In silico analysis of V48A dihydropteroate synthase mutation to dapsone on *Mycobacterium leprae* from Papua

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Abstrak

Latar belakang: *Lepra merupakan penyakit yang disebabkan oleh Mycobacterium leprae. Resistensi obat merupakan salah satu tantangan dalam pemberantasan kusta khususnya di Papua. Adanya mutasi pada gen folP1 penyandi dihydropteroate synthase (DHPS) merupakan dasar untuk deteksi molekuler resistensi dapson pada penyakit lepra. Tujuan penelitian ini adalah mendeteksi mutasi pada gen folP1 Mycobacterium leprae dari Papua, Indonesia dan menganalisis pengaruh mutasi tersebut terhadap dapson dengan metode in silico.*

Metode: *Identifikasi mutasi pada gen folp1 M. leprae dilakukan melalui proses Basic Local Alignment Search Tool (BLAST) di gene bank. Analisis efek mutasi dengan menggunakan server Have (y) Our Protein Explained (HOPE). Prediksi binding pocket menggunakan Computed Atlas of Surface Topography of proteins (CASTp). Homologi modeling struktur 3D DHPS menggunakan server Iterative Threading ASSEmbly Refinement (I-TASSER). Analisis docking dengan menggunakan AutoDock Vina yang terintegrasi dengan aplikasi Python Prescription (PyRx).*

Hasil: *Hasil sekuensing menunjukkan adanya variasi dalam gen folP1 M. leprae yaitu perubahan dari Timin (T) menjadi Sitosin (C) pada nukleotida 143. Residu yang bermutasi (V48A) terletak pada domain yang penting untuk aktivitas protein dan kontak dengan residu di domain lain. Ada kemungkinan bahwa interaksi ini penting untuk fungsi protein secara benar. Mutan V48A tidak banyak mempengaruhi stabilitas dari dihydropteroate synthase M. leprae.*

Kesimpulan: *Berdasarkan analisis molecular docking, mutasi V48A tidak mempengaruhi binding affinity dapson terhadap dihydropteroate synthase M. leprae. Hasil ini menunjukkan mutan V48A kemungkinan tetap rentan terhadap dapson. Dengan demikian perlu dilakukan uji in vivo untuk mengkofirmasi efek mutasi V48A.* ***(Health Science Journal of Indonesia 2020;11(2):70-6)***

Kata kunci: *Mycobacterium leprae*, *folP1 gene, dihydropteroate synthase, dapson*

Abstract

Background: Leprosy is a disease caused by *Mycobacterium leprae*. Drug resistance is one of the challenges in leprosy elimination especially in Papua. The presence of mutations in *folP1* gene that encode dihydropteroate synthase (DHPS) was considered as the exclusive basis for molecular detection of dapsone resistance in leprosy. The objective of this study was to detect mutations in the *folP1* gene of *Mycobacterium leprae* from Papua, Indonesia and to analyze the effect of these mutations on dapsone using the in-silico method.

Methods: Identification of mutations in the *folp1 M. leprae* gene is carried out through the Basic Local Alignment Search Tool (BLAST) process in the gene bank. The analysis of the effects of mutations using the Have (y)Our Protein Explained (HOPE) server. Bindings pocket prediction is done using the Computed Atlas of Surface Topography of proteins (CASTp). Homology modeling 3D structure of DHPS using the Iterative Threading ASSEmbly Refinement (I-TASSER) server. Docking analysis was performed using AutoDock Vina which is integrated with the Python Prescription (PyRx) application.

Results: The sequencing results showed a variation in the *folP1 M. leprae* gene, namely a change from thymine (T) to cytosine (C) in nucleotide 143. The mutated residue (V48A) is in a domain that is essential for the activity of the protein and in contact with residues in another domain. It is possible that this interaction is important for the correct function of the protein. V48A mutants did not significantly affect the stability of DHPS *M. leprae*.

Conclusion: Based on molecular docking analysis, this mutation does not affect binding affinity dapsone against *M. leprae* dihydropteroate synthase. These results indicate that the V48A mutant is likely to remain susceptible to dapsone. Thus, it is necessary to do an in vivo test to confirm the effect of the V48A mutation. ***(Health Science Journal of Indonesia 2020;11(2):70-6)***

Keywords: *Mycobacterium leprae*, *folP1* gene, dihydropteroate synthase, dapsone

Leprosy is a skin infection, membrane and prefers nerve disease.[1] Leprae is a neglected disease that still occurs in about 120 countries with more than 200,000 new cases reported annually. The regional proportions of all new cases in 2019 were: 71.3% (143 787) in South-East Asian Region (SEAR), 14.9% (29 936) in Americas Region (AMR), 9.9% (20 205) in African Region (AFR), 2.1% (4211) in Eastern Mediterranean Region (EMR), 1.9% (4004) in WPR and 42 in European Region (EUR).[2] This disease is still a significant disease in Jayapura City, Papua, furthermore, the burden disease of leprosy in Jayapura is considered as high.[3] Based on the Disability Number, Papua was the highest number in Indonesia in 2013 (26,88), followed by Aceh (18,62), and West Papua (17,72). The data from General Directorate of Disease Control and Health Environment in 2013 reveals that Case Detection Rate (CDR) of Papua was 35,64 in 100.000 people and declined to 30,43/100.000 on 2014.[4,5]

Since 1995, WHO has supplied Multi Drug Therapy (MDT) to all country with leprosy burden. The MDT consists of three antibiotics, contained rifampicin, clofazimine and dapsone for Multibacillary (MB) and rifampicin and dapsone combination for Paucibacillary (PB).[6] Unfortunately, MDT program in Papua and West Papua faces challenges such as the low awareness of patients, geographical barriers to access the health facility and other people's paradigm about drugs. DHPS is an enzyme that plays a role in the biosynthesis of folate in bacteria including *M. leprae*, which targets dapsone by inhibiting p-aminobenzoic acid (PABA).[7] The presence of point mutations in *folP1* gene that encodes dihydropteroate synthase (DHPS) was considered as the exclusive basis for molecular detection of dapsone resistance in leprosy.[8] Dapsone-resistant *M. leprae* isolates have shown mutation at codon 53 or 55 in the *folP1* gene. [9,10,11] The most frequently detected mutation associated with dapsone resistance in *M. leprae* is CCC→CTC in codon 55 of *folP1* resulting in the substitution of leucine for a proline residue (Pro55Leu) in the DHPS.[12] Predictively, the mutation will decrease the effectiveness of dapsone therapy.

The effect of drug resistance due to the point mutation on the amino acid residues of the targeted protein can be studied by bioinformatics simulation (in-silico). This method is relatively accurate, rapid and cost-effective compared to in vitro and in vivo method. Therefore, computational studies can be performed to study drug resistance.[13,14] Many of the molecular docking is successful in predicting the binding form of the ligand in the receptor binding sides.[15] The objectives of this study were to detect mutations in the *folP1* gene of *M. leprae* from Papua Island, Indonesia and to analyze the effect of these mutations on dapsone using the *in silico* method.

METHODS

This study was a cross section. This research was ethically approved by Ethics Committee of National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia number LB.02.01/5.2/KE.065/2016. The samples in this study were the result of an incision in one or both ears skin of leprosy patients. Sampling was carried out at Hamadi Public Health Centers, Jayapura and Bintuni Regency by making direct visits to patients' homes or to patients who were conducting control at health facilities. The total samples were 100 leprosy patients.

a. Identification of mutation of *folP1* gene *M. leprae* from Papua islands

The molecular examination began with DNA extraction process using QIAamp DNA Mini Kit (REF:51306, Qiagen, German), followed by Polymerase Chain Reaction (PCR) process. The primers used to amplify the *folP1* gene *M. leprae* in this study were WHOF15'-GCAGGTTATTGGGGTTTTGA-3' as a forward primer and WHOF2 5'-CCACCAGACACATCGTTGAC-3' as reverse primers. The reagents for PCR are GoTaq® Green Master Mix (REF:M7122, Promega, USA). A touchdown PCR method was performed, preheating was done at 98 °C for 2 minutes, followed by 5 cycles of 98 °C for 20 seconds, 60 °C to 56 °C with decrement 1 °C per cycle for 30 seconds, and 72 °C for 20 seconds. The further cycle was done at 98 °C for 20 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds for 40 times, with a final extension at 72 °C for 5 minutes. The resulting PCR product was 312 bp which is a partial part of the *folP1* gene. The PCR product was purified by Applied Biosystems™ CleanSweep™ PCR Purification Reagent (Thermo Fisher Scientific, USA) with a ratio of 2:5 The purified PCR product proceeded to Sanger Sequencing procedure. The sequencing cycle uses BigDye™ Terminator v3.1 Cycle Sequencing Kit (REF:4336917, Thermo Fisher Scientific, USA) as 4 µL, BigDye Terminator buffer v1.1 / v3.1 5X buffer (REF:4336917, Thermo Fisher Scientific, USA) 4 µL, 1 µL molded DNA, and nuclease free water 7 µL. DNA pGEM -3Zf was used as a positive control and Primary control -21 M13 as a positive control primer. The primers for sequencing are the same as the primers for PCR. The reaction of the sequencing

cycle was carried out under conditions: 96°C 1 min, 96°C 10 sec, 50°C 5 sec, 60 °C 4 min. The cycle was repeated 25 times later the result of the sequencing cycle is purified by XTerminator Solution and SAM solution (REF:4376486, Thermo Fisher Scientific, USA)10:45. The sample volume used is 10 µL. The tube containing the premix and the vortexed sample for 30 minutes then in the centrifuge for 1 minute. The supernatant was inserted into a 20 µL wellbore slab and read by using 3500 Genetic Analyzer. The sequencing results were then processed in the gene bank to identify the presence of mutations in the *folP1* gene *M. leprae*.

b. Variation analysis and dihydropteroate synthase *M. leprae*

DNA sequence from our clinical samples was edited by referring it with reference sequence of *folP1* gene (NC_002677.1) using Bioedit. The edited sequence was aligned to the corresponding sequences in the database using Basic Local Alignment Sequence Tool (BLAST) in NCBI website.

c. Mutation effect of V48A, T53A, P55L on *folP1* gene *M. leprae*

The amino acid sequence of the DHPS compiler was downloaded from Uniprot (P0C0X1). The analysis of structural effects of point mutation in a protein sequence was performed using HOPE web service (http://www.cmbi.ru.nl/hope/). The prediction of binding pocket (wide and volume) was performed using CASTp server (http://sts.bioe.uic.edu/castp).

d. Homology modelling of dihydropteroate synthase *M. leprae*

Homology of 3D dihydropteroate synthase structure was analyzed by using I-TASSER[16] (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Variants in T53A, V48A, and P55L were constructed using the fold-X[17] based on the wildtype structure (P0C0X1).

e. Preparation of drug molecules

The 3D structure of dapsone (CID:2955) was downloaded from Pubchem database, available at https://pubchem.ncbi.nlm.nih.gov/. Dapsone molecule was downloaded in SDF format. Molecule energy was reduced and converted pdbqt using Open Babel software.

f. Molecular docking

Docking analysis was performed by using AutoDock Vina[18] which is integrated in PyRx application.[19] Docking results were visualized using PyMol 1.8.6 and Discovery Studio 2017.

RESULTS

From 100 clinical samples, 53 PCR positive result samples were obtained and qualified to be proceed to sequencing. The BLAST result in NCBI shows that variation was identified in *M. leprae* Papua strain compared with TN strain in *folP1* gene. The type of mutation is missense where Thymine (T) was replaced by Cytosine (C) on nucleotide 143 (Figure 1). This mutation gave rise to the replacement of amino acid Valine become Alanine on DHPS (Figure 2b). The wild type and mutant amino acids differ in size. Alanine is smaller than Valine. The mutation will cause an empty space in the core of the protein. Among the 53 samples that were successfully amplified, there were six samples that show the mutation.

Mutation in the 143 nucleotides *folP1* gene of *M. leprae* was identified as a single peak (Figure 2b) and in a double peak (Figure 2c). This mutation caused a change from the Thymine (Figure 2a) to Cytosine. In some samples this mutation was found in a mixed allele (Figure 2c). As a comparison, we used the P55L, T53A variants which were confirmed as mutations that cause resistance to dapsone.[10,11]

Figure 1. Alignments folP1 gene of M. leprae Papua strain comparing with M. leprae strain TN (NC_002677.1) Variation was identified in folp1 gene of M. leprae Papua strain

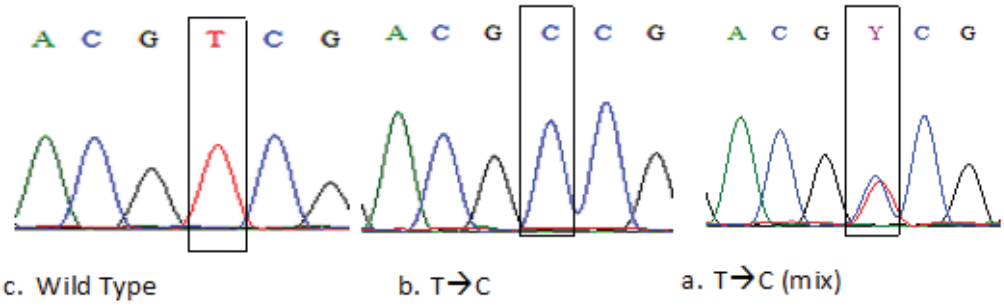


Figure 2. Comparison of electropherogram between wild type and mutant (a), Thymine mutation becomes Cytosine with single pic (b) and mix between mutant and wildtype (c).

Figure 3. Overview of the protein in ribbon-presentation. The protein is colored grey, the side chain of the mutated residue is colored magenta and shown as small balls.

Protein structure analysis of DHPS conformation on mutant of *M. leprae* at V48A, P55L, T53A, showed that each mutation influences the protein function (Figure 3). The mutation on V48A is located within a domain, annotated in UniProt as Pterin-binding. The mutation introduces an amino acid with different properties, which can disturb this domain and abolish its function[20] (Figure 3a). The wild-type residue on 55 DHPS is a proline. Prolines are known to be very rigid and therefore induce a special backbone conformation which might be required at this position. The mutation P55R/L can disturb this unique conformation[20] (Figure 3b dan 3c). The wild-type residue on 53 DHPS is a threonine. The mutation T53A introduces an amino acid with different properties, which can disturb this domain and abolish its function[20] (Figure 3).

The wide and the volume of binding pocket DHPS were predicted using CASTp servers.[21] Binding pocket from DHPS is in the red area (Figure 4). The V48A mutant showed the identical area and volume as wildtype while the binding pocket of the T53A and P55L mutants showed the increasing area and volume (Table 1).

Table 1. The active binding and volume of the *M. leprae* DHPS binding pocket

WT and mutant DHPS	Active binding pocket (Å^2)	Volume of the Binding Pocket (Å^3)
WT	931.323	697.065
V48A	931.323	697.065
T53A	949.217	712.906
P55L	1005.725	803.004

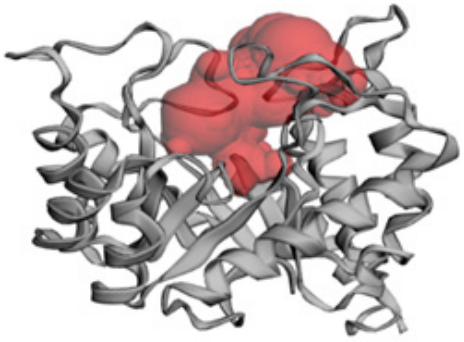


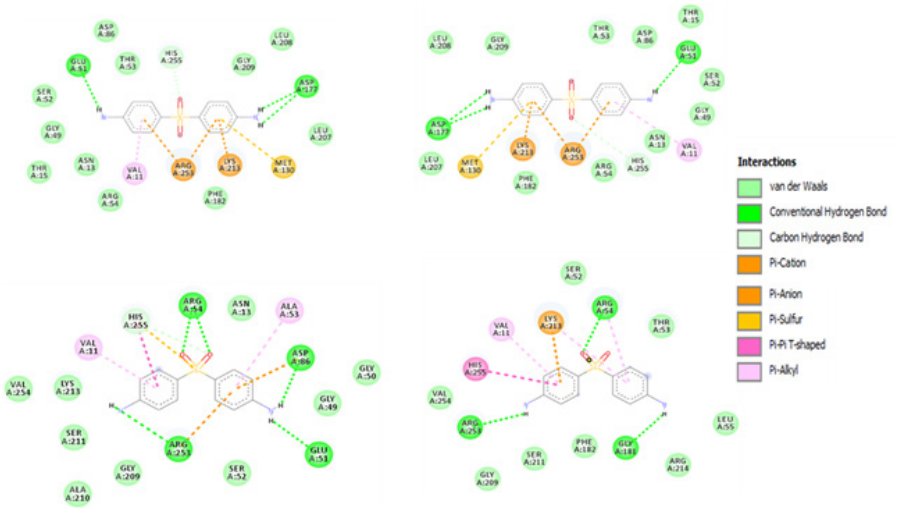
Figure 4. The prediction of the binding pocket of DHPS of M. leprae was labelled by red color zone

The wildtype DHPS (P0C0X1) modeling was performed using I-TASSER, while the mutant DHPS protein structure was derived using fold-X by emphasizing the mutations in the amino acids 48, 53 and 55. The model used in this study was the model with the highest confidence level based on the C- score. [16,22] Mutations in all three amino acids did not significantly alter the stability of the energy of the molecules (Table 2). Molecular docking between dapsone and DHPS showed the ability of dapsone to interact with wild type DHPS and mutant DHPS. Binding affinity between dapsone was higher on different sides of wildtype, specifically on variants T53A and P55L, whereas the binding affinity between dapsone and wild type and V48A of DHPS were equal (Table 2).

Table 2. Stability energy dihydropteroate synthase dan its binding energy to dapsone

Receptor	Binding Energy (kcal/mol)	Stability Energy (kcal/mol)
Wildtype	-6.7	170.39
V48A	-6.7	174.23
T53A	-7.2	167.86
P55L	-6.5	169.91

Interaction between dapsone and DHPS consisted of hydrogen bond, unfavorable donor-donor, Pi-cation, Pi-sulfur, and Pi-Pi T-shaped (Figure 5).



DISCUSSIONS

We found mutations in the *folP1* gene of *M. leprae* from Papua in nucleotide no 143 where there has been a change of amino acids from Thymine (T) to Cytosine (C) (Figure 1). This mutation caused changes in the amino acid Valine to Alanine in the DHPS enzyme (Figure 2b). These mutations exist in the region of Drug Resistance-Determining Regions (DRDR) of the *folP1* gene. The *folP1* gene is the gene that encodes the formation of the DHPS enzyme. This enzyme is the target of dapsone in the treatment of leprosy.[9] WHO has recommended this area for surveillance of drug resistance using PCR-direct sequencing. [12] This mutation was found in a single allele (Figure 2a) as well as multiple alleles (Figure 2a). Confirmation of mixed alleles in the *folP1*, *gyrA,* and *rpoB* genes of *M. leprae* has been reported previously.[23,24] Mutation V48A has been detected by Nakata et al in their clinical samples.[25]

In this study, we analyze V48A mutant using in silico method. Former studies show mutations in the *folP1* gene were missense mutations located at codon 53 (Thr53Ile, Thr53Arg and Thr53Ala) or codon 55 (Pro55Arg, Pro55Leu).[11] Nakata *et al*, found all mutations that cause amino acid substitutions at codon 55 resulted in dapsone resistance and mutations at codon 53 also gave rise to dapsone resistance except for the T53S substitution, which resulted in less resistance to dapsone than the wild-type sequence.[25] Mutation on V48A is located within a domain, annotated in UniProt as Pterin-binding which can disturb this domain and abolish its function.[20] The wild-type residue is very conserved, but a few other residue types have been observed at this position too. The mutant residue was not among the other residue types observed at this position in other, homologous proteins. However, residues that have some properties in common with your mutated residue were observed. This means that in some rare cases mutation V48A might occur without damaging the protein. The mutant residue is located near a highly conserved position important for the activity of the protein and in contact with residues

in another domain. It is possible that this interaction is important for the correct function of the protein (Figure 3). This mutation does not affect the volume binding pocket (Table 1) and protein stability (Table 2). However, the mutation may affect this interaction and as such affect protein function.[20]

On P55L mutant, the mutated residue is located in a domain that is important for the activity of the protein and in contact with residues in another domain. The mutant increased the volume binding pocket of DHPS (Table 1). It is possible that this interaction is important for the correct function of the protein. The mutation can affect this interaction and as such affect protein function.[20] The mutated residue of T53A mutant is located in a domain that is important for the activity of the protein and in contact with another domain that is also important for the activity. The interaction between these domains could be disturbed by the mutation, which might affect the function of the protein.[20]

On wildtype, the binding position of dapsone mutants V48A, T53A and P55L present in the binding pocket. The binding affinity between dapsone in T53A DHPS was stronger than wildtype, but preferably bond to a site that was different from wildtype. Dapsone linked with the T53A mutant through four hydrogen bonds on the residues Q51, E54, D86 and R253 (Figure 5). Dapsone is bound to the mutant P55L with three hydrogen bonds, R54, G181, and R253. Binding affinity formed was lower than that of wildtype, but also on sites that were different from wildtypes (Table 1). This indicated that the variants of P55L and T53A caused Dapsone to experience orientation changes in binding to DHPS. So that dapsone activity in inhibiting the performance of DHPS in both variants had decreased, or resistance to dapsone. T53A mutations, P55L in the *folP1* gene have also been confirmed to cause resistance to dapsone.[10,11] Chaitanya also found the greatest decrease in free energy bindings was present in the T53I and T55V mutants.[8] The energy and changes in the bonding patterns revealed the structural and mechanistic effects of these mutations on inducing dapsone resistance in leprosy.[8]

The binding affinity of V48A mutant was similar to wildtype (Figure 5). Dapson was bound with V48A through three covalent bonds of hidrogen namely 2 hidrogen bonds on residual E51 and 1 on D177 residual. This interaction was similar to the dapsone interaction on the wildtype (Gambar 5). This shows that the V48A mutant might still possess the ability to interact with dapsone. The testing of the effect of mutant 448A based on MIC values has been conducted before and it is known that the V48A mutation effect might give rise to low-level resistance to dapsone in *M. leprae* based on MIC value.[25] Thus it is necessary to do an in vivo test to confirm the effect of the V48A mutation.

In conclusion, based on molecular docking analysis, this mutation does not affect binding affinity dapsone against *M. leprae* dihydropteroate synthase. These results indicate that the V48A mutant is likely to remain susceptible to dapsone. Thus it is necessary to do an in vivo test to confirm the effect of the V48A mutation.[26]

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Comparison of DNA extraction methods for molecular identification of pathogenic *Leptospira* in the urine samples

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Abstrak

Latar belakang: *Leptospirosis merupakan zoonosis penting di dunia, yang masih sering terjadi salah diagnosis. Deteksi laboratorium Leptospira menjadi tantangan karena bakterimea cukup singkat untuk dideteksi molekuler, namun antibodi juga muncul sangat lambat. Urine dapat menjadi sampel alternatif untuk deteksi PCR pada leptospirosis. Pengerjaan PCR membutuhkan DNA berkualitas dan andal, dan diperoleh dari metode ekstraksi DNA yang baik. Penelitian bertujuan untuk mengetahui metode ekstraksi DNA Leptospira terbaik untuk sampel urin, serta mengevaluasi pengaruh waktu penyimpanan dan suhu terhadap kestabilan DNA.*

Metode: *Penelitian ini menggunakan tiga metode isolasi DNA yang berbeda; berbasis silika dengan spin kolom, kromatografi spin column menggunakan resin sebagai matriks pemisah, dan metode larutan dengan guanidine isothiocyanate. Hasil ekstraksi diperiksa konsentrasi dan kemurniannya. Gen SecY pada Leptospira dideteksi dengan PCR real-time. Pengaruh suhu dan lama penyimpanan DNA juga dilihat.*

Hasil: *Hasil isolasi DNA menggunakan resin menunjukkan konsentrasi tertinggi (7,94 + 2,11 µg / mL) dan jumlah salinan amplifikasi DNA Leptospira tertinggi (50167,92 + 1,19). Suhu penyimpanan pada suhu 4°C, -20°C, dan -80°C dan umur simpan 91 hari tidak berpengaruh terhadap kualitas dan kuantitas DNA Leptospira hasil isolasi spike urin.*

Kesimpulan: *Isolasi DNA menggunakan spin column chromatography dengan resin sebagai matriks separasi memiliki kualitas dan kuantitas terbaik berdasarkan kemurnian dan konsentrasi DNA serta jumlah gen SecY yang teramplifikasi.* ***(Health Science Journal of Indonesia 2020;11(2):77-84)***

Kata kunci: *Leptospira, Leptospirosis, ekstraksi DNA, sampel urin, penyimpanan sampel.*

Abstract

Background: Leptospirosis is a worldwide zoonotic disease, which is still often misdiagnosed. Laboratory detection of Leptospira is challenging since the bacteraemia is quite short for molecular detection, however, the rise of the antibody is late to post the infection. Urine can be a potential alternative sample for PCR detection in leptospirosis. The PCR method requires a reliable DNA template, which is obtained from good DNA extracting methods. The study aimed to determine the best method of extraction Leptospira DNA from the urine sample, as well as evaluating the effect of time storage and temperature for its DNA stability.

Methods: This study was utilizing three different DNA isolation methods; silica based with spin column, spin column chromatography using resin as separation matrix, and solution method with guanidine isothiocyanate. The yields were examined for its concentration and purity. Leptospira's SecY gene was detected with real-time PCR. The influences of storage temperature and the life time of the DNA were also studied.

Results: The yield of DNA isolation using resin showed the highest concentration (7.94±2.11 µg/mL) and highest Leptospira DNA amplification copy number (50167.92±1.19). Storage temperature at 4°C, -20°C, and -80°C and life time of 91 days did not have any effect on the quality and quantity of Leptospira DNA isolated from spiked urine.

Conclusions: DNA isolation using spin column chromatography with resin as separation matrix has the best quality and quantity based on the purity and concentration of DNA and the higher number of amplified SecY gene. ***(Health Science Journal of Indonesia 2020;11(2):77-84)***

Keywords: Leptospira, Leptospirosis, DNA extraction, urine sample, sample storage.

Leptospirosis is a worldwide zoonosis and endemic in South East Asia. The disease can cause severe infection in humans. The infectious disease is caused by pathogenic bacteria, *Leptospira interrogans*.[1] In Indonesia, the case fatality rate of leptospirosis is reportedly high and the incidence rate is still uncertain and often under-reported. The mortality and morbidity of the disease are high, however, difficult to predict due to mis-diagnosis.[2] The clinical manifestation of the disease ranges from asymptomatic to severe disease with rapid mortality. It is difficult to differentiate leptospirosis from other diseases that have similar symptoms including fever, headache and myalgia such as dengue, malaria and influenza. A severe manifestation of leptospirosis (Weil's disease), is characterized by fever, jaundice, renal failure and haemorrhage.[3]

There are several methods available to diagnose leptospirosis. Polymerase chain reaction (PCR) is one of the diagnostic methods that has been reported to be able to detect pathogenic *Leptospira* from clinical samples such as blood, urine, cerebrospinal fluid and infected organs. It is also a sensitive, specific and rapid technique[4,] but the sensitivity and results of PCR reaction also depend on the quality of isolated DNA because only a small amount of the original sample is included in the final PCR assay.[5,6] The DNA extraction is included as Nucleic Acid Extraction (NAE) methods can be more broadly characterized to be solid-phase or chemically actuated methods.[7]

Blood, cerebrospinal fluid (CSF) and urine have been known as specimens for *Leptospira* DNA detection. Leptospira's DNA can be found in the blood 48 hours after infection. Meanwhile, CSF could be used to detect *Leptospira* within the first week of illness. The detection of Leptospira in the urine can be done from the early course of the disease before the initiation of antimicrobial therapy[1] the brown rat (Rattus norvegicus. The urine as specimen has advantages over the others. However, urine also has some disadvantages as a DNA isolation specimen. Urine contains factors that inhibit DNA amplification such as urea and nitrite.[8] The other disadvantage is because the bacteria contained in it produce endonuclease which breaks down the DNA.[8,9]

Spiked urine was produced from healthy urine contaminated with certain bacteria. This procedure is performed to make a model for experimental study such as the detection of bacteria in urine and comparison study of urine DNA isolation.[10-12] DNA isolation could be obtained through several ways and currently DNA isolation kits provide different choices of samples, which cover both specific and non-specific specimens including kits for tissue, blood, and urine.[9]

Storage conditions can affect DNA quality and quantity. Degradation of DNA in storage can influence molecular biology testing. One problem is temperature fluctuations over time. Temperatures at 4°C, –20°C or –80°C are reported to be good conditions for DNA storage.[13,14]

Blood and urine samples may serve as reliable specimens; however, urine has advantages since it does not require intervention in the collection process and it contains fewer biological units, therefore it is simpler to be tested. Producing a reliable PCR product means good preparation is required to produce an excellent DNA template. The outstanding quality of the DNA templates can only be achieved through a reliable process of DNA isolation with good storage conditions. A good DNA template is produced from a reliable isolation DNA process. A reliable isolation method and good storage conditions are supposed to be able to produce good DNA isolated product, but unfortunately to this date the method to properly isolate DNA of Leptospira from urine and the best storage conditions have not been well established.

This study aimed to determine the best DNA isolation method that produces good quality and quantity of DNA from *Leptospira* spiked urine and also determine the effect of temperature and shelf life.

METHODS

This study is classified as an analytical study to compare three methods to isolate leptospira DNA from urine samples. The study was conducted from May 2018 to October 2018 at the Institute for Vector and Reservoir Control Research and Development (B2P2VRP), Salatiga, Indonesia. The urine samples were collected from 5 healthy volunteers, who do not have any degeneration diseases and not taking any medicine or food supplements two days before the samples were taken. The urine sample criteria were the second urination in the morning. All the volunteers have signed informed consent before the study. The total numbers of replication attempts were 4 times. Accordingly, the total numbers of the samples were twenty (N=20).

The urine samples were used as media for the spike process. The culture of the pathogenic Leptospira serovar Icterrohaemorrhagie, which is maintained at Bacteriology Laboratory, Institute for Vector and Reservoir Control Research and Development (IVRCRD) Salatiga with a concentration of $2x10^8$ CFU/mL was inactivated at 50ºC. The culture was diluted using Phosphate Buffer Saline (PBS) until its concentration became $1x10^8$ CFU/mL. Then the culture was gradually diluted with the urine until the concentration reached $1x10^6$ CFU/mL as the working concentration. Accordingly, the 5 urine samples (from 5 volunteers) were aliquoted into 4 tubes. Each tube received 1 million CFU of the bacteria and was stored in (-20ºC) freezer for a future experiment.

All the DNA extraction steps and the qPCR tests in this study were performed by one student. Our technicians assisted her only in preparing the serovar (Icterrohaemorrhagie) to be spiked in the urine samples.

DNA isolation from spiked urine

All the urine samples were taken from -20ºC freezer were thawed completely in the ice cubes to let it thaw gradually and avoid the shock heat. The samples then underwent well vortex before the extraction process, as follows:

1. **The silica-based with spin column**
   The urine samples were centrifuged at 14,000 g for 3 minutes. The supernatant was then removed and the pellet was extracted following the protocol from DNA isolation kit by Thermo Fisher Scientific (PureLink™ Genomic DNA Mini Kit catalogue number K1820-01).
2. **The spin column chromatography using resin as the separation matrix**
   The 1.75 ml of urine was adding with 0.25 ml of the binding solution and centrifuged for 1 minute at 6700 g and then continued by following the protocol from Norgen's Urin DNA isolation kit (urine DNA isolation Kit product # 18100).
3. **The guanidine isothiocyanate DNA isolation method**
   The urine samples as much as 1 ml was centrifuged at 14,000 g for 3 minutes. The supernatant was discarded and the pellet was taken to be followed by the protocol of TRIzol ™ Reagent (catalogue number 15596026).

We checked the pH qualitatively using pH paper Merck Universal pH 0 - 14. All the urine samples were in normal pH (6-7).

Storage time and temperature

DNA extracted from urine samples were aliquoted and storage into three different temperatures: -80^{0}C,-20^{0}C, and 4^{0}C. The DNA template also was analyzed every 2 weeks, during 3 months of observation. We did not find any crystal on the samples during cold storage.

Concentration and purity

The DNA concentration (µg/mL) and purity (absorbance ratio at A260/A280) were determined by spectrophotometry using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

***Leptospira* DNA amplification**

The presence of Leptospira DNA was detected using a real time PCR (qPCR) assay, with amplifying *SecY* gene as the housekeeping gene, specific for Leptospira. The *SecY* gene correspond to primer sequences were R- CCGTCCCTTAATTTTAGACTTCTTC and F-ATGCCGATCATTTTTGCTTC.[15] The master mix was prepared in BSC/PCR work station with composition: 5µL Ssofast™ Eva Green® Super mixes, 1 µL forward *secY* Primer, 1 µL reverse *SecY* Primer, 2 µL ddH2O, and 1 µL DNA template. The solution was processed in a regulated PCR machine: pre-denaturation 98^{0}C for 2 minutes, denaturation 95^{0}C for 30 seconds, annealing 60^{0}C for 30 seconds, extension 65^{0}C for 5second. The qPCR cycle was set to 40 times.

A standard curve was made using a known concentration of *Leptospira* DNA. Serial dilutions were performed, starting from 10^0 until $10^7$of *Leptospira*DNA copy. In parallel, the Real Time PCR was performed in duplicates. The calculation of regression was obtained from the serial dilution result. The quantification data of Leptospira DNA were calculated by using the regression equation:

$$10^{(cq-b/r)}$$

where Cq=cycle of amplification, b=y intercept, r= regression coefficient.

Statistical analysis

The variances between DNA isolation results and differences in storage temperature and time were statistically analyzed using ANOVA Tukey's multiple comparison tests. Prior to the ANOVA analysis, the data, which were presented as mean for each group, were checked for its' distribution normality. The software used for statistical analysis in this study was SPSS version 10 and the cut-off value for the

statistical significance was p < 0.01. The reason was that the data obtained from the laboratory study with a high level of accuracy and the study was supported by sophisticated equipment.

Ethical consideration

This study protocol had been approved by the Health Research Ethics Committee, National Institute of Health Research and Development (HERC-NIHRD) no. LB.02.01/2/KE.268/2018.

RESULTS

Total DNA concentration

The total DNA concentration indicated the amount of DNA isolated from the specimen. The total DNA concentration is shown in Figure 1. There was no significant difference among the three methods (p = 0.897). However, DNA isolation using resin showed the highest average yield (7.94 ± 2.11µg/ mL).

DNA purity

The purity of the DNA isolates was shown as a ratio of optical density (OD) at A260/280. The DNA purity of each method is shown in Table 1. The results of DNA isolation using resin showed purity value in recommended range, DNA was considered in good purity when the value of A260/280 is 1.8-2.0 [7]. There was no significant difference between methods (p = 0.085)

The leptospira DNA amplification

The data of DNA amplification showed that the isolation of DNA with resin has the highest amount of DNA copies (50,167.92± 1.19). The result showed a significant difference (p = 0.0025) between each isolation methods. The result of *Leptospira* DNA amplification is shown in Figure 2.

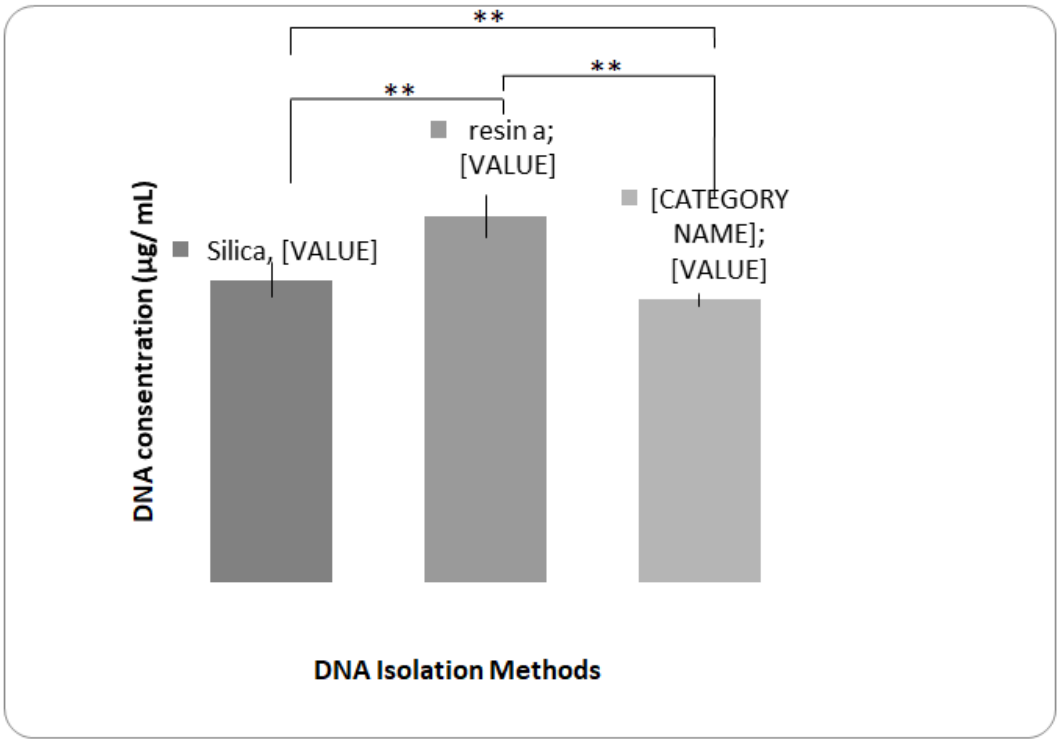

*Figure 1. Total DNA concentration extracted by using three different methods: silica, resin a, and guanidine isolation. p > 0.01 (one way anova followed by t test) N=20. **denoted: p< 0.01 (one way anova followed by t test). N=20.*

Effect of storage temperature to the concentration of leptospira DNA

The DNA concentration showed no significant changes over time. The different temperature storage also did not affect the DNA concentration. This result is indicated by the magnitude of the p >0.01 from day to day. DNA concentration at -80^{0}C (p = 0.997), -20^{0}C (p = 0.936) and 4^{0}C (p = 0.781) remained stable. The result of *Leptospira* DNA stability is shown in Table 2.

Table 1. Leptospira's DNA purity extracted by using three different methods (p = 0.085).

Methods	Leptospira's DNA Purity (mean)	SD
Silica	2.11	0.14
resin	1.57	0.66
Guanidine	2.63	0.23

Effect of storage temperature to the purity of leptospira DNA

The different temperature storage was not affecting the DNA purity over the time significantly. This result is indicated by the magnitude of the p-value > 0.01 from day to day. DNA concentration at -80^{0}C (p-value 0.158), -20^{0}C (p-value 0.035) and 4^{0}C (p-value 0.356) remained stable. Table 3 shows the stability of *Leptospira* DNA purity.

Effect of storage to the yield of leptospira DNA amplification

The variable storage temperature did not affect the result of DNA amplification (Table 4). DNA copy number after qPCR amplification using DNA template which were stored in different temperature at -80^{0}C (p-value 0.894), -20^0 C (p-value 0.741) and 4^{0}C (p-value 0.621) remained stable.

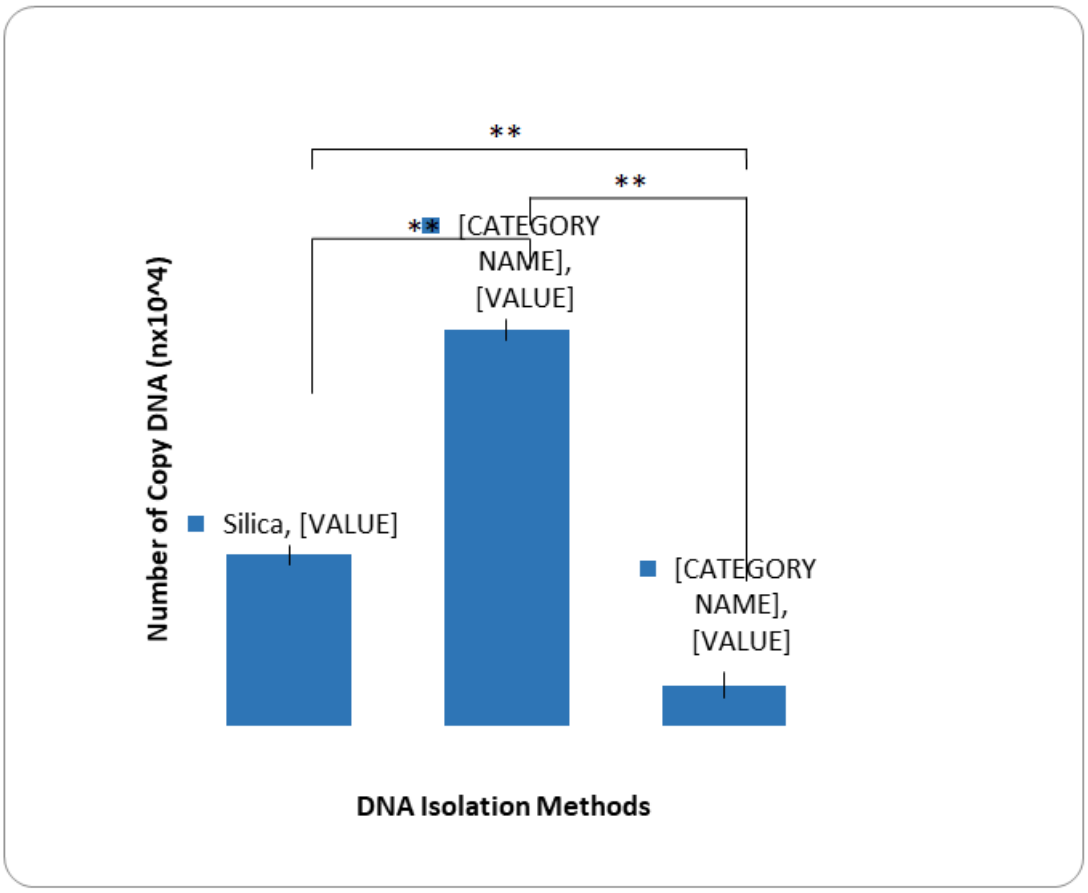

*Figure 2. The Leptospira DNA copy number after real time PCR amplification assay of DNA extracted from three different methods: silica, resin a, and guanidine isolation. ** denoted: overall significant p value at 0.0025 (one way anova followed by t test). N=20.*

Table 2. Concentration of leptospira DNA stored in different temperatures

Storage temperature	Shelf life (Days)	1	14	28	43	91	*p*-value
	Isolation methods	DNA Concentration (ng/µL) (mean)					
-80^{0}C	Silica	6.563	7.238	7.169	7.563	6.931	0.997
	Resin a	7.944	10.488	10.000	9.938	9.539	
	Guanidine	6.131	5.394	6.475	6.694	6.278	
-20^{0}C	Silica	6.563	6.981	7.125	5.575	6.163	0.936
	Resin a	46.470	91.585	81.920	60.350	60.650	
	Guanidine	6.131	6.425	7.244	15.163	6.250	
4^{0}C	Silica	6.563	8.744	8.481	8.225	7.575	0.781
	Resin a	7.944	10.775	16.363	10.919	13.388	
	Guanidine	6.131	7.363	7.338	6.213	5.881	

Table 3. The purity of leptospira DNA stored in different temperatures

Storage temperature	Shelf life (Days)	1	14	28	43	91	p-value
	Isolation Procedure	A 260/280 (mean)					
-80^{0}C	Silica	2.055	2.251	2.125	2.206	2.249	0.158
	Resin a	1.851	1.627	1.684	1.706	1.740	
	Guanidine	2.586	3.230	2.564	2.714	2.656	
-20^{0}C	Silica	2.055	1.9825	1.916875	2.204375	2.325	0.035
	Resin a	1.85125	1.51	1.51625	1.624375	1.815	
	Guanidine	2.585625	2.288125	1.98625	2.285	3.045	
4^{0}C	Silica	2.055	2.455	1.994375	2.159375	2.05125	0.356
	Resin a	1.85125	1.611875	1.65875	1.735625	1.81625	
	Guanidine	2.585625	2.55875	2.149375	2.464375	2.9525	

Table 4. Leptospira's DNA copy number amplified by using real time PCR after stored in different temperatures

Storage temperature	Shelf life (Day)	1	14	28	43	91	*p*-value
	Isolation methods	Amplified DNA (x10^3) (mean)					
-80^{0}C	Silica	21,611	20,674	22,370	21,505	26,594	0.894
	Resin a	50,167	57,816	50,327	55,209	62,734	
	Guanidine	5,115	4,730	5,235	4,821	5,783	
-20^{0}C	Silica	21,611	19,312	15,783	25,087	25,551	0.741
	Resin a	50,167	51,732	51,536	54,119	40,369	
	Guanidine	5,115	4,059	3,837	5,596	4,452	
4^{0}C	Silica	21,611	27,852	33,282	30,057	29,838	0.621
	Resin a	50,167	62,821	42,436	70,726	75,364	
	Guanidine	5,115	5,084	6,143	6,722	5,708	

DISCUSSION

The data showed that the resin method produced higher DNA concentration than the other two methods. However, the result also showed a wider standard deviation than the other DNA isolation methods. Previous research reported that DNA isolation result depends on the ability of researcher's handling procedures.[16]

Isolation with the resin resulted in a better purity although not significantly different. The mean value of DNA purity from resin methods showed an acceptable for further analysis. A ratio of OD A260/280 less than 1.8 is an indication of the presence of residual protein, phenol, or other reagents associated with the extraction protocol, where a ratio of more than 2.0 indicates RNA contamination.[17]

RNA contamination in DNA samples can cause low amplification in the sequencing cycle. Protein and organic solvent contamination will cause interference with the enzyme reaction.[18]

Storage temperature (-80^0, -20^0, and 4^{0}C) and shelf life did not give a significant effect on DNA quality from the first day until day 91. This finding was similar to the result from Permenter *et al.*[13] *temperature (4 °C and room temperature*, who found genomic DNA aliquots stored at -20°C and -80°C were stable for over 24 months. DNA samples stored at 4°C were stable for up to 12 months. The study by Ghatak *et al*.[16] showed that the storage of extracted DNA from urine, blood, hair, and buccal swab at -20^{0}C over one month did not affect the PCR performance. It is important to remind that storage of the DNA extraction is better than storage of the fresh urine sample because Hilhorst *et.al.* (2013) showed that storage of fresh urine at 4°C or lower temperatures effected in significant degradation of human DNA.[19]

Urine is considered one of the specimens that can be used in Leptospira detection. Spiked urine is often representing the real urine from leptospirosis patients in Leptospira diagnosis. A study by Lee *et al*.[10] showed that lyophilized reagent-based PCR can detect Leptospira in spiked urine with as much as 1x10^2 *Leptospira* gene copies that come from 1x10^6 *Leptospira*/mL of spiked urine. The comparative study of three DNA extractions with plant proteinase by Veloso *et al*.[11] showed that cow urine contaminated with Leptospira as much as 10^5 *Leptospira* / mL can be detected by PCR.

The amount of DNA in the sample should be known for further research purposes. Successful detection of *Leptospira* gene with PCR can be achieved when the DNA isolation sample has good quantity and quality.[20] There are various methods that may be used to isolate DNA, but not all methods yield DNA with good quality and quantity. Guanidine isothiocyanate is the DNA extraction method that relies on the biochemical properties of the cellular component. The basic principle of this method is the separation between RNA, DNA and protein by an acidic solution, which yields high DNA purity and concentration. However, the procedure still uses hazardous chemicals like phenol and chloroform. The silica matrix method provides high-purity DNA and is easy to perform. The weakness of this procedure is the inability to recover the small fragments DNA that binds tightly with the silica matrix. Anion exchange matrix or anion exchange resin can extract high pure DNA compare to a silica matrix. However, it requires desalting of high salt concentration produced in the elution step.[7]

The use of q PCR is intended to identify and quantify the number of amplified products during a reaction. By using qPCR, the number of starting templates can be determined.[9] A recent study in Laos found that urine has no difference in sensitivity to blood, and urine is a useful sample for the molecular diagnosis of leptospirosis.[6] In this study, *SecY* gene specific to *Leptospira* was used. The *SecY* gene is significantly better than the other housekeeping genes such as *Lipl32* gene.[15] The lowest Ct obtained in this study was DNA which was extracted using resin. It showed that Leptospira's DNA copy number which was obtained from resin methods remarkably highest among other methods. In addition, the lowest Ct may correspond to the good quality of template DNA extracted using resin. Furthermore, when we want to use clinical urine samples for doing DNA extraction, we need to aware of the sampling time post symptom onset because it may influence the quantity of the DNA for molecular determination.[21]

The limitation of this study was that we did not see the characteristics of the urine samples. Our suggestions for the future study is to provide the information of the urine-analysis conditions, such as macroscopic and sedimentation-microscopic characteristics, such as quantitative pH, albumin, leucosit esterase, and other parameters that may influence the study.

In conclusion, DNA isolation using spin column chromatography with resin as separation matrix has the best quality and quantity based on the purity and concentration of DNA as well as the highest number of amplified *SecY* genes specific to *Leptospira interrogans.* Storage temperature at 4^0, -20^0, and -80^{0}C and life time of 91 days did not affect the quality and quantity of *Leptospira* DNA isolation products from spiked urine samples.

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Author contribution

FDH is the coordinator and designer of the study, developing the concept and main contributor to the study. RAW carried out the experiment and wrote the manuscript under the direction of FDH. RR, MHG, and TW supervised the project and provided critical feedback with inputs to the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare for this study.

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Overexpression of MiR-155-5p and increased number of macrophage population in precancerous prostatic disease

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Abstrak

Latar belakang: *Gangguan regulasi mikroRNA(miR) dan inflamasi kronik dapat mengubah tumor menjadi karsinoma dan kanker dengan metastasis melalui perubahan seluler dan genomik. Lesi prekanker memiliki peluang 33,3 persen menjadi kanker. Penelitian ini bertujuan untuk mengkaji peran miR-155-5p terhadap mRNA SOCS1 dan populasi makrofag terhadap progresivitas penyakit yang berhubungan dengan* Benign Prostate Hyperplasia (BPH), High Grade Prostatic Intraepithelial Neoplasia (HGPIN)*, dan* Prostate Adenocarcinoma (PRAD).

Metode : *Penelitian ini merupakan penelitian potong lintang dengan 3 kelompok, yaitu BPH,HGPIN, dan PRAD. Sampel jaringan didapatkan dari Tindakan TURP. Ekspresi miR-155 dianalisis menggunakan qPCR dan dikalkulasi menggunakan metode Livak. Ekspresi mRNA SOCS-1 dianalisis menggunakan* reverse transcriptase PCR*. Penanda pan makrofag, anti CD-68* monoclonal antibody(MoAb) *digunakan untuk mendeteksi populasi makrofag pada jaringan dengan imunohistokimia.*

Hasil : *Ekspresi miR-155 lebih tinggi pada HGPIN dibandingkan BPH dan PRAD (p=0,14). Ekspresi mRNA SOCS1 pada HGPIN paling rendah diantara ketiga sampel (p=0,96). Terdapat korelasi negative antara miR-155 dan mRNA SOCS1 (p=0,02). Terdapat peningkatan persentase populasi makrofag yang signifikan pada HGPIN (6,03 persen) dibandingkan BPH (0.89 persen) dengan p=0,00.*

Kesimpulan : *Pada penelitian ini, terdapat perubahan persentase makrofag dan miR-155 pada HGPIN. Variasi ekspresi miR-155 dan persentase populasi makrofag dapat disebabkan karena perubahan epigenetik. Oleh sebab itu, perlu penelitian lebih lanjut untuk memvalidasi hasil tersebut dan memahami kemungkinan menjadi biomarker pada penyakit prekanker pada prostat.* ***(Health Science Journal of Indonesia 2020;11(2):85-91)***

Kata Kunci: Prostatic Intaepithelial Neoplasia, miR-155, *Makrofag*

Abstract

Background: Impaired microRNA(miR) regulation and chronic inflammation could transform tumors into carcinoma and cancer by metastasis through cellular and genomic changes. Precancerous lesions have a 33.3 percent chance of becoming cancerous. This study investigated the role of miR-155 related to SOCS1 mRNA and macrophage population in disease progression associated with Benign Prostate Hyperplasia (BPH), High-Grade Prostatic Intraepithelial Neoplasia (HGPIN), and Prostate Adenocarcinoma (PRAD).

Methods: This was a cross-sectional study using three groups of samples, namely BPH, HGPIN, and PRAD. Tissue samples were obtained from TURP Action. The expression of miR-155 was analyzed using real-time qPCR and calculated using the Livak method. The expression of SOCS1 mRNA was analyzed using reverse transcriptase PCR. The macrophage pan-marker, anti-CD68 monoclonal antibody (MoAb), was used to detect macrophage population in tissues by immunohistochemistry.

Results: The expression of miR-155 was higher in HGPIN than BPH and PRAD (p=0.14). The expression of SOCS1 mRNA in HGPIN was the lowest among the three samples (p=0.96). There was a negative correlation between miR-155 and SOCS1 mRNA (p=0.02). There was a significant increase in the percentage of the macrophage population in HGPIN (6.03 percent) compared to BPH (0.89 percent) with p=0.00.

Conclusion: In this study, there were changes in the percentage of macrophage and miR-155 in HGPIN. The variation in miR-155 expression and the percentage of the macrophage may be caused by epigenetic changes. Therefore, further research is needed to validate these results and understand the possibility of being a biomarker in precancerous disease of the prostate. ***(Health Science Journal of Indonesia 2020;11(2):85-91)***

Keywords: Prostatic Intraepithelial Neoplasia, miR-155, Macrophage

Prostate cancer (PCA) is the third-highest cancer in men according to GLOBOCAN in 2012. In Europe, the mortality rate was 94,000 in 2008, while in the USA there were 28,000 deaths in 2012. In Indonesia, it was reported from three major cities (Jakarta, Surabaya, Bandung), the mortality rate was 1,102 patients in 2003. To date, the diagnostic tools for PCA are rectal toucher examination, detection of prostate-specific antigen (PSA) serum, and transrectal ultrasound, but PSA is ultimately not a specific biomarker because will increase for other conditions namely infection and inflammation such as prostatitis. There are several types of prostate cancer and the most common type of PCA is prostate adenocarcinoma (PRAD). Assessment of disease progression using the Gleason score is based on histopathological features.[1]

Benign prostate hyperplasia (BPH) is hyperplasia in the transition zone of the prostate. The risk of BPH emergence was related to chronic inflammation. Chronic inflammation can play a role in carcinogenesis by cellular and genomic changes.[2] Prostatic intraepithelial neoplasia (PIN) is an abnormal progression into atypical cell formation. It is classified into low-grade PIN and high-grade PIN. High-grade PIN (HGPIN) occurs in the peripheral zone of the prostate with a 33.3 percent chance of becoming cancer.[3] Cancer progress is not only regulated by genetic factors and epigenetic factors, but also chronic inflammation that can affect carcinogenesis.[4]

Macrophages located in the tumor area are called tumor-associated macrophages, which function in increased angiogenesis and metastasis. Macrophages are polarized into M1 (classically activated macrophage) as a proinflammatory response, and M2 (alternatively activated macrophage), which are influenced by cytokines released by cells. M1 macrophages related to bacterial infection responses secrete nitric oxide, Interluekin-12 (IL-12), IL-23, and tumor necrosis factor-alpha (TNF-α). In cancer, M1 is responsible for identifying and eradicating cancer cells and provides a good prognosis. Macrophage type M2 is an anti-inflammatory response that releases IL-10, IL-13, IL-6, TGFβ, and vascular endothelial growth factor-beta (VEGF), which play a role in angiogenesis, tissue remodeling, and are related to therapy resistance and poorer prognosis.[5,6] The anti-CD68 monoclonal antibody is a marker for pan macrophage.[7,8] It can be observed in human BPH tissue and is involved in the growth and progression of BPH by an unknown mechanism.[2]

The histopathological features of HGPIN are characterized by abnormal development of epithelial lining of the prostate acini and duct, followed by secretion of cytokines and chemokines from macrophage surrounding the prostate epithelial cells. Further, macrophage could be polarized into pro-inflammatory or anti-inflammatory.[9] There are also prominent nucleoli and reactive atypia due to inflammation, metaplasia, and hyperplasia in HGPIN .[10]

MicroRNA (miR) are small non-coding RNA consisting of 18-25 nucleotides that regulate gene expression and are stable under a variety of conditions. MicroRNA control messenger RNA (mRNA) stability and target mRNA translation in the 3'untranslated region from noncoding RNA and convert tumor expression to cancer and metastatic cancer.[11] MicroRNA-155 can induce the activation of this role in the conversion of macrophage to M1 by degraded SOCS1 (suppressor of cytokine

signaling).[12] This study investigated the role of miR-155 related to SOCS1 mRNA and macrophage populations in the disease progression related to BPH, HGPIN, and PRAD.

METHODS

Sample collection

This research was a cross-sectional study using Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples provided by a private laboratory, histopathologically confirmed as BPH, HGPIN, and PRAD with a total of 30 samples. Each of the 10 samples represents one of the three sample types. Tissue samples were collected from the Transurethral Resection Prostate (TURP) which had been processed into FFPE. This study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (Ref: KE/FK/0888/EC/2018).

RNA isolation from FFPE tissue

Four sections of four micrometers (16 µm) FFPE tissue were used to extract the total RNA. Isolation of RNA using the miRNeasy FFPE Kit, cat. no. 217504 (Qiagen, Germany). The tissue was deparaffinized using xylene, centrifuged at 10,000 RPM for 4 minutes then continued at 12,0000 RPM for 3 minutes to bring the sample to the bottom of the tube. The samples were further analyzed using the protocol from miRNeasy FFPE Kit. MiRNeasy FFPE Kit can effectively purify microRNA and total RNA from FFPE tissue portion. The concentration of total RNA was measured using a Nanovue Plus Spectrophotometer (GE Healthcare Life Science). Total RNA was stored at -80^{0}C until use.

Real-Time Quantitative PCR

Quantification of miR-155 expression in the FFPE tissue using Quantitative real-time PCR in duplicate. Synthesis of cDNA using miRCURY LNA RT Kit (cat.no. 339340, Qiagen) according to the procedure in the kit. Syber green for qPCR is used with miRCURY SYBR Green PCR Kit (cat.no. 339345, Qiagen). The primer sequence for miR-155 was 5'- UUAAUGCUAAUCGUGAUAGGGGU-3' and U6 is 5'- CGCTTCGGCAGCACATATACTA-3'. Samples were run on a real-time Biorad Thermal cycler CFX 96 (Biorad, USA) system. Relative quantification for the miR-155 expression used the Livak method, and U6 was used as the reference gene. All of these samples were standardized with reference genes.

Reverse Transcriptase PCR

SOCS1 mRNA analysis used reverse transcriptase PCR with Select cycler™ II Thermal cycler. Synthesis of cDNA SOCS1 mRNA used ReverTra Ace®qPCR RT Master Mix with gDNA Remover (cat. no. FSQ-301, TOYOBO) and SYBR Green GoTaq® Green Master Mix (cat.no. M7122, Promega) with the protocol as described in the kit, using cDNA template 3 ul. Primer sequences for SOCS1 mRNA are: forward 5'- GACGCCTGCGGATTCTACTG-3', and SOCS1 mRNA reverse 5'-AGGCCATCTTCACGCTAAGG-3', and primer sequences for beta-Actin mRNA were: forward 5'-CGCGAGAAGATGACCCAGATC-3' beta-Actin mRNA Reverse 5'-TCACCGGAGTCCATCACGA-3'. The annealing temperature for SOCS1 was 60^{0}C and for beta-Actin was 56^{0}C. After electrophoresis of PCR product then pictures were taken for mRNA band using gel doc G:BOX Chemi XRQ® and quantified using ImageJ.

Immunohistochemistry

The sections were separated into three alterations of fresh xylene for 5 minutes each followed by dehydration in a series of absolute alcohol for 2 minutes each. Antigen retrieval was done by heating for 15 minutes in a citrate buffer solution. The system was allowed to cool to room temperature and then the slides were washed with Phosphate Buffer Saline (PBS) for 5 minutes. Endogenous peroxidase was blocked with 3% H_2O_2 for 5 minutes at room temperature. The slides were then washed with PBS for 5 minutes. To block endogenous biotin, the sections were incubated with a blocking agent (Background Sniper, Starr Trek Universal HRP Detection, BioCare Medical) for 20 minutes, using parafilm. Then, the sections were incubated with a primary anti-CD68 monoclonal antibody (Abcam), concentration 1:400, and covered with parafilm overnight at temperature 4^{0}C. The next day, the slides were put at room temperature for 30 minutes and washed with PBS. To further enhance staining, the sections were then incubated with secondary antibody (Trekkie Universal Link, Starr Trek Universal HRP Detection, BioCare Medical) using parafilm for 60 minutes followed by three consecutive buffer washes (PBS), each for 5 minutes. Avidin-Horseradish peroxidase tracks (Starr Trek Universal HRP Detection, BioCare Medical) were

added to the sections and incubated for 45 minutes using parafilm. The diaminobenzidine chromogen (Starr Trek Universal HRP Detection, BioCare Medical) was prepared just prior to use by mixing 1µl of chromogen into 200 µl of buffer in a mixing vial and later adding 100 µl over the sections until the sections were brown. The sections were washed with water and counterstained with hematoxylin, dehydration, cleared, and installed. The number of macrophages was counted from 10 microscopic fields in one slide. The percentage was calculated by comparing the number of macrophages per cell population in an area multiplied by 100 percent.

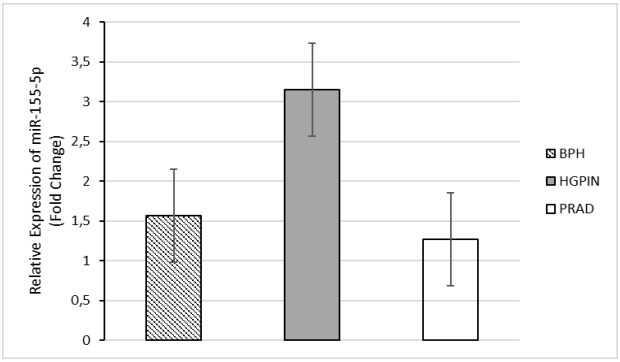
Data analysis

MicroRNA expression was analyzed using Bio-Rad CFX manager 96 software, and the differences between the three samples were analyzed using the Kruskal Wallis tests. Differences in SOCS1 mRNA expression and percentage of pan macrophages were analyzed using ANOVA and post hoc LSD. Correlation analysis used Pearson correlation tests.

RESULTS

MicoRNA-155 expression, SOCS1 mRNA expression, and negative correlation between miR-155 and SOCS1 mRNA in BPH, HGPIN, and PRAD

Relative expression for miR-155 calculation used Livak method $=2^{-\Delta\Delta Cq}$. The results are shown in Figure 1.



Kruskal Wallis test, p significant <0.05

Figure 1. Averages of miR-155 expression in BPH, HGPIN, and PRAD using the Livak method.

In this study, compared to BPH, miR-155 expression in HGPIN was two times higher than BPH, while in PRAD the expression of miR-155 was downregulated. The mean expressions of miR-155 BPH were 1.57 ±1.33, HGPIN 3.15±2.75, and PRAD 1.27±1.45. The difference in miR expression between the three groups was not significantly different with $p = 0.14$. SOCS-1 mRNA expression was analyzed using PCR reverse transcriptase with internal control of beta-Actin mRNA. The expressions of SOCS1 mRNA in this study were not significantly different (p=0.96), as shown in Figure 2. The average SOCS1/beta-Actin ratio in BPH was 2.4±0.89, HGPIN was 2.3±0.95, and PRAD was 2.43 ±1.06.

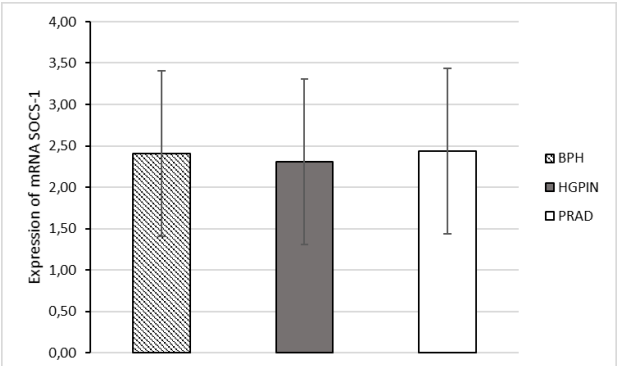


Figure 2. The ratio of SOCS1/beta-Actin in BPH, HGPIN, PRAD. Quantification of mRNA band using ImageJ software.

To determine whether miR-155 and SOCS1 mRNA affected macrophages, a correlation analysis was performed using Pearson correlation tests. The results in Table 1 indicate that miR-155 has a significant negative correlation with SOCS1 mRNA (p=0.02).

Table 1. Correlation between variable

Correlation	Correlation coefficient (r)	*p* value
miR-155 to SOCS1 mRNA	-0.47	0.02
miR-155 to macrophage	-0.05	0.79
SOCS1 mRNA to macrophage	0.22	0.28

Pearson correlation, p significant <0.05

Percentage of macrophage population in BPH, HGPIN, and PRAD

Analysis of data using ANOVA obtained the mean percentages of macrophages in BPH 0.89±0.93, HGPIN 6.03±1.68, and PRAD 7.94±3.07 with $p = 0.00$. The macrophage population between HGPIN and PRAD was not significantly different with $p = 0.075$.

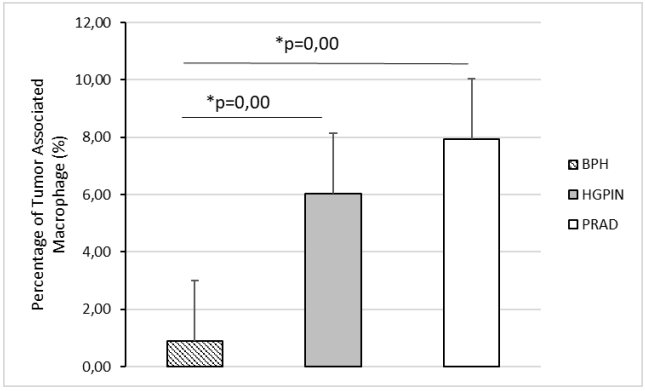


*Figure 3. Percentage of macrophages in BPH, HGPIN, and PRAD. * p < 0.001.*

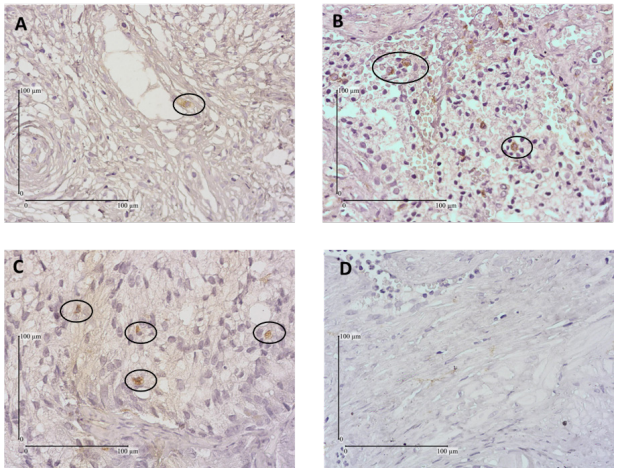


Figure 4. IHC staining, the macrophage is stained brown using an anti-CD68 antibody (in the circle), photomicrograph magnification – 400x, scale bar 100µm; A: BPH, B: HGPIN, C: PRAD, D: negative control without CD68 antibody

DISCUSSION

In humans, miR-155 is encoded by the miR155 gene in the 21st chromosome, which regulates several biological processes including proliferation, invasion, apoptosis, *in vitro* or *in vivo* cell cycles, and inflammation. Changes in miR-155 expression induced by epigenetic changes related to oncogenesis and disease progression are observed in many cancers including PRAD and could be used as a carcinogenesis biomarker and in prognostic evaluation.[13,14] In PRAD, 30 percent of epigenetic silencing regions are placed at the microRNA locus. Epigenetic mechanisms include deletion of microRNA genes, acetylation or methylation of microRNA promoters, alteration in transcription control, and defects in microRNA biogenesis.[14] MicroRNA-155 targets SOCS-1 mRNA and SHIP1 mRNA and increases activation of the AKT and interferon pathways. Decreasing SOCS1 mRNA increases the pro-inflammatory response of M1 macrophages by activating the JAK/STAT pathways. The MiR-155 also manages M2 via CCL18, SERPINE, CD23, and DC-SIGN.[15,16]

In precancerous lesions, such as HGPIN tissue, our findings suggest that miR-155 is increased by two times as much as in BPH because non-carcinoma tissue can be induced by proliferation. The SOCS1 mRNA as a target for miR-155 decreased slightly. However, miR-155 has a significantly negative correlation with SOCS1 mRNA. High-grade PIN is characterized by cell proliferation in the ductus and prostate gland acini with cytological changes observed in PRAD including giant nucleus and nucleoli.[10] Another study stated that miR-155 had a proliferation effect via annexin-7.[17] The sharp increase in miR-155 expression could be due to cell proliferation, not inflammation, leading to cancer progression.

SOCS1 mRNA is a negative regulator of cytokine signaling which attenuates classical antigen signaling and T cell function. In general, the SOCS1 mRNA promoters have a STAT binding site and could be induced by the JAK-STAT pathways via proinflammatory cytokines such as IFN-γ, IL-6, IL-2, IL-4, and IL-7.[18] In the regulatory process, SOCS1 mRNA can be targeted by other microRNAs such as miR-19, miR-30d, and miR-221-5p.[19,20] Based on miRTarBase, SOCS1 mRNA has three binding sites for miR-155. MicroRNA and argonaute form RNA-induced silencing complex (RISC) which binds to the mRNA seed region which triggers translational repression, mRNA decay, and mRNA degradation.[21,22]

Adenocarcinoma of the prostate is considered to have a significant relation with inflammation. Inflammatory stress can lead to gene instability, which in turn affects cell transformation, vascularity, apoptosis, and DNA mutations. Evidence suggests that in normal prostate glands, gland hyperplasia, and adenocarcinoma commonly coincide with inflammation.[7] Therefore, the CD68+ marker is an important marker for pan macrophages. The CD68+ infiltration found in this study indicated that the percentages of macrophages in HGPIN and PRAD were significantly higher than those in BPH. Chronic inflammation that induces inflammatory stress could further stimulate angiogenesis, apoptosis, and gene instability.[7] Macrophages play a role in cancer progression because more than 50% of tumor mass consists of infiltrated macrophages.[5] In this study,

macrophages were significantly increased. To determine polarization, it is necessary to stain the M1 and M2 macrophages. In addition to miR-155, several microRNAs play a role in macrophage activity such as miR-125b and miR 19a-3p. Anti-tumor macrophage induced miR-125b overexpression by targeting IRF-4 mRNA, the promoter of M2 macrophages. Downregulation of miR-19a-3p increases STAT3 signaling leading to suppression of the immune system by macrophages.[23]

In conclusion, miR-155 plays an important role in macrophage polarization by targeting SOCS1 mRNA and inducing polarization of macrophages to M1 or M2 macrophages. In our findings, miR-155 and the percentage of pan macrophages in HGPIN were higher than in BPH. These findings may be related to macrophage infiltration and increased cell proliferation in precancerous lesions. Interestingly, even though miR-155 indirectly regulates pan macrophage activity through SOCS1 mRNA expression, but in this study, SOCS1 mRNA was unlikely to play this role, while other microRNAs might play a role in the precancerous progression. These possibilities need further study.

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Conflict of Interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest concerning this manuscript**.**

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The expression of GLUT-1 and VEGF-A mRNA in the rectal cancer patients with neoadjuvant chemoradiation

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Abstrak

Latar belakang: *Angka kekambuhan dan resistensi pasien kanker rektum mencapai 40 persen. Kondisi tersebut bisa disebabkan karena peningkatan ekspresi GLUT-1 dan VEGF-A, serta mempengaruhi prognosis pasien. Tujuan penelitian ini adalah untuk mengetahui korelasi ekspresi mRNA GLUT-1 dan VEGF-A, serta hubungannya dengan prognosis pasien kanker rektum yang menjalani kemoradiasi.*

Metode: *Penelitian Kohor ini melibatkan 16 orang pasien kanker rektum lokal stadium II atau III yang menjalani kemoradiasi di RSUP Kariadi Semarang. Sampel darah intravena diambil 5 mL pada saat sebelum dan sesudah kemoradiasi. Total RNA diisolasi dari 200* µl *serum, kemudian dilakukan sintesis cDNA. Ekspresi mRNA GLUT-1 dan VEGF-A dikuantifikasi dengan metode Livak menggunakan reference gene β-actin.*

Hasil: *Rata-rata ekspresi mRNA GLUT-1 menurun signifikan 2,14 kali (P=0,044) dan mRNA VEGF-A menurun 1,9 kali (P = 0,03). Ekspresi mRNA GLUT-1 dan VEGF-A berkorelasi positif kuat dan signifikan pada saat sebelum (r = 0,6; R^2 = 0,455; P = 0,013) dan sesudah kemoradiasi (r = 0,8; R^2 = 0,598; P < 0,001). Peningkatan ekspresi mRNA GLUT-1 berhubungan dengan prognosis buruk pasien, dengan resiko 18 kali lebih tinggi (P=0,036; OR=18, 95% CI=1,2 – 261). Peningkatan ekspresi mRNA VEGF-A tidak berhubungan signifikan dengan prognosis pasien (P=0,12; OR=9; 95%CI=0,6-123).*

Kesimpulan: *Ekspresi mRNA GLUT-1 dan VEGF-A berkorelasi positif dan saling mempengaruhi satu dengan lainnya. Peningkatan ekspresi mRNA GLUT-1 berhubungan dengan prognosis buruk pasien. Hasil penelitian ini mengindikasikan bahwa ekspresi mRNA GLUT-1 dari sampel darah berpotensi sebagai biomarker prognosis pada pasien kanker rektum yang menjalani kemoradiasi.* ***(Health Science Journal of Indonesia 2020;11(2):92-9)***

Kata kunci: *kanker rektum, kemoradiasi, GLUT-1, VEGF-A, prognosis*

Abstract

Background: Rectal cancer patients have 40 percent risk of recurrence and resistance, which is triggered by increasing in GLUT-1 and VEGF-A mRNA expression. This condition associate with the patients prognosis. This study aimed to determine the correlation between GLUT-1 and VEGF-A mRNA expression, and analyze its association with the rectal cancer patients prognosis who received chemoradiation.

Methods: This was a Cohort study involving 16 rectal cancer patients with stage II or III undergoing chemoradiation at Kariadi Hospital Semarang. Five milliliters of intravenous blood samples were taken before and after chemoradiation. Total RNA was isolated from 200 µl of blood serum, followed by cDNA synthesis. GLUT-1 and VEGF-A mRNA expression was quantified by the Livak method using β-actin as a reference gene.

Results: GLUT-1 and VEGF-A mRNA expression decreased significantly 2.14 times (P=0,044) and 1,9 times (P=0,03), respectively. Expression of GLUT-1 and VEGF-A mRNA have a significant and strong positive correlation at before (r=0,6; R^2=0,455; P=0,013) and after chemoradiation (r=0,8; R^2=0,598; P<0,001). GLUT-1 mRNA expression enhancement significantly associate with poor prognosis and risk 18 times of worse prognosis (P=0,036; OR=18, 95% CI=1,2–261). VEGF-A mRNA expression did not associate with patient prognosis (P=0,12; OR=9; 95%CI=0,6-123).

Conclusion: Expression of GLUT-1 and VEGF-A mRNA have a significant and strong positive correlation. GLUT-1 mRNA expression enhancement significantly associate with the poor prognosis of the rectal cancer patients. Our finding suggests that GLUT-1 mRNA expression from blood sample was potential as a biomarker to predict rectal cancer patient prognosis who received chemoradiation. ***(Health Science Journal of Indonesia 2020;11(2):92-9)***

Keywords: rectal cancer, chemoradiotherapy, GLUT-1, VEGF-A, prognosis

Colorectal cancer is the third most common cancer worldwide after lung and breast cancer, and it is the second leading cause of cancer-related death. Colorectal cancer prevalence is 12.8 percent and about 55 percent of cases occur in developing countries.[1] In epidemiological studies, the occurrence of the colon and rectal cancers is still not distinguished, because the anatomical location is adjacent and symptoms are generally almost the same. Requires further clinical and biomedical examination to differentiate colon and rectal cancer.[2] About 30 percent of colorectal cancer cases were rectal cancer. Although the incidence rate is lower than colon cancer, the rate of recurrence and therapy resistance in rectal cancer is higher. Standard therapy in rectal cancer known as "trimodality therapy", consists of neoadjuvant chemoradiotherapy, surgery and adjuvant chemotherapy. There is about 40 percent of rectal cancer patients who have recurrence and resistance after treatment.[3,4]

Recurrence and resistance after therapy can be triggered by hypoxic conditions in the tumor microenvironment, especially in locally advanced solid tumors. Hypoxia in the tumor microenvironment is a condition of oxygen lacking supply in cells and tissues, due to uncontrolled cell proliferation, energy metabolism switching and abnormal growth of blood vessels.[5] Cancer cells that have adapted with hypoxia play a role in therapy resistance through inhibition of therapeutic drug delivery and absorption by cells, and low oxygen levels reduce the effects of chemotherapy cytotoxicity.[6,7] Hypoxic conditions could modify cancer cell metabolism by increase glycolysis rate and Glucose transporter-1 (GLUT-1) expression, inhibit oxidative phosphorylation and induce hypoxia-inducible factor-1 (HIF-1) expression.[8] Glucose transporter-1 is a membrane cell receptor that facilitates glucose uptake by the passive transport mechanism into the cytoplasm. Cancer cells dominantly use aerobic glycolysis as the main pathway in glucose metabolism to obtain adequate energy in a fast and short time. This abnormality is known as the Warburg effect.[9] The more glucose transfer into the cell, the higher GLUT-1 expression. Glucose transporter-1expression usually increases in cancer cells and associated with cancer progression.[10]

Hypoxia also triggers pro-angiogenesis factors expression in large numbers, such as vascular endothelial growth factor A (VEGF-A). In cancerous conditions, VEGF-A stimulates new blood vessel formation from nearby capillaries to help tumor cells obtaining adequate oxygen and nutrients.[11] Vascular endothelial growth factor-A also regulates abnormal vascularization in tumor microenvironments such as excessive branching, leaky blood vessels and dead blood end-vessels. Increasing VEGF-A expression is associated with carcinogenesis, metastasis and tumor cell invasion. Vascular endothelial growth factor-A also becomes a cytokine marker of poor prognosis after radiotherapy.[12] Increasing GLUT-1 and VEGF-A expression contribute to maintain tumor microenvironment, so that cancer cells can survive, proliferate continuously, metastasis and resistant to a given therapy.[5] Through this study, the authors want to determine the correlation between GLUT-1 and VEGF-A mRNA expression and analyze its association with the prognosis of the rectal cancer patients who received chemoradiation.

METHODS

Patients and samples

This research used Cohort design and involved 16 patients with locally advanced rectal cancer in 2017 and 2018. Inclusion criteria of patients were having clinical tumor size, node status and metastasis (TNM) stage II or III without metastasis and history of other malignancies based on histopathological examination, colonoscopy and Magnetic Resonance Imaging (MRI) at Kariadi Hospital Semarang. The patients were given chemoradiotherapy at RSUP dr. Kariadi Semarang using capecitabine orally for chemotherapy and radiation dose were 45-50 Gy with 1.8-2 Gy fraction for 6-8 weeks.

Blood samples were taken from peripheral venous as much as 5 mL in the EDTA vacutainer at the time before and after chemoradiotherapy. Blood serum was isolated by centrifugation 3000 rpm for 15 minutes. Blood serum was separated from the blood and aliquoted, then it was stored at -80^{0}C until further analysis.

RNA isolation and cDNA synthesis

Ribonucleic acid (RNA) was isolated from 200 µl of serum using miRCURY RNA Isolation Kit for Biofluids (Qiagen, China), following the procedure from the manufacture's guideline. Ribonucleic acid concentration and purity were counted using Nanodrop (NanoVue Plus, GE Healthcare, Life Science). Pure RNA was eluted in RNAse-free water and stored in the -80^{0}C refrigerator. Synthesis of cDNA was carried out by reverse transcription reactions using ReverTraAce qPCR RT Master Mix with gDNA remover (Toyobo) and performed using

a thermal cycler (Applied BiosystemsTM A24811l) following the manufacturer's guidelines. The results of cDNA synthesis were stored at -20^{0}C until further analysis.

Quantitative real-time PCR (qRT-PCR)

Analysis of mRNA expression was performed using fluorescent SYBR Green (Bioline SensiFAST SYBR No-ROX kit) and samples were run on CFX96 Touch TM Real-Time PCR Detection System (Bio-Rad, USA) in a total volume 20 µl. β-actin was used as a reference gene. The primer sequence of each mRNA was shown in Table 1. The end result of mRNA expression in the form of a cycle of threshold (Ct) or cycle of quantification (Cq) was quantified using Livak's method relative quantification ($2^{-\Delta\Delta Ct}$) to obtain the fold changes.

Table 1. Primer of mRNA

mRNA		Primer Sequence
β-actin	*Forward*	5'-CGCGAGAAGATGACCCAGATC-3'
	Reverse	5'-TCACCGGAGTCCATCACGA-3'
GLUT-1	*Forward*	5'-TCTGGCATCAACGCTGTCTTC-3'
	Reverse	5'-CGATACCGGAGCCAATGGT-3′
VEGF-A	*Forward*	5'-GCACCCATGGCAGAAGG-3'
	Reverse	5'-CTCGATTGGATGGCAGTAGCT-3'

Data analysis

Patients characteristic is shown as total number (n) and percentage, or mean plus/minus and standard deviation, or minimal and maximal score. The normality data was analyzed using P value from Shapiro-Wilk. The mean difference before and after treatment were analyzed using Wilcoxon rank-test. Correlations between variables were analyzed using Spearman's rank correlation. The association of GLUT-1 and VEGF-A mRNA expression to the patient's characteristic was assessed using χ^2 test (Chi-square, Fisher's exact test). The association power was evaluated with Odds Ratio (OR) equivalent to the 95% Confident Interval (CI). Statistically significant was regarded when P-value <0,05.

This study has approved by Medical Faculty of Diponegoro University and Kariadi Hospital Ethics Committee number 14/EC/ FK-RSDK/I/2017, then continued by the Medical, Public Health and Nursing Faculty Ethics Committee, Universitas Gadjah Mada Ref No KE/FK/1008/ EC/2018. All respondents approved the informed consent before register in this research.

RESULTS

Clinicopathological characteristics of the patient

Patient's clinicopathological characteristics were shown in Table 2. Patients were dominated by males, with an average age was 47 years. Based on anatomical pathology results, tumor differentiation mostly good, with the depth of tumor invasion (T stage) mostly at T3-T4 stage which means that tumor cells were invaded the visceral surface of the peritoneum. Tumor spreading to regional lymph nodes (N1-N2) occurs in majority of patients. The average early carcinoembryonic antigen (CEA) level exceeds the normal range (>5 ng/mL). Carcinoembryonic antigen is one of the blood-based biomarkers that used to determine the prognosis of the rectal cancer patient.

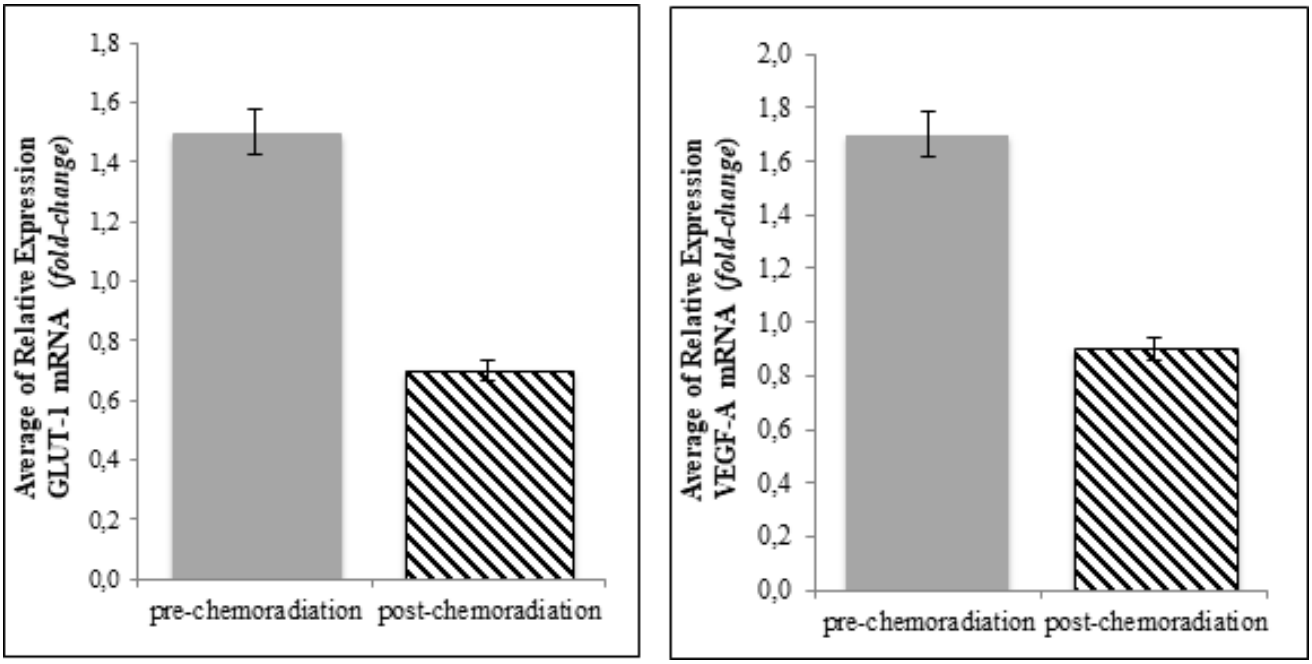
Table 2. Patient's clinicopathological characteristics

Clinicopathological of Patients	
Sex, n (%)	
Male	9 (56,3)
Female	7 (43,7)
Age	
Mean ± SD (min-max)	46,6 ± 12,97 (26-70)
Patological Differentiation, n (%)	
Well	13 (81,3)
Moderate	2 (12,5)
Poor	1 (6,3)
Early T stage, n (%)	
T1	2 (12,5)
T2	2 (12,5)
T3	6 (37,5)
T4	6 (37,5)
Early N stage, n (%)	
N0	1 (6,3)
N1	9 (56,2)
N2	6 (37,5)
Early Clinical TNM stage, n (%)	
II	1 (6,3)
III	15 (93,7)
Early CEA level (ng/mL)	
Mean ± SD (min-max)	42,7 ± 78,8 (0,5 – 201)

SD=standard deviation; n=amount of subject; TNM=*Tumor size, Node status, Metastasis*;
CEA=*carcino embryonic antigen*; min=minimal; max=maximal

Mean difference of GLUT-1 and VEGF-A expression

Expression of GLUT-1 and VEGF-A mRNA were observed before and after chemoradiation. The results were shown in Figure 1.



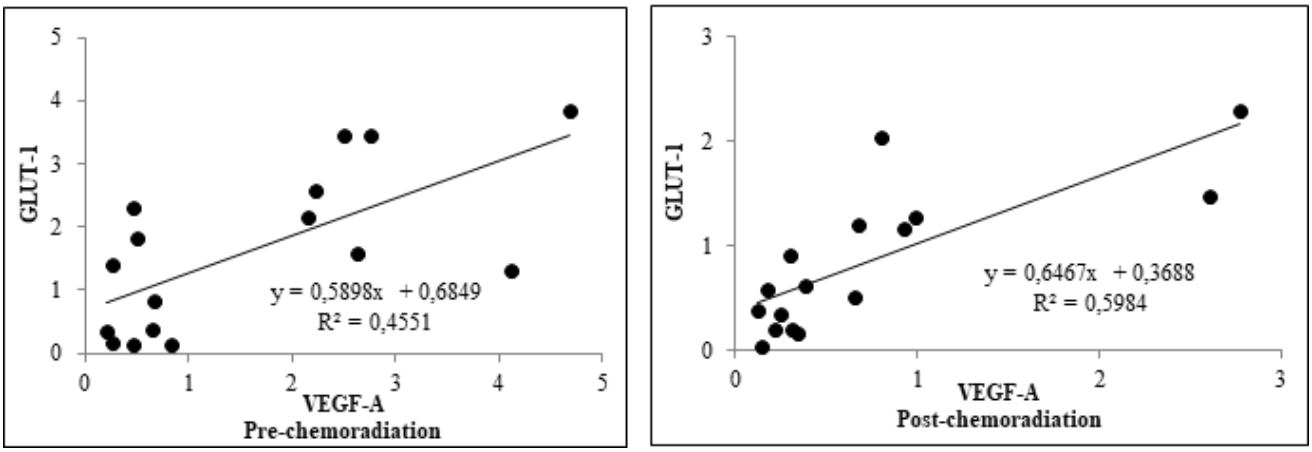
Wilcoxon signed ranks test, *P-value significant < 0,05

Figure 1. Average difference of GLUT-1 (left) and VEGF-A (right) mRNA relative expression at before and after chemoradiation

Based on average difference analysis, GLUT-1 mRNA relative expression significantly decreased 2,14 times after chemoradiation (P=0,044). Meanwhile VEGF-A mRNA relative expression also significantly decreased 1.9 times after chemoradiation (P=0,03).

Correlation between GLUT-1 and VEGF-A mRNA expression

Based on mRNA expression in each patient, the correlation of GLUT-1 and VEGF-A mRNA expression can be determined before and after chemoradiation. The results of correlation analysis are shown in Figure 2.



Spearman's rank correlation test; r = coefficient of correlation; y = equation of correlation; R = coefficient of determination; *P-value significant < 0,05

Figure 2. Correlation between GLUT-1 and VEGF-A mRNA expression at the time before (left) and after (right) chemoradiation

Based on correlation analysis, GLUT-1 and VEGF-A mRNA expression showed a significant and strong positive correlation (P <0.05; r> 0.5), both before and after chemoradiation. This was means that increasing or decreasing of GLUT-1 and VEGF-A mRNA expression will be affected on one each other. The more increase GLUT-1 mRNA expression, the more enhance VEGF-A mRNA expression, and vice versa. Glucose transporter-1 mRNA expression could affect 45,5 percent VEGF-A mRNA expression at before chemoradiation (R^2 = 0,455) and 59.8 percent at after chemoradiation (R^2 = 0,598).

Association of GLUT-1 and VEGF-A mRNA expression with clinicopathology patient

Association between GLUT-1 and VEGF-A mRNA expression with clinicopathology patient is shown in Table 3. There were no significant association between GLUT-1 and VEGF-A mRNA expression with sex, age, patological anatomy differentiation, early T stage, early N stage, early clinical TNM stage, and early CEA level (P>0,05) of rectal cancer patient. More than half of total patient with poor prognosis tend to have elevating GLUT-1 and VEGF-A expression. Patient with poor prognosis have significantly increasing of GLUT-1 mRNA expression 18 times (OR=18; 95% CI=1,2 – 261; P<0,05). Meanwhile, increasing of VEGF-A mRNA expression did not associate significantly with patient prognosis (P>0,05), but majority of patient with poor prognosis have elevated VEGF-A expression.

DISCUSSION

Neoadjuvant chemoradiation as preferable therapy for stage II or III rectal cancer had proven can reduce local relapse, increase long-term survival and optimize surgery. Neoadjuvant chemoradiation in patients with a good response can reduce tumors up to 60 percent and reduce local recurrence. Some of the patients who can not respond to this therapy usually show a worse prognosis.[13] Poor prognosis, resistance and relapsing are associated with the presence of hypoxic conditions in the tumor microenvironment. Hypoxic conditions are related to genomic instability, metastases, sustaining proliferative signaling, avoiding immune destruction, inducing angiogenesis and deregulating cellular energetics.[14]

Table 3. Association of GLUT-1 and VEGF-A mRNA and clinicopathology characteristic of patient

Clinicopathology characteristics	GLUT-1 mRNA Expression N (%)		OR (95% CI) P	VEGF-A mRNA Expression N (%)		OR (95% CI) P
	Decrease	Increase		Decrease	Increase	
Sex						
Male	3 (33,3)	6 (66,7)	1,2 (0,1-10,7)	4 (44,4)	5 (55,6)	0,5 (0,3-0,9)
Female	2 (28,6)	5 (71,4)	0,63	0 (0)	7 (100)	0,09
Age						
< 50 years	2 (20)	8 (80)	0,2 (0,03-2,3)	1 (10)	9 (90)	0,1 (0,01-1,5)
≥ 50 years	3 (50)	3 (50)	0,3	3 (50)	3 (50)	0,12
Differentiation						
Well	3 (23)	10 (77)	0,1 (0,01-2,3)	3 (23)	10 (77)	0,6 (0,04-9,1)
Moderate – Poor	2 (66,7)	1 (33,3)	0,21	1 (33,3)	2 (66,7)	0,61
Early T Stage						
T1 – T2	1 (25)	3 (75)	0,67 (0,05-8,6)	0 (0)	4 (100)	1,5 (1-2,2)
T3 – T4	4 (33,3)	8 (66,7)	0,63	4 (33,3)	8 (66,7)	0,52
Early N Stage						
N0	0 (0)	1 (100)	1,5 (1-2,1)	0 (0)	1 (100)	1,3 (1-1,8)
N1 – N2	5 (33,3)	10(66,7)	0,68	4 (26,7)	11 (73,3)	0,75
Early Clinical TNM Stage						
II	0 (0)	1 (100)	1,5 (1-2,1)	0 (0)	1 (100)	1,4 (1-1,8)
III	5 (33,3)	10(66,7)	0,69	4 (26,7)	11 (73,3)	0,75
Early CEA Level						
Normal (≤ 5 ng/mL)	2 (50)	2 (50)	3 (0,3-31)	2 (50)	2 (50)	5 (0,4-59)
Elevated(>5 ng/mL)	3 (25)	9 (75)	0,36	2 (16,7)	10 (83,3)	0,24
Prognosis after chemoradiation						
Good	4 (66,7)	2 (33,3)	18 (1,2-261)	3 (50)	3 (50)	9 (0,6-123)
Poor	1 (10)	9 (90)	0,036*	1 (10)	9 (90)	0,12

Chi-square test (Fisher's exact test) *P-value significant < 0,05

OR = Odds Ratio; CI = confident interval

Hypoxic conditions drive tumor cells to modify cellular energetic metabolism pathways to survive. Deregulating energy metabolism are needed by cancer cell to obtain adequate nutrition in a stressful environment due to oxygen lack. In normal condition, the normal cell uses oxidative phosphorylation to produce energy, meanwhile under hypoxic conditions, cancer cells switch cellular energetics turn into aerobic glycolysis. Aerobic glycolysis produces only 2 ATP molecules, while oxidative phosphorylation produces 36 ATP molecules. Aerobic glycolysis produces less energy but tends to be faster than oxidative phosphorylation to response increasing energy requirements of cancer cells. Cancer cells need energy in a fast time and large amounts to support fast cell growth and continuous proliferation. [9]

Cancer cells need large amounts of glucose to produce ATP for energy metabolism. Glucose enters the cell by diffusion via glucose transport protein in the cell membrane, which is known as the glucose transporter (GLUT) family. The high demand ~~of~~ for glucose could increase GLUT expression, mainly GLUT-1. Glucose transporter-1 regulates glucose concentration gradient and ensures adequacy for energy metabolism.[15] Its expression increases in various types of solid tumors and associated with tumor aggressiveness, cancer stage and poor prognosis in cancer patients. Increasing of GLUT-1 expression also correlates with cancer cell metastases to lymph nodes.[16] In mouse animal model, increasing of GLUT-1 expression associate with resistance to multiple anti-cancer therapies, including chemotherapy drug vincristine.[17] In this study, increasing GLUT-1 mRNA expression associate with poor prognosis in rectal cancer patients. Our result in line with Brophy et al.[18] Study, which show increase in GLUT-1 expression associate with poor response to chemoradiation and is correlated with a worse prognosis. Tumors with GLUT-1 negative have 70 percent probability of good response to therapy, whereas tumors with GLUT-1 positive only have 31 percent well response. Glucose transporter-1 is potential to be a good predictive biomarker for chemoradiation response in rectal cancer. This study has limitation in small amount of subject sample, so we suggest more amount of subject sample by massive examination will obtain a more precise result.

In this study, we also observe the relationship of VEGF-A mRNA expression with patients prognosis. We did not find any significant association statistically, nevertheless based on the amount of patients we find a tendency that patients with poor prognosis have to elevate VEGF-A mRNA expression. Based on Buka et al.[19] study, elevating VEGF expression is correlated with poor prognosis and a high incidence of liver metastases. Vascular endhotelial growth factor-A expression increase in 50 percent of patients of colorectal cancer and associate with cancer cell metastases to lymph nodes and others organ. Its expressions were found very high in adenocarcinoma, but lower in normal colorectal mucosal tissue. Vascular endhotelial growth factor-A is a heparin-binding glycoprotein, the most potent pro-angiogenesis cytokines of tumor cells. Angiogenesis enables tumor cells to enter the circulation system and metastasis. Vascular endhotelial growth factor-A induces new blood vessel formation and capillaries by increase endothelial cell permeability to support cancer tissue growth rapidly. Decreasing of VEGF-A mRNA expression after chemoradiation was used as a marker for increasing patient survival rate.[20] High expression of VEGF-A associate with tumor-associated macrophage (TAM), which plays a role in tumor microenvironment remodeling after radiation or chemotherapy, so that tumor cells become resistant to the therapy.[21]

This study also showed a significant positive correlation between GLUT-1 and VEGF-A mRNA expression. Glucose transporter-1 plays a role in mediating glucose uptake as an energy source for cancer cells, while VEGF-A initiates angiogenesis to obtain oxygen supply and nutrients delivery to the tumor microenvironment.[22] Vascular endhotelial growth factor-A could increase GLUT-1 expression by influencing translocation of transporters in the cytoplasm to the plasma membrane.[23] Correlation between GLUT-1 and VEGF-A molecularly involves phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway activation. Phosphatidylinositol 3-kinase/Akt pathway becomes over-reactive in tumor conditions and triggers other pathway responses to support tumor cell progression, growth and proliferation to increase resistance and motility of tumor cells. The activation of the PI3K/Akt pathway responsible for radiation therapy resistance because it can modify oncogenes expression to optimize cancer cells growth.[24] Phosphatidylinositol 3-kinase/Akt pathway regulates glucose entry through cell surface and GLUT-1 activity. Activation of the PI3K/Akt pathway also induces VEGF-A expression through activation of HIF-1α (hypoxia-induced factor). Likewise, VEGF-A can activate PI3K/Akt pathway to protect cancer cells from radiation.[25] Inhibition of the PI3K/Akt pathway can reduce GLUT-1 and VEGF-A mRNA expression.[24]

In conclusion, our results study support previous studies that GLUT-1 regulation relates one to each other with VEGF-A. Glucose transporter-1 and VEGF-A mRNA expression have a significant and strong positive correlation in the rectal cancer patients. Increasing GLUT-1 mRNA expression significantly associated with the poor prognosis of the rectal cancer patients. Our finding suggests that GLUT-1 mRNA expression from blood sample was a potential biomarker to predict the prognosis of the rectal cancer patients who received chemoradiation.

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The effect of ethyl acetat fraction of *Caesalpinia sappan L.* wood on PC3 cancer cell line : cell viability and migration study

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Abstrak

Latar belakang: *Tingginya insidensi kanker di Indonesia, termasuk kanker prostat menimbulkan beban ekonomi kesehatan yang tinggi bagi Indonesia. Pengembangan terapi kanker berbasis sumber daya alam lokal dapat membantu meringankan beban negara. Penelitian ini bertujuan untuk mengetahui potensi aktivitas anti-kanker fraksi ethyl acetat Caesalpinia sappan L. terhadap sel line kanker PC3 yang merupakan model in vitro kanker prostat.*

Metode*: Fraksi ethyl acetat kayu secang (Caesalpinia sappan L.) diperoleh melalui proses liquid chromatography. Efek fraksi 9 dari Ffraksi ethyl acetat kayu secang terhadap aktivitas anti-proliferasi dan migrasi sel diuji menggunakan desain uji in vitro. Hambatan proliferasi sel diukur dengan metode MTT assay, sedangkan aktivitas migrasi sel diukur dengan metode migration assay*

Hasil: *Fraksi 9 dari fraksi ethyl acetat kayu secang memperlihatkan hambatan proliferasi sel line kanker PC3 dengan IC50:14.99*µg/ml. *Hasil migration assay menunjukkan pada dosis 10µg/ml fraksi 9 menghambat migrasi sel line kanker PC3, sedangkan pada dosis 100µg/ml sel line kanker PC3 mati.*

Kesimpulan: *Fraksi 9 dari fraksi ethyl acetat kayu secang menunjukkan aktifitas anti-proliferasi dan anti-migrasi yang kuat terhadap pertumbuhan sel line kanker PC3 secara in vitro.* ***(Health Science Journal of Indonesia 2020;11(2):100-5)***

Kata kunci: *ethyl acetat fraction, Caesalpinia sappan, prostate cancer, PC3, migrasi sel*

Abstract

Background: The high incidence of cancer, including prostate cancer, in Indonesia create a high burden on health economic cost. Development of cancer therapy based on local natural resources may help the country to alleviate the burden. This research aimed to find out the potency of selected compound of ethyl acetate fractions of *Caesalpinia sappan* as anti-cancer by using PC3 cancer cell line as an in vitro model of prostate cancer.

Methods: Ethyl acetate fraction of *Caesalpinia sappan L***.** heartwood was prepared using a liquid chromatography method. The effect of ethyl acetate fraction 9 on anti-proliferative and cell migration activities was assessed using MTT assay and migration assay.

Results: Fraction-9 of ethyl acetate fraction of *Caesalpinia sappan L.* wood showed inhibition of PC3 cancer cell line proliferation. The IC50 of the fraction was 14.99µg/ml. The migration assay showed inhibition of cell migration on dose 10µg/ml compared to the 0 doses, while most of the cells cultured was dead when treated with 100µg/ml fraction 9.

Conclusion: Ethyl acetate fraction 9 of *Caesalpinia sappan L.* heartwood possibly has anti-cancer properties based on its anti-proliferative and anti-migration activities against PC3 cancer cell line. ***(Health Science Journal of Indonesia 2020;11(2):100-5)***

Keyword: ethyl acetate fraction, *Caesalpinia sappan,* prostate cancer, PC3, cell migration

The increasing cancer incidence worldwide put the disease on the high rank of global health challenge. The high mortality rate of the cancer patient and the expensive cost of cancer therapy justify cancer as a catastrophic disease.[1] Global cancer observatory (Globocan) reported 11.361 new prostate cancer cases in 2018 in Indonesia. The incidence rate and mortality rate of prostate cancer are in the third rank among other cancer in the male in Indonesia.[2]

The application of the screening method on patients with urologic symptoms increases the detection rate of prostate cancer.[3] Current therapeutic approaches for prostate cancer in Indonesia include surgery, External Beam Radiation Therapy, and Adjuvant Androgen Deprivation Therapy.[4] Although advanced cancer therapy has been available nowadays, the cost of cancer therapy hampers many cancer patients from low-income society to seek immediate and appropriate cancer treatment. This condition urges the need to investigate the potential use of local plant medicine as cancer therapy.

In this study, we report the anti-cancer activity of one of Indonesian plant medicine, known with local name as secang. The Latin name of Secang is *Caesalpinia sappan L.* The heartwood of *Caesalpinia sappan* has been widely used as Javanese traditional medicine.[5] In the field of cancer research, attention has been largely spent to study the potential anti-cancer activity of compounds isolated from *Caesalpinia sappan L*. *Caesalpinia sappan* heartwood contains many active compounds with anti-cancer activities including protosappanin B[6] Brazilein,[7] Brazilin[8]. Brazilin is the major compound of *Caesalpinia sappan L.* heartwood which gives the red color when dissolved in water.[9] This study investigated the anti-cancer activity of the ethyl acetate fraction of *Caesalpinia sappan* on PC3 cancer cell line.

METHODS

The experiment was conducted under the Ethical Approval issued by The Health Research Ethics Committee of Faculty of Medicine Universitas Sebelas Maret No. 59/UN27.6/KEPK/2018.

Preparation of ethyl acetate fraction of *Caesalpinia sappan L.*

Caesalpinia sappan heartwood was obtained from an herbal store in Surakarta, Central Java. An amount of 180 g of *Caesalpinia sappan* heartwood powder was macerated in 1 L of methanol® (Sigma) for 24 hours. The methanolic soluble compound was filtered using Whatman paper. The process was repeated three times. The filtrate of the methanolic soluble compound was then subjected to rotary evaporation to get concentrated crude methanolic extract. The methanolic extract was partitioned using n-hexane® (Sigma): ethyl acetate® (Sigma) (v:v) to separate the non-polar compound from the polar compound. The n-hexane soluble fraction was taken out and filtered. The ethyl acetate soluble fraction was then concentrated and applied to a silica gel column using 1:9 of Chloroform: Ethyl Acetate eluent. The final process yielded 24 separated fractions. All the fractions were subjected to a rotary evaporator to remove ethyl acetate residue. One hundred and twenty (120) mg of fraction-9 with yellowish red color was used in this experiment. The selection of Fraction-9 was based on the physical reference of Brazilin.[10,11] Further identification of Fraction-9 compound was conducted by using comparative HPLC with Brazilin®(Sigma) as standard. The stock solution used in anti-proliferative and migration assay was made by dissolving Fraction-9 with cell culture grade *of Dimethyl Sulfoxide (*DMSO®) from Sigma. The DMSO concentration on the tested fraction was adjusted not to exceed 0.1% for all of the experiments.

Anti-proliferative assay

Anti-proliferative activity of ethyl acetate fraction of *Caesalpinia sappan* wood was measured using MTT assay. PC3 cancer cell line was used as an *in vitro* model of prostate cancer.[12] RPMI 1640 culture medium supplemented with 10% FBS and 1% Penicillin + Streptomycin was used. A number of 1 x 10^4 cells were seeded in 96-well microplates and incubated in 37°C and 5%CO_2. After 24 hours the medium was replaced with 100 µl medium containing—serial concentration (100, 50, 25, 12.5, 6.25, 3.125, 1.55, and 0 µg/ml) of the tested fraction. The cells were incubated for the next 24 hours. After 24 hours the culture medium was replaced with 100 µl MTT stock solution (5 mg/ml, Sigma, St. Louis, MO) and incubated for 4 hours on 37°C and 5%CO_2. Sodium Dodecyl Sulphate (SDS) 10% in 0.1 N HCl was added as stop solution after 4 hours incubation, and the plate was wrapped with aluminum foil and kept at room temperature overnight. The absorbance was measured at 570 nm wavelength using ELISA reader (Biorad®) All

the measurements were performed in triplicate. The cell viability was measured by comparing the optical density of the treated (dose 100 – 1.55 µg/ml) and the non-treated (dose 0 µg/ml) cells as a reference. The data were presented as the percentage of cell viability.

Migration assay

The experiment was conducted using a 6 well plate. Following the standard procedure on migration assay[13], an amount of 7 x 10^5 PC3 cancer cell line/ well was seeded in 6 well plate. RPMI 1640 culture medium supplemented with 10% FBS and 1% Penicillin and Streptomycin were used. The cells culture reached the monolayer, after 24 hours of incubation at 37°C and 5% CO_2. The wound was made by scratching the monolayer cells using a 200µl pipet tip. After scratching, the monolayer was carefully washed using PBS to remove the cell's debris. The culture medium was replaced with a medium containing 0, 10, and 100 µg/ml of fraction-9. The picture of the wound was taken at 0 and 18 hours after the scratching by using an inverted microscope. The cell migration was measured by the narrowing of cell free area (the width of the wound area) during the experiment time. The width of the wound area was measured using ImageJ software (NIH, Bethesda, MD). The cell migration was expressed as the reduction of the width of the cell-free area or the proximity of the wound edge.

RESULTS

Identification of selected compound from ethyl acetate fraction (fraction-9)

The HPLC analysis performed using HPLC system (Shimadzu Corp., Tokyo, Japan). The eluent system consisted of an isocratic mode of 100% (v/v) Methanol, running at a flow rate of 0.5 ml/min at a column temperature of 25°C. The spectrometric result of comparative HPLC showed in the Figure1.

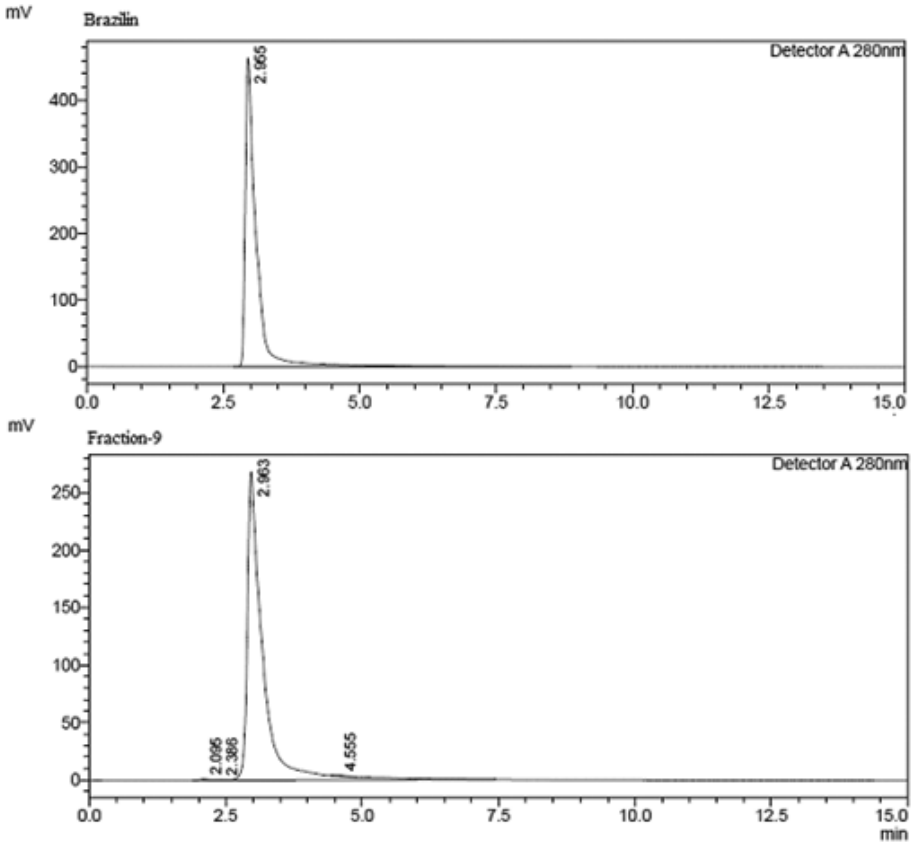

Figure 1. HPLC Chromatograph of Brazilin® and Fraction-9.

The peak of fraction-9 at the same retention time indicates that brazilin is the major compound of fraction-9.

1. Anti-proliferative assay

The result of anti-proliferative assay of fraction-9 on PC3 cancer cell line was shown in Figure 2. The fraction-9 was markedly showed a dose-dependent anti-proliferative effect on the cell. The increase of F9 concentration resulting in a decrease in the percentage of the viable cell. The IC50 of the compound was calculated using the following equation [14].

$$\text{IC50} = \frac{(X2 - X1)x(50 - Y1)}{(Y2 - Y1)} + X1$$

X1/X2 = the higher/the lower concentration bordering the concentration that reduces the cell growth of 50%
Y1/Y2 = percentage of viable cells at concentration X1/X2

The IC50 of fraction-9 acquired by the calculation was 14.99µg/mL.

2. Migration assay

The migration assay showed that fraction-9 of ethyl acetate fraction extracted from *Caesalpinnia sappan* capable of inhibiting the migration of PC3 cancer cell line from the scratch line to the middle part of the wound. Figure 3 and Figure 4 showed that the addition of fraction-9 on PC3 cancer cell line monolayer after scratching inhibits cell migration activity. The migration activity was calculated as a percentage of the closure of the wound edge at 18 hours in respect to those at 0 hours. The percentage of the closure of the wound edge was calculated using the following formula [15]:

$$wound\ closure\ \% = \frac{At0h\ - At\Delta h}{At0h} X100\%$$

A_{t0h} = the area of the wound measured immediately after scratching (t_{0h})
$A_{t\Delta h}$ = the area of the wound measured Δ hours after the scratch is performed

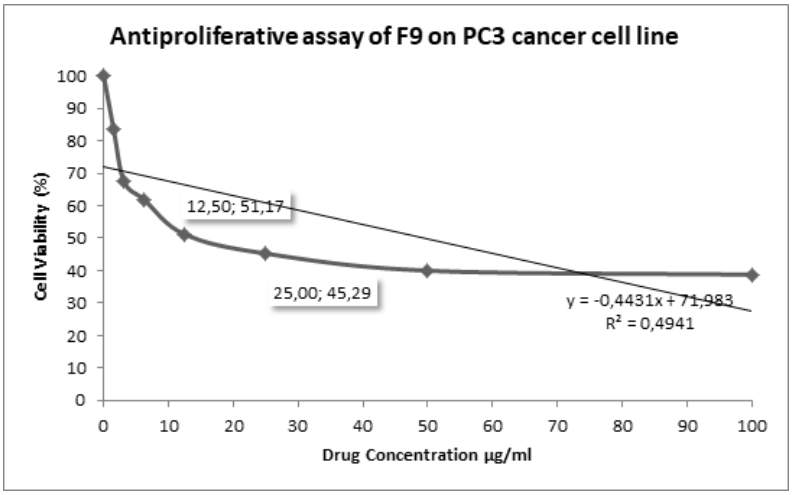

Figure 2. MTT Assay

The graphic showed a dose dependent effect of Fraction-9 on the percentage of PC3 cancer cell line viability. The IC_{50} of the fraction-9 was 14.99µg/ml.

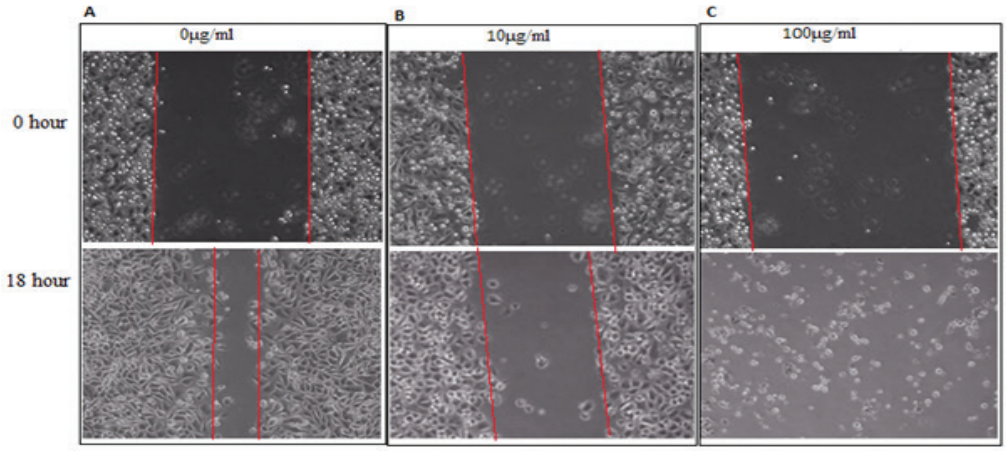

Figure 3 The effect of Fraction 9 on PC3 cancer cell line migration. A. The cell free area of PC3 cancer cell line monolayer without addition of Fraction 9 (0µg/mL) at 0 and 18 hours after scratcing. B. The cell free area of PC3 cancer cell line monolayer treated with 10µg/mL of Fraction 9 at 0 and 18 hours after scratcing. C. The cell free area of PC3 cancer cell line monolayer treated with 100µg/mL of Fraction 9 at 0 hour after scratcing (upper) and at 18 hours after scratching most of the cells died (lower).

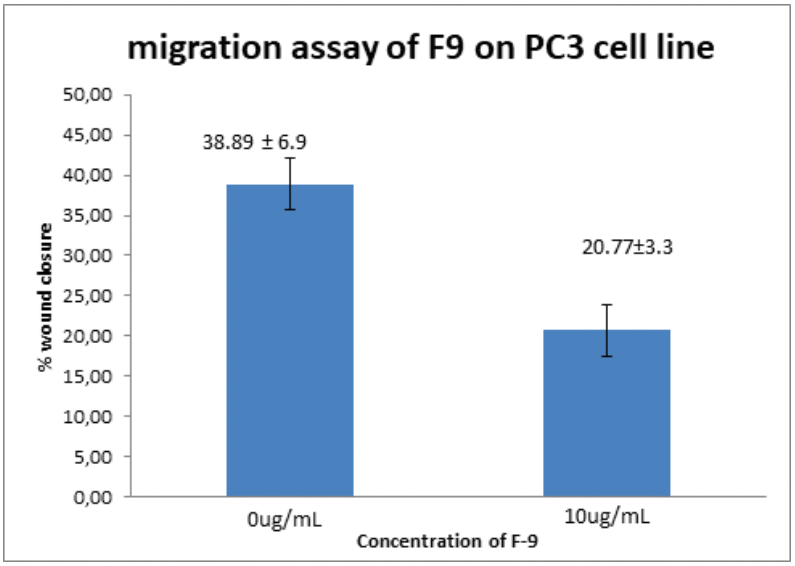

Figure 4. The effect of Fraction 9 of Ethyl acetate fraction of Caesalpinia sappan L. heartwood on PC3 cancer cell line migration. Cell migration was presented as a percentage of wound closure. Treatment of PC3 cancer cell line monolayer after scratching decreases the percentage of wound closure as compared to the control.

Figure 3 showed the highest percentage of cell migration on the untreated cells (0µg/ml). The cell-free area was wider in cells treated with 10µg/ml of fraction-9 than those in the untreated cells. The migration activity presented as the percentage of wound closure were 38.89% ± 6.9 20.7% and 20.77% ± 3.3% for untreated cell and 10µg/ml Fraction-9 treated cell respectively.

DISCUSSIONS

Brazilin is considered the major compound of *Caesalpinia sappan*. heartwood.[10] The data resulted from the comparative HPLC of fraction-9 with Brazilin® as standard compound indicated that Fraction-9 consisted mainly of brazilin. As can be shown in the chromatography of HPLC, many other compounds in small proportion were still mixed in fraction-9. Although fraction-9 was not a purified compound, it showed strong cytotoxic activity toward the growth of the PC3 cancer cell line. The IC50 of fraction-9 in this study was 14.99µg/mL.

The anti-proliferative activity of fraction-9 selected from the Ethyl acetate fraction of *Caesalpinia sappan* heartwood was markedly different from the anti-proliferative activity of methanolic extract of *Caesalpinia sappan* reported in a previous study, where the IC50 of methanolic extract of *Caesalpinia sappan* was 48µg/ml on MCF-7 cell line. Fraction-9 also exhibits a higher cytotoxic activity compared to the cytotoxic activity of ethyl acetate fraction reported on HeLa, MCF-7/Moc, MCF-7/Her2, and T47D cancer cells, where the range of IC50 were 40 – 65µg/mL.[17–19]

Investigation on anti-cancer activity of purified brazilin showed a potential anti-cancer activity of brazilin. Kim *et al.* reported high cytotoxic activity of brazilin on MM cells (U266 and MM1s), leukemia cell line (K562) with the range of IC50 10-80µM.[8] Ren *et al.* also reported the inhibition ratio of brazilin purified from sappan wood on the bladder cancer cell line (T24). The study reported the 50% inhibition ratio of brazilin on the T24 cell line was 25.81µg/mL.[20] Compare to the anti-proliferative activity of purified Brazilin, the result of this study showed a promising potency to inhibit cancer cell proliferation. However, the cytotoxic dosage of fraction 9 on PC3 cancer cell line was higher than the dose of Doxorubicine and Docetaxel reported by Tsakalozou *et al.* where the IC50 of Doxorubicine and Docetaxel on PC3 cancer cell line were 0.598nM and 0.469nM respectively.[21]

The anti-migratory activity of fraction 9 was evaluated on PC3 cancer cell line in this study. The cell migration activity was examined using wound-healing assay. This method measure cell migration activity as the rate of wound closure. The result of this study showed that fraction-9 inhibits wound closure on dosage 10µg/mL. PC3 cancer cell line treatment with 10µg/L of fraction-9 resulting in 22.77% of wound closure, while the wound closure of untreated PC3 cancer cell line was 38.89%. This

anti-migratory effect was comparable with other reported studies on purified Brazilin, where treatment with 12.5μM of Brazilin inhibits up to 16% of MCF/Her2 cell migration.[22]

In conclusion, ethyl acetate fraction 9 of *Caesalpinia sappan* L wood possibly has anti-cancer properties based on its anti-proliferative and anti-migration activities against PC3 cancer cell line. Further study to optimize the compound, dosage, and anti-cancer mechanism of *Caesalpinia sappan* L. is required.

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The expression of GLUT-1 and VEGF-A mRNA in the rectal cancer patients with neoadjuvant chemoradiation

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Abstrak

Latar belakang: *Angka kekambuhan dan resistensi pasien kanker rektum mencapai 40 persen. Kondisi tersebut bisa disebabkan karena peningkatan ekspresi GLUT-1 dan VEGF-A, serta mempengaruhi prognosis pasien. Tujuan penelitian ini adalah untuk mengetahui korelasi ekspresi mRNA GLUT-1 dan VEGF-A, serta hubungannya dengan prognosis pasien kanker rektum yang menjalani kemoradiasi.*

Metode: *Penelitian Kohor ini melibatkan 16 orang pasien kanker rektum lokal stadium II atau III yang menjalani kemoradiasi di RSUP Kariadi Semarang. Sampel darah intravena diambil 5 mL pada saat sebelum dan sesudah kemoradiasi. Total RNA diisolasi dari 200* µl *serum, kemudian dilakukan sintesis cDNA. Ekspresi mRNA GLUT-1 dan VEGF-A dikuantifikasi dengan metode Livak menggunakan reference gene β-actin.*

Hasil: *Rata-rata ekspresi mRNA GLUT-1 menurun signifikan 2,14 kali (P=0,044) dan mRNA VEGF-A menurun 1,9 kali (P = 0,03). Ekspresi mRNA GLUT-1 dan VEGF-A berkorelasi positif kuat dan signifikan pada saat sebelum (r = 0,6; R^2 = 0,455; P = 0,013) dan sesudah kemoradiasi (r = 0,8; R^2 = 0,598; P < 0,001). Peningkatan ekspresi mRNA GLUT-1 berhubungan dengan prognosis buruk pasien, dengan resiko 18 kali lebih tinggi (P=0,036; OR=18, 95% CI=1,2 – 261). Peningkatan ekspresi mRNA VEGF-A tidak berhubungan signifikan dengan prognosis pasien (P=0,12; OR=9; 95%CI=0,6-123).*

Kesimpulan: *Ekspresi mRNA GLUT-1 dan VEGF-A berkorelasi positif dan saling mempengaruhi satu dengan lainnya. Peningkatan ekspresi mRNA GLUT-1 berhubungan dengan prognosis buruk pasien. Hasil penelitian ini mengindikasikan bahwa ekspresi mRNA GLUT-1 dari sampel darah berpotensi sebagai biomarker prognosis pada pasien kanker rektum yang menjalani kemoradiasi.* ***(Health Science Journal of Indonesia 2020;11(2):92-9)***

Kata kunci: *kanker rektum, kemoradiasi, GLUT-1, VEGF-A, prognosis*

Abstract

Background: Rectal cancer patients have 40 percent risk of recurrence and resistance, which is triggered by increasing in GLUT-1 and VEGF-A mRNA expression. This condition associate with the patients prognosis. This study aimed to determine the correlation between GLUT-1 and VEGF-A mRNA expression, and analyze its association with the rectal cancer patients prognosis who received chemoradiation.

Methods: This was a Cohort study involving 16 rectal cancer patients with stage II or III undergoing chemoradiation at Kariadi Hospital Semarang. Five milliliters of intravenous blood samples were taken before and after chemoradiation. Total RNA was isolated from 200 µl of blood serum, followed by cDNA synthesis. GLUT-1 and VEGF-A mRNA expression was quantified by the Livak method using β-actin as a reference gene.

Results: GLUT-1 and VEGF-A mRNA expression decreased significantly 2.14 times (P=0,044) and 1,9 times (P=0,03), respectively. Expression of GLUT-1 and VEGF-A mRNA have a significant and strong positive correlation at before (r=0,6; R^2=0,455; P=0,013) and after chemoradiation (r=0,8; R^2=0,598; P<0,001). GLUT-1 mRNA expression enhancement significantly associate with poor prognosis and risk 18 times of worse prognosis (P=0,036; OR=18, 95% CI=1,2–261). VEGF-A mRNA expression did not associate with patient prognosis (P=0,12; OR=9; 95%CI=0,6-123).

Conclusion: Expression of GLUT-1 and VEGF-A mRNA have a significant and strong positive correlation. GLUT-1 mRNA expression enhancement significantly associate with the poor prognosis of the rectal cancer patients. Our finding suggests that GLUT-1 mRNA expression from blood sample was potential as a biomarker to predict rectal cancer patient prognosis who received chemoradiation. ***(Health Science Journal of Indonesia 2020;11(2):92-9)***

Keywords: rectal cancer, chemoradiotherapy, GLUT-1, VEGF-A, prognosis

Colorectal cancer is the third most common cancer worldwide after lung and breast cancer, and it is the second leading cause of cancer-related death. Colorectal cancer prevalence is 12.8 percent and about 55 percent of cases occur in developing countries.[1] In epidemiological studies, the occurrence of the colon and rectal cancers is still not distinguished, because the anatomical location is adjacent and symptoms are generally almost the same. Requires further clinical and biomedical examination to differentiate colon and rectal cancer.[2] About 30 percent of colorectal cancer cases were rectal cancer. Although the incidence rate is lower than colon cancer, the rate of recurrence and therapy resistance in rectal cancer is higher. Standard therapy in rectal cancer known as "trimodality therapy", consists of neoadjuvant chemoradiotherapy, surgery and adjuvant chemotherapy. There is about 40 percent of rectal cancer patients who have recurrence and resistance after treatment.[3,4]

Recurrence and resistance after therapy can be triggered by hypoxic conditions in the tumor microenvironment, especially in locally advanced solid tumors. Hypoxia in the tumor microenvironment is a condition of oxygen lacking supply in cells and tissues, due to uncontrolled cell proliferation, energy metabolism switching and abnormal growth of blood vessels.[5] Cancer cells that have adapted with hypoxia play a role in therapy resistance through inhibition of therapeutic drug delivery and absorption by cells, and low oxygen levels reduce the effects of chemotherapy cytotoxicity.[6,7] Hypoxic conditions could modify cancer cell metabolism by increase glycolysis rate and Glucose transporter-1 (GLUT-1) expression, inhibit oxidative phosphorylation and induce hypoxia-inducible factor-1 (HIF-1) expression.[8] Glucose transporter-1 is a membrane cell receptor that facilitates glucose uptake by the passive transport mechanism into the cytoplasm. Cancer cells dominantly use aerobic glycolysis as the main pathway in glucose metabolism to obtain adequate energy in a fast and short time. This abnormality is known as the Warburg effect.[9] The more glucose transfer into the cell, the higher GLUT-1 expression. Glucose transporter-1expression usually increases in cancer cells and associated with cancer progression.[10]

Hypoxia also triggers pro-angiogenesis factors expression in large numbers, such as vascular endothelial growth factor A (VEGF-A). In cancerous conditions, VEGF-A stimulates new blood vessel formation from nearby capillaries to help tumor cells obtaining adequate oxygen and nutrients.[11] Vascular endothelial growth factor-A also regulates abnormal vascularization in tumor microenvironments such as excessive branching, leaky blood vessels and dead blood end-vessels. Increasing VEGF-A expression is associated with carcinogenesis, metastasis and tumor cell invasion. Vascular endothelial growth factor-A also becomes a cytokine marker of poor prognosis after radiotherapy.[12] Increasing GLUT-1 and VEGF-A expression contribute to maintain tumor microenvironment, so that cancer cells can survive, proliferate continuously, metastasis and resistant to a given therapy.[5] Through this study, the authors want to determine the correlation between GLUT-1 and VEGF-A mRNA expression and analyze its association with the prognosis of the rectal cancer patients who received chemoradiation.

METHODS

Patients and samples

This research used Cohort design and involved 16 patients with locally advanced rectal cancer in 2017 and 2018. Inclusion criteria of patients were having clinical tumor size, node status and metastasis (TNM) stage II or III without metastasis and history of other malignancies based on histopathological examination, colonoscopy and Magnetic Resonance Imaging (MRI) at Kariadi Hospital Semarang. The patients were given chemoradiotherapy at RSUP dr. Kariadi Semarang using capecitabine orally for chemotherapy and radiation dose were 45-50 Gy with 1.8-2 Gy fraction for 6-8 weeks.

Blood samples were taken from peripheral venous as much as 5 mL in the EDTA vacutainer at the time before and after chemoradiotherapy. Blood serum was isolated by centrifugation 3000 rpm for 15 minutes. Blood serum was separated from the blood and aliquoted, then it was stored at -80^{0}C until further analysis.

RNA isolation and cDNA synthesis

Ribonucleic acid (RNA) was isolated from 200 µl of serum using miRCURY RNA Isolation Kit for Biofluids (Qiagen, China), following the procedure from the manufacture's guideline. Ribonucleic acid concentration and purity were counted using Nanodrop (NanoVue Plus, GE Healthcare, Life Science). Pure RNA was eluted in RNAse-free water and stored in the -80^{0}C refrigerator. Synthesis of cDNA was carried out by reverse transcription reactions using ReverTraAce qPCR RT Master Mix with gDNA remover (Toyobo) and performed using

a thermal cycler (Applied BiosystemsTM A24811l) following the manufacturer's guidelines. The results of cDNA synthesis were stored at -20^{0}C until further analysis.

Quantitative real-time PCR (qRT-PCR)

Analysis of mRNA expression was performed using fluorescent SYBR Green (Bioline SensiFAST SYBR No-ROX kit) and samples were run on CFX96 Touch TM Real-Time PCR Detection System (Bio-Rad, USA) in a total volume 20 µl. β-actin was used as a reference gene. The primer sequence of each mRNA was shown in Table 1. The end result of mRNA expression in the form of a cycle of threshold (Ct) or cycle of quantification (Cq) was quantified using Livak's method relative quantification ($2^{-\Delta\Delta Ct}$) to obtain the fold changes.

Table 1. Primer of mRNA

mRNA		Primer Sequence
β-actin	*Forward*	5'-CGCGAGAAGATGACCCAGATC-3'
	Reverse	5'-TCACCGGAGTCCATCACGA-3'
GLUT-1	*Forward*	5'-TCTGGCATCAACGCTGTCTTC-3'
	Reverse	5'-CGATACCGGAGCCAATGGT-3′
VEGF-A	*Forward*	5'-GCACCCATGGCAGAAGG-3'
	Reverse	5'-CTCGATTGGATGGCAGTAGCT-3'

Data analysis

Patients characteristic is shown as total number (n) and percentage, or mean plus/minus and standard deviation, or minimal and maximal score. The normality data was analyzed using P value from Shapiro-Wilk. The mean difference before and after treatment were analyzed using Wilcoxon rank-test. Correlations between variables were analyzed using Spearman's rank correlation. The association of GLUT-1 and VEGF-A mRNA expression to the patient's characteristic was assessed using χ^2 test (Chi-square, Fisher's exact test). The association power was evaluated with Odds Ratio (OR) equivalent to the 95% Confident Interval (CI). Statistically significant was regarded when P-value <0,05.

This study has approved by Medical Faculty of Diponegoro University and Kariadi Hospital Ethics Committee number 14/EC/ FK-RSDK/I/2017, then continued by the Medical, Public Health and Nursing Faculty Ethics Committee, Universitas Gadjah Mada Ref No KE/FK/1008/ EC/2018. All respondents approved the informed consent before register in this research.

RESULTS

Clinicopathological characteristics of the patient

Patient's clinicopathological characteristics were shown in Table 2. Patients were dominated by males, with an average age was 47 years. Based on anatomical pathology results, tumor differentiation mostly good, with the depth of tumor invasion (T stage) mostly at T3-T4 stage which means that tumor cells were invaded the visceral surface of the peritoneum. Tumor spreading to regional lymph nodes (N1-N2) occurs in majority of patients. The average early carcinoembryonic antigen (CEA) level exceeds the normal range (>5 ng/mL). Carcinoembryonic antigen is one of the blood-based biomarkers that used to determine the prognosis of the rectal cancer patient.

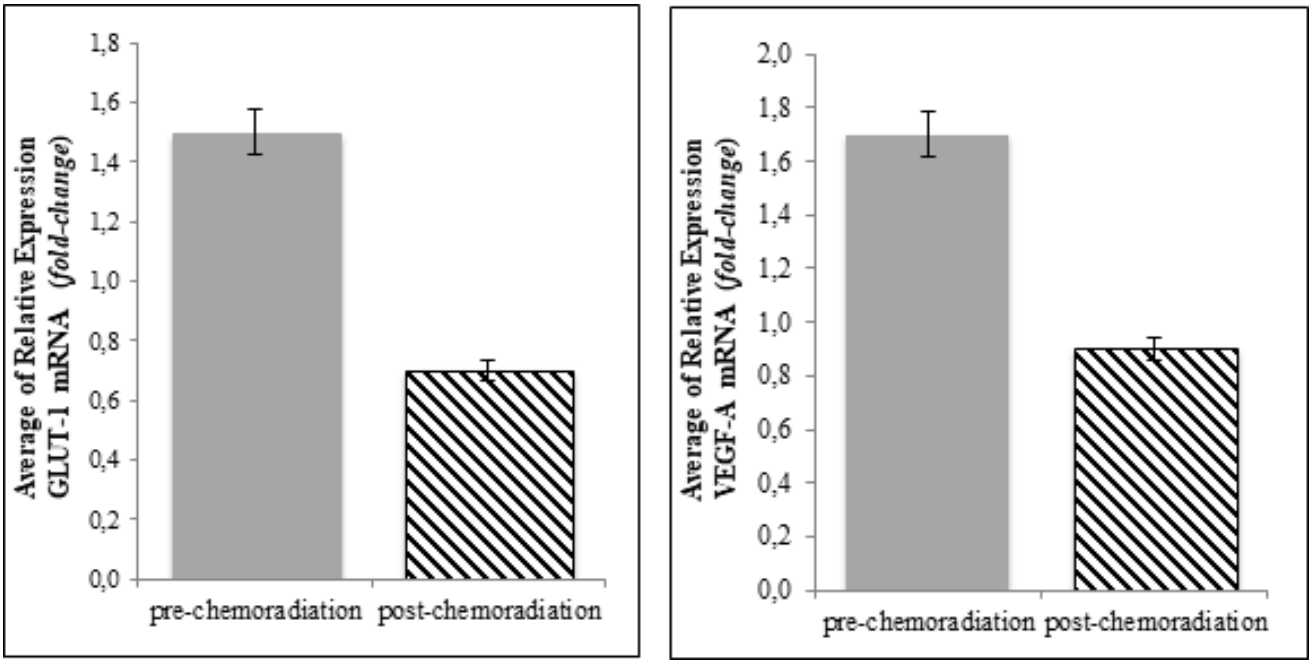
Table 2. Patient's clinicopathological characteristics

Clinicopathological of Patients	
Sex, n (%)	
Male	9 (56,3)
Female	7 (43,7)
Age	
Mean ± SD (min-max)	46,6 ± 12,97 (26-70)
Patological Differentiation, n (%)	
Well	13 (81,3)
Moderate	2 (12,5)
Poor	1 (6,3)
Early T stage, n (%)	
T1	2 (12,5)
T2	2 (12,5)
T3	6 (37,5)
T4	6 (37,5)
Early N stage, n (%)	
N0	1 (6,3)
N1	9 (56,2)
N2	6 (37,5)
Early Clinical TNM stage, n (%)	
II	1 (6,3)
III	15 (93,7)
Early CEA level (ng/mL)	
Mean ± SD (min-max)	42,7 ± 78,8 (0,5 – 201)

SD=standard deviation; n=amount of subject; TNM=*Tumor size, Node status, Metastasis*;
CEA=*carcino embryonic antigen*; min=minimal; max=maximal

Mean difference of GLUT-1 and VEGF-A expression

Expression of GLUT-1 and VEGF-A mRNA were observed before and after chemoradiation. The results were shown in Figure 1.



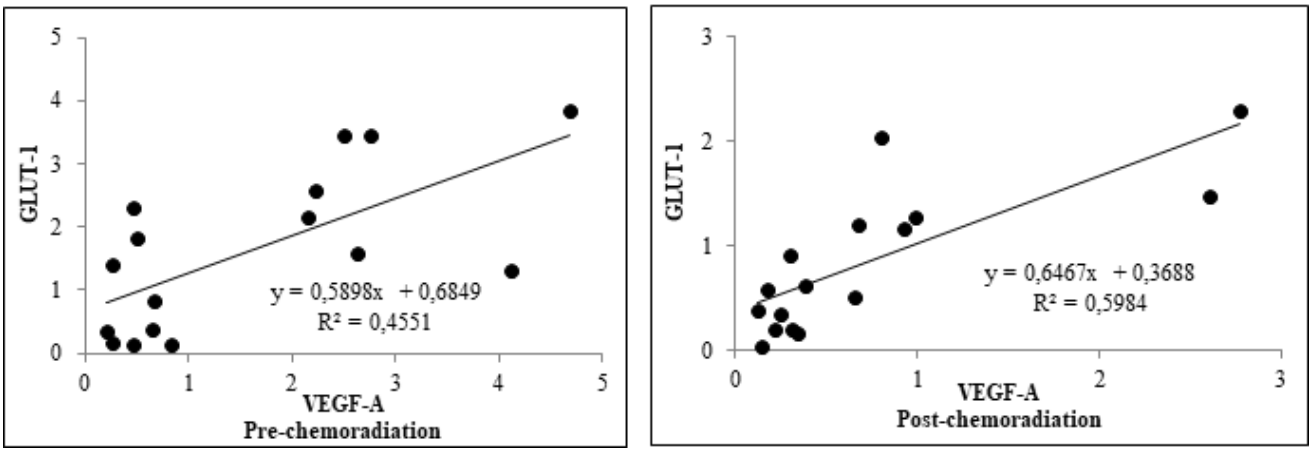
Wilcoxon signed ranks test, *P-value significant < 0,05

Figure 1. Average difference of GLUT-1 (left) and VEGF-A (right) mRNA relative expression at before and after chemoradiation

Based on average difference analysis, GLUT-1 mRNA relative expression significantly decreased 2,14 times after chemoradiation (P=0,044). Meanwhile VEGF-A mRNA relative expression also significantly decreased 1.9 times after chemoradiation (P=0,03).

Correlation between GLUT-1 and VEGF-A mRNA expression

Based on mRNA expression in each patient, the correlation of GLUT-1 and VEGF-A mRNA expression can be determined before and after chemoradiation. The results of correlation analysis are shown in Figure 2.



Spearman's rank correlation test; r = coefficient of correlation; y = equation of correlation; R = coefficient of determination; *P-value significant < 0,05

Figure 2. Correlation between GLUT-1 and VEGF-A mRNA expression at the time before (left) and after (right) chemoradiation

Based on correlation analysis, GLUT-1 and VEGF-A mRNA expression showed a significant and strong positive correlation (P <0.05; r> 0.5), both before and after chemoradiation. This was means that increasing or decreasing of GLUT-1 and VEGF-A mRNA expression will be affected on one each other. The more increase GLUT-1 mRNA expression, the more enhance VEGF-A mRNA expression, and vice versa. Glucose transporter-1 mRNA expression could affect 45,5 percent VEGF-A mRNA expression at before chemoradiation (R^2 = 0,455) and 59.8 percent at after chemoradiation (R^2 = 0,598).

Association of GLUT-1 and VEGF-A mRNA expression with clinicopathology patient

Association between GLUT-1 and VEGF-A mRNA expression with clinicopathology patient is shown in Table 3. There were no significant association between GLUT-1 and VEGF-A mRNA expression with sex, age, patological anatomy differentiation, early T stage, early N stage, early clinical TNM stage, and early CEA level (P>0,05) of rectal cancer patient. More than half of total patient with poor prognosis tend to have elevating GLUT-1 and VEGF-A expression. Patient with poor prognosis have significantly increasing of GLUT-1 mRNA expression 18 times (OR=18; 95% CI=1,2 – 261; P<0,05). Meanwhile, increasing of VEGF-A mRNA expression did not associate significantly with patient prognosis (P>0,05), but majority of patient with poor prognosis have elevated VEGF-A expression.

DISCUSSION

Neoadjuvant chemoradiation as preferable therapy for stage II or III rectal cancer had proven can reduce local relapse, increase long-term survival and optimize surgery. Neoadjuvant chemoradiation in patients with a good response can reduce tumors up to 60 percent and reduce local recurrence. Some of the patients who can not respond to this therapy usually show a worse prognosis.[13] Poor prognosis, resistance and relapsing are associated with the presence of hypoxic conditions in the tumor microenvironment. Hypoxic conditions are related to genomic instability, metastases, sustaining proliferative signaling, avoiding immune destruction, inducing angiogenesis and deregulating cellular energetics.[14]

Table 3. Association of GLUT-1 and VEGF-A mRNA and clinicopathology characteristic of patient

Clinicopathology characteristics	GLUT-1 mRNA Expression N (%)		OR (95% CI) P	VEGF-A mRNA Expression N (%)		OR (95% CI) P
	Decrease	Increase		Decrease	Increase	
Sex						
Male	3 (33,3)	6 (66,7)	1,2 (0,1-10,7)	4 (44,4)	5 (55,6)	0,5 (0,3-0,9)
Female	2 (28,6)	5 (71,4)	0,63	0 (0)	7 (100)	0,09
Age						
< 50 years	2 (20)	8 (80)	0,2 (0,03-2,3)	1 (10)	9 (90)	0,1 (0,01-1,5)
≥ 50 years	3 (50)	3 (50)	0,3	3 (50)	3 (50)	0,12
Differentiation						
Well	3 (23)	10 (77)	0,1 (0,01-2,3)	3 (23)	10 (77)	0,6 (0,04-9,1)
Moderate – Poor	2 (66,7)	1 (33,3)	0,21	1 (33,3)	2 (66,7)	0,61
Early T Stage						
T1 – T2	1 (25)	3 (75)	0,67 (0,05-8,6)	0 (0)	4 (100)	1,5 (1-2,2)
T3 – T4	4 (33,3)	8 (66,7)	0,63	4 (33,3)	8 (66,7)	0,52
Early N Stage						
N0	0 (0)	1 (100)	1,5 (1-2,1)	0 (0)	1 (100)	1,3 (1-1,8)
N1 – N2	5 (33,3)	10(66,7)	0,68	4 (26,7)	11 (73,3)	0,75
Early Clinical TNM Stage						
II	0 (0)	1 (100)	1,5 (1-2,1)	0 (0)	1 (100)	1,4 (1-1,8)
III	5 (33,3)	10(66,7)	0,69	4 (26,7)	11 (73,3)	0,75
Early CEA Level						
Normal (≤ 5 ng/mL)	2 (50)	2 (50)	3 (0,3-31)	2 (50)	2 (50)	5 (0,4-59)
Elevated(>5 ng/mL)	3 (25)	9 (75)	0,36	2 (16,7)	10 (83,3)	0,24
Prognosis after chemoradiation						
Good	4 (66,7)	2 (33,3)	18 (1,2-261)	3 (50)	3 (50)	9 (0,6-123)
Poor	1 (10)	9 (90)	0,036*	1 (10)	9 (90)	0,12

Chi-square test (Fisher's exact test) *P-value significant < 0,05

OR = Odds Ratio; CI = confident interval

Hypoxic conditions drive tumor cells to modify cellular energetic metabolism pathways to survive. Deregulating energy metabolism are needed by cancer cell to obtain adequate nutrition in a stressful environment due to oxygen lack. In normal condition, the normal cell uses oxidative phosphorylation to produce energy, meanwhile under hypoxic conditions, cancer cells switch cellular energetics turn into aerobic glycolysis. Aerobic glycolysis produces only 2 ATP molecules, while oxidative phosphorylation produces 36 ATP molecules. Aerobic glycolysis produces less energy but tends to be faster than oxidative phosphorylation to response increasing energy requirements of cancer cells. Cancer cells need energy in a fast time and large amounts to support fast cell growth and continuous proliferation. [9]

Cancer cells need large amounts of glucose to produce ATP for energy metabolism. Glucose enters the cell by diffusion via glucose transport protein in the cell membrane, which is known as the glucose transporter (GLUT) family. The high demand ~~of~~ for glucose could increase GLUT expression, mainly GLUT-1. Glucose transporter-1 regulates glucose concentration gradient and ensures adequacy for energy metabolism.[15] Its expression increases in various types of solid tumors and associated with tumor aggressiveness, cancer stage and poor prognosis in cancer patients. Increasing of GLUT-1 expression also correlates with cancer cell metastases to lymph nodes.[16] In mouse animal model, increasing of GLUT-1 expression associate with resistance to multiple anti-cancer therapies, including chemotherapy drug vincristine.[17] In this study, increasing GLUT-1 mRNA expression associate with poor prognosis in rectal cancer patients. Our result in line with Brophy et al.[18] Study, which show increase in GLUT-1 expression associate with poor response to chemoradiation and is correlated with a worse prognosis. Tumors with GLUT-1 negative have 70 percent probability of good response to therapy, whereas tumors with GLUT-1 positive only have 31 percent well response. Glucose transporter-1 is potential to be a good predictive biomarker for chemoradiation response in rectal cancer. This study has limitation in small amount of subject sample, so we suggest more amount of subject sample by massive examination will obtain a more precise result.

In this study, we also observe the relationship of VEGF-A mRNA expression with patients prognosis. We did not find any significant association statistically, nevertheless based on the amount of patients we find a tendency that patients with poor prognosis have to elevate VEGF-A mRNA expression. Based on Buka et al.[19] study, elevating VEGF expression is correlated with poor prognosis and a high incidence of liver metastases. Vascular endhotelial growth factor-A expression increase in 50 percent of patients of colorectal cancer and associate with cancer cell metastases to lymph nodes and others organ. Its expressions were found very high in adenocarcinoma, but lower in normal colorectal mucosal tissue. Vascular endhotelial growth factor-A is a heparin-binding glycoprotein, the most potent pro-angiogenesis cytokines of tumor cells. Angiogenesis enables tumor cells to enter the circulation system and metastasis. Vascular endhotelial growth factor-A induces new blood vessel formation and capillaries by increase endothelial cell permeability to support cancer tissue growth rapidly. Decreasing of VEGF-A mRNA expression after chemoradiation was used as a marker for increasing patient survival rate.[20] High expression of VEGF-A associate with tumor-associated macrophage (TAM), which plays a role in tumor microenvironment remodeling after radiation or chemotherapy, so that tumor cells become resistant to the therapy.[21]

This study also showed a significant positive correlation between GLUT-1 and VEGF-A mRNA expression. Glucose transporter-1 plays a role in mediating glucose uptake as an energy source for cancer cells, while VEGF-A initiates angiogenesis to obtain oxygen supply and nutrients delivery to the tumor microenvironment.[22] Vascular endhotelial growth factor-A could increase GLUT-1 expression by influencing translocation of transporters in the cytoplasm to the plasma membrane.[23] Correlation between GLUT-1 and VEGF-A molecularly involves phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway activation. Phosphatidylinositol 3-kinase/Akt pathway becomes over-reactive in tumor conditions and triggers other pathway responses to support tumor cell progression, growth and proliferation to increase resistance and motility of tumor cells. The activation of the PI3K/Akt pathway responsible for radiation therapy resistance because it can modify oncogenes expression to optimize cancer cells growth.[24] Phosphatidylinositol 3-kinase/Akt pathway regulates glucose entry through cell surface and GLUT-1 activity. Activation of the PI3K/Akt pathway also induces VEGF-A expression through activation of HIF-1α (hypoxia-induced factor). Likewise, VEGF-A can activate PI3K/Akt pathway to protect cancer cells from radiation.[25] Inhibition of the PI3K/Akt pathway can reduce GLUT-1 and VEGF-A mRNA expression.[24]

In conclusion, our results study support previous studies that GLUT-1 regulation relates one to each other with VEGF-A. Glucose transporter-1 and VEGF-A mRNA expression have a significant and strong positive correlation in the rectal cancer patients. Increasing GLUT-1 mRNA expression significantly associated with the poor prognosis of the rectal cancer patients. Our finding suggests that GLUT-1 mRNA expression from blood sample was a potential biomarker to predict the prognosis of the rectal cancer patients who received chemoradiation.

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In silico analysis of antihypertensive and hepatotoxicity potential of the n-butanol fraction of the methanol extract of of cantaloupe (*Cucumis melo var. cantalupensis*)

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Abstrak

Latar belakang: *Hipertensi merupakan faktor risiko utama penyakit kardiovaskular. Penduduk Indonesia cenderung mengkonsumsi herbal dalam terapi hipertensi dalam mempertahankan kadar tekanan darah seperti buah blewah (Cucumis melo var. cantalupensis). Namun mekanisme kerja buah blewah dalam menurunkan tekanan darah, dan potensi toksisitasnya jika dikonsumsi dalam jangka panjang masih belum jelas. Tujuan studi ini adalah untuk menganalisis mekanisme antihipertensi dari buah blewah dan potensi toksiknya melalui pendekatan in silico.*

Metode: *Bubuk blewah kering dimaserasi menggunakan metanol absolut, difraksinasi menggunakan n-butanol. Uji fitokimia dilakukan dengan metode LC-MS, kemudian senyawa bioaktif ditelusuri hingga SMILESnya di PubChem. Analisis QSAR untuk analisis potensi antihipertensi dilakukan dengan PASS server. Kelas toksisitas dan potensi hepatotoksisitas dianalisis menggunakan ProTox-II, dilanjutkan dengan analisis networking menggunakan STITCH dan STRINGdb.*

Hasil: *Setidaknya terdapat 434 jenis senyawa yang terdapat pada fraksi n-butanol dari ekstrak metanol buah blewah (FBEMB). Berdasarkan analisis STITCH dan STRINGdb, FBEMB dapat bekerja dalam menurunkan tekanan darah melalui mekanisme aksi seperti senyawa amlodipine, yang menstabilkan saluran kalsium tipe-L yang terisi tegangan dalam konformasi tidak aktifnya. Dengan demikian, mencegah kontraksi myocyte yang bergantung pada kalsium dan vasokonstriksi. FBEMB mungkin berpotensi hepatotoksik melalui mekanisme kerja senyawa seperti itrakonazol yang menghambat enzim sitokrom P450 yang mempengaruhi gangguan pada sintesis ergosterol, dan efavirenz yang memiliki efek neurotoksik. Penghambatan sitokrom P450 dapat menyebabkan toksisitas obat dan kerusakan hati.*

Kesimpulan: *FBEMB dapat bekerja dalam menurunkan tekanan darah melalui mekanisme penstabilan saluran kalsium tipe-L yang terisi tegangan dalam konformasi tidak aktifnya.* ***(Health Science Journal of Indonesia 2020;11(2):106-14)***

Kata kunci: *in silico, antihipertensi, hepatotoksisitas, blewah*

Abstract

Background: Hypertension is a major cardiovascular disease risk factor. Indonesian people tend to consume herbal medicine to maintain hypertension therapy, i.e cantaloupe (*Cucumis melo var. cantalupensis*). However, the mechanism of action of cantaloupe in lowering blood pressure and toxicity potential for long term consumption is unclear. The study aimed to analyze the antihypertensive mechanism of cantaloupe and its toxic potential through the *in silico*

Methods: The dried cantaloupe powder was macerated using absolute methanol, then fractionated using n-butanol. The phytochemical test was done by LC-MS method, then the bioactive compounds were traced to their SMILES in the PubChem. The QSAR analysis of the antihypertensive potential was done using the PASS server. The toxicity class and hepatotoxicity potential were analyzed using ProTox-II, followed by networking analysis using STITCH and STRINGdb.

Results: At least 434 types of compounds were shown in the n-butanol fraction of the methanol extract of cantaloupe (BFMEC). Based on the networking analysis, BFMEC may work in lowering blood pressure through the action mechanism of the amlodipine compound-like, which stabilizes voltage-gated L-type calcium channels in an inactive conformation, thus, prevents calcium-dependent myocyte contraction and vasoconstriction. BFMEC presumably has hepatotoxic through the action mechanism of itraconazole compound-like inhibited cytochrome P450-dependent enzymes, affecting the impairment of ergosterol synthesis, and efavirenz which has neurotoxic effects. The inhibition of cytochrome P450 may cause drug toxicity and liver damage.

Conclusion: BFMEC may work in lowering blood pressure through the action mechanism which stabilizes voltage-gated L-type calcium channels in an inactive conformation. ***(Health Science Journal of Indonesia 2020;11(2):106-14)***

Keywords: *in silico*, antihypertensive, hepatotoxicity, cantaloupe

Hypertension is a major risk factor in cardiovascular disease. Globally, there are about one third of people with hypertension are undiagnosed, and half of those diagnosed do not take antihypertensive drugs. [1] The drug can be considered as a xenobiotic group, which are compounds originating from outside the body that enters the body. [2] The liver plays an important role in the metabolism of xenobiotics, making this organ vulnerable to chemicals that are exposing ubiquitously. Most of the liver damage caused by chemicals begins with the metabolism of chemicals, such as reactive intermediates such as electrical compounds or free radicals, which can alter the structure and function of cellular cell molecules. [3]

Indonesian people tend to consume herbal medicine to maintain good health[4]jamu is still very popular in rural as well as in urban areas. Based on its traditional use jamu is being developed into a rational form of therapy, by herbal practitioners and in the form of phytopharmaceuticals. Jamu has acquired a potential benefit, both economically and clinically. We surveyed the most frequently used plants in jamu that have also been investigated regarding their constituents and pharmacological effects. The Indonesian government has divided the preparation of medicinal plants into three categories, i.e. jamu, standardized herbal medicines and fitofarmaka (phytomedicines, including hypertension therapy.[5] Cantaloupe (*Cucumis melo* L) is often used in hypertension therapy in Indonesia, especially among Javanese people. Some studies showed that *Cucumis melo* L inhibits phenylephrine-mediated vasoconstriction in mice. [6] But it is not yet known the mechanism of action of cantaloupe in lowering blood pressure. It also not known the potential of toxicity if consumed in the long term.

Nowadays, the process of drug discovery which begins with the bioinformatics method is very popular. This is because the method efficiently reduce the occurrence of trials and errors during *in vitro* and *in vivo* studies. Therefore, this study aimed to analyze the antihypertensive and hepatotoxicity potential of the n-butanol fraction of the methanol extract of cantaloupe (*Cucumis melo var. cantalupensis*) (BFMEC) through the bioinformatics approach.

METHODS

Tools and materials

The materials used in extraction are cantaloupe fruits, methanol absolut as maceration solvent, and n-Buthanol as fractionation solvent. Material used in Phytochemical tests were 0.1% Formic acid in Water, 0.1% Formic acid in Acetonitrile as LC-MS Solvent and Hypersil GOLD aQ 50 x 1 mm x 1.9 u. Tools used in maceration were analytical balance, beaker glass, wood stirrer, cloth, funnel, rotary evaporator, and extract bottle. Tools used in phtochemical test were Thermo Scientific Dionex Ultimate 3000 RSLCnano with microflow meter. The tool used for tracing the canonical SMILES was PubChem (https://pubchem.ncbi.nlm.nih.gov). Bioinformatic tools used in this study were Way2Drug (http://www.pharmaexpert.ru/passonline/) to analize antihypertensive potential, STITCH (http://stitch.embl.de/) to analize ligan-protein interactions, STRINGdb (https://string-db.org) to analize target proteins interactions.

Cantaloupe extraction and phytochemical testing

Cantaloupe fruits we obtained in April 2018 from Materia Medica Batu, were cut and air dried for 7 days. The dried cantaloupe was crushed into powder for subsequent maceration. Extraction was carried out by maceration method. Two hundred grams of cantaloupe powder were macerated in 3 liters of absolute methanol in beaker glass for 3 days. The extract is then filtered and dried using a rotary evaporator. Fractionation is done using n-Butanol. The BFMEC stored at 4^{o}C until used. Phytochemical tests were carried out by the method Liquid Chromatography-Mass Spectrometry using Thermo Scientific Dionex Ultimate 3000 RSLCnano with microflow meter. Solvents: A= 0.1% Formic acid in Water; B= 0.1% Formic acid in Acetonitrile. Analytical column: Hypersil GOLD aQ 50 x 1 mm x 1.9 u particle size. Analytical flow rate: 40 uL/min. Flow gradient: Run time 30 minutes; Column oven 30 C.

Analysis of antihypertensive potential

The names of the compounds contained in BFMEC from LC-MS test result are traced to the canonical SMILES via PubChem (https://pubchem.ncbi.nlm.nih.gov). BFMEC antihypertensive potential was analyzed by inputting SMILES in Way2Drug (http://www.pharmaexpert.ru/passonline/), potentially antihypertensive compounds with Pa> 0.3 were selected. The interaction of antihypertensive compounds with their predicted functional partners was analyzed using STITCH (http://stitch.embl.de/). Interactions between target proteins were analyzed using STRINGdb (https://string-db.org).

Analysis of hepatotoxicity potential

The potential of BFMEC hepatotoxicity was analyzed by inputting SMILES on ProTox-II (http://tox.charite.de/protox_II/). In ProTox-II, the hepatotoxicity potential of each compound was indicated by "active" or "inactive" information along with the probability of hepatotoxicity score. We chose compounds that fell into the "active" category. Interactions of antihypertensive compounds with predicted functional partners were analyzed using STITCH. Interactions between target proteins were analyzed using STRINGdb.

Ethical clearance

This research doesn't require ethical clearance due to the absence of human involvement as research subjects.

RESULTS

Cantaloupe extraction and phytochemical testing

Extraction carried out by maceration with methanol solvent on 200 grams of cantaloupe powder simplicia produced 16.03 grams of crude extract and 2.214 grams of n-butanol fraction. Phytochemical tests were carried out using the LC-MS method, showed that there were 434 types of bioactive compounds (Figure 1).

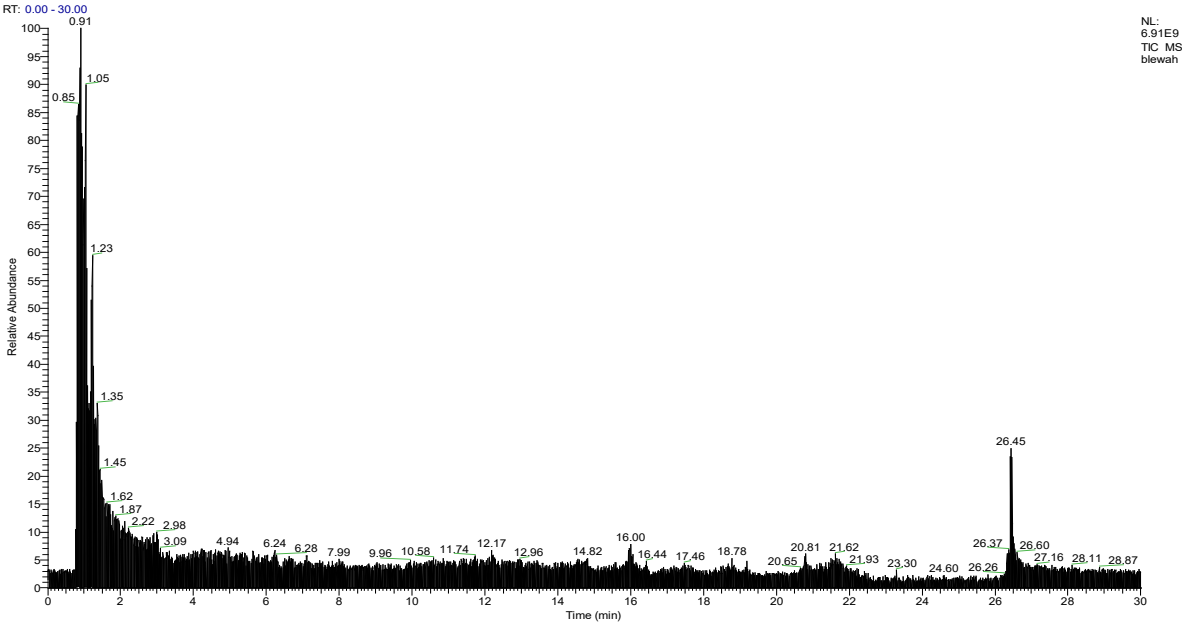

Figure 1. Chromatogram of the n-butanol fraction of the methanol extract of cantaloupe (Cucumis melo var. cantalupensis) (BFMEC) phytochemical testing using LC-MS method

We traced to the canonical SMILES via of the 434 bioactive compounds via PubChem (https://pubchem.ncbi.nlm.nih.gov). With these canonical SMILES we traced their antihypertensive potential. BFMEC antihypertensive potential was analyzed by inputting the canonical SMILES of 434 bioactive compounds in Way2Drug (http://www.pharmaexpert.ru/passonline/), potentially antihypertensive compounds with Pa> 0.3 were selected.

Analysis of antihypertensive potential

Based on the results of the analysis of antihypertensive potential on Way2Drug, there were 24 compounds that had the potential as antihypertensive drugs in BFMEC marked with a value of Pa> 0.3 (data not shown). However, only 3 compounds (Table 1) that displayed direct interaction with the target proteins (Table 2); amlodipine (2162), okadaic acid (446512), and terfenadine (5405), respectively.

Table 1. The active compound in the BFMEC with the anti-hypertensive potential

No	Name	Compound ID	Pa score of antihypertensive
1	[Similar to: Amlodipine; ΔMass: 92.0960 Da]	2162	0,917
2.	[Similar to: Okadaic acid; ΔMass: 540.3353 Da]	446512	0,459
3.	[Similar to: Terfenadine; ΔMass: -18.0060 Da]	5405	0,329

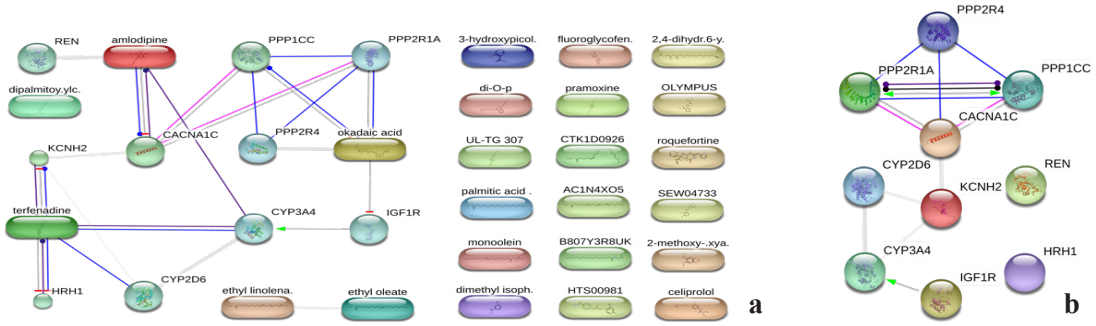

Figure 2a. Interactions of the active compounds in the BFMEC with the antihypertensive potential.
2b. Interactions between predicted functional partners of the active compound in the BFMEC with the antihypertensive potential

Table 2 showed predicted functional partners of the active compound in the BFMEC with the potential of antihypertensive. Figure 2b showed the interaction between proteins that were targeted by BFMEC compounds. There were interrelated interactions between PPP2R1A, PPP2R4, PPP1CC, and CACNA1C. PPP2R1A, PPP2R4, and PPP1CC are a group of protein phosphatase. IGF1R showed positive interaction with CYP3A4. REN and HRH1 didn't show any interaction with other target proteins. In this predicted functional partners showed that KCNH2 interacted with CACNA1C, CYP2D6, and CYP3A4.

Table 2. Predicted functional partners of the active compound in the BFMEC with the antihypertensive potential

No	Protein Name		Action			Score
			Activation	Inhibition	Binding	
1.	KCNH2	potassium voltage-gated channel, subfamily H (eag-related), member 2		V	V V	0,997
2.	CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit		V	V V	0,995
3.	PPP1CC	protein phosphatase 1, catalytic subunit, gamma isozyme		V	V	0,993
4.	HRH1	histamine receptor H1		V	V V	0,991
5.	REN	renin	V			0,991
6.	CYP2D6	cytochrome P450, family 2, subfamily D, polypeptide 6		V	V	0,984
7.	IGF1R	insulin-like growth factor 1 receptor		V		0,975
8.	CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4			V V	0,968
9.	PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha		V	V	0,963
10.	PPP2R4	protein phosphatase 2A activator, regulatory subunit 4		V		0,959

Analysis of hepatotoxicity potential

Based on the results of the analysis of hepatotoxicity potential on ProTox-II, there were 10 compounds in the BFMEC that showed hepatotoxic activity with Pa > 0.3 (data not shown). However, there were only 2 compounds that interacted directly with the target proteins; itraconazole (3793) and efavirenz (64139), mentioned below in the table 3.

Table 3. The active compound in the BFMEC with the hepatotoxicity potential

No	Name	Compound ID	Probability of hepatotoxicity
1.	[Similar to: Itraconazole; ΔMass: 370.0933 Da]	3793	0.88
2.	[Similar to: Efavirenz; ΔMass: 112.9863 Da]	64139	0.83

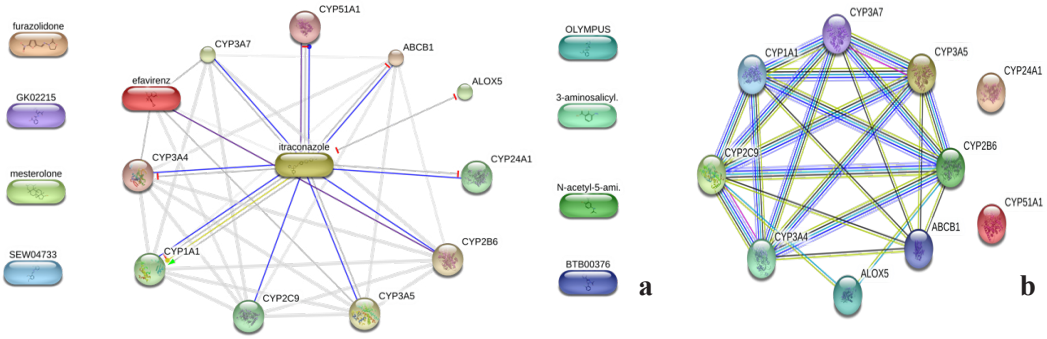

*Figure **3a**. Interactions of the active compound in the BFMEC with the hepatotoxicity potential.*
***3b.** Interactions between predicted functional partners of the active compound in the BFMEC with the hepatotoxicity potential*

Table 4. Predicted functional partners of the active compound in the BFMEC with the hepatotoxicity potential

No	Protein Name		Action				Score
			Activation	Inhibition	Binding	Catalysis	
1.	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1		V	V	V	0,997
2.	CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	V	V	V	V	0,992
3.	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1		V	V		0,988
4.	CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	V	V	V	V	0,979
5.	CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	V	V	V		0,904
6.	CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	V	V	V		0,888
7.	ALOX5	arachidonate 5-lipoxygenase;		V			0,843
8.	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	V	V	V		0,832
9.	CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1		V	V		0,832
10.	CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9		V	V		0,830

Table 4 showed predicted functional partners of the active compound in the BFMEC with the potential of hepatotoxicity. Figure 3b showed the interaction between proteins that were targeted by BFMEC compounds. There were interrelated interactions between CYP51A1, CYP2C9, CYP3A4, ABCB1, CYP2B6, CYP3A5, and CYP3A7. ALOX5 is interrelated to CYP2B6 and CYP2C9.

DISCUSSION

Hypertension is a major risk factor for cardiovascular disease. It's about one third of hypertension sufferers are undiagnosed, and of those diagnosed, about half do not use antihypertensive drugs. The World Health Organization (WHO) estimates that high blood pressure directly or indirectly causes the deaths of at least nine million people globally every year. [7] Antihypertensives can be divided into two major groups, the first group are groups that directly or indirectly block the renin-angiotensin system (RAS), for example, ACE inhibitors, angiotensin receptor antagonists (ARA), direct renin inhibitors (DRI), and β-blockers. Although these drugs have several mechanisms of action, their dominant effect is to cause vasodilation. The second group of drugs works by increasing the excretion of water and sodium and reducing intravascular volume. This drug causing vasodilation via non-RAS pathways, for example, diuretics and calcium channel blockers (CCB). This second group action increases RAS activity through negative feedback. [1]

Cantaloupe extraction and phytochemical testing

Maceration with methanol solvent on 200 grams of cantaloupe powder simplicia produced 16.03

grams of crude extract and 2.214 grams of n-butanol fraction. Phytochemical tests were carried out using the LC-MS method showed that there were 434 types of bioactive compounds (Figure 1). Butanol has more than two carbon atoms and has significant solubility in water [8], shown in Figure 4.

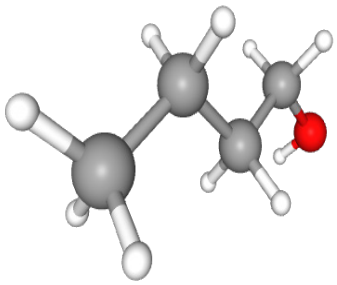

Figure 4. 3D Structure of n-butanol [9]

Butanol is used as a solvent because it can attract semi-polar organic compounds. Semi-polar organic compounds include flavonoids with a slightly lower polarity index below the polarity index of ethyl acetate (polarity index ethyl acetate = 4.3 and n-butanol = 3.9) due to the presence of functional groups attached to it or other compounds that have functional groups are polar. [10]

Anti-hypertensive potential

Based on the results of the analysis of antihypertensive potential on Way2Drug, there were 24 compounds had the potential as antihypertensive drugs in BFMEC marked with a value of Pa> 0.3 (data not shown). However, only 3 compounds (Table 1) displayed direct interaction with the target proteins (Table 2); amlodipine (2162), okadaic acid (446512), and terfenadine (5405), respectively.

Amlodipine has been widely used as 1,4-dihydropyridine calcium channel blockers (DHP-CCBs) as coadministrator with statins for hypercholesterolemia and hypertension. To reduce the myopathy risk, the FDA suggests taking amlodipine caused its ability to interact with CYP3A4 as well as CYP3A5.[11] Other studies also mentioned the amlodipine impact among high-risk blood pressure African-American patients with CYP3A4 genotype.[12] Amlodipine stabilizes voltage-gated L-type calcium channels in an inactive conformation, thus, prevents calcium-dependent myocyte contraction and vasoconstriction.[13] Based on our results, amlodipine interacted directly not only with CYP3A4, but also to REN, and CACNA1C (Figure 2a). Apparently, REN produces angiotensin I from angiotensinogen in plasma. CYP3A4 involve in NADPH-dependent electron transport pathway in the liver microsomes. It has previously been stated that the renin gene is associated with critical hypertension in a number of ethnic groups. [14] A previous study mentioned the effect of amlodipine on REN gene. It was emphasized that blocking of amlodipine calcium channels promotes renin secretion and in vivo expression of the renin gene. These stimulatory effects are almost additive to improvements in renin secretion that arise after renal perfusion pressure decreases unilaterally.[15] CACNA1C is a calcium channel, voltage-dependent, L type, alpha 1C subunit. Downregulation of CACNA1C may lead mitochondrial resilience and oxidative stress in the neuronal cells which is related to affective disorder. [16,17]

Okadaic acid (OA) is a specific inhibitor of fosfoserin. Okadaic acid interacted with PPP2R4, PPP1CC, and PPP2R1A (Figure 2), all three are phosphatases. Because of this, the function of these phosphatases in cells was observed using this class of molecules. When OA binds to the phosphatase protein(s), various proteins inside the infected cell are hyperphosphorylated, which in turn decreases sodium secretion regulation and solvent cell permeability.[18,19] High sodium consumption and elevated blood pressure levels are correlated with water accumulation, increased systemic peripheral tolerance, improvements in endothelial function, changes in large elastic artery structure and function, changes in sympathetic response, and autonomic cardiovascular system neural regulation.[20] While IGF1R, usually overexpresses in the event of cancer.[21] Okadaic acid inhibited IGF1R (Figure 2a) which means it might be able to become an anticancer agent.

Terfenadine interacted with KCNH2, CYP3A4, CYP2D6, and HRH1. Terfenadine inhibits KCNH2 (Figure 2a) which functions to mediate the rapid activation component of delayed rectifying potassium current in the heart. Inhibition of this protein will interfere with calcium transportation. Terfenadine also inhibits HRH1, inhibition of this protein can inhibit nerve transmission mediation in the central nervous system.[22]

Table 2 showed predicted functional partners of the active compound in the BFMEC with the potential of anti-hypertensive. Figure 2b showed the interaction between proteins that were targeted by BFMEC compounds. There were interrelated

interactions between PPP2R1A, PPP2R4, PPP1CC, and CACNA1C. PPP2R1A, PPP2R4, and PPP1CC are a group of protein phosphatase. IGF1R showed positive interaction with CYP3A4. REN and HRH1 didn't show any interaction with other target proteins. In this predicted functional partners showed that KCNH2 interacted with CACNA1C, CYP2D6, and CYP3A4. There was a study found that defects in potassium channel KCNH2 caused numerous congenital and acquired cardiac disease including autosomal-dominant long QT syndrome 2 (LQT2).[23]

Hepatotoxicity potential

Based on the results of the analysis of hepatotoxicity potential on ProTox-II, there were 10 compounds in the BFMEC that showed hepatotoxic activity with Pa > 0.3 (data not shown). However, there were only 2 compounds that interacted directly with the target proteins; itraconazole (3793) and efavirenz (64139) (Table 3).

Some of the adverse effect caused by Itraconazole is inducing heart attack[24], and other effects such as hypokalemia, aspartate aminotransferase elevation, alanine aminotransferase elevation, gastrointestinal disturbances, diarrhea and a skin rash with a high dose >400 mg/day. Itraconazole's mechanism of action triggering heart failure is unknown; however, there were no causes that could lead to heart failures such as asthma, cardiomyopathy and other potential factors in the patient.[25] Based on the networking analysis, itraconazole acts as an inhibitor for CYP51A1, CYP24A1, CYP1A1, CYP3A4, ABCB1, and ALOX5 (Figure 3a). Itraconazole is one of the triazole antifungal agents which inhibit cytochrome P-450-dependent enzymes that affect the impairment of ergosterol synthesis.[26]

The CYP group is the Cytochrome P450 family, in liver microsomes, this enzyme is involved in the NADPH-dependent electron transport pathway.[27] The inhibition of this enzyme by Itraconazole can cause the disruption of calcium homeostasis in cells. Itraconazole also inhibits the ABCB1 protein, an Energy-dependent efflux pump that is responsible for reducing the accumulation of drugs in multidrug-resistant cells. Thus inhibition of this protein causes an increase in drug accumulation in multidrug-resistant cells.[28] Another protein that was inhibited by Itraconazole is ALOX5 (Figure 3). The presence of inhibition in ALOX5 can cause the disruption of the inflammatory process.[29]

Efavirenz interacted with itraconazole and 6 proteins; CYP3A7, CYP3A4, CYP1A1, CYP2C9, CYP3A5, and CYP2B6, respectively. Itraconazole interacted with efavirenz and 10 proteins; CYP51A1, ABCB1, ALOX5, CYP24A1, CYP3A7, CYP3A4, CYP1A1, CYP2C9, CYP3A5, and CYP2B6, respectively. These proteins are in the cytochrome P450 family, except ABCB1 and ALOX5. Efavirenz showed an interaction with CYP2B6 which was marked by a purple line which showed catalytic action with a score of 0.800. As for the Itraconazole compound and other proteins, there was no significant interaction. It was reported that Efavirenz has neurotoxic effects.[30,31]

Figure 3b showed the interaction between target proteins in the hepatotoxicity mechanism. Table 4 showed predicted functional partners of the active compound in the BFMEC with the hepatotoxicity potential. This interaction involved cytochrome P450 family. Cytochrome P450 is classified as hemeprotein responsible in the drug metabolism and xenobiotic.[32] Drugs with common pathway may involve in the drug-drug interaction[33], however not all drugs have Cytochrome P450 activity. Drugs with Cytochrome P450 activity may be inhibitors, inducers, or substrates for a specific Cytochrome P450 enzymatic pathway, which may alter the metabolism of agents simultaneously administrated. Drugs that inhibit enzymatic pathway of Cytochrome P450 may cause increased concentration of other drugs metabolized by the same pathway and resulting in drug toxicity.[32] In conclusion, the BFMEC may work in lowering blood pressure through the action mechanism of the amlodipine compound-like, which stabilizes voltage-gated L-type calcium channels in an inactive conformation, thus, prevents calcium-dependent myocyte contraction and vasoconstriction. The BFMEC may potentially hepatotoxic through the action mechanism of itraconazole which inhibits cytochrome P-450-dependent enzymes that affect the impairment of ergosterol synthesis and efavirenz which has neurotoxic effects.

Acknowledgments

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Risk factors of death among children hospitalized with social insurance (BPJS): a cross sectional study using hospital claim data

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Abstrak

Latar belakang: *Angka kematian di rumah sakit merupakan salah satu indikator yang digunakan untuk mengukur kinerja dan kualitas pelayanan. Tujuan penelitian ini untuk menganalisis faktor risiko kematian pada anak yang dirawat dengan BPJS kesehatan di satu rumah sakit di Jakarta.*

Metode: *Penelitian potong lintang pada satu rumah sakit pemerintah di Jakarta. Sampel menggunakan semua data klaim pasien BPJS selama periode Januari - Desember 2017. Semua pasien BPJS berusia di bawah 18 tahun yang dirawat dimasukkan dalam analisis. Regresi logistik digunakan untuk menganalisis faktor risiko kematian anak.*

Hasil: *Dari total 18,941 jumlah pasien BPJS yang dirawat, sebanyak 3689 data anak yang dianalisis. Proporsi angka kematian anak selama satu tahun sebesar 7,3%. Kasus dengan tingkat keparahan derajat II memiliki risiko kematian 11,51 kali lipat [rasio odds suaian (ORa) = 11,51; IK*=7,45-17,78*; P = 0,000] dibandingkan tingkat keparahan penyakit derajat I, sedangkan kasus dengan tingkat keparahan derajat III beresiko terhadap kematian 33,97 kali lipat (ORa = 33,97;IK*=19,93-57,91*; P = 0,000). Selain itu, anak yang memiliki indikasi dirawat di ICU meningkatkan risiko kematian* 14,21 *kali lipat (ORa =* 14,21*; IK*=9,15-22,08*; P= 0,000) dibandingkan yang tidak ada indikasi ICU. Kondisi tertentu yang timbul pada periode perinatal meningkatkan risiko kematian anak* 7,65 *kali lipat (ORa =* 7,65 *; IK*=1,81-32,35*;P = 0,006) dibandingkan penyakit pada sistem muskuloskeletal dan jaringan ikat.*

Kesimpulan: *Tingkat keparahan penyakit, indikasi ICU dan kondisi tertentu yang timbul pada periode perinatal adalah faktor risiko kematian anak yang paling sering di rumah sakit.* ***(Health Science Journal of Indonesia 2020;11(2):115-20)***

Kata kunci: *faktor risiko, kematian, anak, BPJS*

Abstract

Background: Hospital death rate is one of the indicators used to measure hospital performance and quality of care, especially the overall hospital death rate. This study aims to analyze the risk factors of death among children hospitalized with social insurance (BPJS) in one hospital in Jakarta.

Method: This was a cross-sectional study conducted in one government hospital in Jakarta. The sample was all individual claim data of BPJS patients who were hospitalized during the period of January to December 2017. All BPJS patients aged below 18 years admitted into the pediatric wards were included in the analysis. The logistic regression was used to analyze the risks of children death

Results: A total of 18.941 BPJS inpatients in the hospital was identified, out of the 3689 met the inclusion criteria. The proportion of death in children during one year was 7.3%. Illness severity level II had 11.51-fold [adjusted odds ratio (ORa)=*11.51;CI*=7.45-17.78*; P=0.000]*] meanwhile severity level III had *33.97*-fold higher risk of children death (ORa=*33.97*; *CI*=19.93-57.91*;P=0.000*) compared to children with severity level I. Children who had ICU indicator increase risk of children death at 14.21 -fold (ORa=14.21*;IK*=9.15-22.08*;P= 0.000)* compared to those who did not have. Furthermore the risk of children death in certain conditions originating in the perinatal period increases by 7.65–fold (ORa=7.65*;IK*=1.81-32.35*;P=0.006)* compared to diseases of the musculoskeletal system and connective tissue.

Conclusion: Illness severity level, ICU indicator and diseases in certain conditions originating in the perinatal period are the most common risk factors for children death in the hospital. ***(Health Science Journal of Indonesia 2020;11(2):115-20)***

Keywords: risk factors, death, children, BPJS

A hospital inpatient ward is a place where necessary care is provided to patients whose health conditions require hospital admission for a certain time.[1] Inpatient care has become the core of services at the hospital and has a significant contribution to hospital revenue.[2] Therefore, the demands for excellent service and quality need to be managed properly. The information of inpatient productivity can be measured through several indicators.[3] Hospital death rate is one of the indicators used to measure hospital performance and quality of care, especially the overall hospital death rate.[4,5] It shows the proportion of patients who die during or immediately after the patients were admitted to the hospital.[6]

Shihab et al reported that National Health Insurance (Jaminan Kesehatan Nasional/JKN) program increases the utilization of hospitalization both in the government and private hospitals.[7] It opens wider access to nationwide inpatient utilization.[7] The roll-out of JKN introduced in 2014 aims to extend financial coverage for health care to at least 95 % of the population by 2019. Nearly half of children (47%) have health insurance programs in 2015. Out of those with insurance, as many as 23% were covered by *Badan Penyelenggara Jaminan Sosial* (*BPJS* / Social Security Agency).[8]

There is an increasing number of hospitalization in children, particularly in the urban areas and large community hospitals.[9] Improving the quality of care for seriously ill children is essential for reducing children death. Many factors were associated with the death rate in children such as mother's age at birth, mother's educational level, and mother's household socioeconomic, several diseases (malaria, diarrhea diseases, respiratory infections, and malnutrition), breastfeeding duration, total health care visits, low birth weight.[10, 11] Beside that living in poor households, living in rural areas, birth rank, birth interval, previous death of a sibling, having other children under 5 years old, contraceptive, type of births, complications, history of previous mortality, antenatal care and place of delivery were also considered to contribute with childhood mortality.[12, 13, 14]

Risk factors data for hospital death is widely assessed from socioeconomic factors, and the use of claim data for hospital evaluation is still limited in Indonesia. Although hospital-based mortality reviews may not reflect the multiple causes of all deaths in a large population, it can examine the causes that contribute to deaths occurring in hospital and identify inadequate care. The present study aimed to analyze the risk factors of death among children hospitalized with BPJS insurance in one hospital in Jakarta.

METHODS

This study was a cross-sectional study conducted in one government hospital in Jakarta. The sample was all individual claim data of BPJS patients who were hospitalized during the period of January to December 2017. The inclusion criteria was BPJS patients aged below 18 years admitted into the pediatric wards. The data with incomplete records was excluded from the analysis.

The outcome of the study was children death (death in children occurs both before and after 48 hours of admission over one year period). The independent variables of interest in this study were age, gender, hospital accommodation level, illness severity level, length of hospital stay (LOS), ICU indicator, ICU length of stay, and diseases category.

Age group was divided into two groups: less than 5 / under 5/ Balita and 5-17 (years). Gender was categorized into two groups: male and female. Hospital accommodation level was divided into three categories first class, second class and third class. Illness severity was grouped into severity level I(without complications or comorbidity), II(mild complications and comorbidity), III(major complications and comorbidity).[15] Illness severity level was automatically generated by the Indonesian Case Based Groups (INA-CBGs) software grouper application after entering the principal and secondary diagnosis. Length of hospital stay was categorized into 7 days or less and more than 7 days (the time from patient admission to the hospital until discharge), ICU indicator was grouped as yes (If the patient is admitted to the ICU during the treatment episode) and no. ICU length of stay was divided as 3 days or less and more than 3 days. Principal diagnosis was used to classify the diseases according to ICD 10 Category (2010). There were 20 disease groups identified in this study and several disease groups with a number of cases less than 100 cases were assigned to "other" groups. The diseases groups were certain infectious and parasitic diseases; diseases of the respiratory system; diseases of the digestive system, diseases of the musculoskeletal system and connective tissue; diseases of the genitourinary system, certain conditions originating in the perinatal period, symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified; injury, poisoning and certain other consequences of external causes; factors influencing health status and contact with health services; others (mental and behavioral disorders; diseases of the nervous system; diseases of the circulatory system; diseases of the skin and subcutaneous tissue; diseases of the ear and

mastoid process; endocrine; nutritional and metabolic diseases; pregnancy, childbirth and the puerperium; diseases of the nervous system; diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism; neoplasms)

The data was tabulated according to various factors included in this study and analyses were performed using the STATA version 9. The logistic regression model was used for multivariate analysis. Variable with a significance level (*p*-value) < 0.25 on bivariate analysis was kept in a multivariate model. Stepwise regression and likelihood ratio test was performed to select the final model.

This study was approved by The Ethics Committee, Faculty of Public Health, University of Indonesia with a letter number: 564/UN2.F10/PPM.00.02/2017. The confidentiality of the patient's information was ensured in such a way that the data will be used for the study purpose only.

RESULTS

A total of 18.941 BPJS inpatients in the hospital was identified, out of the 3689 met the inclusion criteria. The proportion of death in children during one year was 7.3% (271/3689).

As shown in Table 1, compared to the respective reference groups, children under five years, LOS more than 7 days, children who had ICU indicator and ICU LOS more than 3 days were more likely to increase the risk of children death. The higher the severity level of diseases also seems to have a higher risk of death in children. Those who had the risk of death and did not have were similarly distributed in terms of gender and accommodation level (P > 0.05).

In respect with diseases group based on ICD, children who had diseases of respiratory system, infectious and parasitic diseases, certain conditions originating in the perinatal period, congenital malformations, deformations and chromosomal abnormalities, as well as factors influencing health status and contact with health services were more likely to increase the risk of children death compared to those with the musculoskeletal system and connective tissue disease groups.

Table 2 the final model demonstrates severity level II had 11,51-fold while severity level III had 33.97-fold higher risk of children death compared to children with severity level I. In addition, children who had ICU indicator increase risk of children death at 14.21-fold compared to those who did not have. Furthermore, the risk of children death in certain conditions originating in the perinatal period; symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified increase by 7.65–fold and 6.05-fold respectively compared to diseases of the musculoskeletal system and connective tissue.

DISCUSSION

This study showed that the greater the severity of illness, the greater the risk of death in children. The severity of illness is a condition of disorder in the organ system that can be assessed using demographic, clinical, physiological, and laboratory results.[16] The severely ill children have a clinical condition with severe organ systems dysfunction. If the condition was not treated properly and immediately, the risk of sequelae and death would have increased significantly.[17] In the case mix system, the severity is affected by the presence of a complication or comorbidity (secondary diagnosis).[18] The severity indicates how sick and what burden of illness has suffered by the patient. Patients often have additional diseases or medical condition which accompanies the main disease. Therefore, the existence of comorbidity is more likely to have worse initial well-being. Additional diseases will worsen the clinical course of the disease. Martins reported that the patients with one comorbidity have a higher mortality rate compared to patients without comorbidity.[19] However Chang LS et al in Chang-Gung Memorial Hospital-Kaohsiung, Taiwan, found the death in pediatric patients without comorbidities in several cases.[20] It was more common in younger children with the central nervous system and cardiovascular infections, consciousness change, and high liver enzyme levels at admission.[20]

This study found when children were admitted to the ICU during the treatment episode, they had a greater risk of death 14.21-fold compared to those who did not. Most of the patients in ICU have at least one organ failure at the time of death. The death may take place suddenly or unexpectedly due to hemodynamic causes or occur later with many organ failures.[21] Children with ICU indicator was in critical condition or unstable or potentially unstable which poses a threat to the life of the patient. Intensive care provides integral management, optimum care with specialized personnel and equipment. Therefore, ICU allows the treatment for critically ill patients who need close vigilance and had potentially recoverable conditions.[22] The patients should be admitted to the ICU before achieving irreversible health conditions.

This study demonstrated that certain conditions originating in the perinatal period had 7.65-fold for children death compared to the musculoskeletal system and connective tissue. Zhu et al in China reported that the majority of death occurred in infants (42%) over a 10 years period with pneumonia as the largest proportion for the immediate cause of death.[9] Lahmini et al in Marrakech mentioned that among pediatric patients, neonatal mortality was predominant followed by postnatal mortality (1 month to 1 year old).[23] Meanwhile the leading cause of pediatric mortality (at all ages) was neonatal pathologies.[23] The differences with this study may be due to the different data sources as well as different research designs and analyses.

This study has limitations, which did not represent the general population. Several determinants were not available in the claim data such as signs and symptoms, laboratory results, medications prescribed by physicians, and referred from other facilities.

Table 1. Several characteristics and risk of children death

Variable	Children death				Crude OR	95 Confidence interval	*P*
	No (n=3418)		Yes (n=271)				
	n		n				
Age groups (Years)							
Above five	1319	94.5	77	5.5	1.00	Reference	
Under five (Balita)	2099	91.5	194	8.5	1.58	1.21-2.08	0.001
Gender							
Male	2002	93.3	144	6.7	1.00	Reference	
Female	1416	91.8	127	8.2	1.25	0.97-1.59	0.081
Accomodation level							
First class	399	93.0	30	7.0	1.00	Reference	
Second class	858	93.6	59	6.4	0.91	0.58-1.44	0.701
Third class	2161	92.2	182	7.8	1.12	0.75-1.67	0.579
Severity level							
I	2635	98.5	40	1.5	1.00	Reference	
II	650	82.9	134	17.1	13.58	9.44-19.53	0.000
III	133	57.8	97	42.2	48.04	31.97-72.21	0.000
Lenth of hospital stay							
7 or less	2336	94.5	135	5.5	1.00	Reference	
More than 7	1082	88.8	136	11.2	2.17	1.69-2.79	0.000
ICU Indicator							
No	2911	97.4	77	2.6	1.00	Reference	
Yes	507	72.3	194	27.7	11.24	8.47-14.91	0.000
Length of stay in ICU							
3 or less	3225	95.2	162	4.8	1.00	Reference	
more than 3	193	63.9	109	36.1	14.47	10.93-19.15	0.000
Grouping ICD							
Diseases of the musculoskeletal system and connective tissue	132	98.5	2	1.5	1.00	Reference	
Diseases of the respiratory system	181	84.2	34	15.8	12.40	2.93-52.52	0.001
Diseases of the digestive system	242	98.4	4	1.6	1.09	0.20- 6.04	0.921
Diseases of the genitourinary system	118	97.5	3	2.5	1.68	0.28-10.22	0.574
Certain conditions originating in the perinatal period	942	91.2	91	8.8	6.38	1.55-26.19	0.010
Congenital malformations, deformations and chromosomal abnormalities	361	93.8	24	6.2	4.39	1.02-18.82	0.047
Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified	159	97.5	4	2.5	1.66	0.30- 9.21	0.562
Injury, poisoning and certain other consequences of external causes	241	96.4	9	3.6	2.47	0.53-11.58	0.253
Certain infectious and parasitic diseases	237	93.3	17	6.7	8.77	2.11-36.44	0.003
Factors influencing health status and contact with health services	414	88.3	55	11.7	4.73	1.11-20.11	0.036
Others	391	93.3	28	6.7	4.73	1.08-20.81	0.040

Table 2. The final model for risk of children death

Variable	Adjusted OR	95 Confidence interval	*P*
Severity level			
I	1.00	Reference	
II	11.51	7.45-17.78	0.000
III	33.97	19.93-57.91	0.000
ICU Indicator			
No	1.00	Reference	
Yes	14.21	9.15-22.08	0.000
Grouping ICD			
Diseases of the musculoskeletal system and connective tissue	1.00	Reference	
Certain conditions originating in the perinatal period	7.65	1.81-32.35	0.006
Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified	6.05	1.09-33.60	0.039

*Adjusted each other between variables listed on this table, age, gender, length of hospital stay, length of stay in ICU.

In conclusion, illness severity level, ICU indicator and diseases in certain conditions originating in the perinatal period are the most common risk factors for children death in the hospital. The clinical pathways could be implemented to standardize patient management and improve hospital cost efficiency, however this must be followed by conducting an audit of clinical pathway compliance as quality control. Expansion of neonatal or pediatric intensive care unit (NICU/PICU) and improvement of the skill of its staff to reduce mortality caused by certain conditions originating in the perinatal period.

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Is hypoalbuminemia a predictor marker of mortality?

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Abstrak

Latar belakang*: Hipoalbuminemia pada pasien rawat inap berkaitan dengan prognosis buruk pasien. Penelitian ini, mengidentifikasi bahwa hipoalbuminemia berat pada awal pasien masuk rawat inap sebagai prediktor andalan penanda laboratorium dalam mortalitas.*

Metode*: Sebuah studi cross sectional pada pasien dewasa dengan hipoalbuminemia (kadar albumin < 3,5 g / dL) pada pasien rawat inap (usia > 18 tahun) pada periode Januari 2013 - Maret 2018. Kami mengevaluasi penanda prediktor kematian. Multivariat dengan regresi logistik diterapkan dalam penelitian ini.*

Hasil*: Dari 747 hipoalbuminemia pada pasien rawat inap dengan rata-rata kadar albumin pada awal adalah 2,0 ± 0,6 g / dL. Sebagian besar pasien (83,4%) memiliki kadar albumin ≤ 2,5 g / dL (hipoalbuminemia berat), 16,6 persen memiliki > 2,5 g / dL (hipoalbuminemia ringan-sedang). Kondisi yang mendasari pasien adalah infeksi HIV / AIDS (26,9%) dan sepsis (26,6%). Proporsi multiple komorbiditas pada kelompok hipoalbuminemia berat adalah 55,1 persen Pada kelompok hipoalbuminemia berat terutama untuk kadar albumin 2,01 - 2,5 g / dL, angka mortalitas adalah 28,3 persen. Berdasarkan model regresi logistik akhir, faktor risiko kematian meliputi kadar albumin pada awal dan lama rawat pasien. Mortalitas lebih tinggi pada pasien dengan hipoalbuminemia berat (rasio odds yang disesuaikan 2,91, 95% CI 1,88-4,50) dibandingkan pasien dengan hipoalbuminemia ringan-sedang.*

Kesimpulan*: Hipoalbuminemia berat pada awal pasien rawat inap sebagai prediktor penanda kematian di rumah sakit.* ***(Health Science Journal of Indonesia 2020;11(2):121-5)***

Kata kunci*: hipoalbuminemia, pasien rawat inap, mortalitas*

Abstract

Background: Hypoalbuminemia in hospitalized patients has been associated with poor prognosis. In this study, we attempted to identify that severe hypoalbuminemia at baseline in hospitalized patients is a reliable predictor of laboratory marker for mortality.

Methods: A cross sectional study on adults of hypoalbuminemia (albumin level < 3.5 g/dL) in hospitalized patients (aged > 18 years old) in period January 2013 - March 2018. We evaluated the predictor marker of mortality. Multivariate with the logistic regression was applied in this study.

Result: Of the 747 hypoalbuminemia in hospitalized patients with the mean albumin level at baseline was 2.0 ± 0.6 g/dL. Most patients (83.4 %) had less than or equal to 2.5 g/dL albumin level (severe hypoalbuminemia), 16.6 percent had over 2.5 g/dL (mild-moderate hypoalbuminemia). The underlying condition of patients was HIV/AIDS infection (26.9%) and sepsis (26.6 %). The proportion of multiple comorbidities in the severe hypoalbuminemia group was 55.1percent. In the severe hypoalbuminemia group especially for 2.01 – 2.5 g/dL albumin level, the mortality rate was 28.3 percent. Based on the final logistic regression model, known risk factors of mortality include albumin level at baseline and length of stay. Mortality was higher among patients with severe hypoalbuminemia (adjusted odds ratio 2.91, 95 % CI 1.88-4.50) than patients with mild-moderate hypoalbuminemia.

Conclusion: Severe hypoalbuminemia at baseline in the hospitalized patients was a predictor laboratory marker of hospital mortality. ***(Health Science Journal of Indonesia 2020;11(2):121-5)***

Keywords: hypoalbuminemia, hospitalized patients, mortality

Hypoalbuminemia is common in hospitalized patients. Hypoalbuminemia is the result of the effects of acute organ dysfunction, inflammatory disorders, and also inadequate protein and caloric intake in patients with chronic disease.[1] As we know that albumin has some important roles in the body, including maintaining oncotic pressure and binding a variety of molecules.[2]

The association between hypoalbuminemia and poor prognosis is well progressively increased morbidity and mortality.[3] The risk of prolonged hospital stay increased by 16 percent and the risk of increased death by 39 percent for each 2.5 mg/L serum albumin drop.[2]

Vincent et al performed hypoalbuminemia as an independent predictor of poor outcomes in patients with acute illness.[4] Severe hypoalbuminemia represents a potential predictor of adverse in hospitalized patients. To our knowledge, the clinical relevance of hypoalbuminemia has not yet been fully evaluated in this clinical setting. The aim of the present study was to evaluate the predictor marker of mortality.

METHODS

A cross sectional study on adults of hypoalbuminemia (albumin level < 3.5 g/dL) in hospitalized patients of Sulianti Saroso Infectious Diseases Hospital (aged > 18 years old) in period January 2013 until March 2018. We had a total sample of 747 patients that completed the variable of data. Data collection from hospital management information system and medical record. Descriptive analysis for baseline characteristic patients based on albumin levels and bivariate analysis to get significance of variable. The variable as predictor marker were age > 65 years old, HIV infection, sepsis condition, Length of Stay (LoS) more 8 days, ICU ward, severe hypoalbuminemia. We evaluated the predictor marker of mortality. Multivariate analysis included independent variable with p-value less than 0.25 and odss ratio (OR) for the logistic regression were applied in this study. Data was analyzed using SPSS version 21. The study protocol was approved by the Ethics Committee at Sulianti Saroso Infectious Diseases Hospital, number: 17/XXXVIII.10/V/2018.

RESULTS

Of the 747 hypoalbuminemia in hospitalized patients with the mean albumin level at baseline was 2.0 ± 0.6 g/dL. Most patients (83.4 %) had less than or equal to 2.5 g/dL albumin level (severe hypoalbuminemia); 16.6 percent had over 2.5 g/dL to 3.5 g/dL (mild – moderate hypoalbuminemia). The underlying condition of severe hypoalbuminemia patients was HIV/AIDS infection (29.9 %) and sepsis (24.6 %).

Table 1 shows that patients with severe hypoalbuminemia were less than or equal 65 years old and more likely to be male, hospitalized for more than 8 days in a non ICU ward and they also had multiple comorbidities (p = 0.000) such as diabetes mellitus, tuberculosis, heart failure, renal disease.

Table 1. Baseline characteristic of the study patients according to the albumin levels

Variabels	Albumin level at baseline		p
	Severe Hypoalbuminemia n (%)	Mild-moderate hypoalbuminemia n (%)	
Age in years			
>65	115 (18.5%)	36 (29.0%)	0.011
18-65	508 (81.5%)	88 (71.0%)	
Sex			
Female	260 (41.7%)	51 (41.1%)	0.980
Male	363 (58.3%)	73 (58.9%)	
HIV Infection			
Yes	186 (29.9%)	15 (12.1%)	0.000
No	437 (70.1%)	109 (87.9%)	
Sepsis			
Yes	153 (24.6%)	46 (37.1%)	0.006
No	470 (75.4%)	78 (62.9%)	
Length of Stay in days			
≥ 8	496 (79.6%)	107 (86.3%)	0.478
4-7	103 (16.5%)	15 (12.1%)	0.201
1-3	24 (3.9%)	2 (1.6%)	Ref
Room			
ICU	91 (14.6%)	42 (33.9%)	0.000
Non ICU	532 (85.4%)	82 (66.1%)	
Amount of Comorbidities			
≥ 3	343 (55.1%)	94 (75.8%)	0.000
< 3	280 (44.9%)	30 (24.2%)	

α=5%

Overall,251(33.6%)patientsdiedduringhospitalization. Mortality was higher with severe hypoalbuminemia (72.5 %) than mild-moderate hypoalbuminemia (27.5 %). In severe hypoalbuminemia group especially for 2.01 – 2.5 g/dL albumin level mortality rate was 28.3 percent followed by 1.51-2.0 g/dL (27%) and 1.01-1.5 g/dL (14%). In-hospital mortality rate for each albumin level are shown in Fig. 1. The patients with severe hypoalbuminemia group died within 16 ± 12 days (mean ± SD).

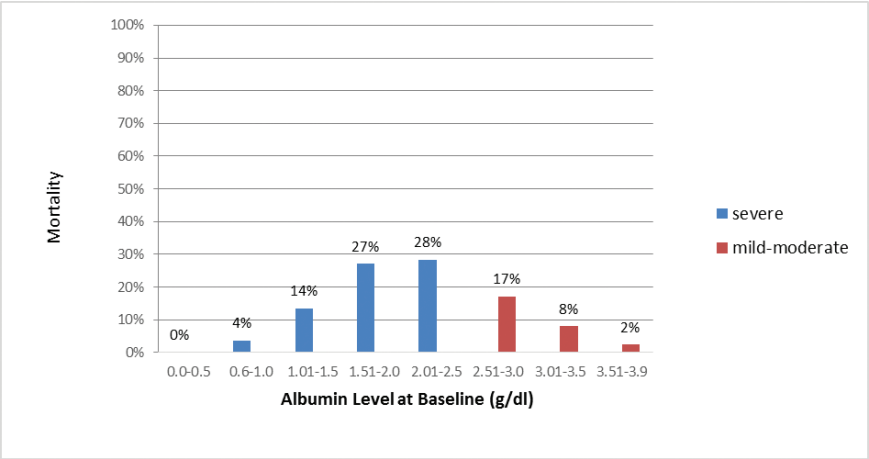

Figure 1. In hospital mortality rate by albumin level

Tabel 2. The bivariable analysis and multivariable logistic regression to validate factors associated with the mortality.

Parameters	Bivariable Analysis Odds Ratio (95% CI)	p	Multivariable Analysis Odds Ratio (95% CI)	p
Age > 65 years old	1.35 (0.98 – 1.80)	0.084	0.67 (0.44 – 1.03)	0.071
Hypoalbuminemia with HIV infections	1.33 (0.93 – 1.96)	0.118	0.48 (0.33 – 0.72)	0.000
Hypoalbuminemia with sepsis	3.46 (2.47 – 4.86)	0.000	0.41 (0.28 – 0.60)	0.000
Length of Stay more 8 days	1.83 (0.78 – 4.29)	0.168	3.20 (1.39 – 7.41)	0.006
In ICU ward	4.98 (3.34 - 7.41)	0.000	0.31 (0.20 – 0.48)	0.000
Severe hypoalbuminemia	0.33 (0.22 – 0.49)	0.000	2.91 (1.88 – 4.50)	0.000

In Table 2. Mortality is dependently related to the following markers, which are ranked in order from the higgest odds ratio (OR) as length of stay more 8 days (OR [95%CI] : 3.20 [1.39-7.41], p = 0.006) and severe hypoalbuminemia (OR[95% CI] : 2.91[1.88-4.50], p = 0.000). In contrast the risk of hypoalbuminemia patients with HIV infections (OR[95%CI]: 0.48[0.33-0.72], p = 0.000), and hypoalbuminemia patients with sepsis (OR [95%CI]: 0.41 [0.28-0.60], p =0.000), also patients in ICU ward (OR[95%CI]: 0.31 [0.20-0.48], p =0.000) was lower.

DISCUSSION

Our data showed that the mortality rate of the severe hypoalbuminemia group at baseline was higher than the mild-moderate hypoalbuminemia. The proportion of patients with 1.01 – 1.5 g/dL albumin level was lower (14 %) than 2.01 – 2.5 g/dL albumin level group (28.3 %). Because the majority of patients with ≤ 1.5 g/dL albumin level were in non ICU ward and less comorbidities rather than patients with 2.01 – 2.5 g/dL albumin level. However, this result assumed that low albumin level and critical ill condition at baseline are probably comparable in predicting mortality.[5] Our hospital mortality rate data was in line with the previous study : around 34 % in severe hypoalbuminemia patients on admission.[6]

Our data showed that low albumin level at baseline in hospitalized patients had a significant association with an increased risk of morbidity and mortality.[7] On multivariable analysis for mortality, severe hypoalbuminemia (≤ 2.5 g/dL albumin level) was a predictive laboratory marker of in-hospital mortality. Similar to the previous study in spesific groups of patients have shown that initial albumin levels were independently associated with in-hospital mortality among adult patients hospitalized with aspiration pneumonia.[8] Another study, of the 707 Necrotizing fasciitis patients with hypoalbuminemi in the emergency department (3.1 ± 0.9 g/dL). Showed that albumin level was significantly lower in the non

survivor group (death occurring in the hospital after admission) than in the survivor group (2.8 ± 0.7 d/dL vs 3.5 ± 0.8 g/dL).[5] Albumin level was significantly predicted with in-hospital mortality (OR [95%CI]: 0.92 [0.88 – 0.96], p <0.001).[5]

In fact, in this study, the risk of hypoalbuminemia in patients with HIV infections, sepsis, and patients in ICU ward was lower. However, the underlying condition of patients was HIV/AIDS infection (26.9%) and sepsis (26.6 %), also the most proportion of patients had multiple comorbidities. Hypoalbuminemia requires careful management of their condition. HIV infection can be caused of hypoalbuminemia that produces chronic inflammation and related to chronic malnutrition.[3,5,9]

The cause of hypoalbuminemia in sepsis patients are often multifactorial, such as decreased albumin synthesis, increased albumin loss, redistribution of albumin to locations outside the intravascular space, and dilution of albumin within the intravascular space.[9] In addition, some drugs, including antibiotics, can bind with plasma protein and form protein-drug complexes.[9] Binding to plasma proteins plays a major role in drug treatment.[5] However, severe hypoalbuminemia may result in potentially life-threatening consequences.[9]

The most important concept in treating hypoalbuminemia patients is to address the underlying problem.[7,8] The appropriate treatment of hipoalbuminemia is needed based on the reason for the low albumin level. In addition, it is uncertain whether correcting hypoalbuminemia with continuously albumin infusion administration is more beneficial or not. However, although the role of albumin infusion therapy for the correction of hypoalbuminemia is still controversial related to the high cost of health facilities, albumin infusion administration is one of an effort to reduce the poor prognosis of patients if the therapy is correctly prescribed and rationally used.[10]

Additionally, the initial albumin level at baseline is important to check on the health care system. It can be a laboratory marker for predicting in -hospital mortality.[11,12,13] Our study has several limitations. We do not have any data on the severity degree of sepsis patients such as SOFA score that can be the prognostic performance of an albumin value.[14] However, the prognostic accuracy of the combination of albumin level and SOFA score was not significantly better than the use of SOFA score alone (p=0,274).[5] Other than that, we do not have information regarding the nutritional status of patients, other than body mass index, which may be of relevance in this case.[15]

In conclusion, severe hypoalbuminemia at baseline in the hospitalized patients was a predictor laboratory marker of hospital mortality. However, although the role of albumin infusion therapy for the correction of hypoalbuminemia is still controversial, the administration of albumin infusion is one of an effort to reduce the poor prognosis of patients if the therapy is correctly prescribed and used rationally in relation to the underlying problem.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Risk factors of soil transmitted helminth infection among primary school students

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Abstrak

Latar belakang: *Infeksi kronis dari soil transmitted helminth (STH) dapat menyebabkan gangguan gizi, pertumbuhan dan kognitif pada anak. Untuk mengurangi dampak infeksi STH, diperlukan identifikasi faktor risiko. Penelitian ini bertujuan untuk mengidentifikasi faktor risiko yang berhubungan dengan infeksi STH pada siswa sekolah dasar di Desa Seraya Timur, Karangasem, Bali.*

Metode: *Penelitian ini menggunakan desain potong lintang dengan mengambil total sampel. Penelitian dilaksanakan pada bulan Januari 2020. Data primer mengenai faktor-faktor risiko infeksi STH dikumpulkan dengan menggunakan kuesioner. Diagnosis infeksi STH dilakukan dengan pemeriksaan tinja dengan metode Kato-Katz. Analisis data menggunakan uji chi-square untuk menentukan faktor risiko yang berhubungan dengan infeksi STH.*

Hasil: *Sebanyak 83 siswa yang berusia 6-12 tahun berpartisipasi dalam penelitian ini. Terdapat 9 siswa (10.84%) yang terinfeksi STH dengan intensitas infeksi ringan. Sebanyak 55.56% terinfeksi Trichuris trichiura, 33.33% terinfeksi Ascaris lumbricoides dan 11.11% terinfeksi cacing tambang. Terdapat beberapa faktor risiko yang memiliki hubungan bermakna dengan infeksi STH diantaranya adalah sering bermain tanah (OR=6.86; 95%CI 1.326-35.494), bermain tanpa alas kaki (OR=10.5; 95%CI 1.249-88.278) tidak mencuci tangan setelah bermain tanah (OR=9.450; 95%CI 1.809-49.358) dan tidak memotong kuku secara rutin (OR=6.462; 95%CI 1.250-33.388). Pemberian obat cacing setiap enam bulan mampu memberikan efek proteksi terhadap infeksi STH (OR=0.085; 95%CI 0.016-0.449).*

Kesimpulan: *Kebersihan diri menjadi faktor risiko yang berhubungan dengan infeksi STH. Direkomendasikan untuk meningkatkan promosi kesehatan terkait kebersihan diri disamping pemberian obat cacing setiap enam bulan.* ***(Health Science Journal of Indonesia 2020;11(2):126-32)***

Kata kunci: *Faktor risiko, infeksi STH, anak sekolah dasar*

Abstract

Background: Chronic soil transmitted helminth (STH) infection might cause nutritional, growth and cognitive impairment in children. Identifying the risk factors of STH infection is crucially needed to minimize the infection effects. This study aimed to identify risk factors associated with STH infections among primary school students in Seraya Timur Village, Karangasem, Bali.

Methods: This study used a cross-sectional design with a total sampling method. The study was conducted in January 2020. Risk factors data were collected using a questionnaire. The diagnosis of STH infection was done by stool examination with the Kato-Katz method. The chi-square test was used to determine the risk factors associated with STH infection.

Results: 83 students with ages ranging from 6-12 years participated in this study. There were 9 students (10.84%) whose infected with mild infection of STH. 55.56% of students were infected by *Trichuris trichiura*, 33.33% were *Ascaris lumbricoides* infections and 11.11% were hookworm infections. There were several risk factors that significantly associated with STH infection including ground's playing (OR=6.86; 95%CI 1.326-35.494), barefoot (OR=10.5; 95%CI 1.249-88.278), did not wash hands after playing soil (OR=9.450; 95%CI 1.809-49.358) and did not routinely cut their nails (OR=6.462; 95%CI 1.250-33.388). Deworming every six months could provide a protective effect against STH infection (OR=0.085; 95%CI 0.016-0.449).

Conclusion: Personal hygiene is a risk factor associated with STH infection. It is recommended to increase personal hygiene promotion besides dewormed every six months. ***(Health Science Journal of Indonesia 2020;11(2):126-32)***

Keywords: Risk factors, STH infection, primary school students

Soil transmitted helminth (STH) infection is an infectious disease caused by worms that require soil as a medium to develop. This infection is associated with poor communities with poor environmental hygiene. The transmission of infection can happen through eggs or larvae that contaminate the soil in poor sanitation areas. Some types of STH that are often found to infect humans include whipworms *(Trichuris trichiura)*, roundworms *(Ascaris lumbricoides)*, and hookworms *(Necator americanus, Ancylostoma duodenale)*.[1] STH infections are classified as neglected diseases. This disease is chronic and often without typical clinical symptoms. However, STH infections in children might cause impaired nutrient absorption and growth disorders which have an impact on decreased cognitive development.[2]

The prevalence of STH infections in Indonesia is still quite high. The Ministry of Health Republic of Indonesia data showed STH infection rates range from 2.5% to 62% in the poor population and poor sanitation.[3] STH infections are associated with careless defecation habits, eating or playing with the ground, not washing hands and playing barefoot. Most of those habits are often found in children and make them a vulnerable group. The prevalence of STH infection among school age students in Indonesia between 2002 and 2009 was 31.8%.[4] Hotez et al also reported that in 2012 there were 16.9 million pre-school age children and 43.5 million school age children infected by STH in Indonesia.[5] The World Health Organization (WHO) estimated that there were 576 million children worldwide need to be prevented with the total coverage would be 75% in 2018.[6]

In controlling STH infections, WHO recommends doing intervention using Albendazole or Mebendazole tablet once a year to children who live in areas with STH infections rates range from 20%-50% and twice a year in the areas with STH infections rates more than 50%.[6] The Minister of Health Regulation number 15 year 2017 stated, the intervention of STH infection control are dewormed, health promotion, risk factor control and helminthiasis surveillance accordingly with WHO recommendation. In addition, the target of national STH infection prevalence is below 10% in the year 2017. Program evaluation is needed by doing surveillance and identification of STH risk factors to optimize the control of STH infections.[3]

The prevalence of STH infection in rural areas in Bali Province is still quite high. Based on several studies between 2017 and 2019 showed that the prevalence of STH infection among primary school-aged children was around 10.1%-31.7% in three villages in the District of Karangasem.[7-9] Those numbers were quite higher than the national target of STH infection prevalence that was set by the Ministry of Health Republic of Indonesia (below 10%). The Seraya Timur Village is one of the villages in the District of Karangasem. The socio-geographical conditions are not much different from other villages in the District of Karangasem, which might also cause the high prevalence of STH infection in Seraya Timur Village. So, identifying the risk factors of STH infections is still needed.

This study aimed to identify risk factors associated with STH infections among primary school students in Seraya Timur Village, District of Karangasem, Bali Province.

METHODS

This research was an observational analytic study with cross sectional design. Sampling was using a total sampling method conducted at the Primary school of SDN 4 Seraya Timur Village, District of Karangasem, Bali Province in January 2020. This research evaluated the risk factors of STH infection as the independent variables by fulfilled the validated questionnaire include gender, frequently playing on soil, hand washing habit, barefoot habit, cut their nails routinely, have a proper toilet at their home, eating while playing on soil habit, nail-biting habit, anthelmintic tablet consumption every six months. The dependent variable was the infection status of STH which was determined by the stool examination.

Before collecting faecal samples, all parents of 1st to 6th grade students were given informed consent of research and signed it as approval to permit their children to participate in this research. All students whose informed consent has been approved by the parent are participating in this study.

The data collection was conducted through stool taken. All students were given 30 mL of faecal containers and a small stick to collect the feces that had been labeled with the student's identity. Stool sampling was taken in the morning before going to school. Stool examination conducted in the Laboratory of Parasitology, Faculty of Medicine, Udayana University used the Kato-Katz method to determine the status and intensity of STH infections. The infection status is defined positive if there are eggs or larvae found in microscopic examination. The intensity of infection is based on the number of worm

eggs per gram of feces (epg). The intensity category of *Trichuris trichiura* infection is mild (1-999 epg), moderate (1.000-9.999 epg) and severe (≥10.000 epg). Category of hookworm infection is mild (1-1.999 epg), moderate (2.000-3.999 epg) and severe (≥4.000 epg). Category of *Ascaris lumbricoides* infection is mild (1-4.999 epg), moderate (5000-49.999 epg) and severe (≥50.000 epg).[10]

The collected data then analyzed using SPSS v.23 Software with Chi-Square Test, Odd Ratio (OR) and 95% Confidence Interval (CI) to determine risk factors that were significantly associated with STH infection. The results were considered to be statistically significant for the p-values of <0.05.

This research has approved by the Research Ethics Commission of the Faculty of Medicine, Udayana University/RSUP Sanglah Denpasar with registration number is 29931/UN14.2.2.VII.14/LP/2019.

RESULTS

There were 83 students who participated in this study with a range of ages are 6-12 years. Based on data in table 1, there were 43 (51.8%) male and 40 (48.2%) female students. Stool examination results are depicted in figure 1. There were 9 (10.84%) students infected with STH. The proportion of STH infections was found higher in females (15%) than in male students (7%). Students of 5th grade had the highest proportion of STH infections (55.56%).

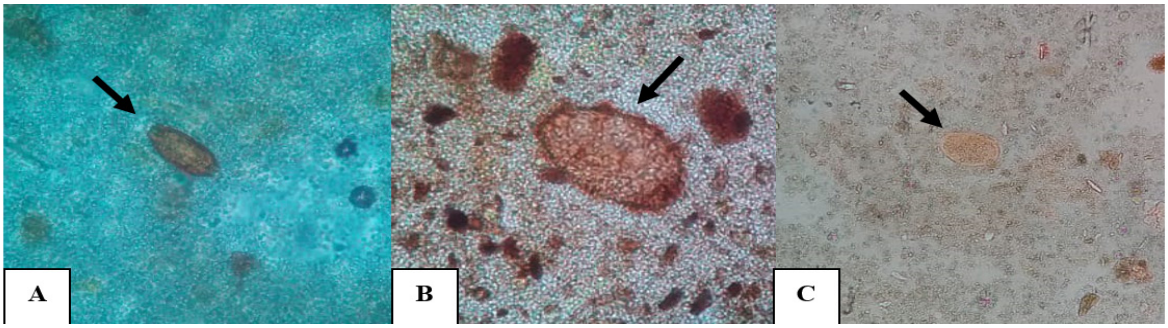

Figure 1. Stool examination results using The Kato-Katz Method (400X Magnification). (A) An egg of Trichuris trichiura. (B) An egg of Ascaris lumbricoides. (C) An egg of Hookworm.

Table 1. Characteristics of subjects

Characteristics	STH Infection (n=83)		
	Positive (n=9)	Negative (n=74)	Total (n=83)
Median age (min-max), years	10 (6-11)	10 (6-12)	10 (6-12)
Gender, n (%)			
Male	3 (7)	40 (93)	43 (100)
Female	6 (15)	34 (85)	40 (100)
Grade, n (%)			
1st grade	1 (16.7)	5 (83.3)	6 (100)
2nd grade	0 (0)	4 (100)	4 (100)
3rd grade	1 (25)	3 (75)	4 (100)
4th grade	1 (4.8)	20 (95.2)	21 (100)
5th grade	5 (23.8)	16 (76.2)	21 (100)
6th grade	1 (3.7)	26 (96.3)	27 (100)
STH infection types, n (%)			
Trichuris trichiura only	5 (55.56)	-	5 (55.56)
Ascaris lumbricoides only	3 (33.33)	-	3 (33.33)
Hookworm only	1 (11.11)	-	1 (11.11)
STH infection intensity, n (%)			
Mild	9 (100)	-	(100)
Moderate	-	-	-
Severe	-	-	-

All of STH infection cases in this study were single infection with mild intensity. This study also found that the infection of *Trichuris trichiura* as the major type of STH infection with a proportion of 55.56%. Based on the data in table 2, playing ground, did not wash hands after playing with the ground, playing barefoot, did not cut nails, and did not take anthelmintic medication every 6 months have become the risk factors of STH infections with playing barefoot as the major risk factor.

Table 2. Risk factors for STH infection

Variables	Infection status		*p*	OR	95% CI
	Positive n (%)	Negative n (%)			
Gender					
Male	3 (7)	40 (93)	0.302	0.425	0.099-1.829
Female	6 (15)	34 (85)			
Frequently playing with or on soil					
Yes	7 (21.9)	25 (78.1)	0.024*	6.860	1.326-35.494
No	2 (3.9)	49 (96.1)			
Unwashed hands after defecation					
Yes	1 (14.3)	6 (85.7)	0.567	1.417	0.151-13.310
No	8 (10.5)	68 (89.5)			
Unwashed hands after playing with or on soil					
Yes	7 (25.9)	20 (74.1)	0.005*	9.450	1.809-49.358
No	2 (3.6)	54 (96.4)			
Playing barefoot					
Yes	8 (20)	32 (80)	0.013*	10.5	1.249-88.278
No	1 (2.3)	42 (97.7)			
Uncut nails regularly					
Yes	7 (21.2)	26 (78.8)	0.026*	6.462	1.250-33.388
No	2 (4)	48 (96)			
Unavailable of proper toilet at home					
Yes	4 (19)	17 (81)	0.221	2.682	0.647-11.119
No	5 (8.1)	57 (91.9)			
Eating while playing with soil or objects covered with soil particles					
Yes	3 (13)	20 (87)	0.703	1.350	0.308-5.918
No	6 (10)	54 (90)			
Nail-biting behavior					
Yes	2 (15.4)	11 (84.6)	0.627	1.636	0.300-8.930
No	7 (10)	63 (90)			
Anti-helminthic tablet consumption every six months					
Yes	2 (3.4)	57 (96.6)	0.002*	0.085	0.016-0.449
No	7 (29.2)	17 (70.8)			

Note: *p<0.05

DISCUSSIONS

STH infection is a disease that is easily found in tropical and sub-tropical regions. Climate can affect soil conditions as a growing medium for STH. All people of all ages are at risk of becoming infected with this disease, but the highest prevalence is found in children. Epidemiologically, the peak occurrence of STH infection at the age of 5-10 years. This is closely related between age and the process of receiving information. The young children (<10 years old) usually do not care about personal hygiene and lack of knowledge to understand the effects of infection. In addition, young children also tend to be more active playing that allows contact with soil that has been contaminated with STH as a medium of transmission. The increasing age can change children's activity pattern and personal hygiene awareness which might decrease the

infection rates in older age children.[11,12] The results of this study indicate that the prevalence of STH infections is 10.84% with the highest proportion is a *Trichuris trichiura*. This result is accordingly but lower than the study conducted by Sofiana et al who also found *Trichuris trichiura* as the major type of STH infection with a proportion of 24.6%.[13] Other research in Karangasem region, Bali showed a varied prevalence of STH infection, namely a study in Gegelang Village found a prevalence of 31.7%, study in Antiga Kelod Village reported a prevalence of 24.8%, while research in Ngis Village depicted the prevalence of 10.1%.[7-9]

The results of this study indicated that there was a significant relationship between frequently playing on soil with STH infection. Similar results were also found in Dewi's study which showed that STH infections were more common in children who were frequently had contact with soil compare to those who were rarely had contact with soil (OR 6.3).[1] Annida et al reported the majority of children in Dayak Meratus community (80.4%) had the habit of playing in the yard and used soil as the game media were associated with hookworm infections.[11] Samad's research also found that 52.5% of the soil contaminated with *Ascaris lumbricoides* eggs in Tembung, Medan was associated with the incidence of STH infections among elementary school students.[14] The STH transmission is easier found among the children who often play or have contact with the ground. The moist soils allow the eggs of *Trichuris trichiura* and *Ascaris lumbricoides* to multiply rapidly. The loose sandy soils in rural areas are well-suited for the growth of hookworm larvae. The transmission can occur due to ingestion of embryonated eggs through contaminated hands, food or drinks, or directly through dust, soil, pets or toy items.[15]

Personal hygiene is a person's effort to maintain the degree of health that can be done by maintaining the cleanliness of the skin, hands, feet, nails, oral cavity and other body organs. Washing hands is one of the important points to do with. Hands to be washed with soap in every dirtiness, for example after holding money or animals, before and after eating, after defecation, after playing, especially after playing with the ground. Hands can be a proper transmission medium for STH infection via oral transmission if the hands are contaminated with STH.[16] This study showed that did not wash hands after playing with the ground was one of the risk factors associated with STH infection. This result is supported by the study of Sofiana et al which showed that children who did not wash their hands had a risk of being infected with STH 2.23 times higher than children who washed their hands.[13] Sandy et al also reported that children who had bad hands washing habits (using water only) had 3.03 times higher risk to be infected by STH than those who had good hands washing habits (using water and soap).[17]

Transmission of STH infections can pass through the skin pores of the feet. Filiform larvae of hookworm can penetrate the skin and enter the blood capillaries and be carried to the heart and lungs until they develop in the small intestine. Hookworms attack all ages with the largest proportion of children due to children's activities which are relatively more unhygienic than adults. One of the STH risk factors is playing barefoot.[16] This study showed that children who were playing barefoot are at risk to develop STH infection 10.5 times higher than children who did not play barefoot. This result is supported by Suryantari's research which showed that elementary school-age children who playing in barefoot tend to have a higher STH infection than those who used footwear while playing (OR 6.5).[9] Fitri et al also found that students who rarely using footwear when doing their activities had 5.5 times higher to be infected STH than those who always using footwear.[18]

Nails can be a medium for the transmission of STH infections. Soil contaminated with STH eggs can be tucked into long nails. Children often unconsciously bite their nails or touch food without washing their hands first which allows the transmission of STH eggs into their mouth. Nail hygiene is also a risk factor for STH infections in children.[19] This study found that children who did not cut their nails routinely had the risk of developing STH infection 6.4 times higher than their counterparts. This result is in line with the study of Wiryadana et al who found that children who did not cut their nails once a week were at risk of developing STH infection 3.3 times higher than children who cut their nails routinely.[7] Halleyantoro et al also reported that primary school students who had dirty nails tend to have higher STH infection than those who had clean nails (OR 5.3).[20]

Broad-spectrum worm medicine such as Albendazole and Mebendazole can kill STH in an infected person and be able to prevent transmission to others. The results of this study indicated that the implementation of the deworming program every six months could have a protective effect on primary school students from STH infection (OR 0.02). In line with this

result, Kartini reported that students who did not take anthelmintic tablet every six months had 11 times higher risk to be infected by STH than those who took it.[21] Suryantari's research also showed that the deworming program was able to prevent STH infections in elementary school children (OR 0.23).[9] Thus, the deworming program for primary school students is expected to be a proper type of intervention to prevent STH infections in elementary school children.

In conclusion, habits of playing in the ground, playing barefoot, not washing hands after playing with the ground and not cutting nails regularly are risk factors for STH infection. It is recommended to increase personal hygiene and health promotion in addition to the deworming program every six months.

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Quality of life among ovarian cancer survivors in Haji Adam Malik General Hospital Medan, Indonesia

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Abstrak

Latar belakang*: Kanker ovarium memiliki angka mortalitas yang cukup tinggi dikarenakan gejalanya yang tidak spesifik, sering ditemukan pada stadium lanjut, dan belum adanya metode deteksi dini yang sudah terbukti. Untuk menilai keberhasilan terapi penyintas kanker ovarium, tidak hanya dinilai dari aspek klinis tetapi juga dinilai dari kualitas hidup penyintas kanker ovarium yang penilaiannya berdasarkan skala fungsional dan skala gejala dalam kuesioner EORTC QLQ C30 dan EORTC QLQ OV28.*

Metode: *Penelitian ini menggunakan desain penelitian cross sectional, menggunakan data primer dari hasil wawancara dengan kuesioner EORTC QLQ C30 dan EORTC QLQ OV28 serta data sekunder yang berasal dari rekam medik di RSUP Haji Adam Malik Medan tahun 2017 - 2018. Sampel penelitian dipilih dengan metode total sampling dari seluruh data rekam medik yang memenuhi kriteria penelitian.*

Hasil: *Hasil penelitian ini didapatkan kualitas hidup global penyintas kanker ovarium 89.36% adalah baik, dan 10.64% adalah sedang serta tidak ada yang memiliki kualitas hidup buruk. Namun, didapatkan adanya gangguan pada skala fungsional berupa: fungsi emosional, fungsi kognitif, fungsi seksual, dan sikap terhadap penyakit, serta adanya permasalahan pada skala gejala berupa: kelelahan, nyeri, neuropati perifer, dan gejala menopause. Didapatkan juga tidak ada hubungan karakteristik usia, jenis histopatologis, stadium, lama terapi dengan kualitas hidup penyintas kanker ovarium, namun terdapat hubungan antara jenis terapi dengan kualitas hidup penyintas kanker ovarium.*

Kesimpulan: *Kualitas hidup penyintas kanker ovarium secara global adalah baik.* ***(Health Science Journal of Indonesia 2020;11(2):133-9)***

Kata kunci: *Kualitas hidup, penyintas kanker ovarium, EORTC QLQ C-30, EORTC QLQ OV-28*

Abstract

Background: Ovarian cancer has a high mortality rate due to nonspecific symptoms, often found at an advanced stage, and also the absence of proven early detection methods. To assess the success of ovarian cancer survivors therapy, it is not only assessed from the clinical aspect but also from the quality of life of ovarian cancer survivors which is based on the functional and symptom scale in the EORTC QLQ C30 and EORTC QLQ OV28 questionnaires.

Methods: This study used a cross sectional study design, using primary data from interviews with the survivors based on the questionnaire EORTC QLQ C30 and EORTC QLQ OV28 as well as secondary data derived from medical records at Haji Adam Malik General Hospital Medan in 2017 - 2018. The research sample was used with a total sampling method from all medical record data that fulfill the research criteria.

Result: The quality of life of ovarian cancer survivors is generally good (89.36%), meanwhile the rest is moderate (10.64%) without the poor quality of life. However, there are disorders on the functional scale in the form of emotional function, cognitive function, sexual function, and attitude toward disease. Likewise on the scale of symptoms, there are problems including: fatigue, pain, peripheral neuropathy, and menopausal symptoms.

Conclusion: The quality of life of ovarian cancer survivors globally is good. ***(Health Science Journal of Indonesia 2020;11(2):133-9)***

Keywords: Quality of life, ovarian cancer survivors, EORTC QLQ C-30, EORTC QLQ OV-28

Ovarian cancer is one of the ten most common cancer suffered by women in Indonesia.[1] According to Globocan 2018, the incidence of ovarian cancer is 5.7 per 100.000 women globally, and the mortality rate is 4.0 per 100.000 women.[2] Although the incidence of ovarian cancer is not as high as those of breast and cervical cancer, the mortality rate is high as most ovarian cancer cases (60-70%) are found in late stages, thus the 5 year survival rate reaches only around 45%.[3]

The quality of life is one of the measurements to assess the outcome of therapy mainly in chronic diseases with a low possibility of recovery. The target of therapy in cancer is not to cure but to improve the quality of life.[4] According to Goncalves's research in 2014 using EORTC QLQ C30 (European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire C30) and FACT G (Functional Assessment of Cancer Therapy - General) questionnaires, the quality of life among ovarian cancer survivors in Portugal was fairly good in terms of physical, psychological and social welfare, but most of the women suffered from waist pain and problems in sexual life.[5]

As in Indonesia, Riska's research in 2017 using EORTC QLQ C30 on the quality of life of gynecological cancer patients undergoing therapy at Dharmais Hospital and Gatot Subroto Hospital, obtained research results indicated that most of the gynecology cancer patients required a high level of quality of life on global health and functional domains. However, the quality of life in the symptom domain was quite low.[6]

The research on quality of life specifically for ovarian cancer survivors was not developed in Indonesia. So this research aims to measure the quality of life among ovarian cancer survivors in Indonesia especially in Haji Adam Malik Hospital, Medan.

METHODS

This research uses a cross-sectional design. The collecting and recording of data were carried out in July-October 2019. This research uses primary data obtained from European Organization of Research and Treatment of Cancer Quality of Life Q-C30 (EORTC QLQ C30) and Quality of Life Questionnaire Ovary Cancer Module (EORTC QLQ OV28) by interviewing the ovarian cancer survivors as well as secondary data to identify the characteristics of ovarian cancer survivors written in medical records at Haji Adam Malik General Hospital, Medan City in 2017-2018.

The EORTC QLQ C30 questionnaire contains 30 questions consisting of 3 scales, the global quality of life, functional and symptom scales. The questionnaire was previously tested in validity and reliability by Rini Noviyani et al.[7] Meanwhile, EORTC QLQ OV28 questionnaire contains 28 questions consisting of 2 scales, functional and symptom scales with tested validity and reliability by the author on 30 respondents in Universitas Sumatera Utara Hospital (count r > table r; 0.361 for 30 respondents with Alpha Cronbach value > 0.7).

Total sampling was used to determine the samples and 47 respondents were selected after meeting the inclusion and exclusion criteria. The inclusion criteria are: 1) Women diagnosed with ovarian cancer have completed primary treatment (surgery, chemotherapy), all stages of ovarian cancer, and all types of ovarian cancer in 2017-2018. 2) From the evaluation in 2019, there was no recurrence. The exclusion criteria is: 1) Survivors of ovarian cancer that can not be contacted by researchers due to data in the form of telephone numbers and addresses that are incomplete in the medical record.

There were two variables used in this research, dependent and independent variables. The dependent variable was the quality of life and the independent variables were age, histopathological results, stage of cancer, type of therapy, and duration of therapy. Quality of Life of Ovarian Cancer Survivors, age, stage of cancer, and duration of therapy variables have an ordinal scale, while histopathological result and type of therapy variables have a nominal scale.
This research had been approved by The Ethical Commission of Medical Faculty, Universitas Sumatera Utara with a letter number 13/TGL/KEPK FK USU- RSUP HAM/2019. The data were analyzed in univariate, bivariate and multivariate tests. Bivariate analysis was by spearman correlation test and the multivariate analysis was by logistic regression test. Bivariate analysis is used to determine whether there is a relationship between ordinal and nominal variables with the quality of life of ovarian cancer survivors. Meanwhile, multivariate analysis was used to find which nominal variables influenced the quality of life of ovarian cancer survivors.

RESULTS

The population in this study was ovarian cancer survivors in Haji Adam Malik General Hospital in 2017-2018, totaling 78 people. However, only 52

people had complete medical record data and of those 52 people only 47 people want to be interviewed, so the number of samples in this study totaled 47 ovarian cancer survivors.

Table 1. Distribution of characteristics of ovarian cancer survivors in Haji Adam Malik Hospital, Medan City in 2017-2018.

Variable	Frequencies (n = 47)	Percentages (%)
Age (Years)		
≤19	1	2.1
20-29	4	8.5
30-39	1	2.1
40-49	17	36.2
50-59	19	40.4
60-69	5	10.6
Histopathological Type		
Epithelial		
Serous adenocarcinoma	8	17.0
Mucinous cystadenocarcinoma	16	34.0
Papillary serous adenocarcinoma	6	12.8
Endometrioid carcinoma	6	12.8
Clear cell carcinoma	5	10.6
Sex Cord Stromal		
Granulosa cell carcinoma	1	2.1
Germ cell		
Teratoma	2	4.3
Yolk sac tumor	1	2.1
Borderline epithelial tumors		
Serous cystadenoma	1	2.1
Mucinous cystadenoma	1	2.1
Stage of Cancer		
I	18	38.3
II	9	19.1
III	20	42.6
IV	0	0
Type of Therapy		
Surgery only	5	12.8
Neoadjuvant Chemotherapy + Surgery + Adjuvant Chemotherapy	4	6.4
Surgery + Adjuvant Chemotherapy	36	80.9
Duration of Therapy		
≤ 3 months	5	10.6
4-7 months	41	87.2
≥ 8 months	1	2.1

From 47 ovarian cancer survivors in 2017-2018, it was found that the most age was in the age group 50-59 years (40.4%), the most histopathological type was an epithelial tumor (87.2%), the highest stage was stage III (42, 6%), the most type of therapy was in the form of surgery + adjuvant chemotherapy (80.9%), the longest duration of therapy was 4-7 months (87.2%).

Table 2. Quality of life of ovarian cancer survivors based on EORTC QLQ C30

VARIABLES	MEAN	±SD
Global Health Status/QOL	**82.44**	**14.03**
Global Health Status/QOL		
Functional Scales	**90.34**	**14.28**
Physical Functioning	93.90	5.53
Role Functioning	100	0
Emotional Functioning	83.68	16.93
Cognitive Functioning	76.95	16.86
Social Functioning	97.16	7.22
Symptom Scales/Items	**8.66**	**15.27**
Fatique	31.91	10.51
Nausea and Vomiting	1.06	4.11
Pain	20.92	15.72
Dyspnoea	4.96	11.99
Insomnia	5.67	16.03
Appetite Loss	2.17	10.77
Constipation	5.67	12.66
Diarrhoea	0.70	4.86
Financial Difficulties	4.96	11.99

On the EORTC QLQ C30 and EORTC QLQ OV28 scoring, all of the scales and single-item measures range in score from 0 to 100. A high scale score represents a higher response level. Thus a high score for a functional scale represents a high/healthy level of functioning, a high score for the global health status / QoL represents a high QoL, but a high score for a symptom scale/item represents a high level of symptomatology/problems.[14]

Table 2 shows a fairly high functional scale with an average score of 90.34 which shows that the level of functional health in terms of ovarian cancer survivors is also quite high, even the role function shows a score of 100 which proves that none of the survivors experienced any disruption in performing role functions such as doing their work, hobbies and daily activities after completing chemotherapy.

The scores of emotional and cognitive functions show a slight disturbance. In emotional function, some of the survivors complain they often feel quickly offended, and feel worried especially about recurrence. In cognitive function also some survivors say they are increasingly difficult to remember things and in terms of concentration while on the move and work.

The symptom scale in table 2 shows a score of 8.66 which means that the level of symptoms or problems experienced by survivors is low, even in the symptoms of nausea and vomiting, dyspnoea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties showing very low scores. It means that most survivors no longer experience these symptoms which are mostly experienced during their chemotherapy.

The symptoms of fatigue show a high enough score (31.91). Many survivors reported that they felt tired quickly and also felt that their bodies were weak so that sometimes they had to take a break while working and doing their daily activities. Some survivors also reported pain in their bodies, especially in the former operation, waist, and joints, so that a little disturbing in their daily activities that can be seen in the pain score (20.92) in table 2 which shows a disturbance in pain symptoms.

Table 3. Distribution of global health status/ quality of life based on EORTC QLQ C30

Quality of Life	Frequency	Percentage (%)
Good	42	89.36
Moderate	5	10.64
Poor	0	0
Total	**47**	**100**

Table 3 shows the Global Health Status/Quality of Life of ovarian cancer survivors is generally good (89.36%), meanwhile the rest is moderate (10.64%) without the poor quality of life.

Table 4. Quality of life of ovarian survivors based on EORTC QLQ OV 28

VARIABLES	MEAN	±SD
Functional Scales	**59.94**	**32.54**
Body Image	84.04	16.28
Sexuality	23.94	25.16
Attitude to Disease/Treatment	71.87	16.19
Symptom Scales/Items	**16.91**	**20.14**
Abdominal/GI Symptoms	10.23	6.19
Peripheral Neuropathy	34.99	20.98
Hormonal/Menopausal Symptoms	20.57	26.52
Other Chemothrapy Side Effects	13.47	10.98
Hair Loss	5.31	14.79

Based on table 4, it can be seen that the functional scale on the EORTC QLQ OV-28 shows an average score of 59.94 which means that the functional level of health in survivors of ovarian cancer is impaired, especially in sexual function and attitudes to disease/ treatment. Many ovarian cancer survivors complain of problems in their sexual function, namely a decrease in interest in sexual intercourse, discomfort in sexual intercourse and pain during sexual intercourse because their vagina is dry during sexual activity.

Likewise on the scale of attitudes to disease/ treatment, it is also said by some survivors that their previous illnesses and treatments are weighing on them especially those concerned about the recurrence of ovarian cancer. Younger survivors often have a disturbance of body image and future perspective. They also often feel burdened about their disease prior to the high expectation about their health. This could affect their attitude to the disease and its treatment.[9]

The results obtained for the symptom scale in table 4 also have a fairly low average score (16.91) which shows that the level of symptoms or problems experienced by survivors based on EORTC QLQ OV-28 is not too problematic. This can be seen in abdominal/GI symptoms, other chemotherapy side effects, and hair loss showing very low scores which proves that most survivors no longer experience these complaints that may be experienced during therapy. However, most survivors experience peripheral neuropathy in the form of numbness, tingling, and weakness in the extremities, especially survivors who undergo chemotherapy. Survivors who did not undergo chemotherapy, did not experience peripheral neuropathy. Likewise on the scale of menopause symptoms, some survivors complain of experiencing hot flushes and night sweats.

Table 5. Bivariate analysis (Relationship between characteristics and quality of life of ovarian cancer survivors based on EORTC QLQ C30)

Variables	Quality Of Life	
	p *value*	R
Age	1.000	0.001
Type of Histopathology	0.043	0.132
Type of Treatment	0.026	0.330
Stage	0.067	0.269
Duration of Treatment	0.064	0.272

Based on Table 5 to see the relationship between the independent variable and the dependent variable, the result obtained p value 0.026 (p value < 0.05) on the type of treatment variable, and type of histopathology with p value 0.043 (p value < 0.05), which means that the type of therapy and type of histopathology have relationship with the quality of life of ovarian cancer survivors. However, there is no significant association between age, stage of cancer, duration

of therapy and the quality of life of ovarian cancer survivors because p value > 0.05.

Table 6. Multivariate analysis (Relationship between characteristics and quality of life of ovarian cancer survivors based on EORTC QLQ C30)

Variables	Quality Of Life		
	p *value*	R	R^2
Age	1.000	0.001	
Type of Histopathology	0.999	0.132	
Type of Treatment	0.026	0.330	0.215
Stage	0.067	0.269	
Duration of Treatment	0.064	0.272	

This research shows that there is no significant association between age, histopathological results, stage of cancer, duration of therapy and the quality of life of ovarian cancer survivors. However, there is a significant relationship between the type of therapy and quality of life in ovarian cancer survivors (p=0.026) with R square value of 0.215 or 21.5%, which means the types of therapy affect 21.5% on quality of life while the remaining 78.5% is influenced by other factors.

Compared to Bughwandass, the types of therapy underwent by the survivors affect their quality of life. Survivors who received surgery and chemotherapy have a lower quality of life than the survivors who only went through surgery, especially on the scale of symptoms such as fatigue, peripheral neuropathy, attitude toward disease, and financial terms. These symptoms, otherwise not found in survivors with surgical treatment alone, greatly affect the quality of life.[10]

Table 7. Type of treatment and mean global quality of life of ovarian cancer survivors

Type of Treatment	Mean Global Quality of Life	Frequency n(%)
Surgery only	96.67	5 (12.8)
Neoadjuvant Chemotherapy + Surgery + Adjuvant Chemotherapy	66.67	4 (6.4)
Surgery + Adjuvant Chemotherapy	82.23	36 (80.9)

Table 7 shows the more types of treatment obtained by survivors will make the quality of life decreases. The results showed that the highest mean quality of life was found in survivors who only received treatment only surgery and the lowest quality of life in survivors who received neoadjuvant chemotherapy + surgery + adjuvant chemotherapy.

DISCUSSION

According to the American Cancer Society, the incidence of ovarian cancer peaked in women aged 55-64 years old.[11] In Indonesia, the peak age group in gynecologic cancer especially the cervix, uterus and ovary were between 45-54 years old.[1] Over 90% malignant ovarian cancer were derived from epithelium, 5% - 6% from sex cord-stroma and 2% - 3% were germ cell tumors.[1] Currently, two-third of the patients are only diagnosed when they reach the third or fourth stage because the symptoms of ovarian cancer are not specific and there is no screening instrument with high specificity, sensitivity and cost-effectiveness.[12]

The choice of treatment is cytoreductive surgery followed by chemotherapy. The great development of advanced chemotherapy is due to the increasing number of studies on the appropriate dosage, schedule, sequence and duration of chemotherapy in ovarian cancer patients.[13] Dinkelspiel's research stated that 55.8% of ovarian cancer patients had a therapy duration of 4 – 8 months and 44.2% patients underwent a duration of ≤ 3 Months.[9]

In EORTC, high scores on the Global Quality of Life Scale and Functional Scale define the better quality of life and functionality in health term, meanwhile the higher scores obtained in symptom scale means higher of the level of symptoms or problems experienced by the survivors.[14]

The physical, role and social functions of ovarian cancer survivors are fairly good and getting improve in long-term survival. But the physical and role functions are decreasing in ≥ 70 years old patients because of the aging process.[15]

In emotional function, some of the survivors often experienced irritability and anxiety. These were related to family, especially from spouse support. Low scores on physical function, fatigue, and pain are also associated with emotional function, coupled with disturbances in body image, sexual dysfunction, fear of death and recurrence. These associations are more common found on the young survivors.[16]

Some of the survivors also stated that it was harder to remember things and to concentrate on activities due to chemotherapy induced cognitive decline, though the mechanism is still unclear.[17] More therapies received by cancer patients will cause more side effects, including fatigue. Fatigue is also influenced by emotional function and worries. Thus, it will affect

their sleep cycles.[18] In this study it was found that most survivors of ovarian cancer did not experience insomnia. However, Greimel Research reported that some survivors of ovarian cancer also experience insomnia, especially in survivors of ovarian cancer that has been long (> 10 years).[15] The survivors also experienced long-term pain in their first and second post-therapy years as a result of surgical scars and side effects of chemotherapy such as joint pain.[19]

Sexual function disturbances are also found. In pre-menopause women diagnosed with ovarian cancer and removed of both ovaries (oophorectomy), estrogen and androgen production is decreased as well as common symptoms such as hot flushes, vaginal dryness and increased sensitivity of local pain. In the latter stage, it will lead to atrophy of the vaginal wall. These factors contribute to dyspareunia or pain during intercourse. Hopkins stated that there were 5 factors affecting sexual function: disinterest in sexual relations, physical disorders, not having a partner, fatigue, and couples who are not interested in sexual intercourse.[20]

Previous chemotherapy induced peripheral neuropathy. It was proved in Buhgwandass research that showed survivors kept on suffering neuropathic symptoms even 12 years after the treatment. Chemotherapy Based on platinum compounds (cisplatin, oxaliplatin, carboplatin and analogues) damage the dorsal root ganglia of neurons by forming adduction with nuclear and mitochondrial DNA. Platinum chemotherapy tends to show similar sensory peripheral neuropathy consisting of numbness, tingling and paresthesia in the hands and/or feet. Although Carboplatin is found to be much less neurotoxic than cisplatin or oxaliplatin.[21] Surgery and chemotherapy also cause iatrogenic menopause.[10]

There is no association between age, histopathological results, stage of cancer, duration of therapy and quality of life of ovarian cancer survivors. The same results obtained in Flora's study stated that there was no significant difference between age, stage, histopathological type, and duration of therapy on the quality of life of ovarian cancer survivors. Flora's study also states that the most important role in assessing the quality of life of survivors of ovarian cancer is the functional scale, especially the physical function and the role and symptom or problems experienced by survivors after chemotherapy, and added to the psychosocial factors of the survivors.[22]

The results obtained are also in accordance with Wright's research which examined 64 women survivors of ovarian cancer with an age range of 40-83 years. The results also found no difference in the quality of life of survivors of ovarian cancer in young and old-age women, although in young women there were disturbances in emotional function and body image, this did not affect the global quality of life.[23]

Beale's research in 2009 found that there was no significant difference between the quality of life for ovarian cancer survivors diagnosed at an early stage or an advanced stage. Although side effects such as abdominal pain and symptoms are more common in the early stages, they do not affect the overall quality of life. Beale said that these side effects were associated with early-stage ovarian cancer survivors being diagnosed more often at a young age, so they have higher hopes for a full recovery.[24]

The histopathological results of ovarian cancer survivors also had no relationship with the quality of life of the survivors. In accordance with the research of Jianguang, it was stated that the histopathological results only function as a prognostic factor when diagnosed so that it does not affect the quality of life which is more influenced by psychological factors and side effects of therapy.[25]

Dienkelspiel's study also suggested that chemotherapy duration and cancer stage had no relationship with the quality of life for ovarian cancer survivors. There was no improvement in the quality of life at chemotherapy duration> 3 months and <3 months. This is because the duration of chemotherapy does not always determine the number of chemotherapy cycles a patient undergoes, so it cannot be used as a determinant of the type of therapy that the previous survivor received.[9]

However, in this study there is a significant association between type of therapy and quality of life in ovarian cancer survivors. Compared to Bughwandass, the types of therapy underwent by the survivors affect their quality of life. Survivors who received surgery and chemotherapy have a lower quality of life than the survivors who only went through surgery, especially on the scale of symptoms such as fatigue, peripheral neuropathy, attitude toward disease, and financial terms. His study also used linear regression analysis which showed that more chemotherapy cycles, recurrences, and short duration of survival were associated with higher neuropathy scores. In fact, 51% of women with ovarian cancer who received chemotherapy experienced neuropathy symptoms up to 12 years after treatment ended, and this greatly affected their quality of life.[10]

In conclusion, the quality of life of ovarian cancer survivors globally is good. There is no association between age, histopathological results, stage of cancer, duration of therapy and quality of life of ovarian cancer survivors. However, there is a significant association between type of therapy and quality of life in ovarian cancer survivors. The more types of therapy obtained by survivors, it will make the more quality of life decreases.

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Laboratory preparedness to support the Covid-19 pandemic respond in Indonesia

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Abstrak

Latar belakang: *Penyakit jenis baru COVID-19 yang disebabkan oleh virus corona menjadi sebuah pandemic di akhir tahun 2019. Kota Wuhan (China) merupakan lokasi pertama terdeteksinya kasus COVID-19. Tanpa adanya kecurigaan apapun penyakit ini dengan cepatnya menyebar ke seluruh dunia mengikuti alur mobilitas manusia. Dalam kondisi tersebut sistem kesehatan di setiap negara tampak kelabakan khususnya dalam pengendalian transmisi penyakit. Studi ini ingin mengidentifikasi kesiapan jejaring laboratorium kesehatan di Indonesia.*

Metode: *Penilaian cepat dilakukan terhadap ketersediaan dan kesiapan laboratoriaum dalam pennanganan pandemi Covid-19. Pengumpulan data dilakukan melalui pengisian questioner yang dikirim secara elektronik. Waktu pelaksanaan adalah minggu ketiga dan keempat, Maret 2020. Terdapat 44 laboratorium jejaring laboratorium dibawah Kementerian Kesehatan yang menjadi subjek penelitian, dan sebanyak 33 yang merespon secara lengkap Variabel ketersediaan, kecukupan dan kebutuhan bahan dan alat.*

Hasil: *Jejaring laboratorium kesehatan dibawah Kementerian Kesehatan sudah terbentuk sejak tahun 2009. Dengan terjadinya pandemic COVID-19 Surat Keputusan Menteri Kesehatan telah direvisi hingga dua kali agar dapat meningkatkan kapasitas dan memperluas jejaring ke seluruh wilayah NKRI. Hasil studi menunjukkan, laboratorium jejaring dibawah Kementerian Kesehatan belum siap dalam menghadapi pandemic COVID-19. Dua jenis laboratorium jejaring yaitu laboratorium surveillans maupun laboratorium diagnostic memiliki kondisi yang sama. Ketersediaan bahan dan alat laboratorium standar masih tergolong rata-rata, bahkan dari sisi kecukupannyapun masih jauh dibawah kapasitas kebutuhan dalam penanganan specimen COVID-19. Kondisi yang sama juga tampak untuk bahan pendukung laboratorium termasuk alat pelindung diri untuk petugas.*

Kesimpulan: *Kesiapan laboratorium sebagai bagian dari system kesehatan dalam kondisi pandemic masih lemah. Keberadaan alat penunjang diagnose khususnya untuk penyakit menular harus dilengkapi sesuai dengann type laboratorium. Pandemi COVID-19 menjadi alarm dalam menghadapi era baru dan antisipasi masalah dimasa yang akan datang.* ***(Health Science Journal of Indonesia 2020;11(2):140-8)***

Kata kunci: *Kesiapan laboratorium, COVID-19, Indonesia*

Abstract

Background: A novel coronavirus disease called COVID-19 has become pandemic in late 2019. Wuhan City was the first place detected as the source of the pandemic. Without suspicion, it spreads over the world, along with human mobility. In such a condition, every country seems quite stuttering to prepare its health system to prevent its people from the possible transmission. This study aimed to describe the preparedness of the networking laboratory in Indonesia.

Methods: We conducted a rapid assessment of laboratory availability and preparedness to respond to the Covid-19 pandemic. We held the data collection on the third and fourth week of March 2020 by sending an electronic questionnaire to all 44 networking laboratories under the Ministry of Health structure. The variables assessed in this study were the availability and the requirements of the Covid-19 related laboratory's substances, including reagents and other equipment types.

Results: The Ministry of Health established the networking laboratory in 2009, but due to the COVID-19 pandemic, it has renewed twice to enhance and expand the laboratory capacities over the country. Our studies showed preparedness among networking laboratories in Indonesia regarding this new emerging COVID-19 condition was quite devastating. Both surveillance and diagnostic laboratories have a similar situation. The availability of their primary materials was mediocre, but the adequacy was far beyond the capacity in handling the COVID-19 specimen. We found a similar case in the laboratory, supporting materials, and personal protective equipment (PPE).

Conclusion: Laboratory preparedness during initial period of time of the COVID-19 pandemic as part of the health system is still weak. The availability of the necessary equipment, supporting materials, and personal protective equipment are far beyond the requirements. The COVID-19 has alarmed the laboratory and the whole health system in Indonesia into a new era with better future preparedness. ***(Health Science Journal of Indonesia 2020;11(2):140-8)***

Keywords: laboratory preparedness, COVID-19, Indonesia

The etiology of emerging COVID-19 as a new type of Corona Disease is unspecified yet when it started identified at Wuhan City, Hubei Province, China in late 2019. On January 7, 2020, China's scientist has called this new emerging disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SAR-CoV-2). Along with the fast spread escalation of the case number, mortality, and import cases in other continents outside China, the WHO declared COVID-19 a public health emergency of international concern.

The role of the laboratory in examining the evidence of Covid-19 transmission is vital. The National Institute of Health Research and Development (NIHRD) under the Ministry of Health is appointed as the diagnostic center for COVID-19 samples. Indonesia faces a stuttering condition in handling this new pandemic as an immense archipelago consisting of more than 17 thousand islands with a 265 million population (CBS).[1]

The Indonesian Presidential Decree number 11/2020 on March 31 stated COVID-19 as Public Health Emergency in Indonesia. The escalation and spread of the disease over the country impacted not only the community health status and mortality but also economics, politics, sociocultural, and security defense.[2]

Resilience planning has forced the central government to work hand in hand to cope with those cascading impacts encompassing the central budget.[3] Following the President Decree, the first step of government preparedness was budget reallocation for the COVID-19 control program. The Ministry of the Finance Republic of Indonesia directly takes into action with the policy of all cost related to the procurement of medical devices supporting health examinations, isolation room, medical protective equipment; free of charge medical treatment for COVID-19 suspect, probable and positive cases including other prevention tools such as face mask, and others; which in consequences would swelling budget deficit of the country.

A rapid assessment is needed to enhance and empower laboratory networking concerning all costs related to medical devices' procurement supporting specimen examinations called laboratory preparedness. This assessment referred to the Ministry of Health (MoH) Statement number HK.01.07/Menkes/214/2020 and HK.01.07/Menkes/216/2020.[4] This study aimed to identify the laboratory preparedness to examine COVID-19 specimens so that the central government can improve the laboratory capacity and provide the specified devices needed.

METHODS

The study design was a Quantitative Study using a cross-sectional type of data collection.[5] We intended this study as assessment research to empower and improve the existing laboratory's standard capacity of the laboratory for examining the COVID-19 specimens. A similar assessment at this data collection focusing on the laboratory capacity has published at the other journal.[6]

We conducted primary data collection to check the current availability of all equipment under study. The variables examined in this study were the availability and the requirements of the necessary materials such as reagents, viral transport media (VTM), and Dacron swab. We were also collecting the availability of supporting materials that are all components needed to do the viral examination.

Personal protective equipment (PPE) such as hazmat suit, N95 face mask, google-glasses, etcetera; were also taken into account for this study variables.

We conducted this study in the 3rd to the 4th weeks of March 2020. The study location was 34 provinces in Indonesia involving 44 Laboratories in the networking categorized as National Referral Laboratories and Surveillance and Diagnostic laboratory.[7]

The data were collected using a standardized questionnaire sent over through emails, followed by a webinar discussion. The person in charge of the laboratories fulfilled the questionnaire, returning by application (email and WhatsApp). Clarification of the data done when there was unclear information given by the respondents. Data compiled by the researchers at the National Institute of Health Research and Development (NIHRD) Ministry of Health (MoH) and Indonesia One Health University Networking (INDOHUN). Incoming data were entered into the data template and analyzed descriptively.

Ethical clearance provided by the Ethics Commission of the NIHRD, MoH under the letter number of LB.02.01/2/KE/303/2020.

RESULTS

Before we move on to the study results, let be understood first the differences of the functions and authorities of the National Referral Laboratory and the Diagnostic and Surveillance Laboratory based on the MoH Regulation number 658/Menkes/Per/VIII/2009.[5] The MioH updated this regulation during the COVID-19 pandemic with Indonesia's MoH number HK.01.07/Menkes/2020 concerning laboratory networking of Covid-19 examination.[4] Both laboratories have a diagnostic function, but the National Referral Laboratory has broader authority to develop the standard operating procedure to be used by the Diagnostic Laboratory.

Table 1. The function of National Referral and Surveillance and Diagnostic Laboratory of COVID-19*

National Referral Laboratory	Surveillance and Diagnostic Laboratory
Receive specimens for COVID-19 assay from hospitals/ COVID-19 Diagnostic laboratory, health offices, and or other health laboratories	Receive specimens for COVID-19 assay from hospitals/COVID-19 Diagnostic laboratory, health offices, and or other health laboratories
Develop standard operating procedures regarding the collection, management, and inspection of COVID-19 specimens	Conduct screening checks on COVID-19 specimens using the form and standard operating procedures established by the Referral laboratory, i.e., the National Referral Laboratory under the National Institute of Health Research and Development, Ministry of Health.
Confirming the results of a positive COVID-19 assay conducted by the COVID-19 Diagnostic Laboratory and report to the Director-General of Disease Prevention and Control and the Director-General of Health Services with a copy to the Minister of Health	Send all specimens (after partly taken for assay) to the COVID-19 national referral laboratory immediately without waiting for the results of the assay
Quality assessment/Assurance/Control including technical supervision and guidance to COVID-19 Diagnostic Laboratory	Send the assay results (positive and negative COVID-19) when ready, to the Center for Research and Development of Biomedical and Basic Health Technology, NIHRD, and the Public Health Emergency Operation Center (PHEOC), Directorate General of Disease Prevention and Control Ministry of Health.
Sending COVID-19 assay panel tests to COVID-19 Diagnostic Laboratory	Inform all the results to the hospital parallel to the Directorate General of Disease Control and Prevention and Provincial Health Office. Due to confidentiality, the positive assay's results can only be issued by the National Referral COVID-19Laboratory
Conduct technical supervision and guidance to the COVID-19 Diagnostic Laboratory Perform recapitulation of assay's results from all COVID-19 Diagnostic Laboratories	Provide feedback to hospitals/health services other health laboratories if there is an error in the use of materials or media on the specimens received

* Indonesia MoH Regulation number 658/Menkes/Per/VIII/2009

Indonesia is a big archipelago country consisted of 34 provinces. To deal with the laboratory issues on COVID-19, the MoH quickly setting up the coordination system through an official statement of the Minister of Health Number HK.01.07/ Menkes/182/2020. This statement pointed out 12 regional laboratories, and each laboratory responsible for two to six provinces depend on the distances and the area's width. Table 2 provides the regional laboratory and the areas under its responsibility.[4]

Table 2. Regionalization of the COVID-19 laboratory in Indonesia

No	Regional Laboratory	Provinces under Responsibility
1	Jakarta Health Laboratory Center	Maluku, South Maluku, West Sumatera, North Sumatera, Aceh
2	Palembang Health Laboratory Center	Bengkulu, Bangka Belitung, South Sumatera, Jambi, Lampung
3	Makassar Health Laboratory Center	Gorontalo, North Sulawesi, West Sulawesi, Central Sulawesi, South Sulawesi, South-East Sulawesi
4	Surabaya Health Laboratory Center	South Kalimantan, Central Kalimantan, North Kalimantan, East Kalimantan
5	Papua Health Research and Development Center	Papua and West Papua
6	Jakarta Center for Environmental Health Engineering and Disease Control Engineering	Riau, Riau Isles, West Java, West Kalimantan, Banten
7	Surabaya Center for Environmental Health Engineering and Disease Control Engineering	Bali, East Java, East Nusa Tenggara, West Nusa Tenggara Barat
8	Yogyakarta Center for Environmental Health Engineering and Disease Control	Yogyakarta and Central Java
9	DKI Jakarta Regional Health Laboratory	DKI Jakarta
10	Eijkman Institute for Molecular Biology	DKI Jakarta
11	Faculty of Medicine of Indonesia University	Central Hospital Dr. Cipto Mangunkusumo and Hospital of Indonesia University
12	Faculty of Medicine Airlangga University	Provincial Hospital of Dr. Soetomo and Hospital of Airlangga University

By March 25, 2020, there are 69 health laboratories distributed over the country. Among these laboratories, 44 laboratories having the capacity for COVID-19 surveillance and diagnostic, and the remaining 25 laboratories haven't yet such capacity.

General condition of the laboratories providing the COVID-19 assays

Among the 44 networking laboratories, 37 laboratories were fulfilled and sent back the questionnaire. Among those, 11 laboratories (29,7%) acted as surveillance laboratories and the remaining 26 (70,3%) as examiner laboratories. Nevertheless, some laboratories did not fill out the questionnaire that considered as missing data. Results found the mean capacity of specimen assay per day was 55 specimens with the same mode and median values of 48. From the 44 networking laboratories total estimated specimen assay per-day that can be conducted all over the country until March 25, 2020, was 4293 specimens. The surveillance laboratory had a higher mean capacity of 70,3 compare to 48 specimen examination for the diagnostic laboratory. Those laboratories depended on the laboratory's human resources, both on their education and training experiences. All 33 laboratories fulfill the questionnaires, 76.5% of the laboratory's human resources having technical skills in a virus laboratory assay. Concerning these skills, of the ten surveillance laboratories, all human resources (100%) had virus examination training skills compared to 66.7% of those in 24 diagnostic laboratories.

The availability and adequacy of laboratory's basic materials of COVID-19 assay

Lessons learned from the H5N1 epidemic in 2007 became references in assessing the laboratory capacity in Indonesia. The basic and necessary materials referred to the reagent, viral transport media (VTM), and Dacron nasal swab. Our study found that almost all of the 33 laboratories in this study have those.

Table 3. The availability and adequacy of laboratory's basic materials of Covid-19

No	Basic Materials	N	Availability		Adequacy	
			n	%	n	%
1	Reagent	33	10	30.3	1	3.8
2	Viral Transport Media (VTM)	33	21	61.8	4	15.4
3	Dacron swab	33	19	57.6	2	7.7

Table 4. Percent availability and adequacy of laboratory's basic materials of COVID-19 between surveillance and diagnostic's laboratory

No	Basic Materials	N	Surveillance Laboratory		Diagnostic Laboratory	
			Availability (%)	Adequacy (%)	Availability (%)	Adequacy (%)
1	Reagent	33	55.6	0	20.8	5
2	Viral Transport Media (VTM)	33	55.6	16.7	64	15
3	Dacron swab	33	55.6	16.7	58.3	5

Table 3 depicted the laboratories' limited availability of necessary materials intended to assay the COVID-19 specimens, yet even limited in its adequacy.

Table 4 indicated quite a different percentage of availability and adequacy of the necessary materials for both types of laboratories. Comparing the availability and adequacy of supporting materials for COVID-19 between the surveillance and the diagnostic laboratory was a bit different. The surveillance laboratory has better adequacy than the diagnostic one.

The availability and adequacy of laboratory's supporting materials of COVID-19 assay

Supporting components are essential for laboratory testing. The laboratory preparedness on supporting materials on Covid-19 assay was based on the past evidence of the H5N1 epidemic. Table 5 showed the types, availability, and adequacy of the supporting materials at the laboratories under study.

Table 5. The availability and adequacy of laboratory's supporting materials of COVID-19

No	Basic Materials	N	Availability		Adequacy	
			n	%	n	%
1	Micro pipet	32	30	93,8	14	58,3
2	Micro-centrifuge refrigerator	32	23	71,9	10	45,5
3	Water bath	33	22	66,7	13	56,5
4	Vortex Mixer	33	29	87,9	10	43,4
5	Freezer reagent – 20	33	27	81,8	22	81,5
6	Freezer sample -20	33	24	72,7	18	66,7
7	Cryotube	30	19	63,3	2	8,3
8	Ice pack	33	30	88,2	7	28,0
9	Micro-centrifuge tube 1,5 mL	31	25	80,6	5	26,3
10	PCR tube	27	16	59,3	3	14,3
11	Aerosol barrier micropipette tips 1000 uL	30	19	63,3	3	14,3
12	Aerosol barrier micropipette tips 200 uL	30	19	63,3	3	14,3
13	Aerosol barrier micropipette tips 10 uL	30	17	56,7	3	14,3
14	RNA extraction kits	30	10	33,3	1	4,3
15	Para-film	31	22	71,0	6	26,1
16	UN Transport box	31	9	29,0	5	20,8
17	Alcohol 70%	30	21	70	4	17,4
18	Biohazard plastic	31	21	67.7	3	13,6

Table 5 depicted the availability and adequacy of supporting materials for COVID-19. Most of the availability was relatively good except for the RNA extraction kits and UN Transport box, which were only 33.3% and 29%, respectively. However, the adequacies were unexpectedly low, and only for the first six equipment showed the percentage of slightly more or less than 50%

The availability and adequacy of Personal Protective Equipment (PPE) for laboratory

Biosafety and biosecurity are an essential standard for networking laboratories. The laboratory technical staff has to be protective safely when managing the assay such a contagious substance or specimen of COVID-19. The personal protective equipment refers to the Hazardous material suit (Hazmat), hair cap, boot shoes or shoe protective gear, appropriate masker a like N95, gloves as well as google glasses. Table 6 provides the availability and adequacy of such equipment.

Table 6 showed the personal protective equipment (PPE) availability at the laboratories was quite good, although it has scared inadequacy. Hazmat suit was available at half of the total laboratory under study. Shoes protective gear was barely available. From the table, it can be seen that the adequacy of all those equipment was very poor.

Table 6. The Availability and Adequacy of Personal Protective Equipment in Laboratory

No	Basic Materials	N	Availability		Adequacy	
			n	%	n	%
1	Hazmat suit	35	17	48.6	2	7.1
2	Other types of laboratory suit	32	18	56.3	2	8
3	N95 Face Mask	36	31	86.1	3	10
4	3-ply surgical mask	32	30	93.8	3	11.5
5	Google glasses	36	30	83.3	5	17.2
6	Gloves	34	33	97.1	5	17.9
7	Boot shoes	34	21	61.8	4	14.3
8	Shoes protective gear	34	10	29.4	2	6.9
9	Hair cap	32	18	56.3	6	23.1

DISCUSSION

The laboratory preparedness in Indonesia at the early pandemic of Covid-19 is relatively struggling. The primary and supporting equipment was far from adequate both at the diagnostic and surveillance laboratories. Procurement of primary and supporting the laboratory's material and personal protective equipment (PPE) enhanced the laboratory capacity for the COVID-19 pandemic. Reallocation of the government budget in laboratory capacity for COVID-19 covered three budget sources; first, refocusing budget at each laboratory; second, budget allocation from the province as well as district and municipality; and third, the budget of the central government as well as the third party such as international agencies, community, and private sectors. The central government and the third-party budget delivered to the National Task Force of COVID-19 for the acceleration of COVID-19 pandemic control at the national, province, and district/city levels.

One of the five critical actions done during the surge pandemic situation is a better preparedness of the health system. In Indonesia, the central government quickly took action since the Covid-19 declared a national pandemic. A national action group called G*ugus Tugas* developed at the national until village level. These groups worked and concerned with the social situation to maintain community health and prevent Covid-19 transmission. At the central level, the *Gugus Tugas* also helped maintain the laboratory preparedness, such as distributing the laboratory equipment simultaneously with the other things delivered by the NIHRD. These actions accordance with the expert thought as mentioned by Lancet.[8]

The availability and adequacy of basic and supporting laboratory materials for COVID-19 assay are fulfilled adequately either at the surveillance or diagnostic laboratories—the availability of reagents for RNA extraction and PCR kits prioritized by the laboratory refocusing the budget. Also, the Ministry of Health and Central Government involved the non-government laboratories such as universities and private laboratories as diagnostic laboratories based on the Circular Latter of the Ministry of Health on April 7, 2020. RT-PCR, as well as guideline on SARS-CoV-2 testing, are equipped these laboratories.[9]

The surge of the COVID-19 pandemic made the Indonesian health system stuttering. The patient buildup in hospitals simultaneously with the swab specimen is queued at the laboratories. An alternate rapid diagnostic test (RDT) was utilized to screen the COVID-19 suspects and prevent such conditions, in addition to waiting for the supply of PCR kits. The test took the blood specimen's fingertip to detect IgG and IgM antibodies as signs of infection. This test procedure was more applicable for mass screening among people at risk, such as those having contact history with a positive case of COVID-19, COVID-19 suspects, and the health worker in hospitals. Yet this RDT that had been widely used at Jakarta's cluster area Jakarta was more likely to give a false-positive result. Therefore, a positive RTD result then still be confirmed and followed by PCR assay for robust diagnosis.[10]

The Center of Research and Development for Biomedical and Basic Health Technology, NIHRD provides and distributes other basic material such as viral transport material (VTM) and Dacron nasal swab. Nonetheless, for further rapid prevention, the Dacron swab's limitation was temporarily replaced by the Diphtheria nasal swab as long as this nasal swab stick was not made by wood and cotton bulb.

The availability and adequacy of supporting materials for COVID-19 comparing to handle the epidemic of Avian Influenza (H5N1). Utmost the laboratories had the materials respectively, but they were in minimal numbers. In the hospital's laboratory, the pseudo materials unavailability or inadequacy was not a problem as they can directly request from the hospital's supporting medic division at any time. The previous experienced told us the essential to conduct the laboratory assessment for new emerging and re-emerging diseases. However, it was unfortunate that the laboratory networking developed in 2009 was still tricky to accomplish the laboratory task and readiness when a new-emerging pandemic such as COVID-19 happened. This situation was again evident after the outbreak of H5N1.[11]

The availability of personal protective materials was minimal at the laboratories under this study. The estimation of the standard requirement was far beyond the adequacy. For the contagious specimen assay, PPE was absolute and standardized. The availability of a Hazmat suit and hair cap approximately only 48-56% for all laboratories under this study. They neglected availabilities of boot and shoes protective gear. Other PPE availability such as masker, gloves, and google glasses were far from required. The technical laboratory persons should implement all biosafety required including PPE for virus examination and utilize laboratory algorithm of SAR-CoV2 as recommended by WHO.[12]

The standard number of PPE needed varied depending upon the capacity of the laboratory. WHO did not address the PPE standard for laboratory, but hospital and patients. For patients per day, the PPE needed were: gown and medical mask (25 units), gloves – non-sterile (50 units), and google-glass or face shield (1 unit).[13]

Supporting assay devices is urgently in need either at the surveillance or examiner of COVID-19 laboratory. It was evident that all of the networking laboratories had standard supporting materials. Nonetheless, the adequacy, based on the laboratory's capacity assay estimation was far beyond the standard referring to an overview by Taipe's scientists, saying that for diagnosis on Covid-19, a well-equipped laboratory having level 3 biosafety level.[14] Nevertheless, a well-equipped laboratory is not merely the important things, but a well documentation of infected cases are simultaneously in need to prevent the transmission of the Covid-19.[15]

In conclusion, the laboratory's networking was developed in 2009. However, in this new pandemic of COVID-19 evident, lack of laboratory preparedness was portrayed at the early time of pandemic evidence in March 2020. The existing laboratory networking seemed to lack maintenance. However, based on study findings, the central government rapidly tackled problems for helping Indonesia's society control the COVID-19 pandemic. The networking system was officially rapidly expanded. The government also set budget refocusing for laboratory hard-wares and soft-wares. This policy prioritized all costs related to the procurement of laboratory and medical devices.

The basic materials barely available in the laboratories under study include reagents, viral transport media, Dacron swab, and other supporting materials. The government quickly fulfills all the required devices identified in this study either by local or imported products. By April 15, 2020, the number of the laboratory capable of doing COVID-19 assays using RT-PCR increased by more than 70 laboratories compared to 44 laboratories when this study carried out. Although the numbers of those laboratories kept growing up, it was still a significant homework for Indonesia to maintain the laboratory capacity to dawn recent and future pandemics.

Study limitation

This paper focused on the laboratory's preparedness, equipment availability, and stuff, excluding human resources. This study kept going to leverage the need for human resources in escalating the laboratory capacity during the surge of Covid-19 pandemics as already published in the other journal.[6]

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Availability of the data and materials

All data kept protected by the Data Management Section at the NIHRD, MoH, Republic of Indonesia.

Author contribution

NKA is the coordinator of this study under the research umbrella investigated by HR. Both NKA and HR have an equal contribution to this article.

Competing interest

We declare that there is no competing interest in this study

Consent for publication

Not applicable

Ethics approval

Exempted ethical clearance declared by The Ethical Committee, NIHRD, MoH, Republic of Indonesia number LB.02.01/2/KE/303/2020.

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