

IDENTIFICATION OF THE LEVULINATE DEHYDRATASE (ALAD) GENE POLYMORPHISM AND WHOLE BLOOD HEMOGLOBIN IN THE STUDENTS OF ELEMENTARY SCHOOL IN KALIDERES, JAKARTA, INDONESIA

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Abstract– Lead (Pb) is a toxic pollutant and no organism can decrease the natural concentration. In Kalideres, there is a bus station and industries that are adjacent to settlements, exposing children around to Pb. One of the characteristics of Pb particulates is that they are found in the bottom layer of air, ± 1 meter above ground, equivalent to the height of children aged 6 to 9 years old (grade 1 to 2 elementary school students) whose height range between 100-140 cm, increasing the risk of the children being exposed to Pb. The aim of this study was to identify the ALAD gene polymorphism, to discover the frequency of the ALAD gene polymorphism genotype, and to discover the relationship between the ALAD gene polymorphism genotype and the hemoglobin concentration. The method used in this study was a comparative-descriptive method with laboratory test techniques using a digital hemoglobinometer, PCR and PCR-ARMS (Amplification Refractory Mutation System), and the completion of questionnaires by the parents for respondent behavioral characteristics and routines. The samples were 60 students who were 51.7% were ALAD wild types, 48.3% heterozygotic genotypes, and no mutants were found in the population of elementary school children in Kalideres, Jakarta, and there was no connection between the ALAD gene polymorphism genotype and the hemoglobin concentration category (anemic and non-anemic). The results of this research also predict profile level of lead environmental present as well.

INTRODUCTION

The heavy metal Pb, both from natural sources and from human activities, cannot be degraded (Sly L. and Marie N.B.D., 2013). The sources of the pollutant Pb from human activities are mainly from transportation and industry (Sun *et al.*, 2012). Dense traffic and the volume of vehicles causes traffic congestions which lead to the decrease in air quality. The ambient air quality in November in Kalideres, West Jakarta showed a Pb concentration of 1,220 $\mu\text{g}/\text{m}^3$ (Environmental Management Agency of the

Special Capital City District of Jakarta, 2014). The data showed an increase from 2013 which was 0.585 $\mu\text{g}/\text{m}^3$. The situation in Kalideres where there are a bus station and industries located near settlements exposes children to more Pb. In addition, Pb particulates are always found in the bottom layer of air (Puspitaningrum *et al.*, 2016). This characteristic puts children aged 6 to 9 years old, who have heights ranging between 100 and 140 cm, at a higher risk of being exposed to Pb. The children's height which is equivalent to the height where the Pb pollutants are found would allow more Pb to be

absorbed through their respiratory tract.

Exposure to the heavy metal Pb in the environment could have negative effects by causing ALAD gene polymorphism in humans. The polymorphism in the *ALAD* gene results in two alleles, *ALAD-1* and *ALAD-2* (Ozturk *et al.*, 2012). Polymorphism is due to the *ALAD-2* sequence composition which undergoes a transversion from the base G to C at the 177th nucleotide of the coding site. If the cellular DNA repair system malfunctions, it will cause an error in reading during protein synthesis (Fatchiyah *et al.*, 2011). This would cause a change in the synthesis of amino acids, from the amino acid lysine to asparagine.

ALAD gene polymorphism produces the ALAD enzyme which is more electronegative to Pb. The ALAD enzyme-2 has stronger bonds with Pb than the ALAD-1 enzyme. This would cause a competition between Zn ions and Pb ions. Zn ions in the ALAD enzyme active site play a role in catalyzing and stabilizing the enzyme's tertiary structure. As a result of the competition with Pb, the ALAD enzyme is unable to bind the Zn cofactor, causing the ALAD enzyme function to be impeded (Palar, 2012). The inhibition of the function of the ALAD enzyme (delta-aminolevulinate dehydratase) could result in the inhibition of heme biosynthesis, causing the decline in hemoglobin production and leading to anemia (Bemmela *et al.*, 2011). The symptoms of anemia have similarities with those of hypoxia, such as weakness, fatigue, difficulty in concentrating, forgetfulness, loss of appetite, paleness, and proneness to illnesses (Azhari, *et al.*, 2014). Detection of the polymorphism gene is begun with tissue samples that are easily collected such as peripheral blood, saliva, buccal mucosa cells, and hair follicles (Fatchiyah *et al.*, 2011). Polymorphism is detected using the PCR-ARMS (Amplification Refractory Mutation System). PCR-ARMS allows simultaneous amplification of mutant and normal alleles using two pairs of primers (Chen *et al.*, 2007). The results of the PCR-ARMS in this study was for the differentiation of the ALAD gene polymorphism genotypes, namely the wildtype, heterozygotic, and mutant.

METHODOLOGY

This study was conducted between May -November 2016. The study was conducted at the Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, Jakarta State University and the Laboratory

of Biochemistry, Faculty of Medicine and Health Sciences, Syarif Hidayatullah Islamic State University, Jakarta. This study was designed with a descriptive-comparative method. The samples used in this study were samples of DNA collected from 60 elementary school (SD) students in Kalideres, West Jakarta. The samples were selected from the population using the multistage sampling technique. The study subjects were those with the following criteria: aged 6-9 years, have lived in the area of study for the past three years, and do not have hereditary blood abnormalities. The collection of samples had received approval from the Medical Research Ethics Commission, Faculty of Medicine, University of Indonesia.

The isolated genome DNA was amplified via PCR using a specific forward and reverse ALAD primer. The forward ALAD primer was F 5'-GCCTCAGTCTTCCCTCCTATTTAGT-3' and the ALAD reverse primer R 5'-TCCCTTCTTAGCCCTTCCTTTGATT-3' (Yamashiro *et al.*, 2014). The total volume of PCR mix was 25 µL consisting of 12.5 µL Kappa Taq Polymerase, 1 µL ALAD Forward and Reverse Primer, 5.5 µL Nuclease-Free Water, and 1 µL DNA sample. The PCR lasted for 30 cycles with the PCR program consisting of pre-denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and the final extension at 72°C for 1 minute. The concentration and temperature used were already the results of an optimization. The PCR product was 306bp. The results of the DNA isolation and amplification were analyzed quantitatively using electrophoresis with 1.5% agarose gel. The electrophoresis lasted for 30 minutes with a potential difference of 100 V in buffer TAE. Next, the PCR-ARMS was conducted to detect the ALAD gene polymorphism. The PCR product was diluted using SDW for the DNA template during the PCR-ARMS phase by adding 1 µL of the PCR product to 99 µL of SDW. The DNA template was amplified using the ALAD C and N primer for normal and ALAD C and M primer for mutants. The PCR-ARMS product was then put through electrophoresis by placing the normal primer and mutant primer in a row. The PCR product was along 168bp, then identification of the ALAD gene polymorphism genotype was done.

RESULTS AND DISCUSSION

This study used buccal mucosa as the source of

DNA through gargling technique. The buccal mucosa genomic DNA produced a concentration equivalent to blood genomic DNA (Ghatak *et al.*, 2013). Buccal mucosa genomic DNA can be used in studies about genotype polymorphism identification (Kuchler *et al.*, 2011). In this study, it was meant to identify the ALAD gene polymorphism in children aged 6-9 years old. Therefore, a non-invasive DNA sampling technique was selected.

The Phenol Chloroform conventional method was used to isolate DNA which was then detected both quantitatively and qualitatively. The detection of buccal mucosa genomic DNA quantitatively resulted in the concentration, purity, and relative purity (Boesenberg-Smith *et al.*, 2012). The results of the buccal mucosa genomic DNA isolation which had a mean genomic DNA concentration of 26.87ng/ μ L. The requirement for amplification of DNA fragments is a genomic DNA concentration of at least 100ng/ μ L is needed (Ghatak *et al.*, 2013). Analysis of the reason for the low concentration could be seen from the purity and relative purity values. DNA purity could indicate protein contamination. Table 6 presents the mean Buccal mucosa genomic DNA purity at 1.52, whereas the optimum DNA purity ranges between 1.8 and 2.0 (Ghatak *et al.*, 2013). This range demonstrates that the DNA sample is free from protein contamination (Nemoda *et al.*, 2011). DNA purity below 1.8

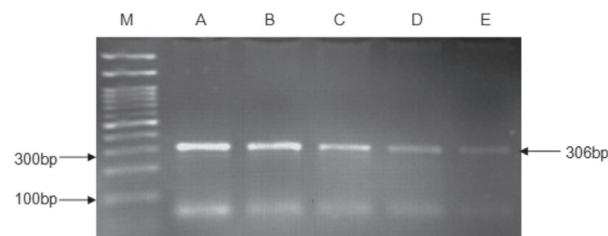


Fig. 1. The results of the phase 1 PCR in the 2% agarose gel electrophoresis with variations in the primer concentration. M is the Geneaid *Ladder* 100bp Cat.DL007 DNA marker. Column A (5 pmol), B (2.5 pmol), column C (2 pmol), column D (1.5 pmol), and column E (1 pmol).

Table 1. The results of the ALAD gene polymorphism genotype screening in the population of children in Kalideres, Jakarta

Genotype	Frequency	Percentage (%)
Wildtype	31	51.7
Heterozygotic	29	48.3
Mutant	0	0
Total	60	100

demonstrates that there is protein contamination (Ghatak *et al.*, 2013). This is explained further that sample storage did not affect the DNA amplification by PCR.

In PCR phase 1 the concentration of the primer was varied between 1 and 5 pmol which can be seen in Figure 2 to find the optimum primer concentration. Based on the visualization, the 1 pmol concentration (column E) was chosen. The visualization was in the form of a 306bp-long band which demonstrated the PCR amplification phase 1 process in all the samples. In column E there was a band beneath 100bp, even though it was faint in the visualization. The band formed in the agarose gel below 100bp was a primer dimer reaction (Lorentz, *et al.*, 2012). This primer dimer is not a problem if the target product can still be seen as a good quality band (McPherson and Moller, 2006). Therefore, this study applied the 1 pmol concentration in PCR phase 1.

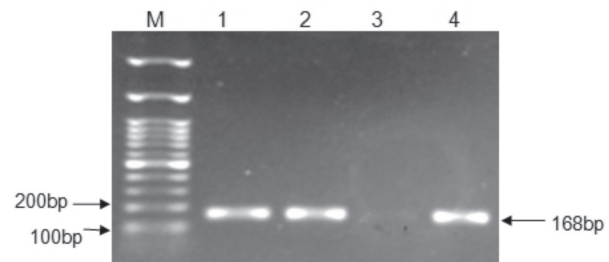


Fig. 2. The PCR phase 2 in the 1.5% agarose gel electrophoresis. M is the Geneaid *Ladder* 100bp Cat.DL007 DNA marker. Column 1 and 3 used the mutant primer (sample no. A33), column 2 and 4 used the normal primer (sample no. A49)

The results of the PCR phase 2 presented in Figure 3 show a positive band signifying a positive band for ALAD gene polymorphism at 168bp (Yamashiro *et al.*, 2014). If there is a band in both columns, the sample is heterozygotic as presented by sample no.A33 (Figure 2, Column 1 and 2). If there is a band in the column using the normal primer, the sample has a wildtype genotype as demonstrated by sample no.A49 (Figure 2, Column 4). Genotypes could be identified through bands because the primers were designed specifically in the sequence that experienced a mutation and vice versa (Huo, Xia, *et al.*, 2014). Using this method, all the ALAD gene polymorphism genotype could be identified in all samples.

The results of the ALAD gene polymorphism screening are presented in Table 7, with a wildtype

genotype frequency of 51.7%, heterozygotic genotype 48.3%, and no mutants were found in the population of children in grade 1 and 2 SDN Kalideres 03 academic year 2015/2016, West Jakarta. There were no mutant genotypes in other populations in Asia, for example, in Taiwan there it only 0.002% (Hsieh *et al.*, 2000), it was not found in Japan (Miyaki *et al.*, 2009) or in China (Yang *et al.*, 2012). This differs from the Caucasian population which has a mutant ALAD gene polymorphism genotype frequency of 1% (Kelada *et al.*, 2001). It could be concluded that the Asian population has a low ALAD-2 allele frequency, ranging between 0 and 1% (Kelada *et al.*, 2001), which is why ALAD gene polymorphism genotype mutants are rarely found.

The three types of ALAD gene genotypes occur due to the expression of two ALAD alleles: the ALAD-1 and ALAD-2 alleles (Battistuzzi *et al.*, 1981). The cause of polymorphism is the composition of the ALAD-2 sequence undergoing a transversion from base G to C in the 177th nucleotide in the coding area, in the 4th exon in the nucleotide base which codes the amino acid lysine (AAG). This causes a change in amino acid synthesis from the amino acid lysine to asparagine (Wetmur *et al.*, 1991). Biosynthesis of enzymes is under the control of genes that are related to the sequence of nucleotide bases in the nucleic acid (DNA). If there are any changes in one of the nucleotide bases in the area of the gene that expresses a protein, there will be changes in the synthesis of the amino acid (Puspitaningrum and Adhiyanto, 2016). This is because the sequence of amino acids in a protein is very specific.

The ALAD gene polymorphism produces an ALAD enzyme which is more electronegative to Pb

(Hegazy *et al.*, 2010). Gene of ALAD-2 allele which codes the ALAD enzyme is more electronegative than the ALAD 1 allele, so the enzyme with the ALAD-2 allele bonds more strongly to Pb than ALAD-1 (Kelada *et al.*, 2001). This condition causes the heterozygotic genotypes and mutants to have a higher risk of exposure to Pb compared to the wildtype genotypes. Individuals with the ALAD-2 allele, with either the heterozygotic or mutant genotype, are more sensitive to Pb exposure, inhibiting the ALAD enzyme activity and increasing the ALA molecule (Moreira *et al.*, 2012). This could cause oxidative damage and cellular structure changes (Patil *et al.*, 2006).

Based on Table 2, the percentage of heterozygotic individuals was 48.3%, showing that the children in grades 1 and 2 SDN Kalideres 03 academic year 2015/2016 West Jakarta are more sensitive to exposure to Pb in the air. In a study conducted on the general population in Japan, in a population of 101 people, 16.8% had the heterozygotic genotype and in the population of children in Bangladesh, with a population of 222 people, 17% were heterozygotic genotypes. These results need to be proven further as there were no mutant genotypes found, by increasing the size of the population in subsequent studies. The size of the findings of altered ALAD gene genotypes in this study was probably due to the presence of industries, a bus station, and densely populated settlements. The industries found in this area were copper, machinery, chemical, and motorbike equipment industries. Another strong assumption is that the source of Pb in Jakarta is the emissions from petroleum that contain lead (Albalak *et al.*, 2003). Data showed that all the respondents had been living in Kalideres area for the past 3 years and had

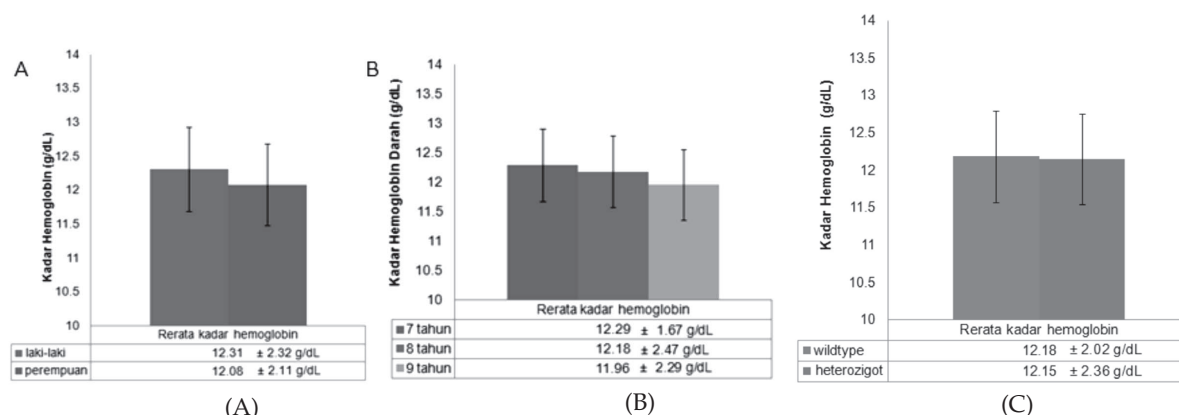


Fig. 3. A. The mean hemoglobin concentration based on sex; B. The mean hemoglobin concentration based on age; C. The mean hemoglobin concentration based on the ALAD gene polymorphism genotype.

to go through busy traffic in the morning using motorized vehicles. Another factor that increased the risk of Pb exposure to children was that the elementary school grade 1 and 2 students were between 100 and 140 cm tall, and the characteristic of Pb particulates is they stay in the lowest layer of air. Accumulation of Pb in the air, even in low concentrations, could exacerbate damage to cells in the body (Kelada *et al.*, 2001). These factors allow Pb to be resorbed and accumulate in children.

The results of the Hb concentration screening in children aged 6-9 years old are presented in Table 2. The percentage of children who were not anemic was 61.7% and 38.4% of the children were anemic. The children who suffered from anemia could be broken down into 10% having mild anemia, 26.7% having moderate anemia, and only 1.7% having severe anemia (Table 2). The classification referred to WHO standards (2001) that the Hb concentration in children between 5 and 11 years old is 11.5 g/dL. The percentage of children with anemia was categorized as low in the population of children in grades 1 and 2 SDN Kalideres 03 academic year 2015/2016, West Jakarta. This study is needed as an effort to discover the differences in Hb concentration in children based on sex, age, and ALAD gene polymorphism genotype.

Table 2. The blood hemoglobin screening results for the population of children in Kalideres, Jakarta

Hemoglobin Classification	Frequency	Percentage (%)
Normal	37	61.7
Mild Anemia	6	10
Moderate Anemia	16	26.7
Severe Anemia	1	1.7
Total	60	100

The respondents in this study consisted of 61.7% girls and 38.3% boys. The results of the study revealed a 12.31 ± 2.32 g/dL mean Hb concentration in boys and 12.08 ± 2.11 g/dL in girls (Figure 3A). This was further supported by the analysis that there was no significant difference in Hb concentration between the sexes ($P > 0.05$). The results of the analysis also revealed that there was no correlation between the sexes and the Hb concentration category (anemic and non-anemic) ($P > 0.05$). The results of this study were supported by the study conducted by Konishi *et al.* (2014) who declared that there was no significant difference between sexes and the Hb concentration in school

children.

Based on the age, the respondents of this study were divided into those aged 6-7 years old (31.7%), 8 years old (46.7%), and 9 years old (21.6%). The mean Hb concentration based on age is presented in Figure 4B. The mean Hb concentration in children aged 7 years old was 12.29 ± 1.67 g/dL, aged 8 years old 12.18 ± 2.47 g/dL, and aged 9 years old 11.96 ± 2.29 g/dL. The mean Hb concentration in children between 6 and 9 years old was 12.21 g/dL³¹. This was further supported by the analysis that the mean Hb concentrations based on age were not significant.

Figure 3 C presents the mean Hb concentration based on the ALAD gene polymorphism genotype. The wildtype genotype had a mean Hb concentration of 12.18 ± 2.02 g/dL and the heterozygotic genotype 12.15 ± 2.36 g/dL. The results of the analysis demonstrated that there was no significant difference between the ALAD gene polymorphism genotype and hemoglobin concentration. The ALAD gene polymorphism genotype and hemoglobin concentration was no significant in workers in a chemical industry in Japan who had blood Pb concentrations of $4.5 \mu\text{g/dL}$ ²⁰. These results were supported by the fact that there was no correlation between the ALAD gene polymorphism genotype and the hemoglobin concentration categories (anemic and non-anemic) ($P > 0.05$).

This study also utilized a questionnaire completed by interviews with the student's parents which was done to discover the relationship between the learning process and anemia. The results of the study revealed that 11.7% (7 subjects) were easily tired and felt sleepy at school and 21.7% of the children (13 subjects) had scores that were below the standards (Figure 4A). When perused more carefully, out of the all the children who were easily tired and felt sleepy, one had moderate anemia, 3 had mild anemia, and 3 were not anemic. These results could not yet provide a description that children suffering from anemia had problems in learning. The subsequent studies should address the relationship between anemia and children's learning behavior in a more detailed way.

Good learning achievements are influenced by a number of factors such as breakfast habits (Widyastuti, 2014). The questionnaire in this study played a role in discovering the children's breakfast habits and nutrient intake (Figure 4B). The results of the study revealed that 70% of the children had

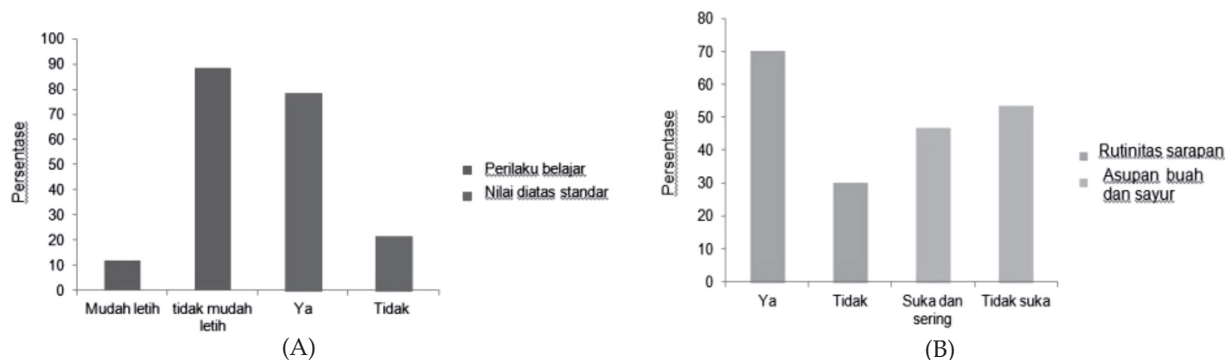


Fig. 4. The results from the questionnaire given to the respondents pertaining to the learning behavior and above-standard school grades (A), breakfast habits and fruit and vegetable intake (B)

breakfast regularly and 30% did not have breakfast before school. The children should eat breakfast to fulfill their dietary needs as a source of energy for their activities at school (Zalilah, 2010). It could be assumed that most of the parents are aware of the importance of breakfast in improving students' ability to follow the teaching and learning activities at school.

The results of the study revealed that 51.7% of the children disliked fruits and vegetables compared to only 48.3% who liked fruits and vegetables (Fig. 4B). One of the ways to reduce the effect of Pb to the body is by consuming fruits and vegetables that are rich in vitamins (Melse-Boonstra *et al.*, 2016). Vitamin C was highly effective in reducing the toxicity of Pb in the body (Kim *et al.*, 2015). This needs to be noted by the parents so that they pay more attention to their children's fruit and vegetable intake.

Based on this study's results, it could be concluded that children in the first and second grade of State Elementary School Kalideres 03, West Jakarta, in the 2015/2016 academic year had a tendency to be prone to Pb exposure because 48.3% of the sample population were found to be heterozygotic. The results also demonstrated that ALAD gene polymorphism and hemoglobin concentration did not have a significant difference. This still needs to be noted because the effect of ALAD gene polymorphism is related to health issues.

CONCLUSIONS

The identification method using PCR-ARMS (Amplification Refractory Mutation System) can be applied for the detection of ALAD gene polymorphism in children. The frequency of the

ALAD gene polymorphism with wildtype genotype was 51.7% and heterozygotic genotype was 48.3%, and no mutants were found in the population of children in grades 1 and 2 Kalideres 03 State Elementary School, West Jakarta, in the academic year 2015/2016. There was no relationship between the ALAD gene polymorphism genotype and hemoglobin concentration categories (anemic and non-anemic). There needs to be further studies with a larger sample population to map a wider ALAD gene polymorphism and a more selective filtering of children above 9 years old to classify lower hemoglobin concentrations so that the cause of anemia could be determined.

The results of this research also predict profile level of its lead environmental present and future as well. Recommendations for the government of the Special Capital District of Jakarta is to significantly reduce the concentration of Pb in the air by planting trees that could absorb Pb, educate the population to take preventive measures against Pb exposure through a balanced diet supplemented with vitamins, high proteins and supported people by the use of a mask when on the road.

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