

## SCREENING OF HEMOGLOBIN E IN STUDENTS OF BIOLOGY UNIVERSITAS NEGERI JAKARTA USING HYBRI-PROBE GENOTYPE METHOD\*

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**Abstract** - Hemoglobin E (Hb E) is a variation of hemoglobin due to mutations in  $\beta$ -globin gene, codon 26, and can cause severe anemia when combined with Thalassemia. Detection of Hb E using DNA sequencing requires a long time and expensive cost. In 2012, Yamashiro *et al.*, developed the hybridization probe methods or Hybri-Probe that are more highly sensitive, accurate, excellent in cost and time effectiveness. The aim of this study was to detect Hb E using the Hybri-Probe Genotype method to determine the number of probability cases of Hb E in students of Biology UNJ. The samples that used in this study was one DNA sample of patient with Hb E heterozygous as positive controls and DNA samples of 50 students of Biology UNJ that were selected randomly. We modified the method to be used in detecting Hb E. In this research, we designed the probes for detection of Hb E, and measure the product Hybri-Probe by the Tm using LightCycler. It is very simple, gives a good performance result and favorable time efficiency. The results of this study showed that the frequency cases of Hb E carrier in students were 6%.

### INTRODUCTION

Haemoglobin E (Hb E) is one of the variations of haemoglobin, due to mutations in the gene  $\beta$ -globin, the codon 26, which causes the amino acid glutamic acid is substituted by lysine (Olivieri *et al.*, 2011). This disorder is autosomal recessive, inherited from parents to their children, and its existence can be in the form of heterozygous, homozygous, or in combination with other abnormal haemoglobin. Patients with Hb E most commonly found in the population of Southeast Asia including Indonesia (Fucharoen *et al.*, 2012).

Clinical manifestations of Hb E are very diverse, ranging from asymptomatic to acute anaemia (Fucharoen *et al.*, 2012).

The diversity of the clinical manifestations of Hb E is highly it whether patients carry only one abnormal allele (Hb E heterozygous), both allele are abnormal (Hb E homozygous), or patients having Hb E allele in combination with other abnormal haemoglobin.

Hb E might contribute to be one factor that will reduce the quality of human resources in Indonesia. Patients with Hb E homozygotes experienced a mild anaemia. Patients with Hb E trait (Hb E heterozygous) are generally asymptomatic (Zimmermann *et al.*, 2008). Problem will occur when Hb E is found in combination with other abnormal haemoglobin such as Thalassemia. If any such indication

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exists, then the patient will experience a very severe/acute anaemia and require blood transfusions for the rest of his life (Olivieri et al., 2008). Patients with Hb E/Thalassemia will not be able to perform activities of life well, considering the conditions and ultimately reduce the productivity and quality of Indonesian youth. Therefore, early detection of the presence of Hb E is important.

Until now, detection of Hb E is done by analysing DNA for hemoglobinopathies and DNA sequences (Moiz et al., 2012). The method requires large cost and long time. Therefore, the detection of haemoglobin E requires other methods which are faster but still result in precise and accurate result. Hybridization Probe Melting Curve Genotype (Hybri-Probe) method is the latest method developed to detect the presence of mutations in the DNA quickly and accurately by using DNA probes. The probe is a nucleic acid molecule in the form of single-stranded DNA that has been labelled with a marker which has fluorescent reaction principle (Aquino, 2005).

Hybri-Probe method is expected to be feasible to be applied to detect Hb E quickly and at lower cost, but still gave accurate results. This research program aims to assist the government program in reducing the incidence of Hb E cases, which in turn can improve the quality of life for the young generation of Indonesia. The purpose of this study was to determine the frequency of patients with Hb E in the student population of Universitas Negeri Jakarta, Department of Biology with Hybridization Probes Melting Curve Genotype methods.

## **MATERIALS AND METHODS**

This study was conducted from January to March 2014. Experiment was carried out at the Laboratory of Biology and Biochemistry Faculty of Medicine and Health Sciences, Universitas Islam Negeri Syarif Hidayatullah, Jakarta. The samples used in this study were DNA

samples obtained from 50 students of UNJ Biology Department. Control was done by using a DNA sample Hb E trait. Sampling in this study was approved by ethics of the Health Research Ethics Committee, Faculty of Medicine and Science Health, Universitas Islam Negeri Syarif Hidayatullah Jakarta.

This research was carried out by designing specific primers and probes for Hb E. The primers and probes designed were then used to detect and screen for Hb E with Hybri-Probe techniques. Hb E screening result on UNJ Biology Department student was analysed using the Hardy-Weinberg Equilibrium.

### **Isolation of genomic DNA from whole blood**

Blood samples were taken from the 50 students of UNJ Biology Department respectively 3-5 mL. DNA isolation from whole blood was performed based on the protocol of Promega Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI).

### **Amplification of DNA fragments by the Polymerase Chain Reaction (PCR)**

PCR used in this study was Gradient PCR Applied Bio systems®. The total volume of PCR mix comprising as much as 25 mL of KAPA 2G 5X Buffer A containing 1.5 mM Mg in 1X Buffer A; dNTP mix; Primary forward and reverse primer; KAPA 2G Fast; dH<sub>2</sub>O; and DNA templates. PCR program as follows: pradenaturation at 94 °C for 5 minutes, followed by 40 cycles denaturation at 94° C for 2 minutes, annealing at 62 °C for 30 seconds, extension at 72 °C for 1 minutes, dan post extension at 72 °C for 2 minutes.

### **Detection and screening Hb E with Hybri-Probe Genotype method**

The mutation was detected using specific probes, which consists of sensor probes and anchor probes. Hb E detection process was done by using Hybri-Probe Genotype method using Real Time PCR; PCR instruments Light Cycler® 480 II

Roche. The total reaction volume was 19  $\mu\text{L}$  consisting of 2  $\mu\text{L}$  formamide, 1  $\mu\text{L}$  mixture sensor and anchor probes with each probe concentration was 5  $\mu\text{M}$  and the final concentration of the probe in the solution was 0.2  $\mu\text{M}$ , 12  $\mu\text{L}$  of DNA template from amplification product, and 4  $\mu\text{L}$  SDW ( $\text{dH}_2\text{O}$ ).

The materials were mixed and put in a multiwell plate Roche Light Cycler® and closed with Roche Sealing Foil. The presence or absence of mutations in the sample will be visible on the Melting Curve Genotype on Light Cycler machine that shows the difference between the melting curve of the normal sequence and the mutant sequence.

#### **Analysis of Hb E screening results using the Hardy-Weinberg Equilibrium (HWE)**

Hb E screening results were analysed using Hardy-Weinberg Equilibrium (HWE) to obtain the frequency of patients with haemoglobin E. Screening results were analysed by the equation  $p + q = 1$  and  $p^2 + 2PQ + q^2 = 1$ . Symbol of  $p$  indicates the frequency of dominant allele and  $q$  indicates frequency of recessive allele.

#### **Haemoglobin E primer**

Forward primer in this study are named  $\beta$ -forward and reverse primer named  $\beta$ -reverse. Nucleotide sequences of both primer pairs:

$\beta$ -forward: 5'

AAGAGCCAAGGACAGGTACGGCT  
GTCATCA-3'

$\beta$ -reverse: 5'

CTCCCCTTCCTATGACATGAACTT  
AACCAT-3'.

#### **Haemoglobin E probe for Hybri-Probe**

Hybri-Probe consists of two pairs of probes which are sensor probes and anchor probe. Mutation position is in the area of the sensor probe on the 26<sup>th</sup> codon. Sensor Probe and anchor probes in this study are named  $\beta$ -sensor probe and  $\beta$ -anchor probe. Probe for Haemoglobin E

detection shown in the following sequence:

$\beta$ -sensor probe: 5'-GCC AGG  
5LTC640N-CCC CCA TCA CC-3'

$\beta$ -anchor probe = 5'-CCT TAA ACC  
ACC GTA TTG CTT TGA ATA CC-3'

The Hybri-Probe process consists of 4 stages: denaturation, annealing, melting and cooling. Denaturation process was carried out at a temperature of 95 °C for 5 min. The reaction temperature range for annealing and melting temperature was 20-80 °C for 2 min, followed by cooling temperature of 20 °C for 1 min. Hybri-Probe process was done as much as 1 cycle.

## **RESULTS**

### **Haemoglobin E detection of the positive control samples**

Hybri-Probe was trial tested for Hb E detect in Hb E positive control sample. Wild-type DNA samples was used as a comparison. DNA samples were amplified using conventional PCR and continued with Hb E detection process by using a probe on Light Cycler machine. Hb E detection results can be analysed at the graph on Melting Curve Genotype. The results of the Hb E mutation detection in the positive control DNA samples of Hb E and wild type DNA is shown in Melting Curve below:

Figure 1 is the result of the analysis of the Hybri-Probe for Hb E. There are two graphs on the image. Blue graph represents the wild-type DNA has only one melting peak probe. Red graph representing Hb E mutant DNA (heterozygous) have two melting peak probes. Melting peak probes on wild-type DNA is at a temperature of 55 °C, while the melting peak probes on Hb E mutant DNA (heterozygous) is at a temperature of 45 °C and 55°C.

DNA sequencing analysis was performed on positive control DNA samples to support the results of the Hb E detection by Hybri-Probe. Hb E positive control DNA samples were amplified by PCR and produce a product of 713 bp. DNA fragments were analysed by DNA sequencing.

The results of Hb E positive control DNA samples sequencing showed

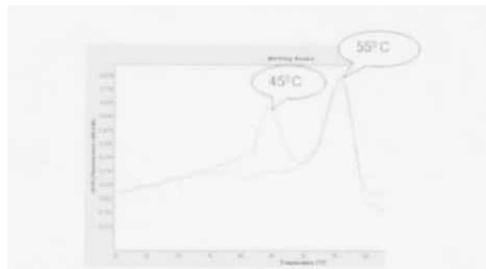


Fig. 1 Melting Curve Genotype detection of Hb E. Blue graph shows the wild-type DNA samples and the red graph shows mutant DNA (Hb E heterozygous).

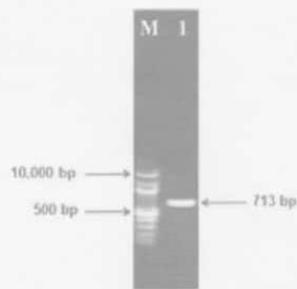


Fig. 2 Results of PCR products of electrophoresis. M is the DNA marker lane KAPA™ Universal Ladder. Lane 1 is a band that shows the results of PCR products for  $\beta$ -forward and  $\beta$ -reverse primers cutting area. PCR product size was 713 bp.

nucleotide G turns into A, so that GAG turns into AAG codon, in codon 26. DNA sequencing results that one allele has a mutation in globin chain on codon 26 and 1 other allele is normal. Sequencing analysis showed that DNA samples that used as trial tested of Hb E detection by Hybri-Probe is a Hb E trait (Fig. 3).

### Detection and screening of Haemoglobin E (in Biology Department Student, Universitas Negeri Jakarta)

Results of Hb E screening on UNJ Biology Department student population showed that as many as 3 out of 50 DNA samples, were positive as carrier of Hb E (Hb E trait). Melting Curve Genotype graph shows that in wild-type DNA there is a melting peak probe temperature at 55°C. In different case, Hb E heterozygotes DNA samples showed two melting temperature peak probe at 45°C and 55°C. Melting Curve Genotype graph of DNA screening samples that has

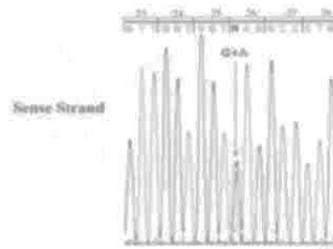


Fig. 3 DNA sequencing of Hb E positive control DNA samples. Red arrows indicate mutation areas.

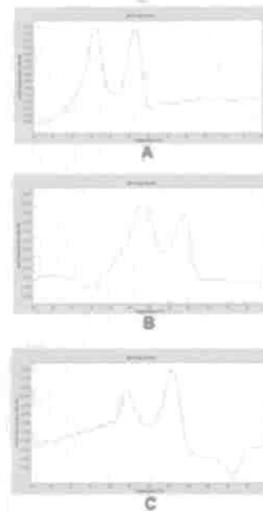


Fig. 4 Melting Curve Genotype graph of Hb E detection and screening results on three DNA samples were detected as Hb E trait. (A) sample H 17; (B) sample H 19; (C) sample H 33.

been detected as Hb E trait can be seen in Fig. 4.

The frequency of the wild-type allele is symbolized by p and the allele frequency of Hb E is symbolized by q. The frequency of heterozygous Hb E is symbolized by pq. Homozygous dominant, homozygous recessive, and heterozygous written in the equation  $p^2 + 2PQ + q^2 = 1$  (Horaguchi, 2013). Calculation shows that 6% of the UNJ Department of Biology student population carrying Hemoglobin E mutation. The results of the interviews with 3 subjects who had positively detected as Hb E carrier showed that 33% of them were suffer from anaemia and 66% is asymptomatic.

### DISCUSSION

#### Haemoglobin E detection of the positive control samples



sequencing techniques. Besides the Hybri-Probe techniques and sequencing, mutation detection can also be done by using a restriction enzyme, however, based on data from New England Biolabs, no restriction enzymes that cut area on f-globin gene in codon 26.

### **Detection and screening of Haemoglobin E**

Hybridization Probe Melting Curve Genotype (Hybri-Probe) method was applied to detect and screening of the Hb E mutation on a population. In this study, a population that was used as the subject of research was the student population of UNJ Biology Department. Selection of the student population UNJ Department of Biology as a subject of research was conducted on the basis of, first, UNJ is located on the island of Java which is a region that is at threat of malaria. Haemoglobin E most commonly found in the population of Southeast Asia, including Indonesia, because the area is a malaria endemic region. Hb E is the body's defence forms of the malaria-causing *Plasmodium falciparum* invasion in early evolution. Second, UNJ Biology Department students consist of various tribes. The prevalence of genetic diseases are closely related to ethnic (ethnic-related genetic). As a gathering place for many tribes, UNJ Biology Department, potentially have a large mutation carrier frequency of Hb E.

All fifty samples were detected on the presence of Hb E mutation through several process stages. Stages of the process, namely the isolation of genomic DNA from whole blood with the detection of genomic DNA isolated quantitatively and qualitatively. The next stage was the detection of haemoglobin E mutation which includes the process of DNA amplification using conventional PCR followed by detection of haemoglobin E by Hybri-Probe using Light Cycler PCR. The results were analysed using Melting Curve Genotype followed by analysis of the results of screening Hb E in the UNJ Department of

Biology student population using Hardy-Weinberg Equilibrium.

Hb E screening results showed that as many as 3 out of 50 DNA samples, were positively carry Haemoglobin E (Hb E heterozygous). Heterozygous Hb E/Hb E trait carry one allele Hb E and one wild-type allele. Probe imperfectly attached (curved) on Hb E allele mutation in the area so that the probe melts faster and eventually emerged a melting peak at a temperature of about 45°C. In contrast to the wild-type allele, probe can bind perfectly, so the longer the probe melting temperature and the melting peak appears at a temperature of about 55°C.

Screening results were analysed using the Hardy-Weinberg Equilibrium to calculate the frequency of patients with Hb E in a population of students of UNJ Department of Biology. Based on calculations, it was known that as many as 6% of the student population of the UNJ Department of Biology were Hemoglobin E carrier (Hb E trait). UNJ Biology Department student population numbered for 448 people. That means, it can be found about 26 students who has carry Hb E.

Individuals who carry haemoglobin E trait when married to individuals who carry thalassemia, it is 25% likely to give birth to children who suffer from Haemoglobin E/Thalassemia (Olivieri et al., 2011). Individuals suffering from Haemoglobin E/Thalassemia experienced clinical manifestations in the form of severe anaemia.

The only treatment that can be performed on clinical manifestations caused by Hb E/Thalassemia is a blood transfusion (Olivieri et al., 2011). Hb E patients who experience severe anaemia need blood transfusions in order to maintain sustainable survival. Giving repeated transfusions can cause complications such as hemosiderosis and hemochromatosis, which cause accumulation of iron in the body that cause tissue damage to organs such as the

liver, spleen, kidney, heart, bone and pancreas (Fucharoen et al., 2012). Patients with Hb E/Thalassemia will not be able to perform activities of life well, considering the conditions he suffered anaemia, and ultimately reduce the productivity and quality of the individual.

Hb E trait carrier number in Indonesia ranges between 1.5 to 36% and the carrier rate of Thalassemia trait in Indonesia is 3-5%, even in some areas reaches 10% (Health Technology Assessment Indonesia, 2010). This means, taking into account the birth rate and population of Indonesia, the number of patients Hb E/Thalassemia are born each year with fantastic figures of about 2,500 patients. The cost of treatment such as blood transfusions and lifetime iron chelation on the people with Hb E/Thalassemia is very large, which ranges from 200-300 million dollars/child/year (Health Technology Assessment Indonesia, 2010). In addition, it is also a psychological burden that must be borne by the patient and his family. The amount of Hb E patients rate will reduce the productivity and quality of the young generation of Indonesia. Therefore, early detection of the presence of Hb E is important.

Clinical manifestations of patients with Hb E are very diverse, ranging from asymptomatic to acute anaemia (Vichinsky, 2007). The diversity of phenotypes in patients with Hb E greatly depending on whether the patient is only carrying one abnormal allele (Hb E heterozygous), both allele is abnormal (Hb E homozygous), or patients having Hb E allele in combination with other abnormal haemoglobin. Patients who only carry Hb E (Hb E trait) are generally asymptomatic (Vichinsky, 2007).

Haemoglobin E trait or Hb E heterozygotes experienced by a person who inherits haemoglobin E allele from one parent and haemoglobin A allele from the other parent (Vichinsky, 2007). These individuals are referred to as Hb E carrier. Such individuals do not

experience health problems or generally asymptomatic. However it is important for the individual to know the status of blood disorders in the body due to the high risk of lowering Hb E next to their children.

The questionnaire in this research was aimed to determine whether patients with Hb E asymptomatic or have symptoms of mild anemia. Hb E carriers that have been detected in this study, 33% had clinical manifestations suffering from mild anaemia and 77% asymptomatic. This is consistent with the theory Vichinsky, (2007), which states that the carrier of Hb E trait (Hb E heterozygous) is generally asymptomatic.

Many studies show that Hb E/Thalassemia prevention programs would be more beneficial than treating patients who continue to grow (Health Technology Assessment Indonesia, 2010). In addition, the WHO stated that the cost of the annual national program of Hb E/Thalassemia prevention equal to the cost of medical treatment needed for 1 patient for 1 year. In outline form of prevention of Hb E may be educating the public, screening, premarital genetic counselling and prenatal diagnosis (Health Technology Assessment Indonesia, 2010).

One way of prevention that can be applied to reduce the incidence of Hb E cases is through Hb screening E. Hb E screening aims to identify individuals and couples careers, informing the possibility of obtaining offspring suffering from Hb E, and provide education in the form of options that can be done to avoid it. Screening for mutations in a population combined with prenatal diagnostics has dramatically reduced the incidence of mutations (Forget, 2000). Screening programs Hb E must be supported by accurate, rapid and inexpensive method.

This research result showed that Hybridization Probe Melting Curve Genotype (Hybri-Probe) method was

very good method to be applied in detecting and screening of Hb E for the reason of accuracy and feasibility on detection process compare to hemoglobinopathi analysis and DNA sequencing.

### CONCLUSION

1. Hybridization Probe Melting Curve Method Genotype (Hybri-Probe) method can be applied and worked out very well for the detection and screening of Hemoglobin E.
2. Hemoglobin E carrier frequency on UNJ Biology Department students was 6%.

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