No. AB914687 and AB914688) for IIIS6 VGSC gene mutation. The susceptibility status of Ae. aegypti to cypermethrin was resistant in high dengue endemic areas and moderately resistant in low dengue endemic areas. It was found double point mutation at S989P and V1016G in Ae. aegypti from high and low dengue endemic areas in Palu City and there was a single point mutation only in high dengue endemic area at target site V1016G. Aedes aegypti from both high and low dengue endemic areas were resistant to cyperpethrinn and the two alleles had a major role in the occurrence of cypermethrin resistance in Palu City.

Keywords: *Aedes aegypti, resistance, pyrethroid, VGSC gene mutation*

ABSTRAK

Telah dilaporkan bahwa nyamuk Aedes aegypti di Kota Palu telah resisten terhadap insektisida sipermetrin, tetapi mekanisme resistensinya belum diketahui dengan baik. Penelitian ini bertujuan untuk mengetahui status resistensi Ae. aegypti terhadap sipermetrin dan untuk menentukan apakah mutasi voltage gated sodium channel (VGSC) dikaitkan dengan resistensi piretroid di daerah endemis dengue yang tinggi dan rendah di Kota Palu. Aedes aegypti yang dikumpulkan dari masing-masing desa dipelihara sampai dewasa dan diuji untuk uji kerentanan terhadap sipermetrin menggunakan metode CDC botol bioassay. Primer PCR AaSCF1 dan AaSCR4 digunakan untuk skrining mutasi gen IIS6 VGSC. Primer PCR AaSCF7 dan AaSCR7 digunakan untuk skrining gen IIIS6 VGSC. Untuk identifikasi lokasi mutasi disekuensing dan disejajarkan dengan bank Gene (akses No. AB914689 dan AB914690) untuk IIS6 VGSC dan Gene bank (akses No. AB914687 dan AB914688) untuk mutasi gen IIIS6 VGSC. Status kerentanan Ae. aegypti terhadap sipermetrin resisten di daerah endemik dengue tinggi dan resisten sedang di daerah endemik dengue rendah. Ditemukan titik ganda mutasi pada S989P dan V1016G Ae. aegypti dari daerah endemik dengue tinggi dan rendah di Kota Palu, dan ada satu titik mutasi hanya di daerah endemik dengue tinggi pada target site V1016G. Aedes aegypti dari daerah endemik dengue tinggi dan rendah resisten terhadap sipermetrin, dan kedua alel memiliki peran besar dalam terjadinya resistensi sipermetrin di Kota Palu.

Kata Kunci: *Aedes aegypti, resisttensi, pyrethroid, mutasi gen VGSC*

INTRODUCTION

Aedes aegypti is the most efficient vector for arboviruses because it is highly anthropophilic, frequently bites, and thrives in close proximity to humans.[1] Indonesia is a hyperendemic area with the spread of cases in both urban and rural areas.[2,3] DHF often causes outbreaks in several districts/cities in Indonesia. The number of dengue cases always increase every year. In 2016, Palu City had the highest number of DHF cases in Central Sulawesi Province, 2 people were died from Balaroa Village. Balaroa Village is categorized as high dengue endemic area while Siranindi Village is categorized as low dengue endemic area of DHF. Almost all Primary Health Facilities in Palu City had problems with DHF every year, and there are reported cases of death.[4]

Various DHF prevention policies and strategies have been programmed. The Government of Palu City issued Regional Regulation No. 2, 2016 about concerning the control of DHF as proof of the seriousness in DHF control efforts, but the results are still not optimal until now.[4] *Aedes aegypti* resistance to insecticides is a global phenomenon. Resistance is inherited and a single obstacle to the success of chemical vector control.[5] The using of insecticides from the community is a factor that triggers resistance.[6] The Health Office of Palu City used malathion insecticides for vector control since its establishment in 1997, but the using of malathion was stopped in 2013. The using of cypermethrin insecticide began in 2014.[4]

The latest information from several areas in Palu City is that adult mosquitoes of *Ae. aegypti* have been resistant to cypermethrin 0.05% based on the conventional method of impragnated paper.[7] Resistance mechanism of *Ae. aegypti* to cypermethrin insecticide has not been identified using this method. There are several methods to determine the resistance mechanism, including biochemical testing to detect the mechanism of metabolic resistance and molecular tests to determine the target site resistance mechanism to insecticides. An example of the target site resistance mechanism to pyrethroids is known as knockdown resistant (kdr)/voltage gated sodium channel (VGSC) gene. The molecular method can determine mutations in the VGSC gene due to selection pressure of insecticides in the organochlorine and pyrethroid groups.[8]

This study was aimed to determine the resistance status of *Ae. aegypti* to cypermethrin and to determine whether the mutation of the voltage-gated sodium channel (VGSC) gene was associated with pyrethroid resistance in high and low dengue endemic areas in Palu City.

MATERIALS AND METHODS

The observational study with cross-sectional analytical design[9] was approved by the Medical and Health Research Ethics Committee of Faculty of Medicine of Universitas Gadjah Mada number KE /FK/262/EC/2017.

One hundred and twenty ovitraps were installed in high and low dengue endemic area (Balaroa and Siranindi Villages). The coordinates of sampling locations are recorded using GPS (Global Positioning System). The ovitraps were installed for 3-4 days and the ovistrips were carefully dried, labeled and inserted in clear plastics and labeled to be stored.

The mosquito eggs were colonized in the insectarium of Department of Parasitology of the Faculty Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. The eggs hatch into larvae were kept until they became pupae. They were taken with a pipette and kept in a cage. Pupae developed into adult mosquitoes after 2 days. Adult mosquitoes were fed 10% sugar solution which was absorbed in cotton. The temperature of test room was 27 ± 2°C, humidity was 75 ± 10% and photoperiod consisted of 12 hours of light: 12 hours of darkness.[10] The Adult mosquitoes were identified to determine *Ae. aegypti* mosquito and were colonized to the F1 Generation mosquito

CDC Bottle Bioassay

Each testing was involved 125 female adults of *Ae. aegypti* F1 generation, aged 3-5 days. Female mosquitoes were fed only with 10% sugar solution the day before testing. The test used 1 control bottle, and 4 test bottles. Each bottle was labeled (4 test bottles, 1 control bottle). The test bottle was filled with 1ml of cypermethrin 10µg/ml solution and the control bottle was filled with 1ml of acetone, then the bottle was tightly closed and the solution was coated on the wall, bottom and bottle cap. Bottles were dried at room temperature for 24 hours. Using an aspirator 25 mosquitoes were introduced into each test bottle and control bottle. A number of knockdown and or alive mosquitoes were recorded every 5 minutes during the diagnostic time of 30 minutes of exposure. Observation was continued until all are dead or up to 2 hours. Mortality was corrected with Abbott's Formula if the mortality at 2 hours in the control bottle is between 3% and 10%. The result should be discarded if the mortality in the control bottle > 10%. Mosquitoes were moved to an insecticide-free recovery cage and were administered with 10% sugar for 24 hours and recorded the number of dead mosquitoes.

$$\text{Abbott's Formula} = \frac{\%\ \text{mortality in test} - \%\ \text{mortality in control}}{100 - \%\ \text{mortality in control}} \times 100\%$$

The resistance status was classified in three categories according to WHO as follows: 98–100% mortality at the recommended diagnostic time indicates susceptibility; 80–97% mortality at the recommended diagnostic time suggests the possibility of resistance that needs to be confirmed; <80% mortality at the recommended diagnostic time suggests resistance.[11]

The Molecular Test

The isolation of genomic DNA was done individually using Genomic DNA mini Kit Geneaid™ Cat No. GB100. Lot No.JM02202 according to the manufacturer's

instructions.[12] PCR primers AaSCF1 (AGA CAA TGT GGA TCG CTT CC) and AaSCR4 (GGA CGC AAT CTG GCT TGTTA) were used for screening of IIS6 VGSC gene mutation. PCR primers of AaSCF7 (GAG AAC TCG CCG ATG AAC TT) and AaSCR7 (GAC GAC GAA ATC GAA CAG GT) were used for screening of IIIS6 VGSC gene mutations.[13] The PCR mixtures contained 15 µl of Mix PCR (Go Taq® Green Master mix. 2x), 11 µl of ddH2O (nuclease-free water lot. 0000123190. Promega), 2 µl of R & F primers (20 µM), 2 µl of the DNA template in a total volume of 30µl. PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 35 cycles each of 94°C for 15s, 55°C for 30s, and 72°C for 30s; and a final elongation step at 72°C for 10 min.[13]

The DNA amplification results were separated according to the size of the base pair by electrophoresis technique. Electrophoresis technique used 2% agarose gel which added 1 µl gel red. The Gel was inserted in chamber electrophoresisis which already contained solution buffer 50X TAE to cover surface gel. Product PCR was taken up 7 µl and inserted on gel well. Standard molecules were used 100 kb ladder marker. Power supply was run with potential difference of 100 volts for approximately 45 minutes. Observation of the DNA bands was done below UV light on Gel doc. The electrophoresis results were read on the target band and documented. The samples which showed bands of target DNA were sent to PT. Genetica Science Indonesia. Samples would be sent to 1st Base Laboratories Singapore for sequencing.

For an identification of mutation sites were sequenced and aligned to Gene bank (access No. AB914689 and AB914690) for IIS6 VGSC gene mutation (S989P, I1011M/V, V1016G/I) and aligned to Gene bank (access No. AB914687 and AB914688) for IIIS6 VGSC gene mutation (T1520I and F1534C)[13] using mega version 7.0.18 and bio edit version 7.2.6.

RESULTS AND DISCUSSION

Result of CDC Bottle Bioassay

It was shown the susceptibility status of *Ae. aegypti* to cypermethrin based on CDC bottle bioassays in high and low dengue endemic areas. The result of statistical analysis through bivariate test using independent T-test, obtained that the result the susceptibility status based on mortality rate between high and low dengue endemic area were significant differences with p value = 0.000 (p <0,05).

It was indicated that there was knockdown resistance (kdr) of *Ae. aegypti* mosquitoes from high and low dengue endemic areas, because there were reduction of mortality, about 26% dan 19,5% after 24 hours in recovery cages (Table 1).

Result of Molecular Assays and Sequencing Analysis

The amplification results of IIS6 and IIIS6 VGSC gene were visualized with 2% agarose gel electrophoresis and read under UV obtained specific band, 619 bp and 748 bp respectively (Figure 1 and Figure 3).

The PCR product was sequenced to determine the mutation of IIS6 VGSC gene (Figure 2). The point mutation of S989P at IIS6 site occurred because one of nucleotide base changed from thymine to citocin at codon TCC → CCC caused the amino acid changed from serine to proline.

Figure 2 showed, there were double point mutation in target site S898P and V1016G and there was single point mutation in target site V1016G.

The PCR product was sequenced to determine the mutation of IIIS6 VGSC gene (Figure 4).

Table 1. The result of CDC bottle bioassay of *Ae. aegypti* to cypermethrin 10 µg/bottle (diagnostic dose)

Location (villages)	Generation	Mortality (%)			Category
		30 minutes	2 hours	Holding 24 hours 24 jam	
High endemic dengue area (Balaroa)					
Test bottle-1	F1	65	98	66	resistant
Test bottle-2	F1	59	100	82	resistant
Test bottle-3	F1	66	99	71	resistant
Average high endemic		63,33	99	73	resistant
Control bottle	F1	0	0	0	
Low endemic dengue area (Siranindi)					
Test bottle-1	F1	91,2	95,2	75,2	moderate resistant
Test bottle-2	F1	89,6	95,2	80,8	moderate resistant
Test bottle-3	F1	90,4	97,6	73,6	moderate resistant
Average low endemic		90,4	96	76,5	moderate resistant
Control bottle	F1	0	0	0	
Laboratory Strain	F1057	99	100	100	susceptible

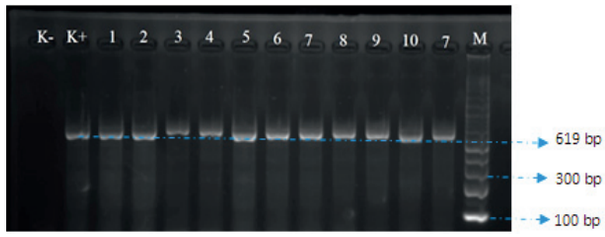

Figure 1. Visualization of IIS6 VGSC gen amplification of *Ae. aegypti* from Balaroa (1-6) and Siranindi (7-10), M (100 lader DNA marker), K- (negative control, without VGSC gene DNA), K+ (positive control of VGSC gene)

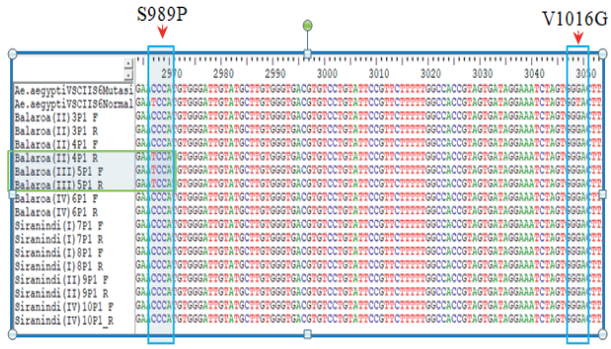

Figure 2. Result sequensing aligned with gen bank access AB914689 and AB914690 indicated mutation of IIS6 VGSC gene of *Ae. aegypti* target site Serin (TCC) 989 Prolin (CCC) and Valin (GTA) 1016 Glycin (GGA) (Mega version of 7.0.18 and Bio edit version of 7.2.6)

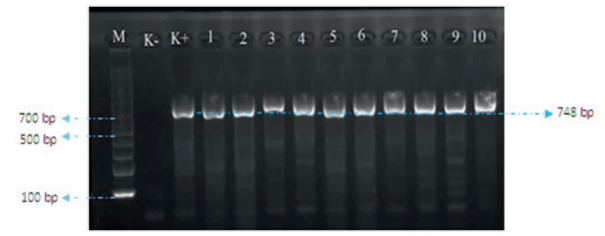

Figure 3. Visualization of IIIS6 VGSC gene amplification of *Ae. aegypti* from Balaroa (1-6) and Siranindi (7-10), M (100 lader DNA marker), K- (negative control, without VGSC gene DNA), K + (positive control of VGSC gene)

DISCUSSION

It was shown that the susceptibility status of *Ae. aegypti* to cypermethrin insecticide was resistant in high endemic dengue area and moderate resistant in low endemic dengue area.

Differences in susceptibility status between regions can occur because they are influenced by differences in knowledge, education, control efforts and frequency of insecticides used for health and agricultural purposes.[14] Cypermethrin insecticide began to be used for the benefit

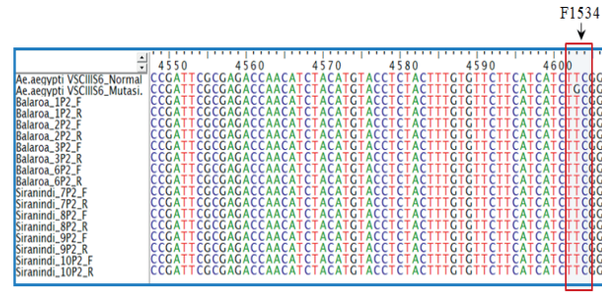

Figure 4. Results of sequencing aligned with Gene bank access AB914687 and AB914688. There weren't change TTC (phenilalanine) to Cystein (TGC) (Mega version of 7.0.18 and Bio edit version of 7.2.6)

of the program in Palu City, in 2014. Insect resistance to insecticides generally occurs after 2-20 years of use. The use of insecticides on a large scale, continuously for a long period of time and high frequency can cause a decrease in susceptibility to mosquitoes targeted.[15]

The process of the occurrence of vector resistance to certain insecticides is influenced by multiple factors, namely genetic (presence of specific gene frequencies), operational (insecticide type and application) and biological (size and characteristics of vector populations).[16] The different of susceptibility status in these two regions due to operational factors, namely vector control through fogging from the program until now is still being used. Fogging is carried out when there is a DHF case report. Dengue endemic areas have a higher fogging frequency compared to low dengue endemic areas.

Another factor that triggers a decrease in the susceptibility status of *Ae. aegypti* mosquitoes from these two villages were the use of household insecticides by the local community. Some household insecticides such as aerosol formulations and other formulations were used in Balaroa and Siranindi Villages, 28.2% and 36.95% respectively. The active ingredients used were malation and other active ingredients such as propoxur (bendiocarb) which can cause multiple resistance. *Aedes aegypti* were still undergoing selection pressure on organophosphate insecticides in Palu City, but selection pressure was higher for cypermethrin insecticides because of the use of the program and the effect of exposure from household insecticides. The data of research through structured interviews showed that household insecticides in Balaroa and Siranindi Villages were equal to 76.67%. Most household insecticides were pyrethroids. The percentage of household insecticides which was quite high affects the susceptibility status to cypermethrin insecticides in both regions.

Another factor that influences the increase in resistance status is the ability of mosquitoes to adapt and evolve well. Mosquitoes have a high reproductive speed and a short generation period so that mosquitoes are susceptible to genetic mutations.[17] This is evidenced by research in Malaysia which shows an increase in *Ae. aegypti* resistance to permethrin is 5-18 times after five generations.[18,19]

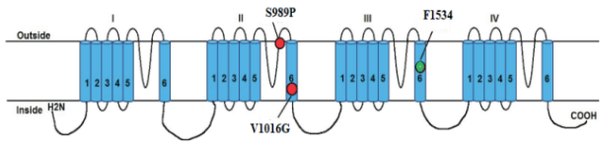

Figure 5. IIS6 VGSC double point mutation target site S989P and V1016G associated to resistance to pyrethroid from *Ae. aegypti* were mostly detected in this study

Aedes aegypti resistance against cypermethrin insecticide occurs in West Venezuela,[20] and several regions in Central Java (Semarang, Grobogan, Purbalingga and Kendal).[21] The resistance of *Ae. aegypti* against deltamethrin is also reported in Central Java (Semarang, Jepara, Blora, Salatiga, Surakarta, Tegal, Magelang and Purwokerto).[22]

The mechanism of resistance to pyrethroid insecticides can be detected molecularly. This study indicated that there was a target site mutation in the VGSC gene. The target site mutation in VGSC gene regarding resistance to pyrethroids suggests that there is an ongoing resistance mechanism. Detection of VGSC gene mutations can directly assess the transformation of target cells which are the target of insecticides. Gene mutation causes conformational changes in the sodium channel because it can not be opened by insecticide molecules.

Mutations like this can only be detected by molecular methods. The basic principle of molecular detection of resistance in vectors is identify genes.

The results showed that most of the samples of *Ae. aegypti* from Balaroa and Siranindi villages, Palu City experienced double-point mutations (two-point mutations simultaneously) at S989P, and V1016G in IIS5-S6 and IIS6 VGSC genes respectively (Figure 5). The results of susceptibility test gave a very specific description of phenotypic resistance events and were supported by mutations found in *Ae. aegypti* mosquito in VGSC gene (genotypic resistance). A valine to glycine transversion in domain II of the VGSC (V1016G) is associated with resistance to type I and II pyrethroids, such as permethrin and deltamethrin, respectively.[23]The V1016G mutation is often found with a serine to proline mutation (S989P) in domain II. They have also been found in several other regions of Asia. Mutations were reported at these points in Thailand, Myanmar, Vietnam, Taiwan and Indonesia.[24]

In this study, the only one sample experienced a single point mutation at the V1016G target site in Balaroa Village. It was also reported in Klaten, Central Java.[23] The results of study by Rajatileka[25] and Srisawat[26] found a point mutation at target site V1016G in VGSC gene of *Ae. aegypti* that was associated with pyrethroid synthetic resistance in Thailand.

The V1016G allele seems limited in Southeast Asia, but recently the V1016G allele was found in Mecca.[27] Different substitutions of V1016I are found in *Ae. aegypti* populations from Brazil V1016I.[28,29] The V1016I allele was distributed in South and North America (Alvarez *et al*., 2013)[20], but the V1016I allele was also found in Palembang-Indonesia (Ghiffari *et al*., 2013).[30]

Transformation in valine to glycine IIS6 VGSC (V1016G) were associated with resistance to type I and II pyrethroids, such as permethrin and deltamethrin.[23] Pyrethroids mainly affect the peripheral and central nervous system in insects by binding to the VGSC target site in the nerve membrane. Some of the advantages of insecticides from this group include low levels of toxicity to humans and mammals in general and easily decompose in the soil (Martins *et al*., 2009).[28]

Pyrethroid is divided into 2 types based on chemical structure & the effects, such as pyrethroid Type I and Type II. Pyrethroid type II contains parts of α-cyano-3-phenoxybenzyl alcohol such as cypermethrin, sifulthrin, deltamethrin, fenvalerate, esfenvalerate and lamellalhalrin (Ishak *et al*, 2015).[31] It was reported by Al Nazawi[27] that the *Ae. aegypti* sample was resistant to deltamethrin. The strain from Mecca experienced a point mutation simultaneously S989P and V1016G. Point mutations found simultaneously were also reported in *Ae. aegypti* populations from Latin America in different substitutions and alleles, I1011V and V1016I (Plernsub *et al*., 2016).[32]

Mutation is a marker for monitoring resistance (Ishak et al., 2015).[31] According to Widyastuti *et al.* (2015)[33] that VGSC gene mutations in several positions can occur simultaneously in one individual mosquito and the possible effect will be greater on mosquito resistance properties. *Aedes aegypti* V1016G strains (occurring with and without S989P) and F1534C mutations are common and widespread throughout Asia. The G1016 allele was known to be associated with resistance to type I and II pyrethroids. The C1534 allele was mainly associated with resistance to pyrethroid type I and known as recessive alleles (Ghiffari *et al*., 2013.[30] F1534 allele of this study are similar to those conducted by Stenhouse.[24]

CONCLUSION

Aedes aegypti from high and low dengue endemic areas were resistant to cyperpethrinn, and the two alleles (V1016G and S989P) had a major role in the occurrence of cypermethrin resistance in Palu City.

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Research Report

ACID-FAST BACILLI CONVERSION OF *BEIJING* AND NON-*BEIJING* STRAIN OF PULMONARY TUBERCULOSIS IN SOUTH SULAWESI

Syahridha[1], Ni Made Mertaniasih[2], Sustini Florentina[3], Soedarsono[4]
[1]Magister of Tropical Medicine, Faculty of Medicine, Universitas Airlangga
[2]Department of Microbiology, Faculty of Medicine, Universitas Airlangga
[3]Departemen of Publich Health Science and Prevent Medicine, Faculty of Medicine, Universitas Airlangga
[4]Departement of Pulmonology and Respiratory, Universitas Airlangga-Dr, Soetomo General Hospital
[5]Doctor of Soetomo General Hospital Surabaya
[a] Corresponding author: Email: nmademertaniasih@gmail.com, ni-made-m@fk.unair.ac.id

ABSTRACT

Beijing strains are a major part of the Mycobacterium tuberculosis Asian phylogenetic lineage. Beijing strains represent about 50% of all TB strains in East Asia and at least 13% of strains worldwide. Beijing strain of Mycobacterium tuberculosis is presumed as the factor of the increase in bacteria virulence and drug resistance, and the contributor in treatment failure. The aim of this study was to analyze the association between acid-fast bacilli conversion with strain genotipe Beijing and non-Beijing of pulmonary tuberculosis in South Sulawesi. The design of research was observational analytic with prospective approach. The sampling technique used consecutive sampling. Data were taken from active pulmonary tuberculosis patients' medical record in Balai Besar Kesehatan Paru Masyarakat Makassar (Pulmonary Health Center of Makassar) and Community Health Center in Gowa Regency, South Sulawesi from March to June 2018. Collected sputum samples were screened for AFB and identified as Beijing strain and non Beijing strains using Multiplex PCR in Tropical Disease Institute of Universitas Airlangga. The results is showed that the characteristics of the respondents consisted of 12 respondents (33.3%) aged 56-65 years, 25 respondents (69.4%) men and 28 respondents (77.8%) had low category gradation of AFB smear. Univariate analysis showed 6 respondents (16.7%) with Beijing strains, 30 respondents (83.3%) with non-Beijing strains, 32 respondents (88.9%) conversion sputum AFB and 4 respondents (11.1%) non conversion sputum AFB. Bivariate analysis with Chi-Square statistical test shows that p value 0.022 < 0,05, that means there was association of Beijing strains with BTA conversion. Microscopic examination of BTA can be used to monitor and evaluate the treatment of new pulmonary TB patients undergoing treatment and the Beijing Mycobacterium tuberculosis strain has a significant correlation with the treatment failure of anti-tuberculosis drugs in South Sulawesi.

Keywords: *AFB conversion, Beijing strain, Mycobacterium tuberculosis, pulmonary tuberculosis, Treatment failure*

ABSTRAK

Strain Beijing merupakan bagian utama dari garis keturunan filogenetik Mycobacterium tuberculosis Asia. Strain Beijing mewakili sekitar 50% dari seluruh strain TB di Asia Timur dan setidaknya 13% dari strain seluruh dunia. Strain Beijing dari Mycobacterium tuberculosis diprediksi penyebab peningkatan virulensi bakteri, resistensi terhadap pengobatan, dan berkonstribusi dalam kegagalan pengobatan. Penelitian ini bertujuan menganalisis asosiasi konversi BTA mikroskopis dengan strain Beijing dan non Beijing pada pasien tuberculosis baru di Sulawesi Selatan. Jenis penelitian adalah analitik observasional dengan rancangan penelitian prospektif. Teknik pengambilan sampel menggunakan Konsekutif sampling. Data diperoleh dari rekam medis pasien dengan TB paru aktif yang dirawat di Balai Besar Kesehatan Paru Masyarakat Makassar dan Puskesmas di Kabupaten Gowa, Sulawesi Selatan pada Maret sampai dengan Juli 2018. Sampel sputum yang dikoleksi dilakukan pemeriksaan BTA kemudian diidentifikasi strain Beijing dan non Beijing dengan metode Multiplex PCR di Institute penyakit tropis Universitas Airlangga. Hasil penelitian menunjukkan karakteristik responden terdiri dari 12 responden (33,3%) berusia 56-65 tahun, 25 responden (69,4%) laki-laki dan 28 responden (77,8%) memiliki

gradasi BTA kategori Low. Analisis univariat menunjukkan 6 responden (16,7%) dengan strain Beijing, 30 responden (83,3%) dengan strain non-Beijing, 32 responden (88,9%) mengalami konversi sputum BTA dan 4 responden (11,1 % tidak mengalami konversi sputum BTA). Analisis bivariat dengan uji statistik Chi-Square menunjukkan p value 0,022, terdapat assosiasi strain Beijing dengan konversi BTA. Pemeriksaan BTA dengan mikroskopis dapat digunakan untu monitoring dan evaluasi pengobatan pasien TB Paru yang baru mejalani pengobatan serta strain Beijing Mycobacterium tuberculosis memiliki korelasi yang signifikan dengan kegagalan pengobatan obat anti tuberculosis di Sulawesi Selatan.

Kata Kunci: *Konversi BTA, Strain Beijing, Mycobacterium Tuberculosis, TBC Paru, Kegagalan Pengobatan*

INTRODUCTION

Pulmonary tuberculosis (TB) is both an acute and chronic disease which caused by *Mycobacterium tuberculosis* that attacks the lungs. The disease spreads from one individual to another through the respiratory system. In 2015, it was estimated that there were 10.4 million new cases of TB worldwide; of which 5.9 million are found in adult male, 3.5 million in adult female, and 1 million in children. Some countries in South and East Asia such as The Democratic Republic of Korea, Indonesia, Myanmar, Bangladesh, India, and Thailand comprise 46.5% of the total global number. India and Indonesia alone make up one-third of the worldwide TB number, each having 23% and 10% respectively[1].

Global efforts on TB control were required integrated and coordinated responses to monitor and assess global outbreak of TB, drug resistance surveillance, and high-risk population. An integrated approach is a must, combining networks of laboratories, TB control programs, and application of state-of-the-art molecular techniques to identify and report tuberculosis bacilli clones occurring around the world[2].

Tuberculosis occurrence in Indonesia is estimated at 1,020,000 cases, with only 330,729 of those are reported. Of all the cases in Indonesia, 93% are pulmonary TB with 64% of those are diagnosed through microscopic evaluation of acid-fast bacilli smear. The large number of untreated TB will become the source of further infections, creating urgency in having continuous preventive and curative measures. The success of a TB countermeasure program is linked to the success in DOTS treatment. Such treatment is deemed to be a success if it is evidenced by sputum AFB conversion at the final phase of the intensive treatment (2 months) reaching at least 80% and recovery rate at the end of treatment reaching more than 85%[3]. Acid Fact Bacilli conversion is a strong predictor and the initial success of therapy in pulmonary TB[4]. Sputum conversion in pulmonary TB was determined based on the absence of Acid Fact Bacilli (AFB) in sputum taken at the end of the 2nd month of treatment. Sputum conversion in pulmonary TB cases occurred at the end of the first month (60-80%) and at the end of the second month (95%)[4].

AFB conversion is one of the methods used in Indonesia which have high accuracy, fast and economical costs. Another method was Chest X Ray but the results are not specific to diagnosis tuberculosis[5]. In addition there are culture methods that have a higher sensitivity but the cost not effective and needed long time for know the result of diagnosis. The research of Wang X, et al was showed that by using direct smear method Ziehl Nielsen staining obtained a sensitivity of 40%, then in the same sample culture method was carried out so the sensitivity value would increase by 65% [6].

Sputum AFB conversion can be affected by previous medical history. *Mycobacterium tuberculosis* exposed with antibiotics might undergone genetic mutation as a result of its biological trait to defend itself. Mutation might occur to a specific gene targeted by the medication, creating risk of drug resistance, which in turn resulting in sputum AFB conversion failure[7].

Another factor correlating with sputum AFB conversion is the *Mycobacterium tuberculosis*' genotype. The genotype of *Mycobacterium tuberculosis* that attack TB patients in Indonesia are different from one province to another as a result of the geographical differences of each province[8]. Different strain has different characteristics; one of which is the tendency to develop antibiotics resistance. *Mycobacterium tuberculosis complex* (MTBC) strains which are known to be easier to treat are *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium canneti* and *M. tuberculosis*[9].

Molecular epidemiology studies have pointed out that *Mycobacterium tuberculosis*' genotype is varied based on geographical distribution[10]. Some pathogen hosts have undergone adjustments while specific genotype has evolved to increase its pathogenicity and virulence. In some East Asian countries such as China and Hong Kong, 86% of the occurring genotype is *Beijing* strain[11]. In contrast, Indonesia has more varied distributions. South Sulawesi, for instance, is still dominated by *Beijing* strain. Beijing strain genotypes have specific characteristic such as contribute to the spread of drug-resistant TB and clinically related to treatment failure[12]. Thai studies is showed infection with Beijing genotype is a significant risk factor for bacterial persistence in treatment that results in treatment failure or relapse inside 2 years[13].

Lisdawati's research on *Beijing* strain distribution in Indonesia is showed that in Palembang and Lampung, *Beijing* strain occurrence number is 31.48%; Serang, Jakarta, Bandung, and Surabaya have 28.83%; Banjarmasin and Pontianak have 16.18%; and Makassar has 25.93%. *Beijing* strain distribution in male is 24.2%, while in female is 15.3%. Specifically in Makassar, other occurring

strains are sub-type EAII (1 pattern), H1 (1 pattern), LAM (2 patterns), EA12 (2 patterns), U (3 patterns), UlikeS (1 pattern), EA15 (1 pattern), Ulikely LAM (1 pattern), MANU2 (1 pattern), and H3 (2 patterns). The sample also holds 1 isolate which was showed pattern of *M. bovis*[14].

Identifying *Beijing* genotype can be done through various methods, such as spoligotyping, IS6110 RFLP, PCR, and sequencing[15]. These methods focus on various types of protein in specific target genes. Nikajima's research points out that *Mycobacterium tuberculosis genome H37Rv* is able to identify *Beijing* strain, specifically the Rv0679c protein with the size of 163bp[15]. Data from South Sulawesi Provincial Health Office in the year 2016 shows that there were 13,659 TB cases with 12,965 cases of TB (7,180), 97 cases of MDR TB, and 597 cases of TB in children. In the cases of AFB positive TB, treatment success rate was 86%, less than the targeted 90%. Beside that, success rate just 82,5% (target more > 90%)[16].

METHOD

This is an analytical observational study with cross-sectional analysis design. The research was done in the City of Makassar and Gowa Regency from March to June 2018. The samples used were 36 new patients diagnosed with pulmonary TB, with an age of 15 to 65 years. Ethical clearance was issued by Ethical Committee of Universitas Airlangga numbered 82/EC/KEPK/FKUA/2018. Tuberculosis patients were diagnosed using Zielh Neelsen AFB smear method. Conversion occurred if there was a change in the result of AFB smear: from positive to negative during the 2-month treatment. Detection of *Beijing* strain was done by decontamination using Petroff alkali method and extraction of DNA using Qiagen kits. Amplification of *Beijing* strain was done with Multiplex PCR. The PCR Mix had a total volume of 20 µl which were comprised of 10 µl of PCR Buffer, 1 µl of ON-1002 (FW), ON-1258 (R1)$^{\alpha}$, and ON-1127 (R2) primers, 6 µl of DW, and 2 µl of template DNA. The next step was a 25-cycle of pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 10 seconds, annealing at 66°C for 10 seconds, and extension at 72°C for 15 seconds. Upon finishing, a final extension was done at 72°C for 3 minutes and an elongation at 15°C for 5 minutes[16]. Data analysis was performed using SPSS 17. Correlation between sputum AFB conversion with *Beijing* strain was assessed using bivariate analysis by Chi-square test.

RESULT

A preliminary survey was performed to collect data of respondents' characteristics such as age, sex, and AFB gradation. The results are presented in the Table 1:

Mycobacterium tuberculosis was isolated from 50 patients are AFB positive, but only 36 fit the criteria. *Beijing* strain is identified through Multiplex PCR with the result showing 6 samples (16.7%) are *Beijing* strain and 30 samples (83.3%) are non-*Beijing*. Treatment success rate is identified by microscopic examination after 2 months of treatment. As much as 32 patients (88.9%) are found to have undergone conversion, while the other 4 (11.1%) are not. The results are presented in the Table 2.

Chi-square test yields p-value of 0.010 < 0.05, meaning that there is a significant correlation between *Beijing* strain genotype with sputum AFB conversion. The results are presented in the Table 3.

Table 1. Respondent distribution based on age, sex, and AFB gradation (N=36)

No	Characteristic	Frequency (n)	Percentage (%)
1	Age (years old)		
	16-25	6	16.7
	26-35	5	13.9
	36-45	5	13.9
	46-55	8	22.2
	56-65	12	33.3
2	Sex		
	Male	25	69.4
	Female	11	30.6
3	AFB Gradation		
	Low	28	77.8
	Medium	3	8.3
	High	5	13.9

Table 2. Frequency distribution of genotype strain and AFB smear conversion

No	Research variables	Frequency (n)	Percentage (%)
1	Genotype		
	Beijing Strain	6	16.7
	non-*Beijing* Strain	30	83.3
2	AFB Conversion		
	Conversion	32	88.9
	Non-conversion	4	11.1

Table 3. Correlation between genotype and sputum AFB conversion

MTB Genotype	AFB Conversion						p-value	OR
	Non-conversion		Conversion		Total			
	N	%	N	%	N	%		
Beijing	3	75.0	3	9.4	6	16.7	0.010	15.0 (95%, CI 1.8-120.8)
Non-*Beijing*	1	25.0	29	90.6	30	83.3		
Total (n)	4	11.1	32	89.9	36	100		

DISCUSSION

This research was showed that AFB conversion rate of new patients with AFB positive is 89.9%. According to WHO, the conversion rate must be more than 80%[17]. Diagnosis and conversion are performed with AFB method using microscopes. Examination on two sputum specimens is sufficient to identify the majority (95-98%) of AFB-positive TB patients. the policy of WHO recommend to using two spesimen sputum on case finding using microscopy to get setting with appropriate external quality assessment and high-quality documentation. The microscopy of both specimens must be screened. In settings with appropriate quality assurance procedure, a case is defined as a person with one positive smear—meaning at least 1 AFB in 100 microscopic fields[18]. In 2010, WHO confirmed the accuracy of microscopy of two consecutive sputum specimens on the same day for TB diagnosis, allowing treatment to take place since the first visit to a health center facility[17].

Microscopy benefits in terms of simplicity and cost efficiency, allowing immediate detection of the most infectious pulmonary TB. Sputum specimen from patients with pulmonary TB, especially those having cavity diseases, often have enough bacteria to be detected using microscopes. Microscopy is also suitable for periphery-level laboratories, or higher. It can be performed safely even in laboratories with low alert value, reducing the risk of TB infection in laboratories. It is a simple, speedy, and inexpensive test needed as a follow up measure for patients with high risk of TB. Despite the benefits, microscopy also has limitation for AFB. It is unable to distinguish *Mycobacterium tuberculosis complex* from non-tuberculous mycobacteria. It cannot tell the difference between organisms that should be exterminated from those that actually pose no threat. It also cannot differentiate drug-sensitive strains from the resistant ones[18].

Sputum AFB conversion in pulmonary TB patients takes place after intensive phase of the treatment. AFB conversion is the change in sputum smear from AFB positive to AFB negative after undergoing intensive treatment[19]. Based on Table 2, it can be seen that AFB conversion occurs in 32 samples (88.9%) and does not occur in 4 samples (11.1%). This research was showed non conversion rate 11,1 %, based on intervew with health worker in the Public Health Centre, the pasient who non conversion after 2 month treatment have comorbid diseaeses such as Diabetes Melitus. Garrido research was showed that there was relationship between Diabetes Melitus with treatment failure[20]. This research was showed a conversion rate of 88.9%, fitting the indicator from the Ministry of Health of >80%. Conversion acts as an indicator of treatment success. Success of a treatment is also influenced by age, sex, medical history, presence of antibiotic resistance and bilateral radiological lession[21, 22]. Smear acid bacilli, retreatment, comorbid diseases, and education[23].

Beijing genotype strains have specific properties such as the effects of the BCG vaccine, increased virulence, and risk of treatment failure. clinically, there were no differences in signs and symptoms of the Beijing and non-Beijing genotype strains [24], but Parwati research is showed that the proportion of patients in the Beijing strain who had fever symptoms was higher than the strain non Beijing[25]. At the molecular level, Beijing strains have specific properties in terms of protein and lipid structure and their interaction with the human defense system. Finally, the Beijing genotype has polymorphisms in immune genes, showed the co-evolutionary of human-mycobacteria. The emergence of the Beijing genotype family may represent the response of M tuberculosis to vaccination or antibiotic treatment, with a negative reaction to tuberculosis control[26].

The findings are showed that the result of sputum Multiplex PCR of the respondents mostly (30 samples, or 83.3%) are non-*Beijing* strain and only a few (6 samples, or 16.7%) are *Beijing* strain. Research done by Octavian using spoligotyping shows that there are 8% *Beijing* strain in Papua[19]. Research by Lisdawati points out a proportion of *Beijing* strain in Makassar to be as much as 25.93%[13]. According to researcher's assumption , the percentage of Beijing in Makassar is quite high compared to other regions in the Eastern Region of Indonesia, Makassar society has a higher level of mobilization and geographically located in Central Indonesia making it easier to move to other area. Data analysis using Chi-square test yields p-value of 0.022 < 0.05, meaning that there is a significant correlation between *Beijing* strain genotype and sputum AFB conversion. This finding is also advocated by Parwati, claiming that there is a correlation between *Beijing* strain and treatment failure[26]. This research is different from Hang's which focuses on *Beijing* strain's sublineage of modern, ancient, and non-*Beijing*. Hang's research also shows that there is no correlation between the strain of *Mycobacterium tuberculosis* and treatment failure[27].A study examining clinical pathways and treatment outcomes depends on the genotype of the Mycobacterium (MTB) strain among various age groups of TB patients. This study was conducted on 6 strains including Beijing, LAM, Haarlem, Ural / Uganda I, S, Africanum and showed that *Beijing* was included in the group with a low treatment success rate[28]. Mourik's research is showed that there is still a presence of active bacteria in the first two months of treatment on test animals infected with Mycobacterium tuberculosis Beijing strain[29]. Beijing strains was also associated with the incidence of MDR and recurrence of TB patients. Vyazovaya's research was showed mutations in rpoB531 were most commonly found among Beijing isolates (60 of 72 isolates with rpoB mutations), five isolates (four of which were Beijing) were showed multiple or triple mutations in rpoB. INH resistance mutations, the katG315 mutation is also the most common in Beijing isolates[30]. Studies from Estonia, China, Korea and Taiwan were showed a significantly higher MDR frequency among Beijing descendants[31]. A study conducted in Sweden which identified 13% of Beijing strains for 15 years. The proportion of strains with MDR was significantly higher among Beijing strains than in non-Beijing strains[31].

Different with Rutaihwa's research, the existence of beijing strains in Africa is not related to anti-tuberculosis drug resistance [32]. This study was supported Soolingen's study of the relationship between genotype and resistance between unclear treatment failure[33].

CONCLUSION

Although it is true that AFB microscopy is vital in monitoring and evaluating one-line anti-tuberculosis treatment in pulmonary TB patients in TB endemic areas, early care management for pulmonary TB patients also needs to consider the lineage of the *Mycobacterium tuberculosis* strain, e.g. *Beijing* strain. At the molecular level, Beijing strains have specific properties in terms of protein and lipid structure and their interaction with the human immune system. This becomes imperative given the fact that the strain of *Mycobacterium tuberculosis* is a determinant of treatment success of the patients and examine the implications for future strategies.

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Research Report

ANTI-DENGUE TYPE 2 VIRUS ACTIVITIES OF ZINC (II) COMPLEX COMPOUNDS WITH 2-(2,4-DIHYDROXYPHENYL)-3,5,7-TRIHYDROXYCROMEN-4-ONE LIGANDS IN VERO CELLS

Teguh Hari Sucipto[1,α], Harsasi Setyawati[2], Siti Churrotin[1], Ilham Harlan Amarullah[1], Sri Sumarsih[2], Puspa Wardhani[1], Aryati[1], Soegeng Soegijanto[1]
[1] Dengue Study Group, Institute of Tropical Disease, Universitas Airlangga, Indonesia
[2] Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Indonesia
[α] Corresponding author: teguhharisucipto@staf.unair.ac.id

ABSTRACT

Dengue virus (DENV) is a disease that is transmitted through Aedes aegypti and Aedes albopictus mosquitoes, and is spread in tropical and sub-tropical regions. Now, dengue or antiviral vaccines for humans do not yet exist, but there are great efforts to achieve this goal. Complex compounds are reported to fungicidal, bactericidal and antiviral activity. Antiviral activity against DENV is an important alternative to the characterization and development of drugs candidate. The purpose of this study was to study zinc(II) compounds with 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one ligand on DENV-2 replication in Vero cells. Vero cell lines (African green monkey kidney) was used in this study, maintained and propagated in Minimum Essential Eagle Medium containing 10% fetal bovine serum at 37°C in 5% CO_2. The activity of dengue virus was carried out by enzyme-immunosorbent assay (ELISA) method and CellTiter96® Non-Radioactive Proliferation. The value of activity inhibition (IC_{50}) of complex compounds with variations of mol metal: ligand 1:2, 1:3, and 1:4 against dengue virus type 2 (DENV2) was 2.44 µg/ml, 2.75 µg/ml, respectively and 2.00 µg/ml, also the toxicity value (CC_{50}) of complex compounds with variation mol metal: ligand 1:4 for Vero cells is 3.59 µg/ml. The results of this study were indicate that these properties have been shown to inhibit anti-dengue type 2 virus (DENV-2), but are also toxic in Vero cells. Including previous study about complex compound interaction with dengue virus type 2 activity, Zn(II) more reactive compound then Cu(II), and Co(II). The comparison with Cu(II) complex compound, it has been revealed that Co(II) and Zn(II) is more toxic, was found to be nontoxic to human erythrocyte cells even at a concentration of 500 µg/ml.

Keywords: *Anti-DENV2, Complex Compounds, Zinc(II), 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one*

ABSTRAK

Virus Dengue (DENV) adalah penyakit yang ditularkan melalui nyamuk Aedes aegypti dan Aedes albopictus, serta didistribusikan di daerah tropis dan sub-tropis. Kini, vaksin dengue atau antivirus untuk manusia belum disetujui secara klinis, meski telah ada upaya besar untuk mencapai tujuan ini. Senyawa kompleks dilaporkan menunjukkan aktivitas fungisida, bakterisida, dan antivirus. Aktivitas antivirus melawan DENV merupakan alternatif penting untuk karakterisasi dan pengembangan obat-obatan. Tujuan dalam penelitian ini adalah untuk investigasi aktivitas senyawa kompleks logam seng(II) dengan ligan 2-(2,4-dihidroksifenil)-3,5,7-trihidroksikromen-4-on terhadap replikasi DENV-2 pada sel Vero. Sel Vero (African green monkey kidney) yang digunakan dalam penelitian ini, dipelihara dan diperbanyak dalam Medium Essential Eagle yang mengandung 10% serum janin sapi pada 37°C dalam 5% CO2. Aktivitas senyawa kompleks virus dengue dilakukan dengan metode enzyme-immunosorbent assay (ELISA) dan toksititas dengan metode CellTiter96® Non-Radioactive Proliferation. Nilai penghambatan aktivitas (IC_{50}) senyawa kompleks dengan perbandingan mol logam:ligan 1:2, 1:3, dan 1:4 terhadap virus dengue tipe 2 (DENV2) masing-masing adalah 2.44 µg/ml, 2.75 µg/ml, dan 2.00 µg/ml, serta nilai toksisitas (CC_{50}) senyawa kompleks dengan perbandingan mol logam:ligan 1:4 untuk sel Vero adalah 3,59 µg/ml. Hasil penelitian ini menunjukkan bahwa senyawa kompleks tersebut menunjukkan aktivitas penghambatan anti-dengue virus tipe 2 (DENV-2), tetapi juga bersifat toksik pada sel Vero. Termasuk penelitian sebelumnya tentang interaksi senyawa kompleks dengan aktivitas virus dengue tipe 2, Zn (II) lebih

reaktif senyawa kemudian Cu (II), dan Co (II). Perbandingan dengan kompleks Cu (II), telah diketahui bahwa Co (II) dan Zn (II) lebih toksik, ditemukan tidak beracun pada sel eritrosit manusia bahkan pada konsentrasi 500 µg / ml.

Kata kunci: *Anti-DENV2, Senyawa Kompleks, Seng(II), 2-(2,4-dihidroksifenil)-3,5,7-trihidroksikromen-4-on*

INTRODUCTION

A major public health concern worldwide in recent years is a most prevalent mosquito-borne viral pathogen dengue virus (DENV), was transmitted through *Aedes aegypti* and *Aedes albopictus* mosquitoes, and is spread in tropical and sub-tropical regions.[1,2,3] Presently around the world dengue is endemic in 112 countries.[4] The incidence of DENV has increased approximately 30-fold over the past 50 years.[5] Dengue virus, the causal agent of dengue, has been shown to induce apoptosis in vitro and in vivo.[6,7] The mechanisms that trigger the apoptotic cellular responses, however, have not been thoroughly investigated.[8]

Micronutrient homeostasis is a key factor in maintaining a healthy immune system. Trace element zinc is a critical cofactor for many proteins involved in cellular processes like differentiation, proliferation and apoptosis that zinc is a nutritionally fundamental trace element and is second most abundant trace metal in the human body after iron.[9] In previous study, Zn^{2+} was suggested that DENV-2 infection of Vero cells and Human breast adenocarcinoma cell line resulted in the induction of apoptosis.[8,10]

The complex compound from metal and organic compound reaction can be used an anti-DENV-2, especially Cu(II) with 2,4,5-triphenylimidazole exhibited adsorption inhibitory activity at IC_{50} = 2.3 µg/ml.[11] A significant inhibitory activity to that of the complex Co(II) with 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen -4-one ligand was reported against the tested pathogenic DENV-2 in Vero cells 3.08 µg/ml.[12]

In the present study, the inhibitory activity of Zinc(II) with 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one ligand against the replication of DENV-2 in Vero cells was investigated.

MATERIAL AND METHODS

Chemicals and Medium

The chemical reagents used in this research were the Zinc(II)–2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen -4-one complex compound, dimethyl sulfoxide by Merck 99.98%, Germany, Minimum Essential Eagle Medium by Sigma-Aldrich (Germany), dengue virus type 2 Surabaya Isolate (KT012513), Vero cell by African green monkey kidney, CellTiter96® Non-Radioactive Proliferation reagent by Promega (USA), and DENV antibody (4G2) for enzyme-linked immunosorbent assay (ELISA).

Vero Cells Preparation

Vero cell lines (African green monkey kidney) was used in this study, maintained and propagated in Minimum Essential Eagle Medium containing 10% fetal bovine serum. Cultured Vero cell lines were incubated at 37 °C, respectively in 5% CO_2. Confluent monolayer of Vero cells were detached with trypsin-EDTA and incubate cells at 37 °C for 5 minutes. Add Minimum Essential Eagle Medium containing 10% fetal bovine serum, pipetting gently to break up any clumps of cells and counted using a Hemocytometer. Add cells in 96-well plate with 1×10^6 cells/10 ml and incubated in 37°C incubator with 5 CO_2. Monitor cells daily or every other day, cells reach a >90 % confluent monolayer.[13,14]

Anti-dengue Type 2 Virus Assay

Confluent monolayers of Vero cells were prepared on a 96-well plate (1×10^6 cells/10 ml), and the titer of DENV-2 (2×10^4 FFU/well). The 50% inhibitory concentration (IC_{50}) was calculated as follows: IC_{50} = (NC − AC) 100/ NC after incubating at 37°C for 2 days in 5% CO_2, where NC is the mean of the absorbance of negative controls and AC is the absorbance of the compound tested. The DENV-2 inhibition of replication by compound was further investigated by using quantitative ELISA at 415 nm.[15]

Cytotoxicity assay

The dye of CellTiter96® Non-Radioactive Proliferation reagent by Promega is a modification of MTT assay method by Mosmann. The assay is suitable to use in adherent and suspension cells. Assay is very sensitive, it can detect 1,000 cells/well of a plate reader. Vero cells (1×10^5 cells/ml) were seeded in plate at 37°C in 5% CO_2 overnight. A total of 100 µl of serial delusion compound were incubated with Vero cells for 24 h. A total of 100 µl of Cell Proliferation Reagent was added into each well, incubated for 4 hour at 37°C. The plate was read at 570 nm using ELISA reader (iMark™ Microplate Absorbance Reader).

RESULT AND DISCUSSION

Anti-dengue Type 2 Virus Activity

Metal complex compounds are a promising class of drug leads, and the associated studies have attracted more attention. However, the systematic basic research of metal complex compounds is lagging behind, partly because very few efforts have been made to establish a set activity screening and subsequent evaluation system for the comprehensive investigations on the structure and activity relationship of metal complex compounds.

The in vitro anti-dengue virus activity of the Zinc(II)–2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one complex compound was tested against one type dengue virus (dengue virus type 2) by enzyme-linked immunosorbent

assay (ELISA).[15] The susceptibility of the strains toward the present compounds was judged measuring the size of inhibition. Zinc(II)– 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one complex compound was further studied for their inhibitory effect on replication of the dengue virus type 2 in Vero cells. The IC_{50} (inhibitory concentration 50) was determined from the dose response curve with variations of mol metal:ligand 1:2 (Figure 1), 1:3 (Figure 2), and 1:4 (Figure 3).

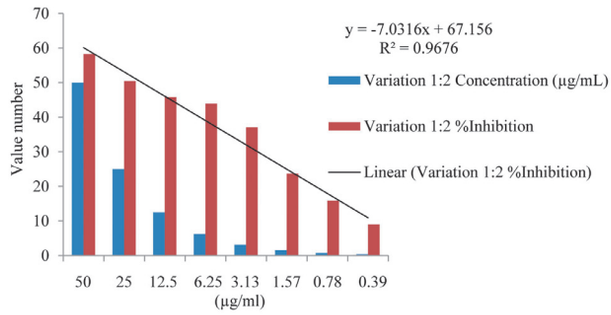

Figure 1. Inhibition curve of dengue virus type 2 at several concentrations of Zn(II) complex with variations of mol metal:ligand 1:2

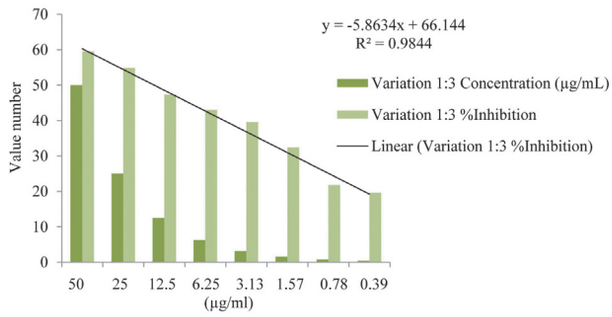

Figure 2. Inhibition curve of dengue virus type 2 at several concentrations of Zn(II) complex with variations of mol metal:ligand 1:3

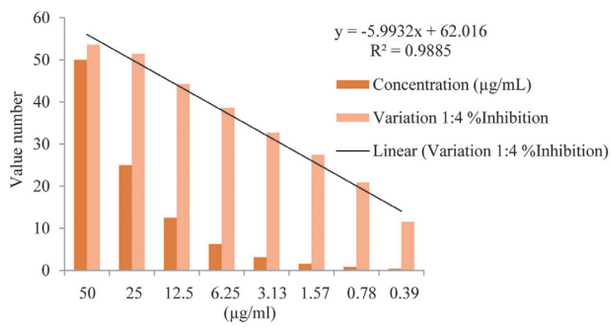

Figure 3. Inhibition curve of dengue virus type 2 at several concentrations of Zn(II) complex with variations of mol metal:ligand 1:4

The IC_{50} value with variations of mol metal:ligand 1:2, 1:3, and 1:4 against dengue virus type 2 was 2.44 µg/ml, 2.75 µg/ml, respectively and 2.00 µg/ml. The comparison of the complex compounds and the known anti-dengue virus type 2 activity showed that the 1:4 variation mol metal:ligand was more effective than 1:2 and 1:3. The bulky 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen -4-one ligand on binding to the metal cation reduces the polarity of the metal ion due to the ligand orbital overlap with the metal orbitals, resulting in a delocalization of positive charge.[16] This increases the lipophilic character of the metal favors its permeation through the lipoid layer of the virus membranes.[17] The effects of other compounds against cellular RNA polymerases and formation of the complex with RNA have reported suggesting that compound could also affect the similar replication enzymes.[18]

Previous research was reported anti-dengue type 2 activity, especially Cu(II) with 2,4,5-triphenylimidazole exhibited adsorption inhibitory activity at IC_{50} = 2.3 µg/ml.[11] A significant inhibitory activity to that of the complex Co(II) with 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen -4-one ligand was reported against the tested pathogenic dengue virus type 2 in Vero cells 3.08 µg/ml. The comparison of the other complex, Zn(II) complex compound more significant activity for inhibit dengue virus type 2 replication then Cu(II) complex, and Co(II) complex. As for the central ion (M^{2+}), when chelated with ligand to form the complex, it has the following order in stability: $Zn^{2+} > Cu^{2+} > Co^{2+}$. Besides that, Cu(II) free ligand more reactive to dengue virus type 2 up to 0.13 µg/ml because Cu^{2+} has stronger oxidative activity[19] and react with cysteine residues on the surface of the protease.[18] At molecular scale such complex compound interact directly with proteins and DNA, leading to dysfunction and cleavage of the structure of macromolecular.[20]

Cytotoxicity Activity

This compound was tested for cytotoxicity by modification of MTT assay method by Mosmann assay on Vero cell lines, Zinc(II)–2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one complex compound showed cytotoxicity with CC_{50} at 3.59 µg/ml. The CC_{50} value was found to increase with an increasing concentration of the test compound, as shown in Figure 4.

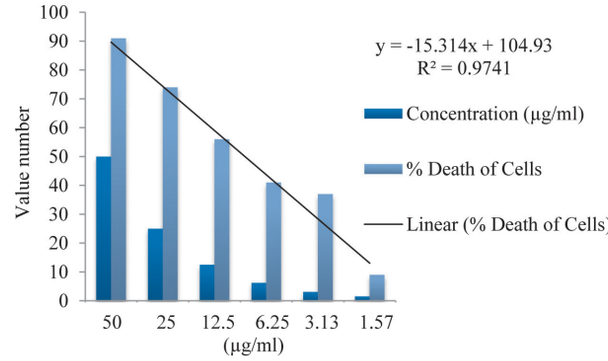

Figure 4. Cytotoxicity of Zn(II) complex curve for Vero cell lines at several concentrations

Previous research was reported cytotoxicity other complex compounds to Vero cell lines, Cu(II) with 2,4,5-triphenylimidazole at CC50 = 44.74 µg/ml11 and the complex Co(II) with 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxycromen-4-one ligand at 3.36 µg/ml.12 The comparison with Cu(II) complex compound, it has been revealed that Co(II) and Zn(II) is more toxic, was found to be lower toxic to human MCF7 cell proliferation.[21]

CONCLUSION

Metal complex compounds are a promising class of drug leads, and the associated studies have attracted more attention. Including previous study about complex compound interaction with dengue virus type 2 activity, Zn(II) more reactive compound then Cu(II), and Co(II).

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Research Article

OVERVIEW OF NUCLEAR FACTOR-κB (NF-κB) AND NON-STRUCTURAL PROTEIN 1 (NS1) IN PATIENTS WITH DENGUE FEVER IN PREMIER HOSPITAL, SURABAYA

Ni Nyoman Budiutari[1a], Yoes Prijatna Dachlan[2], dan Jusak Nugraha[3]
[1]Department of Immunology Postgraduate School, Universitas Airlangga, Surabaya, East Java, Indonesia
[2]Departement of Parasitology, Faculty of Medicine, Universitas Airlangga Surabaya, East Java, Indonesia
[3]Departement of Patology Clinic, Faculty of Medicine, Dr. Soetomo Hospital, Surabaya, East Java, Indonesia
[a]Corresponding author: budiutari2212@gmail.com

ABSTRACT

Dengue fever (DF) is an acute viral fever caused by RNA virus that is transmitted by Aedes aegypti and Aedes albopictus mosquitoes. DF is also called viral arthropod-borne disease and is accompanied by headaches, joint and muscle pain. The main target of dengue infection is macrophages or monocytes and dendritic cells (DC). Infected DC is caused the viral replication and the endocytosis into endosomal, easier, thus inducing the activation of NF-κB transcription factor to produce proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), IL-6, IL-12 and chemokine. NF-kB is one of the transcription factors involved in the regulation of the expression of various cytokines, chemokines and anti/pro-apoptotic proteins during infection and act as indicator of disease severity. Infected DC cells are secreted NS1 protein which is the co-factor needed for viral replication and can be detected in the first eight days. The level will be higher in the initial phase of fever. The purpose of this study was to analyze the description of NF-kB and NS1 levels in the serum of patients with dengue fever through observational analytic studies through a cross-sectional approach. This study was done on 40 patients with dengue fever and 10 healthies people as negative controls. NS1 was analyzed in serum of Panbio rapid test and NF-kB level were measured by sandwich ELISA. The results are showed positive and negative NS1 results in dengue fever patients. The average NF-kB serum level in dengue fever patients was found to be higher than the control. NF-κB level in negative NS1 was higher than the NS1 positive group. It is showed that NS1 is detected both in the acute phase. The detection of NF-κB is showed the involvement of transcription factors in the development of dengue virus infection and has a protective role for host cells.

Keywords: *Dengue Fever, Nuclear Factor-κB, NS1 Protein, Viral Infection, Tropical Disease.*

ABSTRAK

Demam Dengue (DD) merupakan penyakit demam virus akut yang disebabkan oleh virus RNA, ditularkan oleh nyamuk Aedes aegypti dan Aedes albopictu. DD disebut juga sebagai penyakit arthropod-borne viral dan disertai sakit kepala, nyeri otot dan sendi. Target utama infeksi dengue adalah makrofag/monosit dan sel dendritik (DC). DC yang terinfeksi akan mempermudah replikasi virus dan endositosis ke dalam endosomal, dimana hal tersebut dapat menginduksi aktivasi faktor transkripsi NF-κB untuk menghasilkan sitokin proinflamasi seperti Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), IL-6, IL-12 dan kemokin. NF-kB merupakan salah satu faktor transkripsi yang terlibat dalam regulasi ekspresi berbagai sitokin, kemokin, protein anti/pro-apoptosis selama infeksi dan menjadi tanda tingkat keparahan penyakit. Sel DC yang terinfeksi akan mengsekresikan protein NS1 yang merupakan co-factor yang diperlukan untuk replikasi virus. NS1 terdeteksi baik dalam delapan hari pertama serta kadarnya akan lebih tinggi pada fase awal demam. Tujuan penelitian ini adalah menganalisis gambaran kadar NF-kB dan NS1 pada serum pasien demam dengue melalui studi observasional analitik melalui pendekatan cross sectional. Pada penelitian ini menggunakan 40 sampel penderita demam dengue dan 10 sampel orang sehat sebagai control negatif. NS1 dianalisa pada serum menggunakan Panbio rapid test dan kadar NF-kB menggunakan sandwich ELISA. Hasil menunjukkan pada pasien demam dengue ditemukan hasil NS1 positif maupun negatif. Rerata kadar NF-kB serum pada pasien demam dengue ditemukan lebih tinggi daripada kontrol dan kadar NF-κB pada NS1 negatif lebih tinggi dibandingkan

kelompok NS1 positif. Hal ini menunjukkan bahwa NS1 terdeteksi baik pada fase akut dan terdeteksinya NF-κB menunjukkan adanya keterlibatan faktor transkripsi pada perkembangan infeksi virus dengue dan mempunyai peran proteksi untuk sel host.

Kata kunci: *Demam Dengue, Nuclear Factor- κB, Protein NS1, Infeksi Virus, Penyakit Tropis.*

INTRODUCTION

Dengue is an endemic disease caused by RNA viruses which transmitted by Aedes aegypti and Aedes albopictus mosquitoes.[1] This disease is endemic throughout tropical and subtropical regions which are affected by rainfall, temperature and unplanned urbanization.[2] World Health Organization (WHO) is recorded from 1968 to 2009 Indonesia as the country with the highest DHF cases in Southeast Asia. In Indonesia, it was first discovered in the city of Surabaya in 1968 with 58 people infected and 24 people dead. In 2015 there were 126,675 sufferers in 34 provinces in Indonesia.[3] In Surabaya in 2015 there was an increase in the number of cases by 46%.

Dengue Fever (DD) is an endemic disease which is also called viral arthropod-borne disease. The first infection to the host's body raises various pathological levels, ranging from asymptomatic mild symptoms (dengue fever), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS).[4] Dengue is classified as a pediatric disease in Southeast Asia, but at this time dengue patients are recorded not only in children but also in adults.[5]

Dengue virus (DENV) is a genus of Flavivirus in the family Flaviviridae which has four different antigenic serotypes namely DENV-1, DENV-2, DENV-3 and DENV-4.[6] The DENV genome consists of positive single-stranded RNA with a length of about 11 kb which encodes ten proteins, ie no protein structural capsules (C), envelope (E), pre-membrane (preM) and seven non-structural proteins (NS1, NS2A, NS3, NS4, NS4A, NS4B, NS5).[2]

Non-structural 1 (NS1) protein is an important glycoprotein and a co-factor needed for viral replication, although its role in replication is largely unknown. NS1 is detected during the acute phase and will slowly decrease to an undetectable level on days 5-6. The presence of this protein is associated with the severity of the disease and the progression towards DHF.[6] The main target in dengue infection is towards macrophages/monocytes and dendritic cells.[7] Dendritic cells were infected with dengue virus will facilitate viral replication and endocytosis to endosomal, which can induce activation of the NF-κB transcription factor.[8]

NF-κB is a transcription factor that is involved in the regulation of gene expression to encode cytokines, chemokines, proproteins and antiapoptosis. Activation of NF-κB during viral infection is explained as a host protective response to pathogens. NF-κB is a weapon used by hosts to control viruses, but viruses can use them differently to block apoptosis and increase viral replication. Activation of the NF-κB pathway produces various cytokines such as Tumor Necrosis Factor-a (TNF-α), Interleukin-1 (IL-1), IL-6, IL-12. NF-κB is one of the most widely exploited pathways for gene regulation by DENV, but how the role of the NF-κB pathway in dengue pathogenesis is not completely clear.[9]

This study is based on the picture of NF-κB levels and NS1 protein in serum of dengue fever patients and controls, where an increase in NF-κB levels is indicated as a sign that a more severe prognosis is Dengue Shock Syndrome (DSS).

MATERIAL AND METHOD

Study Population

This study was done in the Institute of Tropical Diseases, Universitas Airlangga on August 2018. The study was included 40 patients with dengue fever and 10 healthy people as control who were selected from Surabaya Premier Hospital. Patients who were selected were in accordance with the inclusion criteria in patients with clinical symptoms such as that had onzet fever day 1–4 and without age limitation. Patients who had only clinical symptoms of dengue, but dengue infection had not found in diagnosis laboratory were excluded because of the difficulty of evaluating the extent of the disease.

The control group was included 10 apparently healthy subject with comparable age characteristics, no positive history of dengue infection disease, no fever for 1 month prior to the study.

The clinical disease severity was classified according to the 2011 World Health Organization (WHO) dengue diagnostic criteria.[10] Patients with sharp temperature and is frequently associated with a flushed face and headache, the body temperature is between 39-40°C and lasting 5-7 days in the majority of cases and the other common symptoms including anorexia were classified as DF. Patients with increase of hematocrit greater than 20% compare with the baseline hematocrit or clinical/ultrasound scan evidence of plasma leakage were classified as having DHF. Shock was defined as having cold clammy skin, along with a narrowing of pulse pressure of 20 mmHg. According to the WHO 2011 disease classification, 40 patients were classified as DF.

Ethics Statement

Ethics approval was obtained by Ethical Review Committee at Faculty of Dental Medicine, Universitas Airlangga. All patients were recruited following informed written consent.

Blood Samples

Samples were collected into tubes. Serum from patients was obtained after centrifugation for 10 minutes at 10,000

rpm. Then the samples were stored and frozen at -80°C until used.

Laboratory Diagnosis

Dengue serum NS1 were detected using Panbio rapid test (immunochromatography) from Panbio Dengue Early Rapid Test with positive and negative qualitative results. NF-κB levels were measured using the Human NF-κB p65 Sandwich-ELISA kit from Elabscience followed procedures in the protocol. This is a commercial enzyme-linked immunosorbent assay for detecting NF-κB level against dengue virus in human serum or plasma.[11,12]

Statistical Analysis

In this study, were we analyzed the levels of NF-κB and NS1 in patients with dengue fever are determined clinically. We are use the Mann-Whitney test to see the difference between NS1 positive and NS1 negative in patients with dengue fever. Statistic Package for Social Sciences (SPSS) was used for data entry, processing and statistical analysis at the end of the study. P-values less than 0.05 were considered significant.

RESULT AND DISCUSSION

Study Population

A total of 40 patients suffering from dengue fever and fulfilling the inclusion criteria were enrolled in the study. Serum NS1 examination results from 40 patients found 30 patients with positive NS1 and 10 NS1 patients negative. As a control, 10 healthy people without dengue infection with various ages.

Table 1. Age and gender distribution of the participants (n=50)

	Control (n=10)/%	NS1 positive (n= 30)/%	NS1 negative (n=10)/%	Total /%
Age (mean±SD)				4.66±1.722
Age group				
0-5	0/ 0	1/ 3.3	0/ 0	1/ 2.0
6-11	0/ 0	2/ 6.7	1/ 10	3/ 6.0
12-16	1/ 10	5/ 16.7	0/ 0	6/ 12.0
17-25	8/80	8/ 26.7	3/ 30	19/ 38.0
26-35	1/ 10	5/ 16.7	1/ 10	7/ 14.0
36-45	0/ 0	4/ 13.3	2/ 20	6/ 12.0
46-55	0/ 0	2/ 6.7	2/ 20	4/ 8.0
56-65	0/ 0	2/ 6.7	1/ 10	3/ 6.0
>65	0/ 0	1/ 3.3	0/ 0	1/ 2.0
Total	10/100	30/ 100	10/ 100	50/ 100
Gender				
Male	3/30	13/14.3	6/60	22/44
Female	7/70	17/56.7	4/40	28/56
Total	10/100	30/100	10/100	50/100

Table 1 gives the age and gender distribution of the participants. The age grouping used was based on the Ministry of Health (2009) (mean 4.66 and SD = 1.722). Majority of the dengue fever were between the age group of 17 to 25 years (38.0%), the lowest were toddlers (0-4 years) and elderly (> 65 years old), namely 3.3%. There were 28 (56.0%) males and 22 (44.0%) females with various age, the females was found more than males.

NS1 Serum

NS1 was examined in the serum of dengue-infected patients collected from days 1-4 of the onset of fever and also in healthy controls. The method used for NS1 examination uses the Panbio Rapid Test Of the 40 serum samples of dengue fever patients, 30 NS1 samples were positive and 10 NS1 samples were negative. In 10 controls NS1 was negative.

Examination the levels of NF-κB

NF-κB levels were determined in serum samples in dengue fever patients collected from fever patients day 1 to 4 onsets of fever.

The level of NF-κB in serum from negative control is very low (Table 2). NF-κB levels in serum from dengue fever patients were found to be high (>10 ng/mL); higher than the NF-κB level of control in the negative control. However, the average emission in the group of patients with negative NS1 (mean=13.165) was found to be higher compared to patients with NS1 positive (mean=10.013), but there were no significant differences in NF-κB levels from 40 patients (Figure 1). In the negative control sample, there was low NF-κB level with mean value of 1.646. NF-κB levels tend to increase linearly in patients of all clinical values when the disease develops, but there is no significant difference between cases of dengue fever with NS1 positive and NS1 negative (p=0.187) (ρ>0.05).

Table 2. NF-κB in dengue fever patient

	Control (n=10)/%	NS1 positive (n= 30)/%	NS1 negative (n=10)/%	Total/%
NF-κB (mean ± SD)	1.646 ± 1.294	10.013 ± 4.808	13.165 ± 5.805	9.002±5.919
0,1–5,0 ng/mL	10/100	2/6.7	0/0	12/24
5,1–10 ng/mL	0/0	14/46.7	3/30	17/34
>10 ng/mL	0/0	14/46.7	7/70	21/42
Total	10/100	30/100	10/100	50/100

The average emission value of NF-κB levels in positive NS1 was found to be higher than in the negative NS1 group. The highest NF-κB levels were found to reach 25000 ng/mL. In the box plot the control group is at the minimum value of the dengue fever group.

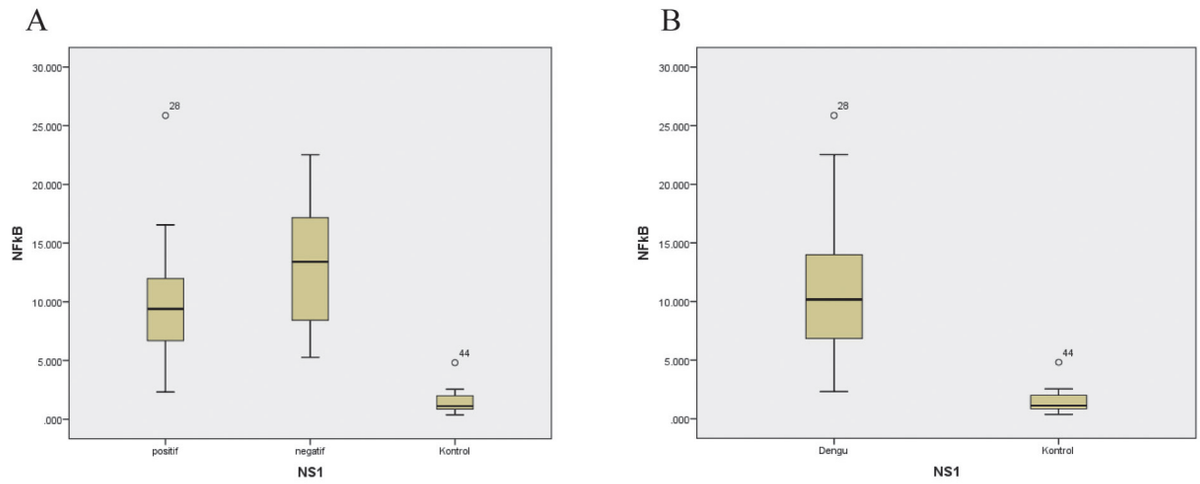

Figure 1. (A) NF-κB levels in serum from patients with dengue fever (n = 30) were determined clinically and in control (n = 10). (B) The mean value of NF-κB levels in patients with dengue fever NS1 is positive and NS1 is negative

DISCUSSION

Our results were showed that in patients with dengue fever did not always show positive results on NS1 examination in serum, this was clear in this study of 40 samples of dengue fever patients found 30 patients with NS1 positive results and 10 patients with NS1 negative. The results in NF-κB showed that the mean levels in serum of dengue fever patients were higher compared to controls.

Homogeneity of the sample in this study was carried out by limiting the duration of fever patients with days 1-4 fever. This restriction is based on a theory which states that in dengue fever patients will experience a fever phase for 2-7 days and on days 1-4 is the first fever phase where fever will appear high enough to 40°C and is the time well in the diagnosis of dengue fever where the antigen from the dengue virus can be detected at a higher level in the acute phase or in the first eight days.[13,14]

Dengue diagnosis is difficult to enforce at the beginning of the disease because the signs and symptoms are not specific so it is often difficult to distinguish from influenza virus infection, measles and typhoid fever. Viremia or the dengue virus in the bloodstream will last for 1 week. NS1 examination is an examination that detects the body parts of the virus and does not wait for the body's response to infection, so the best time to do the best examination is day 0 to day 4 and can be detected before the decline in platelets.[13] In our study there were several patients with negative NS1 results when examined using a Panbio rapid test. In, patients with dengue fever 1-4 days with NS1 positive results show that there is a dengue infection in the body. Conversely, patients dengue fever with NS1 negative do not close the possibility of dengue infection but NS1 is detected at low levels causing false negative and further examination is needed. To detect the viral protein is needed sufficient levels of the amount of virus circulating, while in the initial phase there are not enough viruses, but if it takes the sample after the appearance of antibodies the level of dengue virus will also decrease.[15]

Dengue virus (DENV) has 4 serotypes DENV-1, DENV-2, DENV-3, and DENV-4. This can cause the emergence of negative results on the examination of NS1 in patients with dengue fever is suspected to be associated with the dengue virus serotype that infects. It recommends that NS1 examination must be repeated on the seventh day later to provide more valid results.[16] NS1 is associated with the duration of the disease and is less sensitive to illness that lasts over 3 days or the level detected will decrease. They associate NS1 with the severity of the disease and the risk of progression leading to dengue hemorrhagic fever (DHF).[6] NS1 is a glycoprotein secreted by cells infected with the dengue virus, mainly in the serum supernatant of infected patients, but not in the virus.[17] NS1 is found in primary and secondary infections and can be detected in the first eight days of fever and levels will be high at the start of fever and decrease before defervescence and become negative in some cases. In Tambunan et al. they found that NS1 antigen sensitivity was higher on the third day of fever.[18] Variable antigen levels were showed that NS1 secretion is not persistent (stable). The results of research by Blacksell and Dussart stated that NS1 which binds to soluble endothelium (soluble) and collected in the hepatocyte causes circulating NS1 secretion of blood to become slow.[19,20] This can affect the level of NS1 detected in the blood.[14] NS1 is found in high concentrations in patients with severe degrees.[17]

As a reaction from the host to a viral infection that occurs in the body is the activation of one of the transcription factors namely NF-κB which will secrete various cytokines and chemokine which play a role in infection. The activation of NF-κB is triggered by the genome and protein of the virus which induces the NF-κB signaling pathway recognized by the host PRR through TLR3, TLR7, TLR8 which then activates MyD88 and IRAK-4 which subsequently leads to IRAK-1 bonding with TRAF- 6 and lead to phosphorylation

and degradation of IkB and cause translocation of NF-κB to the nucleus.[21] Activation of NF-κB will cause the secretion of proinflammatory cytokines such as IL-1, IL-6, TNF-α.

Under normal conditions without infection, NF-κB is bound to the IκB in various cells and is inactive.[22] In the event of NF-κB stimulation will be activated and detached from IκB. Because of protein exposure and viral genome NF-κB activation can also trigger extrinsic apoptotic pathways directly or with their products and cause endothelial cell death which results in bleeding. This is consistent with the results of Lin et al. an experimental study using mice and they found dengue virus protease induce cell apoptosis through its interaction with IκBα, IκBβ and cause the development of bleeding.[23]

Dengue viral protease was found to cleave IκBα and IκBβ, activating IKK and triggering activation of NF-κB which causes caspase-mediated endothelial cell apoptosis. Activation of NF-κB usually leads to the production of antiapoptotic proteins and protected cells from apoptosis. In dengue infection, NF-κB has played a pro-apoptotic role. The possibility of NF-κB-mediated endothelial apoptosis is triggered by dengue virus proteases through increased recruitment of macrophages and through increased sensitivity of endothelial cells to TNF by expressing TNF receptors.[23] However, the underlying mechanism activation of NF-κB caused by infection with the dengue virus or viral protein remains were not defined yet.

In Yi-Lin Cheng et al. states that there is an increase in NF-κB activation found in hepatomas induced by dengue virus. Research using RAW264.7 cells is showed that dengue virus protein is needed for NF-κB activation found in endothelial cells and cells undergo apoptosis within 48 hours after infection. In addition, they were found two pathways that could increase NF-κB activation in dengue infection through excessive TNF-α production and through host PRR, TLR3.[24]

Based on the results of data analysis were showed that the levels of NF-κB in dengue fever patients were higher than those of healthy people. This shows that patients with dengue fever experience more NF-κB activation than healthy people. This occurs because of the large presence of dengue viruses that can activate NF-κB which plays a role in innate defenses which then secrete various proinflammatory cytokines and chemokine that function to fight dengue virus and lead to activation of the humoral immune response. In addition, it was found that patients with negative NS1 had higher NF-kB levels than positive NS1, but did not have a significant difference. In patients who showed negative NS1 results, it did not rule out the possibility that dengue infection was occurring in the body, but the number of dengue viruses and NS1 levels in the blood was low, so that there was no detection and further examination was needed. This is not uncommon in some case and the virus can directly exploit the immune system by activating NF-κB and replicating. In this study, 13 cases of patients who died because of dengue infection, it was showed that neither dengue NS1 or dengue NS1 antibodies were detected in the endothelium, questioning the role of NS1 antibodies in the pathogenesis of acute dengue.[25]

Adikari et al. showed that acute NS1 infection was found to correlate with serum IL-10 levels and that NS1 contributed to the pathogenesis of dengue infection by inducing the production of immunosuppressive cytokines from primary monocytes.[26] Based on this, that high amounts of NS1 can induce the production of anti-inflammatory cytokines and vice versa, proinflammatory cytokines which result in an increase in the number of dengue virus replication. But how the NF-κB pathway in the pathogenesis of dengue is still unclear.

CONCLUSION

In conclusion, this study was analyzed the picture of serum NF-κB and NS1 in dengue patients where there was an increase in NF-κB activation and this is known from the high levels of NF-κB detected in serum which is thought to occur due to host cell apoptosis. And NS1 as a marker of early diagnosis of dengue infection that has been widely used was showed good results in the initial infection. But how the path of activation and involvement of NF-κB is still need further research.

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Research Report

DETECTION OF TUMOR NECROSIS FACTOR-α (TNF-α) GENE PROMOTERS POLYMORPHISM AMONG LIVER CIRRHOSIS PATIENTS WITH CHRONIC HEPATITIS B VIRUS (HBV) INFECTION IN SURABAYA, INDONESIA

Citrawati Dyah Kencono Wungu[1,α], Mochamad Amin[2], S. Eriaty N. Ruslan[2], Priyo Budi Purwono[3], Ulfa Kholili[4], Poernomo Boedi Setiawan[4], Maria Inge Lusida[2,3], Soetjipto[1,2], Retno Handajani[1,2]

[1] Department of Medical Biochemistry, Medical Faculty of Universitas Airlangga, Surabaya
[2] *Institute of Tropical Disease*, Universitas Airlangga, Surabaya
[3] Department of Medical Microbiology, Medical Faculty of Universitas Airlangga, Surabaya
[4] Department of Internal Medicine, Medical Faculty of Universitas Airlangga - Dr. Soetomo General Hospital, Surabaya
[α] Corresponding author: cicit.biokimia@gmail.com

ABSTRACT

Polymorphisms in TNF-α gene promoter region are known of its role in the production of TNF-α which may influences the pathogenesis of liver disease. SNPs in positions 238 and 308 of TNF-α gene promoters may affect the production of these cytokines. This study was aimed to detect Single Nucleotide Polymorphism (SNP) on -238 and -308 positions in the TNF-α gene promoter among liver cirrhosis patients with HBV infection in Surabaya, Indonesia. This was descriptive exploratory research with cross sectional study design using serum liver cirrhosis patients with HBV infection in Endoscopy Outpatient Clinic Dr. Soetomo General Hospital, Surabaya from April-May 2017. SNPs at -238 and -308 on TNF-α gene promoter (rs361525 and rs1800629 respectively) were detected using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) with primers specific for the TNF-α promoter region and restriction enzymes NcoI and MspI. The genotypes of TNF-α gene promoter were assessed according to the length of the fragments produced in RFLP. Serum TNF-α levels was measured by commercial ELISA. In this study, as much as 149 positive HBsAg patients was found in Endoscopy Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya. From those amount, as much as 30 liver cirrhosis patients with positive HBsAg were obtained. From 2/30 (6.7%) patients showed the GA heterozygote SNP either position -238 or -308. No patient had the AA genotype. Median blood TNF-α level in women (38 ng / L) was higher than in men (33 ng / L). TNF-α levels in patients with GA heterozygote genotype at -238 and -308 in this research was not different than wild-type (GG genotype). Among patients with liver cirrhosis due to chronic HBV infection in Surabaya, Indonesia, Surabaya, we found GA polymorphisms the TNF-α promoter gene at positions -238 and -308 in 6.7% patients, and did not find homozygous AA polymorphisms. Further studies including larger numbers of patients from various ethnic backgrounds in Indonesia are needed to provide robust data on TNF-α gene promoter polymorphisms and their role in the pathogenesis of liver cirrhosis with HBV infection in this country.

Keywords: *Liver Cirrhosis, Hepatitis B Virus, SNP, TNF-α, PCR-RFLP*

ABSTRAK

Polimorfisme pada promotor gen TNF-α diketahui berperan pada produksi TNF-α yang selanjutnya berperan dalam patogenesis penyakit hepar, penyakit infeksi, serta inflamasi. SNP gen TNF, terutama pada posisi 238 dan 308 dari promotor gen TNF-α telah diidentifikasi dapat memengaruhi produksi sitokin tersebut. Penelitian ini merupakan penelitian pendahuluan yang dilakukan untuk mendeteksi Single Nucleotide Polymorphism (SNP) promotor gen TNF-α posisi -238 dan -308 dari sampel penderita sirosis hati dengan infeksi VHB di Poli Endoskopi RSUD Dr. Soetomo, Surabaya. Jenis penelitian ini adalah penelitian deskriptif eksploratif laboratorik dengan rancangan penelitian cross sectional study yang mengambil sampel pasien penderita sirosis hati dengan infeksi VHB di Poli Endoskopi RSUD Dr. Soetomo Surabaya dari bulan April-Mei 2017. SNP posisi -238 dan -308 (rs361525 dan rs1800629) promotor gen TNF-α dideteksi menggunakan teknik Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

dengan primer yang spesifik untuk daerah promotor gen TNF-α dan enzim restriksi endonuklease NcoI dan MspI. Selanjutnya dilakukan penentuan genotipe promotor gen TNF-α sesuai dengan panjang fragmen yang dihasilkan pada RFLP. Kadar TNF-α serum diukur dengan menggunakan metode ELISA. Dalam penelitian ini, didapatkan sebanyak 149 penderita dengan HBsAg positif di Poli Endoskopi RSUD Dr. Soetomo Surabaya. Dari jumlah tersebut, didapatkan sebanyak 30 penderita sirosis hati dengan HBsAg positif. Dari 30 serum sampel penelitian ini, didapatkan sebanyak 6,67% menunjukkan genotipe SNP heterozigot GA untuk promotor gen TNF-α baik posisi -238 maupun -308. Tidak ditemukan genotipe AA pada penelitian ini. Median kadar TNF-α pada pasien wanita (38 ng/L) lebih tinggi dibandingkan dengan laki-laki (33 ng/L). Kadar TNF-α pada pasien dengan genotipe SNP heterozigot GA pada posisi -238 dan -308 tidak berbeda secara signifikan dibandingkan dengan wild-type (genotipe GG). Pada penderita sirosis hati dengan infeksi VHB di Poli Endoskopi RSUD Dr. Soetomo, Surabaya ditemukan SNP heterozigot GA (6,67%) dan tidak ditemukan SNP homozigot AA promotor gen TNF-α (-238 dan -308). Studi lebih lanjut pada berbagai ras diperlukan untuk memberikan data yang jelas mengenai SNP promotor gen TNF-α pada sirosis hati dengan infeksi VHB.

Kata kunci: *Sirosis hati, Virus Hepatitis B, SNP, TNF-α, PCR-RFLP*

INTRODUCTION

About 240 million people of the world are infected with chronic HBV and about 600,000 people die each year from diseases related to HBV infection and Hepatocellular Carcinoma (HCC)(1). The 5-year cumulative incidence of cirrhosis ranges from 8–20% in untreated chronic HBV patients and, among those with cirrhosis, the 5-year cumulative risk of hepatic decompensation is 20%[2].

Although it is not fully understood, there are several factors suspected to affect the progression of HBV infection, including viral factors, environmental factors, and host genetic factors[3]. From these various factors, research on host factors has begun to be widely developed to understand the difference in progression and outcome of HBV infection in each patient[4].

Under conditions of chronic HBV infection, Th1 cytokines primarily played by TNF-*α* play a dominant role, especially in the immune clearance, inactive carrier, and reactivation phases[5]. Chronic inflammation and hepatic infiltration of leukocytes increase the production of cytokines including TNF-α that trigger cell death thus increasing hepatic damage. High production of TNF-α can cause liver fibrosis through upregulation of TIMP-1 and prevent apoptosis of hepatocytes[6].

Polymorphisms in TNF-α promoter genes are known to play a role in the pathogenesis of liver disease, infectious diseases, and inflammation[3]. Several previous studies have identified the presence of multiple Single Nucleotide Polymorphisms (SNPs) in the TNF gene group. TNF-α promoter polymorphisms can affect the transcriptional rate and, consequently, TNF-α cytokine production. G nucleotide transition to A in the promoter position -238 and -308 is known to affect the production of TNF-α[7,8].

Data on factors related to the incidence of liver cirrhosis in patients with chronic HBV infection in Indonesia are limited, eventhough this data is needed to understand the pathophysiology of liver cirrhosis development, so this study was conducted to detect the TNF-α gene promoter SNP in liver cirrhosis patients due to chronic HBV infection in Indonesia.

MATERIALS AND METHODS

Sampling. This research was a descriptive cross-sectional study and the samples were from liver cirrhosis patients with positive HBsAg who visited the Endoscopy Outpatient Clinic Dr. Soetomo General Hospital, Surabaya in April-May 2017.

The inclusion criterias of this study were: Chronic hepatitis B patients with a history of positive HBsAg ≥ 6 months, with ultrasound diagnosed results from an internist who showed cirrhosis of the liver, the willingness to participate in all study subjects and must sign informed consent, adult patients (š16 years), in conscious condition, and not in an emergency condition. Exclusion criterias in this study were: Patients coinfected with HCV or HIV, and received immunosuppressant therapy.

The study was conducted after obtaining approval from the Research Ethics Committee of Dr. Soetomo General Hospital, Surabaya. Blood collection was taken from cubital vein with 4 mL of blood put on a venoject tube with EDTA and 3 mL of blood put on a venoject tube without EDTA. Blood samples were then taken to the Laboratory of Hepatitis In Institute of Tropical Disease (ITD) of Universitas Airlangga for laboratory examination. Blood samples in the venoject tube with EDTA, plasma separation, PBMC isolation, and host genome extraction for PCR-RFLP examination were performed, while Blood samples in the venoject tube with EDTA, serum separation was performed for the TNF-α ELISA examination.

PBMC Isolation. After plasma separation, the remaining part of blood was performed PBMC isolation using Phosphate Buffer Saline (PBS) and Ficoll-Histopaque-10779. The obtained PBMC was then transferred into a 1.5 mL eppendorf tube and stored in the -30°C at freezer.

Examination of Serum TNF-α levels. Serum TNF-α was examined using diagnostic kit: Human TNF-α ELISA Kit with Cat. No. E0082Hu (Bioassay Technology Laboratory, China). ELISA was performed according to the procedures listed in the kit. Optical Density was measured using Microplate Reader: iMark (Biorad) S / N

12908. TNF-α serum level was calculated by using online software: elisaanalysis.com.

DNA Host Extraction. The DNA host was extracted using the QIAamp DNA Extraction kit (Qiagen, Inc., Hilden, Germany) with Cat.No.51104 using procedures in accordance to the kit. Controls were treated as the same as the sample.

PCR TNF-α Gene Promoters. A total of 5 µL DNA was used for amplification by PCR-RFLP technique, using a PCR 2x PCR Master mix solution (iNtRON®) kit with Ref No.25027. The PCR-RFLP process for TNF-α gene promoter was carried out using: forward: 5'- AGGCAATAGGTTTTGAGGGCCAT -3 'and reverse primers: 5'-TCCTCCCTGCTCCGATTCCG-3' to identify -238 SNP as well as the forward: 5'-AGAAGACCCCCCTCGGAACC-3 'and reverse primers: 5'- ATCTGGAGGAAGCGGTAGTG -3' to identify -308 SNP. Reaction mixture was made in 0.2 mL eppendorf tube with total volume of 50 µL for 1 sample. PCR was performed using the DNA thermal cycler: Applied Biosystem Veriti 96 Well. For -238 SNP, in the initial stage an initial denaturation was performed with 94°C for 5 min, followed by 40 PCR cycles in accordance to Jamil et al with modifications: denaturation at 94°C for 30 s, annealing at 60°C for 30 seconds and elongation at 72°C for 40 seconds. At the end of the process, the final extension was done at 72°C for 7 minutes. For -308 SNP, at the initial stage an initial denaturation was performed at 94°C for 5 minutes, was followed by 40 PCR cycles with the following details: denaturation at 94°C for 30 seconds, annealing at 58.5°C for 30 seconds and elongation at 72°C for 40 seconds. At the end of the process, the final extension was performed at 72°C for 7 minutes[9].

Detection of PCR Products with Electrophoresis. PCR product was examined by electrophoresis using 2% agarose gel which indicated the expected band, ie 107 bp for -308 SNP and 152 bp for -238 SNP. 100bp ladder marker, the negative control, and the samples were put into agarose gel. Electrophoresis results were visualized in the UV light and documented.

Incubation with Restriction Endonucleases. The PCR products of TNF-α gene promoter -238 was incubated with *MspI* restriction enzyme, while TNF-α gene promoter -308 was digested with *NcoI* restriction enzyme. Incubation with restriction enzyme used protocols from manufacturers (New England Biolabs) with a total reaction volume of 50 µL. Incubation was performed at 37°C overnight.

Detection of PCR-RFLP Products with Electrophoresis. PCR-RFLP products were examined using 3% agarose gel. 20bp and 100bp ladder markers, PCR-RFLP products from samples, as well as negative control were incorporated into agarose gel wells. Electrophoresis gel apparatus was run on 100 volts for approximately 25 minutes, then viewed under UV light and documented with Doc Printgraph Gel AE-6933FXCF.

SNP Analysis of TNF-α Gene Promotor. PCR-RFLP product of TNF-α gene at -238 region showed a fragment of 152 base pair (bp) if there is SNP (A allele) and fragment 132 and 20 bp if it is normal allele (G). If a band of 152 bp was found, the sample had homozygous AA allele. But, if the samples showed 152, 132, and 20 bp bands, the sample has GA heterozygous alleles. When two bands 132 and 20 bp were found, the sample has GG homozygous allele.

PCR-RFLP product of TNF-α gene at -308 region showed a fragment of 107 base pair (bp) if there is SNP (A allele), and fragment 87 and 20 bp if it has normal allele (G). When there was a 107 bp band in the sample, the sample has homozygous AA allele. While the samples were showed 107, 87, and 20 bp, the samples has GA heterozygous alleles. If the sample showed two bands 87 and 20 bp, the sample has GG homozygous allele.

RESULTS

In this study, as many as 149 positive HBsAg patients were cared for in the Endoscopy Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya. They were further screened to meet the inclusion and exclusion criteria resulting in blood samples of 30 liver cirrhosis patients with positive HBsAg for more than 6 months

Table 1. Sex and age characteristics of the patients

Sex	Number of patients	Median age	Age distribution			
			≤ 40	41–50	51-60	>60
Male	24 (80%)	49	6	7	6	5
Female	6 (20%)	54	1	2	1	2
TOTAL	30 (100%)		7	9	7	7

Among the patients with cirrhosis of the liver with HBV infection in the Endoscopy Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya in this study the youngest patient was 30 years old and the oldest patient was 71 years old. As shown at Table 1, male patients were dominating (80%), especially in the 41–50 years age range. Female patients in this study had a higher median with age than male, yet the number of female patients in this study was lower than male.

Table 2. Ethnicity of the patients and time diagnosed with cirrhosis

Sex	Ethnicity		Time diagnosed (years)		
	Javanese	Other	< 1	1–3	>3
Male	23	1	12	9	3
Female	6	0	4	2	0
TOTAL	29	1	16	11	3

As shown at Table 2, samples in this study were predominantly obtained from patients of Javanese ethnicity (97%). Only 1 male patient was non-Javanese (Batak). Most

patients, 16/29(53%), had also just recently been diagnosed with liver cirrhosis (<1 year).

After separation of PBMC and HBV DNA isolation, PCR was performed to amplify the TNF-α gene promoter of the -238 and -308 positions. All samples were showed positive PCR results. The result of PCR on TNF-α -238 region gene promoter gave positive result of 152 bp band (Figure 1), while the result of PCR on TNF-α -308 region gene promoter gave positive result of 107 bp band (Figure 2).

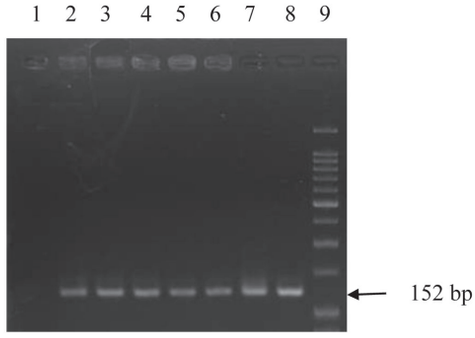

Figure 1. Example of electrophoresis product of PCR promoter gene TNF-α position -238
Description: lane 1: negative control; lane 2-8: samples with positive results; lane 9: marker

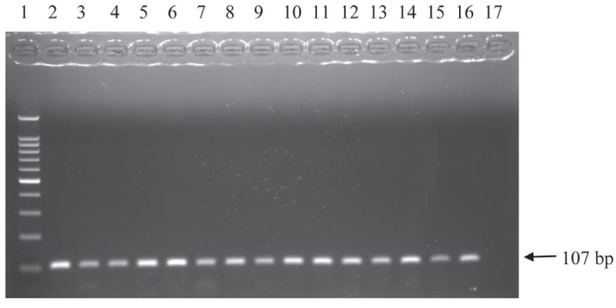

Figure 2. Example of electrophoresis product of PCR promoter gene TNF-α position -308
Description: lane 1: marker; lane 2-16: samples with positive results; lane 17: negative control

Furthermore, all samples were incubated with endonuclease restriction enzyme using *MspI* for -238 SNP, and *NcoI* for -308 SNP. Incubation was performed overnight at 37°C, then the RFLP product was visualized in 3% agarose gel. The determination of TNF-α gene promoter genotypes both in the -238 and -308 positions was based on the DNA fragment formed. In the PCR-RFLP position -238, the perfect cutting of *MspI* result that yielded the 132 and 20 bp fragments was showed the wild-type GG homozygous alleles, *MspI* partial cutting yielded three fragments 152, 132, and 20 bp were indicated GA heterozygotes, while intact 152 bp DNA fragments which was not digested with *MspI* indicated homozygous AA. As shown at Figure 3, in this study, we found samples with GG genotype (132 and 20 bp fragments) and GA genotype (152, 132, and 20 bp fragments), while no AA genotype was found.

At the -308 PCR-RFLP position, the perfect *NcoI* cutting yield of 87 and 20 bp fragments showed a wild-type GG homozygous allele, *NcoI* partial cutting yielded of three fragments 107, 87 and 20 bp were indicating GA heterozygotes, while intact 107 bp DNA fragments undigested with *NcoI* showing homozygous AA. As shown at Figure 4, in this study, we found samples with GG genotype (87 and 20 bp fragments) and GA genotype (107, 87, and 20 bp fragments), while no AA genotype was found.

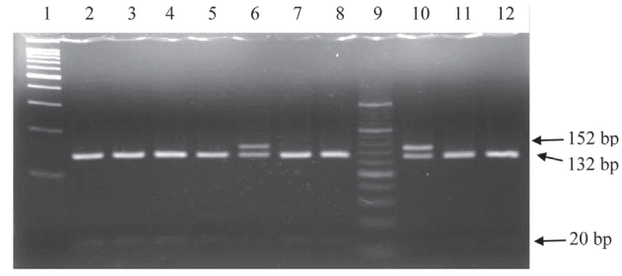

Figure 3. PCR-RFLP products of TNF-α promoter on -238 position
Description: Lane 2-5, 7, 8, 11 and 12: GG genotype with 2 fragments (132 bp and 20 bp); lane 6 and 10: GA genotype with 3 fragments (152 bp, 132 bp, and 20 bp); lane 1: DNA marker 100 bp; lane 9: DNA marker 20 bp

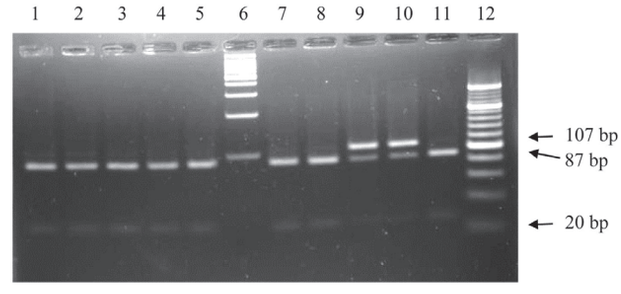

Figure 4. PCR-RFLP products of TNF-α promoter on -308 position
Description: Lane 1-5, 7, 8, and 11: GG genotype with 2 fragments (87 bp and 20 bp); lane 9 and 10: GA genotype with 3 fragments (107 bp, 87 bp, and 20 bp); lane 6: DNA marker 100 bp; lane 12: DNA marker 20 bp

From 30 patients in this study, 2 (6.7%) patients were showed GA heterozygote genotype on -238 TNF-α promoter SNP. No sample was found with AA homozygous SNP. The remaining 28 samples were showed genotype of wild-type GG. For -308 positions, 2 of 30 samples (6.7%) showed GA heterozygote SNP genotype. These were not the same patients that had GA at position -238. No sample was found with AA homozygous SNP genotype. The remaining 28 samples were showed a genotype of wild-type GG alleles. GA heterozygote samples for the -238 and -308 SNP were sampled with different numbers. No sample was found with two SNP-228 and -308 simultaneously in this study.

Table 3. TNF-α levels by sex in liver cirrhosis patients with HBV infection

Sex	Number of patients	Median (ng/L)	IQR
Male	24 (80%)	33	31,85
Female	6 (20%)	38	627,525

Table 4. TNF-α levels based on SNP genotype of TNF-α gene promoter in liver cirrhosis patients with HBV infection

Genotype	Median (ng/L)	IQR
-238 GG	35	30,51
GA	41	-
-308 GG	35	28,36
GA	30	-

In this study, higher levels of TNF-α were obtained in women than in men. In 1 sample, TNF-α level even reached 1118.78 ng/L (Table 3). Patients with liver cirrhosis with GG genotype for both -238 and -308 positions in this study had no different levels of TNF-α than GA genotypes (Table 4).

DISCUSSION

We were able to detect the presence of TNF-α promoter SNP -238 and -308 in patients with liver cirrhosis due to chronic HBV infection using PCR-RFLP. Tumor Necrosis Factor-α is a major cytokine in the inflammatory response to infection. TNF-α normally functions to activate cellular immunity and to provide protection against microbes, but excessive levels of this cytokine will result in severe tissue damage, septic shock and even death[10]. Schwabe[11] suggests that in liver, TNF-α can induce cell death as well as hepatocyte proliferation. In HBV infection, TNF-α levels tend to increase and are associated with inflammation, fibrosis, and hepatic damage. It is also said that TNF-α can be used as a predictor of liver inflammation[12].

It was found that most liver cirrhosis patients with HBV infection in this study was male (80%). This is consistent with the demographic data in previous studies suggesting that patients with cirrhosis of the liver due to chronic HBV infection are more frequent men than women[13–15]. This may due to a protective role of estrogen that is prevents damage to the liver because estrogen inhibits the proliferation of hepatic stellate cells and fibrogenesis that play an important role in the course of cirrhosis. In animal models with liver cirrhosis, estradiol administration leads to decreases in type I and III collagen and stellate cell proliferation[16].

Polymorphisms at TNF-α promoters -238 and -308 have been associated with various diseases associated with severe inflammation, infection, and malignancy. Researches on SNP of TNF-α gene promoters in patients with HBV infection reported conflicting results. A study in China showed a low A allele frequency, which was 4.6% for position -238 and 7.4% for position -308[17]. Another study in China also showed that no AA genotype found[18]. This distribution is different from that observed in Tunisia, where the GA and AA genotype frequencies in liver cirrhosis with HBV infection are quite high, ie 38.8% G/A and 44.5 A/A for -308 SNP, and 44.5% G/A and 33.3% A/A for -238 SNP[19]. A study in Turkey showed that AA genotype was found in healthy control individuals (6.7%), but none in hepatitis patients (0%).[20] TNF promoter polymorphism was known to be ethnic-specific, so each region might has unique distribution of TNF-α SNPs[21].

In a recent study in Brazil, among patients with Hepatitis C (HCV) infection with mild fibrosis, a -308 A/A SNP was found in 1.9% of the cases, and a G/A SNP in 26.1%. In patients with severe fibrosis, -308 A/A SNP in 0.8% while G/A SNP was present 21% patients[22]. Genotype AA is a rare genotype compared to genotype GG and GA[23]. Also in this study the AA genotype was not found. It is said that the frequency of A alleles is much lower in Asia than in other regions of the world.[24]

For Indonesia itself, this is the first time that TNF-α promoter genes polymorphism was studied in hepatitis patients. However, there have been several studies on TNF-α promoter gene SNP (-238, -308) in patients with other diseases such as acne vulgaris[25], Chronic Obstructive Pulmonary Disease (COPD) [26], and Down Syndrome[27]. In these studies, the frequency of AA genotype was also very low, even undetectable in in certain cases. Also, the GA genotype was found in frequently. However, in all those studies, the level of TNF-α was not measured. The low frequency of SNP in these researches could be due to the influence of Indonesian race and ethnicity[27].

Several previous studies on TNF-α SNP in cirrhosis gave controversial results. Some studies were showed that the G-308 allele is associated with low levels of TNF-α both in vitro [28] and in vivo[29] However, some other studies did not support this phenomena[30–32]. Studies of SNP at position -238 have likewise produced conflicting results with regards to TNF-α levels. Some studies were showed that having an A allele at this position is associated with elevated levels of TNF-α[33], but other studies were showed that there is no relationship between the two[34]. In addition, studies have shown that A allele is associated with lower TNF-α levels[17,35]. The expression of TNF-α, just like other cytokines is tightly regulated both at the transcriptional and post-transcriptional level. The polymorphisms located within the regulatory regions of TNF-α have been reported to influence the expression and secretion of this cytokine[35]. In TNF-α -238 and -308 SNP, the presence of the A-allele increases the binding of transcription factor to the promoter region of TNF-α, thereby altering its expression[36]. Nonetheless, several studies in various infectious diseases have shown the importance of TNF G>A in disease susceptibility, albeit with currently unknown molecular mechanism[37].

In this study, patients with liver cirrhosis with the GG genotype both for the -238 and -308 positions actually had

levels of TNF-α comparable to those with the GA genotype. Despite the associations between TNF promoter SNPs and disease, a direct impact of TNF promoter polymorphisms upon TNF transcription has not been conclusively demonstrated.[35] It was also said that TNF-α expression may not be directly related to TNF-α promotor gene polymorphism, but requires further variation in adjacent genes. This is due to the location of the TNF-α gene close to the HLA allele. As a result, pathological conditions might be caused by the variation in a linked gene that regulates the expression of this cytokine rather than due to polymorphism within the TNF-α gene itself.[24] The presence of other polymorphisms in the TNF-α promoter region of the gene may also contribute to TNF-α expression[34]. Finally, contradictory results may also be due to ethnic differences.[17,28,34] Given that in Indonesia harbours various ethnic groups, there is a possibility of variation based on ethnic groups studied in Indonesia, so further research is needed on the subject.

CONCLUSION

In this pilot study, 2/30 of liver cirrhosis patients with chronic HBV infection in Dr. Soetomo General Hospital, Surabaya were GA heterozygote for the TNF-α promoters at positions -238 and -308. 2 patients with SNP at position -238 and 2 patients with SNP at position -308 were different samples. No genotype AA was found. TNF-α level in this study was found higher in women than men. TNF-α level in patients with GA heterozygote genotype at -238 and -308 TNF-α gene promoters in this study was not different than wild-type (GG). Different results might also be due to racial or ethnic differences in each study. Further studies including larger numbers of patients from various ethnicities are needed to provide solid evidence on the prevalence TNF-α promoters polymorphisms in liver cirrhosis patients with HBV infection in Indonesia, and their relationship with the expression of TNF-α.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest in this study.

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Research Report

MICROBIAL PATTERN AND ANTIBIOTIC SUSCEPTIBILITY IN PEDIATRIC INTENSIVE CARE UNIT DR. SOETOMO HOSPITAL, SURABAYA

I Wayan Putra[1a], Arina Setyaningtyas[1], Dwiyanti Puspitasari, Irwanto[1], Agung Dwi Wahyu[2], Ira Dharmawati[1], Abdul Latief Azis[1], Kuntaman[2]
[1]Department of Child Health, Faculty of Medicine, Universitas Airlangga /Dr.Soetomo Hospital,Surabaya-Indonesia
[2]Department of Clinical Microbiology, Faculty of Medicine, Universitas Airlangga /Dr.Soetomo Hospital,Surabaya-Indonesia
[a]Corresponding author: iwayandewana@gmail.com

ABSTRACT

Gram-negative bacterial are known as common pathogen caused infection in Pediatric Intensive Care Unit (PICU). Microbial Pattern and Antibiotic Susceptibility are needed as clinical data for selected appropriate antibiotic therapy. In PICU Dr. Soetomo hospital until now still lacking of Microbial Pattern and Antibiotic Susceptibility data. This descriptive study is to recognized Microbial Pattern and Antibiotic Susceptibility in PICU patients from blood, urine, sputum, stool, cerebrospinal fluid, endotracheal tube, pus swab and pleural fluid culture specimens. Patients whose admitted into PICU without signs of infections were excluded from the study. The inclusion criteria are patients with sign infection as follows: fever < 36,5°C or > 37.5°C, leukocyte < 4000/mm³ or > 10000/mm³, marker infections CRP >10 mg/L or PCT >0,3 ng/mL, bradycardia or tachycardia, tachypnea, infiltrates on chest X-ray, turbid urine, dysuria, thrombophlebitis, abdominal pain or tenderness, and mucous or skin lesion. Medical record data from 2011 to 2016, revealed 1138 patients had positive microbial culture result, wherein positive result came from blood 44.46%, urine 19.15%, sputum 11.59%, stool 8.96%, cerebrospinal fluid 7.50%, endotracheal tube 4.04%, pus swab 2.89%, and pleural fluid 1.41%. The microorganisms found in PICU Dr. Soetomo was dominated with gram negative bacteria. Commonest bacterial that recognized from blood was B. cepacea, urine was E. coli, sputum was P. aeruginosa, Stool was E. coli, Cerebrospinal fluid was S. cohnii, endotracheal tube was K. pneumoniae ESBL, pus swab was S. aureus, and pleural fluid was S. maltophilia. Both gram-negative bacteria and gram-positive bacteria isolates revealed multiple drug resistance to commonly used antibiotic, but still had good susceptibility for antibiotic such as; amikacin, cefoperazone-sulbactam, linezolid, vancomycin and carbapenem group.

Keywords: *PICU, Microbial Paterrn, Dr. Soetomo Hospital, Bacteria, Antibiotic.*

ABSTRAK

Bakteri gram negatif merupakan patogen tersering penyebab infeksi di ruang rawat intensif anak. Pola bakteri dan kepekaan antibiotik diperlukan sebagai data klinis dalam pemilihan terapi antibiotik yang sesuai. Di ruang rawat intensif anak RS.Dr. Soetomo hingga saat ini masih sangat kekurangan data mengenai pola bakteri dan kepekaan antibiotik. Penelitian deskriptif ini bertujuan untuk membuat pola bakteri dan kepekaan antibiotika pada pasien yang dirawat di ruang rawat intensif anak dari spesimen darah, urin, sputum, feces, cairan serebrospinal,tabung endotrakeal (ETT), pus luka dan cairan pleura. Pasien yang masuk ke PICU yang tidak menunjukkan tanda dan gejala infeksi di eklusi dari penelitian. Kriteria inklusi pada penelitian ini adalah ditemukannya tanda dan gejala infeksi, antara lain ;demam < 36,5°C or > 37.5°C, kadar leukosit darah < 4000/mm3 or > 10000/mm3, marker infeksi CRP >10 mg/L or PCT >0,3 ng/mL, bradikardi atau takikardi, takipneu, gambaran infiltrate pada radiologi paru,urine yang keruh, nyeri berkemih, tromboplebitis, nyeri perut, dan lesi pada mukosa atau kulit. Dari data rekam medis dari tahun 2011 sampai 2016 didapatkan 1138 pasien dengan hasil kultur mikrobiologi positif, dimana 44.46% dari spesimen darah, 19.15% dari urin, 11.59% dari sputum,8.96% dari feces,7.50% dari cairan cerebrospinal, 4.04% dari ETT, 2.89% dari pus luka, dan 1.41% dari cairan pleura. Mikroorganisme terbanyak yang ditemukan di rawat intensif anak adalah bakteri gram negatif. Bakteri tersering dari spesimen darah adalah B. cepacea, E. coli pada urine, P. aeruginosa pada sputum,E. coli pada feces,S. cohnii pada cairan serebrospinal, K.

pneumoniae ESBL pada ETT,S. aureus pada pus luka,S. maltophilia pada cairan pleura. Isolat bakteri gram negatif maupun gram positif yang telah didapatkan menunjukan adanya resistensi berberapa golongan antibiotik yang umumnya sering digunakan tetapi beberapa jenis antibiotik lain masih menunjukan kepekaan yang baik terhadap antibiotic seperti amikasin, cefoperazone-sulbactam, linezolid, vancomycin dan grup karbapenem.

Kata kunci: *Rawat Intensif Anak , Pola Bakteri, Rumah Sakit Dr. Soetomo, Bakteri, Antibiotik.*

INTRODUCTION

In this two decade nosocomial Infections are special health problem concerned in terms of morbidities, mortalities and economic consequences.[1] Especially eventful in pediatric intensive care units (PICU) that have more eminent incidence rate than another ward in hospital.[2] These outcome were correlated with prolonged hospital stay, severity of diseases in PICU patients, excessive use of antibiotic and patients often exposed to medical intervention tools such as; peripherals intravenous or central venous lines, urinary catheterization, mechanical ventilation, etc.[2-3] Respiratory tract infections, and bloodstream infections are considerably occurring infection in PICU.[3] Both gram-positive bacteria (GPB) and gram-negative bacteria (GNB) have been reported as commonly pathogen causing infection. Recently, GNB have been presented more often than GPB in this setting.[4]

Knowledge updated about prevalence of the causative agent's infections and antimicrobial susceptibility patterns in PICU are important for proper management of nosocomial infections,[4-5] There were lack quantity of published studies on microbial pattern and antibiotic susceptibility in PICU patients from Indonesia. This study was brought to determine it, especially from PICU patients in Dr. Soetomo Hospital Surabaya. This hospital provides tertiary health care as referral hospital from primary health care or secondary health care in East Java and East Indonesia region

METHODS

This descriptive study was carried out in Dr. Soetomo General Hospital. The data were collected from medical record from January 2011 to January 2016. Ethical clearance issued by Medico-legal Committee Soetomo Hospital. Information collected include the demographic data, Primary diseases diagnosis, specimen, causative agent, and antibiotic sensitivity pattern. Patients admitted into PICU without signs of infections were excluded from the study. The inclusion criteria are patients with sign infection as follows: fever < 36,5°C or > 37.5°C, leukocyte < 4000/mm^3 or > 10000/mm3, marker infections CRP >10 mg/L or PCT > 0,3 ng/mL, bradycardia or tachycardia, tachypnea, infiltrates on chest X-ray, turbid urine, dysuria, thrombophlebitis, abdominal pain or tenderness, mucous or skin lesion. SPSS 17 version was used to process descriptive statistics data.

RESULT

Over period of 5 years, 4144 patients admitted in the PICU were analyzed. There were 1138 (27.46%) patient with positive culture result (Table 1), girls (59.92%) are dominant than boys (40.07%) with mean age 4 ± 0.8 years.

Primary diseases admitted patients in PICU with culture positive result were dominated with respiratory tracts infection and followed by nervous system diseases (Table 2). Microbial culture also undertaken in patients such as; Congenital Heart Diseases (CHD), Acute Leukemic Lymphoblastic (ALL), Dengue Hemorrhagic Fever (DHF), Acute diarrhea, and others, because while being treated show clinical signs or symptom suggested of infections.

Blood culture result were dominated with gram-negative bacteria (GNB) (14 species bacteria), followed 16 species gram-positive bacteria (GPB). The commonest GNB were *B. cepacea* (Table 3) and GPB were *S. haemolyticus* (Table 4).

Table 1. Positive culture result from various specimen in PICU patients

Specimen	Total Sample	Positive Result	(%)
Blood	1345	506	37.62
Urine	824	218	26.45
Sputum	643	132	20.52
Stool	582	102	17.52
Cerebrospinal fluid	348	86	24.71
Endotracheal tube	213	46	21.59
Pus	102	32	31.37
Pleural	87	16	18.39

Table 2. Primary diseases distribution of positive culture result in PICU Patient.

Primary Diseases	(f)	(%)
Pneumonia	341	29.96
Encephalitis	248	21.79
s. Meningoencephalitis	149	13.09
Bronchopneumonia	124	10.89
Congenital Heart Diseases	97	8.52
Oncologic Diseases	49	4.30
Renal Diseases	44	3.86
Post-surgery procedure	38	3.33
Diarrhea	32	2.81
Dengue Hemorrhagic Fever	25	2.19
Diabetic ketoacidosis	17	1.49
Biliary atresia	5	0.43

Table 3. GNB species finding in blood culture

Bacteria Species	(n=334)	(%)
B. cepacea	57	17.06
K. pneumoniae (ESBL+)	56	16.76
A. baumannii	44	13.17
K. pneumoniae	37	11.14
P. aeruginosa	33	9.88
E. coli	29	8.68
E. cloacae	21	6.28
S. marcescens	15	4.49
M. catarrhalis	12	3.59
S. typhi	9	2.69
E. coli (ESBL+)	9	2.69
P. alcalifaciens	7	2.09
Pasteurella spp	3	0.89
S. paratyphi	2	0.59

Table 4. GPB species finding in blood culture

Bacteria Species	(n=172)	(%)
S. haemolyticus	55	31.97
S. hominis	35	20.35
S. epidermidis	16	9.30
S. saprophyticus	14	8.13
S. aureus	13	7.55
MRSA	12	6.97
S. intermedius	5	2.90
S. cohnii	4	2.32
E. faecalis	3	1.75
Corynebacterium spp.	3	1.75
M. lylae	3	1.75
S. gallinarum	2	1.17
S. kloosii	2	1.17
S. warneri	2	1.17
S. ureolyticus	2	1.17
S. parasanguinis	1	0.58

Table 5. GNB species finding in urine culture

Bacteria Species	(n=159)	(%)
E. coli	81	50.94
E. coli (ESBL+)	33	20.75
K. pneumoniae (ESBL+)	13	8.18
E. cloacae	11	6.92
B. cepacea	5	3.14
P. aeruginosa	5	3.14
A. baumannii	3	1.89
E. aerogenes	3	1.89
S. marcescens	2	1.26
P. rettgeri	1	0.63
P. mirabilis	1	0.63
Aeromonas spp.	1	0.63

Urine culture specimen were dominated with GNB (10 species) followed GPB (8 species). The commonest GNB were *E. coli* (Table 5) and GPB were *S. haemolyticus* (Table 6).

Sputum culture specimen were dominated with GNB (8 species) followed GPB (9 species). The most common

Table 6. GPB species finding in urine culture

Bacteria Species	(n=59)	(%)
Gram Positive Bacteria :		
S. haemolyticus	11	18.64
S. epidermidis	10	16.94
S. cohnii	9	15.26
E. faecalis	9	15.26
MRSA	8	13.56
E. faecium	5	8.47
S. warneri	3	5.09
S. ureolyticus	2	3.39
S. parasanguinis	2	3.39

Table 7. GNB species in sputum culture.

Bacteria Species	(n=88)	(%)
P. aeruginosa	42	47.72
K. pneumonia	22	25.00
E. coli (ESBL+)	9	10.22
A. baumannii	6	6.82
S. maltophilia	3	3.42
E. cloacae	3	3.42
S. marcescens	2	2.27
S. fonticola	1	1.13

Table 8. GPB species in sputum culture.

Bacteria Species	(n=44)	(%)
S. epidermidis	23	52.27
MRSA	8	18.18
S. capitis	6	13.64
S. haemolyticus	6	13.64
S. pneumonia	1	2.27

GNB were *P. aeruginosa* (Table 7) and GPB were *S. epidermidis* (Table 8). Higher rate of *S. epidermidis* in this study might be caused by contaminant at recruitment sampling process.

Stool culture specimen were also dominated with GNB (8 species) followed GPB (6 species). The commonest GNB species were *E. coli* (Table 9) and GPB were *Enterococcus spp.* (Table 10).

Cerebrospinal fluid (CSF) culture was dominated with GPB (8 species) followed GNB (7 species). The commonest GPB species were *S. cohnii* (Table 11) and GNB were *A. baumannii* (Table 12). CSF culture with *S. cohnii* and *A. baumannii* in the study result, might be considered as contaminant bacteria while recruitment process because 52 patients with surgery history with device insertion. It is connected the intracerebral area with outer enviroment from External Ventriculo Drainage (EVD) device while sampling process.

Endotracheal tube (ETT) aspirate culture specimen was dominated with GNB (6 species) followed GPB (4 species). The commonest GNB species were *K. pneumonia (ESBL+)* (Table 13) and GPB were *S. haemolyticus* (Table 14).

Pus/ wound swab culture specimen were dominated with GPB (8 species) followed GNB (5 species). The

Table 9. GNB species in stool culture

Bacteria Species	(n=65)	(%)
E. coli	34	52.30
E. cloacae	21	32.32
E. coli (ESBL+)	2	3.07
E. aerogenes	2	3.07
K. pneumoniae (ESBL+)	2	3.07
C. youngae	2	3.07
C. jejuni	1	1.55
C. testosteroni	1	1.55

Table 10. GPB species in stool culture

Bacteria Species	(n=37)	(%)
E. cloacae	19	51.35
S. aureus	10	27.03
S. epidermidis	3	8.11
MRSA	3	8.11
S. paratyphi	1	2.70
C. difficile	1	2.70

Table 11. GPB species in CSF culture

Bacteria Species	(n=56)	(%)
S. cohnii	11	19.64
S. epidermidis	9	16.07
S. haemolyticus	8	14.28
S. aureus	7	12.50
E. faecium	7	12.50
E. faecalis	7	12.50
A. viridans	4	7.15
MRSA	3	5.36

Table 12. GNB species in CSF culture

Bacteria Species	(n=30)	(%)
A. baumannii	10	33.33
E. cloacae	7	23.34
P. aeruginosa	6	20.00
E. coli (ESBL+)	3	10.00
B. diminuta	2	6.67
P. stutzeri	1	3.33
B. cepacea	1	3.33

Table 13. GNB species in ETT aspirate

Bacteria Species	(n=37)	(%)
K. pneumonia (ESBL+)	16	43.24
P. aeruginosa	11	29.72
A. baumannii	6	16.22
E. coli (ESBL+)	2	5.42
S. marcescens	1	2.70
B. cepacea	1	2.70

commonest GPB were *S. aureus* (Table 15) and GNB were *P. aeruginosa* (Table 16). Over 32 wound positive culture isolate in our study were undertaken from 37 pediatric patients with history surgical site infection.

Table 14. GPB species in ETT aspirate

Bacteria Species	(n=9)	(%)
S. haemolyticus	6	66.67
MRSA	1	11.11
S. epidermidis	1	11.11
S. capitis	1	11.11

Table 15. GPB species in pus wound swab

Bacteria Species	(n=23)	(%)
S. aureus	9	39.13
S. epidermidis	7	30.43
S. haemolyticus	2	8.69
S. constellatus	1	4.35
S. acidominus	1	4.35
E. faecalis	1	4.35
MRSA	1	4.35
S. capitis	1	4.35

Table 16 GNB species in pus wound swab

Bacteria Species	(n=9)	(%)
P. aeruginosa	5	55.56
K. pneumonia (ESBL+)	1	11.11
P. mirabilis	1	11.11
C. testosteroni	1	11.11
C. striatum	1	11.11

Table 17. GNB species in pleural fluid.

Bacteria Species	(n=10)	(%)
S. maltophilia	4	40.00
P. putida	3	30.00
L. adecarboxylata	1	10.00
C. farmeri	1	10.00
K. pneumonia (ESBL+)	1	10.00

Table 18. GPB species in pleural fluid.

Bacteria Species	(n=6)	(%)
S. epidermidis	3	50.00
S. haemolyticus	1	16.66
S. capitis	1	16.66
MRSA	1	16.66

Pleural fluid culture specimen was dominated with GNB (5 species bacteria) followed GPB (4 species bacteria). The commonest GNB were *S. maltophilia* (Table 17) and GPB were *S. epidermidis* (Table 18).

Antibiotic sensitivity pattern of GNB (Table 19) are showed that almost all of the isolate are resistant to; penicillin cephalosporin, tetracycline, chloram- phenicol, sulfa and quinolones groups.

Among GNB isolate, Cefo-sulbactam has the highest susceptibility rate (87.71%) for *B. cephacea* in blood, nitrofurantoin (97.53%) for *E. coli* in urine, cefo-sulbactam (88.09%) for *P. aeruginosa* in sputum, both of amikacin

Tabel.19. Antibiotics sensitivity pattern of gram negative bacteria isolate from different samples

Antibiotics	The most gram negative bacteria (GNB) species in isolate samples								
	Blood	Urine	Sputum	Stool	CSF Fluid	ETT	Pus/Wound	Pleural Fluid	
	B. cepachea n=57	E. coli n=81	P. aeruginosa n=42	E. coli n=34	A. baumanii n=10	K. pneumonia n=16	P.aeruginosa n=5	S.maltophilia n=4	GNB Sensitivity
Amikacin	50 (87.71%)	79 (97.53%)	33 (78.57%)	33 (97.05%)	7 (70.00%)	15 (93.75%)	4 (80.00%)	3 (75.00%)	84.95%%
Tobramycin	39 (68.42%)	49 (60.49%)	23 (54.76%)	22 (64.70%)	5 (50.00%)	11 (68.75%)	2 (40.00%)	2 (50.00%)	57.14%
Gentamycin	37 (64.91%)	47 (58.02%)	32 (76.19%)	20 (58.82%)	5 (50.00%)	13 (81.25%)	3 (60.00%)	3 (75.00%)	65.52%
Astreonam	20 (35.08%)	32 (39.50%)	27 (64.28%)	14 (41.17%)	0 (0%)	8 (50.00%)	3 (60.00%)	2 (50.00%)	42.50%
Amoxicillin - Clavulanic	18 (31.57%)	38 (46.91%)	0 (0%)	15 (44.11%)	0 (0%)	8 (50.00%)	0 (0%)	0 (0%)	21.57%
Ampicillin	4 (7.17%)	13 (16.04%)	0 (0%)	5 (14.70%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4.74%
Ampicillin- Sulbactam	25 (43.85%)	32 (39.50%)	5 (11.90%)	13 (38.23%)	7 (7.00%)	7 (43.75%)	1 (20.00%)	1 (25.00%)	28.65%
Pippe - Tazobactam	35 (61.40%)	59 (72.83%)	27 (64.28%)	25 (73.52%)	4 (40.00%)	10 (62.50%)	3 (60.00%)	3 (75.00%)	63.69%
Cefazolin	13 (22.80%)	23 (28.39%)	0 (0%)	10 (29.41%)	0 (0%)	8 (50.00%)	0 (0%)	0 (0%)	16.33%
Ceftazidime	21 (36.84%)	38 (46.91%)	36 (85.71%)	16 (47.05%)	4 (40.00%)	9 (56.25%)	4 (80.00%)	4 (100%)	61.60%
Cefotaxime	22 (38.59%)	34 (41.97%)	0 (0%)	14 (41.17%)	4 (40.00%)	7 (43.75%)	0 (0%)	0 (0%)	25.69%
Ceftriaxone	11 (19.29%)	9 (11.11%)	12 (28.57%)	4 (11.76%)	2 (20.00%)	2 (12.50%)	1 (20.00%)	1 (25.00%)	18.53%
Cefo – Sulbactam	54 (94.73%)	76 (93.82%)	37 (88.09%)	32 (94.11%)	8 (86%)	15 (93.75%)	4 (80.00%)	4 (100%)	91.31%
Cefepime	27 (47.36%)	35 (43.20%)	26 (61.90%)	15 (44.11%)	4 (40.00%)	7 (43.75%)	3 (60.00%)	3 (75.00%)	51.92%
Cotrimoxazole	32 (56.14%)	29 (35.80%)	7 (16.66%)	12 (35.29%)	8 (80.00%)	12 (75.00%)	1 (20.00%)	1 (25.00%)	42.99%
Tetracycline	21 (36.84%)	22 (27.16%)	0 (0%)	10 (29.41%)	4 (40.00%)	8 (50.00%)	0 (0%)	0 (0%)	22.93%
Chloramphenicol	30 (52.63%)	58 (71.60%)	12 (28.57%)	25 (73.52%)	0 (0%)	12 (75.00%)	1 (20.00%)	1 (25.00%)	43.29%
Ciprofloxacin	31 (54.38%)	32 (39.50%)	27 (64.28%)	14 (41.17%)	5 (50.00%)	9 (56.25%)	3 (60.00%)	2 (50.00%)	51.95%
Levofloxacin	33 (57.89%)	35 (43.20%)	30 (71.42%)	15 (44.11%)	6 (60.00%)	9 (56.25%)	3 (60.00%)	3 (75.00%)	58.48%
Fosfomycin	37 (64.91%)	79 (97.53%)	20 (47.61%)	32 (94.11%)	1 (10.00%)	7 (43.75%)	2 (40.00%)	2 (50.00%)	55.99%
Nitrofurantoin	-	80 (98.76%)	0 (0%)	33 (97.05%)	0 (0%)	12 (75.00%)	0 (0%)	0 (0%)	38.69%
Imipenem	46 (80.70%)	69 (85.18%)	32 (76.19%)	29 (85.29%)	7 (70.00%)	15 (93.75%)	4 (80.00%)	3 (75.00%)	80.76%
Meropenem	45 (78.94%)	73 (90.12%)	32 (76.19%)	31 (91.17%)	7 (70.00%)	14 (87.50%)	4 (80.00%)	3 (75.00%)	81.12%

Tabel.20. Antibiotics sensitivity pattern of gram positive bacteria isolate from different samples

Antibiotics	The most gram positive bacteria (GPB) species in isolate samples								GPB Sensitivity
	Blood	Urine	Sputum	Stool	CSF Fluid	ETT	Pus/Wound	Pleural Fluid	
	S. B-haemolyticus n = 55	S. B-haemolyticus n = 11	S. coagulase negatif N = 23	E. cloacae n = 19	S. cohnii n = 11	S. B-haemolyticus N = 6	S. aureus n = 9	S. haemolyticus n = 4	
Gentamicin	16 (29.09%)	2 (18.18%)	12 (52.17%)	8 (42.10%)	6 (54.54%)	2 (33.33%)	8 (88.89%)	3 (75.00%)	(49.16%)
Ampicillin	1 (1.81%)	1 (5.56%)	8 (34.78%)	4 (21.05%)	1 (5.56%)	0 (0%)	1 (1.11%)	1 (25.00%)	(11.86%)
Ampicillin-sulbactam	9 (16.36%)	2 (18.18%)	6 (26.08%)	3 (15.78%)	1 (5.56%)	1 (16.66%)	7 (77.78%)	2 (50.00%)	(28.30%)
Penicillin	2 (3.36%)	1 (5.56%)	4 (17.39%)	4 (21.05%)	2 (18.18%)	1 (16.66%)	1 (1.11%)	1 (25.00%)	(13.54%)
Oxacillin	12 (21.81%)	3 (27.27%)	10 (43.47%)	8 (42.10%)	6 (54.54%)	1 (16.66%)	6 (66.67%)	3 (75.00%)	(43.44%)
Cotrimoxazole	4 (7.72%)	2 (18.18%)	11 (47.82%)	8 (42.10%)	5 (45.45%)	1 (16.66%)	8 (88.89%)	1 (25.00%)	(36.48%)
Tetracycline	29 (52.72%)	6 (54.54%)	6 (20.08%)	7 (36.84%)	3 (27.27%)	0 (0%)	0 (0.00%)	1 (25.00%)	(27.06%)
Chloramphenicol	25 (45.45%)	5 (45.45%)	8 (34.78%)	9 (47.36%)	3 (27.27%)	3 (50.00%)	6 (66.67%)	2 (50.00%)	(45.87%)
Erythromycin	18 (32.72%)	3 (27.27%)	13 (56.52%)	11 (57.89%)	6 (54.54%)	3 (50.00%)	6 (66.67%)	2 (50.00%)	(49.45%)
Clindamycin	21 (38.18%)	5 (45.45%)	16 (69.56%)	12 (63.15%)	4 (36.36%)	2 (33.33%)	6 (66.67%)	2 (50.00%)	(50.34%)
Ciprofloxacin	38 (69.09%)	7 (63.63%)	0 (0.00%)	12 (63.15%)	6 (54.54%)	3 (50.00%)	0 (0.00%)	2 (50.00%)	(43.80%)
Levofloxacin	31 (56.36%)	9 (81.81%)	14 (60.86%)	13 (68.42%)	7 (63.63%)	4 (66.67%)	3 (33.33%)	2 (50.00%)	(60.14%)
Moxifloxacin	49 (89.09%)	9 (81.81%)	16 (69.56%)	12 (63.15%)	6 (54.54%)	4 (66.67%)	3 (33.33%)	3 (75.00%)	(66.64%)
Fosfomycin	48 (87.27%)	10 (90.90%)	19 (82.60%)	11 (57.89%)	9 (81.81%)	3 (50.00%)	8 (88.89%)	3 (75.00%)	(76.80%)
Nitrofurantoin	46 (83.63%)	9 (81.81%)	20 (96.95%)	14 (73.68%)	6 (54.54%)	3 (50.00%)	8 (88.89%)	3 (75.00%)	(75.56%)
Meropenem	8 (14.54%)	2 (18.18%)	18 (78.26%)	10 (52.63%)	5 (45.45%)	2 (18.18%)	8 (88.89%)	2 (50.00%)	(45.77%)
Vancomycin	52 (94.54%)	11 (100.00%)	22 (95.65%)	16 (84.21%)	9 (81.81%)	5 (83.33%)	9 (100.00%)	4 (100.00%)	(92.44%)
Linezolid	53 (96.36%)	11 (100.00%)	20 (86.95%)	17 (89.47%)	11 (100.00%)	5 (83.33%)	8 (88.89%)	4 (100.00%)	(93.13%)
Daptomycin	52 (94.54%)	10 (90.91%)	21 (91.30%)	16 (84.21%)	9 (81.81%)	4 (66.67%)	8 (88.89%)	4 (100.00%)	(87.29%)

and nitrofurantoin (97.05%) for *E. coli* in stool isolate. Amikacin, Cefo-sulbactam and imipenem (93.75%) had highest sensitivity for *K. pneumonia* in ETT isolate and at the last cefo-sulbactam (100%) also had highest sensitive for *S. malthopnilia* in pleural isolates.

GPB antibiotic sensitivity pattern (Table 20), are showed that almost all of isolate resistant for aminoglycoside, penicillin, macrolide, tetracycline, and carbapenem antibiotic groups. In GPB isolate, Linezolid (100%) has highest susceptibility rate for *S. cohnii* in CSF fluid and vancomycin (100%) high sensitive for *P. aeruginosa* in pus/wound swab isolated.

DISCUSSION

In this study totally 4144 PICU patients were followed the study and only 27.46% had positive culture result, female higher than male in distribution gender, with mean age 4 ± 0.8 years. Primary diseases distribution was dominated with respiratory tracts infection. Previous study by *Camilla et.al*, positive culture result in PICU patient dominant respiratory tract infection as primary diseases (33.26%), female more often than male, with higher age incidence at less than five years.[5] Other study in PICU Mohammad Hoesin Palembang stated that commonest respiratory tract infection diagnosis was broncho-pneumonia (33.3%).[6]

In our present study, the frequency Gram-Negative Bacteria (GNB) isolates was slightly higher than that Gran-Positive Bacteria (GPB) isolates. GNB constituted the majority of bacterial pathogens associated with the 6 major specimen, blood (66.01%), urine (72.69%), sputum (66.67%), stool (63.73%), ETT aspirate (80.43%) and pleural fluid (62.50%). The predominance of GNB is a relevant reminder that these pathogens were once the most common human pathogens.[3,7] For approximately the past 2 decades, GNB have been the pathogens most frequently associated with respiratory system diseases and Urinary tracts infections(UTIs).[4,7]

Blood culture result of our study demonstrated GNB (66.01%) were the most common organisms causing blood stream infection, various literatures from the world are showed these phenomena such as; *Gupta et.al, Haeusler et.al, and Kirsty et.al,* showed GNB as predominant pathogen for blood stream infection.[7,8,9] Our study is gained commonest GNB species was *B. cepacea* (11.26%). *B. cepacea* has emerged as a serious human pathogen in the last two decades, causing fatal necrotizing pneumonia and bacteremia. *B. cepacea* has been associated with out breaks involving infections of the bloodstream, respiratory tract, and urinary tract in intensive care unit setting.[7,8,10] *Antony et.al.* stated that the intensive care unit bloodstream infections in tertiary hospital often caused by *B. cepacea* infections.[10]

Urine culture demonstrated positivity rate for 26.45%, clinically with urinary tract infection cases. Several study are showed vary positivity of the urine culture e.g. *Salar et.al* 17%. '*Kaur et.al* 15.7% are showed an occurrence of urinary tract infection among PICU patients[11,12] This difference could possibly due to various antibiotic prescribing practices, variations in sample collection, culture technique and susceptibility testing practices in our hospital than others. Our study was also shown that GNB, *E. coli* (50.94%) was the most common organisms in urinary tract infection. This finding similar with microbial pattern in adult patients in same hospital, that *E. coli* is the most common cause UTIs.[13]

Sputum culture revealed positivity rate 20.52% of respiratory tract infection cases. Its majority caused by GNB with dominant *P. aeruginosa* (47.72%). *Piyush et.al* is stated 34.23% patient had *P. aeruginosa* etiology from sputum sample in respiratory tract infection.[14] *P. aeruginosa* is a gram-negative aerobic rod. It became considered as most challenging pathogen bacteria globally because of its high rate of resistance to antimicrobial agent.[3,15] It was also reported that *P. aeruginosa* is one of the most common nosocomial pathogen and a leading cause of nosocomial respiratory tract infection.[5,15]

Stool culture is obtain positivity rate was 17.52% majority caused by GNB (63.73%) were dominant *E. coli* (52.30%). *E. coli* has been reported as the most frequently identified pathogen in other study throughout the world like China.[16] Some country reported different bacteria as the leading entero-pathogen, such a *Salmonella spp* in South Korea,[17] *and Aeromonas spp*. in Singapore. [18] Some of these regional differences may be related to study population or stool culture techniques.

The endotracheal tube aspirated is performed 21.59% positivity rate which were dominated with GNB dominantly *K. pneumonia (ESBL+)* (43.24%). In contrast to our study *Rehman et.al* are reported 93.65% culture positive in ETT tips, they also revealed that *K. pneumoniae* (41.93%) was the most common isolate.[19] *Kalanuria et.al* are stated that Pseudomonas *spp* was common isolate from ETT tips.[20] This differences result may be most of these microorganism acquired from environment and their concentrations varying depend on hospital geographical distribution and their ability to survive in particular conditions. The lumen of ETT in patients using mechanical ventilation usually became colonized with GNB which commonly appeared to survive within a biofilm.[21] While it appears that colonization of the ETT may begin from as early as 12 hours, it is most abundant at 96 hours.[20,21]

Pleural fluid culture is attained positivity rate for 18.39% and were dominated with GNB (62.50%) with majority species *S. maltophilia* (40.00%). *Jones et.al.* in their study are got positivity culture of pleural fluid rate was 11.50% with *S. maltophilia* (59.16%) are commonest bacterial.[22] *Chawla et.al* are stated that S. *maltophilia* often cause pneumonia infection.[23] In our study these microbes may affect pleural fluid after infected lower respiratory tract such as pneumonia by organ lesion caused diseases progression. At present, the incidence of nosocomial infections cause by *S. maltophilia* is increasing; in

particular, intensive care units are leading areas with high risk of these infections.[23,24] These organisms also resistance to many broad-spectrum antibiotics including carbapenem causes an increase in the mortality and morbidity rates in the intensive care units.[22,24]

Cerebrospinal fluid culture had gram positive bacteria (65.12%) as the common microorganism with majority species S. *cohnii* (52.30%). Previous study conducted by *Jiang et.al.* are showed (50.8%) acute bacterial meningitis in pediatric caused GPB infections.[25] *Zhu et.al.* are found GPB predominant pathogen in pediatric patients caused purulent meningitis were *E. coli* and *Staphylococcus spp.*[26] In our finding has similar perform with the other literature, its suggest that the development of nosocomial staphylococcal meningitis may subsequent to central nervous system conditions and neurosurgery interventions, which include ventriculo-peritoneal shunts, or other embedded devices. In this study over 52 patients also known had surgery history for inserting neurosurgery device. Generally, as is common in other surgical practice, the risk factor of inserting device infection, the venue of procedure and the surgical technique are know by surgeon's experience.[27]

Wound culture were dominated with GPB (71.87%), with isolate was *S. aureus* (39.13%) and followed GNB *P. aeruginosa* (55.56%). *Negi et.al* are found 96,4% surgical site infection yielding bacteria growth with *S. aureus* (54.4%), *P. aeruginosa* (21.7%) and *E. coli.*[28] These infections are usually caused by exogenous or endogenous microorganisms that enter the operative wound during the course of the surgery.[29] In our study over 32 of 37 patients with history surgical site infection had positive result culture. These wound infections may have occurred at hospital and recognized to be associated with an infection before-after or during surgery, extended length of hospital stays and prolonged or permanent disability.

Antibiotics susceptibility pattern of GNB isolates (blood, urine, sputum, stool, ETT and pleural fluid) in our study finding were resistant to three or more groups antimicrobial agents and therefore consider multidrug resistant (MDR), almost all of the isolate are resistant to; penicillin, cephalosporin, tetracycline, chloram- phenicol, sulfa and quinolones groups, The development of antibiotic resistant in our hospital might be caused by unnecessary, inappropriate, or suboptimal prescribed antibiotic therapy from community before, previous health care and our hospital itself. Previous study similar that, find very high level of resistance penicillin derivate, approximately one half isolate in infants and young children.[30] Other study in Africa 75% isolate are MDR to ampicillin, chloramphenicol and cotrimoxazole.[31] WHO in 2014 report that five out of the six WHO regions had more than 50% resistant to third generation of cephalosporin and fluoroquinolones in hospital setting.[32] In GPB isolates (CSF and Wound swab) also found multidrug resistance, over two third of antibiotic testing had resistance. Only vancomycin, linezolid and daptomycin had highest susceptibility for all GPB isolates. *Sarangi et.al* and *Singh et.al.* were also found that vancomycin and linezolid had highest antibiotic susceptibility NICU setting.[33,34] Highest prevalence isolates with multiple drug resistance that observed in our study may cause our hospital is a tertiary care center with large range health service not only in east java but also in east region Indonesia, Patient adjoining provinces are admitted for treatment that before attending the hospital, most of the patient get different antibiotic from low level heath care centers or due to over the counter sell of antibiotics often in improper dose. Limited population in some specimen and obtain of some pathogen or contaminant bacteria were all limitation in our study, multicenter prospective studies are needed to validated our finding.

CONCLUSION

Our study revealed GNB isolates as the predominant pathogen in all PICU isolates sampling, with most microorganism found were *B. cepacea* in blood, *P. aeruginosa* in sputum, *E. coli* in urine and stool, *S. cohnii* in CSF fluid, *K. pneumoniae ESBL* in ETT aspirate, *S. aureus* in pus, and *S. maltophilia* in pleural fluid culture. Both GNB and GPB isolates showed multiple drug resistance to commonly used antibiotic but still had good susceptibility for amikacin, cefoperazone-sulbactam, linezolid, vancomycin and carbapenem group.

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