CHAPTER III

METHODOLOGY

RESEARCH DESIGN

The design of this study is descriptive, cross-sectional for a diagnostic test. The modified O.F.T. and the previous O.F.T were compared blindly (Sackett D.L. *et al.*, 1991) and separately with a standard screening procedure (see appendix 3) to determine the diagnostic performance of each test in order to detect hemoglobinopathies (α thalassemia trait, β thalassemia trait, Hb E trait and Hb E disease) among asymptomatic adult people in a general population.

POPULATION

Target population of this study was asymptomatic adult people in a general population in Indonesia who fulfill these eligibility criteria. The inclusion criteria are (1) agree to participate in this study and (2) healthy as well as aged more than 14 years, whereas (1) pregnant woman and (2) known cases of hemoglobinopathies were excluded.

OBSERVATION AND MEASUREMENT

Data were collected using the form shown in appendix 1. Inter observer agreement of modified O.F.T. was measured by Kappa (K) value. Two observers were involved in this measurement. There were 66 heparinized blood samples run parallel and blindly by them and the result was analyzed by a 2 X 2 table. The Kappa value (K) was calculated by the formula below :

$$\text{Kappa (K)} = \frac{Po - Pe}{1 - Pe}$$

Po   = proportion of observed agreement.
Pe   = proportion of chance-expected agreement.
Po-Pe = actual agreement beyond chance.
1 - Pe = potential agreement beyond chance.

The reliability of those quantitative tests such as Hb A_2 determinations and the previous O.F.T. were controlled by measuring intra class correlation (ICC). Reliability of MCV and MCH measurements were determined by coefficient of variation (CV). These two parameters were measured by automated hematology analyzer so that variation among observer very small and could be ignored.

SAMPLE SIZE CALCULATION

Sample size was calculated by this formula :

$$n = \frac{P\,(100-P)}{\blacktriangle^2} \times f\,(1-\alpha)$$

$$n = \frac{90\,(100-90)}{10^2} \times 3.842 = 34.58 \longrightarrow 35$$

n was the number of subjects who suffer from hemoglobinopathies. P was estimated sensitivity that was 90 %. f (1-α) was square of the upper 1/2 α percentage point of the standard normal distribution. Confidence interval was 95 %, thus α = 0.05 and f (1-α) = 3.842. ▲ was the desired width of the confidence interval sensitivity. Acceptable sensitivity 80-100 %, thus ▲ = 10 % (Dobson, 1984). Number of subjects whom suffer from hemoglobinopathies was 35. The prevalence of hemoglobinopathies was 7.5 % therefore, total subjects in this study was 467.

THE MODIFIED O.F.T.

Material

1. Heparinized blood

2. 0.1 % buffered saline solution

3. 0.36 % buffered saline solution

4. 0.45 % buffered saline solution

<u>Equipment</u>

1. 96 wells microplate ("V" shape)

2. Hamilton pipette (2 μl)

3. 200 μl micropipette

<u>Method</u>

1. Put 200 μl of 0.1; 0.36 and 0.45 % of saline into three wells separately.

2. Add 2 μl heparinized blood into each well and mix gently.

3. Let stand at room temperature for 2 hours.

4. Read the test.

<u>Interpretation</u> : If the button formation present in well containing 0.36 % buffered saline solution was interpreted as a positive test result conversely, if the button formation not present was interpreted as a negative test result. The wells containing 0.45 % and 0.1 % buffered saline play role as control.

Table 3.1. Interpretation test result of the modified O.F.T.

	Saline concentration (%)			Interpretation
	0.45	0.36	0.10	
Button formation	+	+	+	Positive
"	+	+	0	Positive
"	+	0	0	Negative
"	0	0	0	Negative

THE PREVIOUS O.F.T.

Material

1. Heparinized blood

2. Stock solution of 10 % buffered saline

    NaCl   9.000 gram

    Na_2HPO_4 1.365 gram

    $NaH_2PO_4.2H_2O$ 0.243 gram

    Dissolved with double distilled water up to 100 ml.

3. 0.9 %, 0.36 % and 0.1 % buffered saline

Equipment

1. Spectrophotometer

2. Centrifuge

3. 10 ml reaction tube

4. Micropipette

Method

1. Put 5 ml of 0.9 %, 0.36 % and 0.1 % buffered saline separately into three tubes.

2. Add 50 µl heparinized blood into tube and mixed gently.

3. Let stand at room temperature for 20 minutes

4. Mixed again and centrifuged at 3,000 rpm for 5 minutes.

5. Absorbance of supernatant of 0.36 % and 0.1 % mixture were read by a spectrophotometer in 540 nm wavelength.

The hemolysis level was calculated by the formula described below :

$$Hemolysis = \frac{Abs.\ of\ supernatant\ of\ 0.36\ \%\ mixture}{Abs.\ of\ supernatant\ of\ 0.1\ \%\ mixture} \times 100\ \%$$

Interpretation : Those samples which had 91 - 100 % hemolysis was interpreted as negative test result and those which had less than 91 % hemolysis was interpreted as positive test result.

There were 20 tubes containing 200 ul heparinized-blood collected from one person. 10 tubes were given to laboratory technician and the other 10 tubes to another technician. These samples were tested blindly by the two

laboratory technicians by using the previous O.F.T. Reliability in this measurement between the two technicians was expressed by intraclass coefficient correlation (ICC).

GOLD STANDARD

Since hemoglobinopathies are genetic disorders the best method to diagnose these diseases is genetic (DNA) analysis. Unfortunately this technique is expensive so that it could not be carried out in this study as a gold standard.

β/α globin synthesis ratio is a technique that is very useful to differentiate α and β thalassemia but not for Hb E. Therefore, another approach was using a screening procedure that includes several laboratory tests : MCV and or MCH; Hemoglobin (Hb) electrophoresis on cellulose acetate membrane (CAM); Hemoglobin (Hb) A_2 quantification; Hemoglobin (Hb) F quantification and serum ferritin determination.

This screening procedure basically was adopted from a study done by Cao A. *et al.*, 1984. They reported that it was successful during screening thalassemia trait and only missed a few cases of silent carrier cases. Since in their country (Sardinia, Italy) there were no hemoglobin E cases so that in this screening procedure was added by the criteria to diagnose either Hb E trait or Hb E disease (homozygous). The procedure

to diagnose α thalassemia trait and iron deficiency was changed because the test to measure β/α globin synthesis ratio and ZnPP level were not available in the study setting. Therefore, another procedure that was introduced by Pearson H.A. *et al.*, 1973 was substituted to the Cao's procedure.

1. MCV and/or MCH values

Red blood cell indices including MCV and MCH were measured by automated hematology analyzer. The value of MCV used for this screening was less than 79 fL. in males and less than 77 fL. in females and MCH value was less than 27 pg. and 26 pg. in males and females respectively (Cao A. *et al.*, 1984). In Hb E trait or Hb E disease, MCV and MCH values could be either low or normal.

2. Hemoglobin electrophoresis on CAM

Material

1. Hemolysate

2. Electrophoresis buffer

Anode : Tris Borate EDTA (TBE) buffer pH 8.9
Tris hydroxymetiaminometane   60.5 g
Etilen diamine tetra acetic acid   6.0 g
Boric acid   1.6 g
Solved with DDW up to 1 liter.

Cathode : Barbital buffer pH 8.6

      Na-dietil barbiturate 10.30 g

      Dietilbarbiturate acid 1.84 g

      Solved with DDW up to 1 liter.

3. CAM

4. Ponceau solution stain

    Ponceau-S   5   g

    Trichloracetic acid   7.5 g

    Sulfosalisilic acid   12.5 g

    Solved with DDW up to 250 ml.

5. Washing solution (5 % acetic acid)

6. Whatman paper no. 3

Equipment

1. Electrophoresis tank

2. Forceps

3. Applicator

4. Power supply

Method

1. Cut 2.5 X 10 cm CAM and dip into mixture of equal volume of anode and cathode buffer.

2. Take CAM and dry up on filter paper with shining face.

3. Put 175 ml of anode and cathode buffer into anode and cathode chamber of electrophoresis tank. Add 175 ml of DDW into each chamber.

4. Put Whatman paper on wick as conductor.

5. Arrange coded CAM between wick. 2 µl of hemolysate samples are applied vertically on 3/4 part of anode.

6. Fix both sides of CAM.

7. Run electrophoresis with constant voltage (300V) for 35 minutes.

8. Stain with Ponceau-S for 15 minutes.

9. Wash with 5 % acetic acid at least three times until the color of CAM is really white.

3. Hemoglobin A_2 determination

Hemoglobin A_2 level was measured by microchromatography technique according to Helena Laboratory cat. no. 5341. This technique is more precise than elution method. Coefficien of variation of column chromatography method and elution technique are respectively 2.6 % and 3.7 % (Miale, 1982). Principle of this technique is an anion exchange chromatography. The anion exchange resin is a preparation of cellulose covalently coupled to small positively charged molecules. The positively charged cellulose attracts negatively charged molecules. Protein, such as hemoglobin, hemoglobins, contain many positive and negative charges due to the ionizing properties of the component amino acids. In the anion exchange chromatography of Hb A_2, buffer and pH level are controlled to cause different hemoglobins to possess

different net negative charges. These negatively charged proteins are attracted to the positively charged cellulose and bind accordingly. Following binding, the proteins are remove selectively from the resin by altering the pH or ionic strength of the elution buffer. Due to the pH of resin and the ionic strength of the Hb A_2 developer, Hb A_2 does not bind to the positively charge cellulose and is eluted as the developer moves through column. The other normal and most abnormal hemoglobins are retained by the resin. The Hb A_2 The Hb A2 fraction is compared to a total hemoglobin fraction by determining the absorbance of each using a spectrophotometer and then calculating the percentage of Hb A_2.

Interpretation

Table 3.2. Interpretation of Hb A_2 level

Descriptions	Hb A_2 (%)
Normal	1.5 - < 3.5
β thalassemia	≥ 3.5 - < 15
α thalassemia	< 1.5
δβ thalassemia	< 3.5
Hb E trait	> 15

There were 10 tubes contained EDTA-blood collected from one person. 5 tubes were given to laboratory technician and the other 10 tubes to another technician. These samples were tested blindly by the two laboratory technicians using the microchromatography technique. Reliability in this measurement between the two technicians was expressed by intra-class coefficient correlation (ICC).

4. Hemoglobin F determination

Material

1. Hemolysate

2. Cyanide solution

   Potassium ferricyanide 0.2 g
   Potassium cyanide   0.2 g
   Solved DDW up to 1 liter.
   Keep in dark bottle at room temperature.

3. Saturated ammonium sulfate solution

4. 1.2 N NaOH solution

5. Whatman paper no. 1

Equipment

1. Reaction tube

2. Vortex

3. Spectrophotometer

4. Glass funnel

Method

1. Make 5 ml of 0.5 % Cyanmethemoglobin.

2. Put 2.8 ml of 0.5 % Cyanmethemoglobin into tube.

3. Add 0.2 ml of 1.2 N NaOH and mix gently. Two
    minutes later, add 2 ml of ammonium sulfate.

4. Filter by Whatman paper.

5. The absorbance of filtrate was read with a
    spectrophotometer in 540 nm wavelength.

6. Total hemoglobin was measured on mixture of 0.4
    ml of 0.5 Cyanmethemoglobin and 6.75 ml DDW.

Hemoglobin F (Hb F) level was calculated by this formula :

$$\text{Hb F (\%)} = \frac{\text{Abs. Hb F}}{\text{Abs. total Hb}} \times 100\ \%$$

Interpretation

Table 3.3.   Interpretation of Hb F level

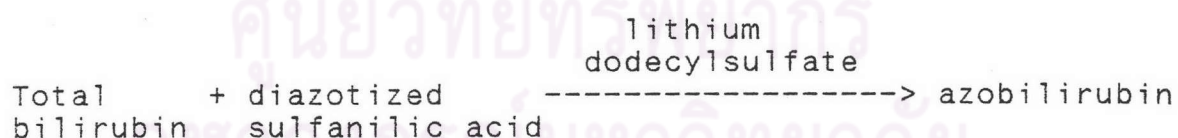| Disease	Hb F (%)
HPFH	1.5 - 100
β thalassemia	> 1.5
δβ thalassemia	> 1.5
Hb E trait	> 1.5
α thalassemia	< 1.5

5. Plasma ferritin level determination

Ferritin level was determined from plasma heparin by microparticle enzyme immunoassay technique. There were 89 plasma heparin samples run by this test. 86 of them had MCV value less than 80 fL. whereas the other 3 samples had MCV value equal or more than 80 fL. (80 - 80.5 fL.).

Normal value for adult males (18-30 yrs.) is 30 - 233 ng/mL and 32 - 284 ng/mL for age group 31 - 60. Normal value for pre-menopausal females is 16 - 81 ng/mL and 14 - 186 ng/mL for post-menopausal females. Ferritin level less than 10 ng/mL is diagnosed as iron deficiency anemia.

OTHER VARIABLES TO BE MEASURED

Hemoglobin level was measured by automated hematology analyzer and bilirubin level by modified Jendranssik-Grof method. The reaction of this method is as follows

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                              lithium
                          dodecylsulfate
Total      + diazotized    ----------------> azobilirubin
bilirubin    sulfanilic acid
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Formation of azobilirubin is proportional to the concentration of total bilirubin in the sample. Azobilirubin was quantified by absorbance measurement taken at a reading wavelength of 550 nm.

DIAGNOSTIC CRITERIA

Table 3.4. Diagnostic criteria for hemoglobinopathies

Description	MCV (fl)		MCH (pg)		Hb A_2(%)	electro-phoresis pattern
	♂	♀	♂	♀		
Normal	≥ 79	≥ 77	≥ 27	≥ 26	>1.5-<3.5	A-A_2
β thal trait	< 79	< 77	< 27	< 26	≥3.5-<7.5	A(F)A_2
Hb E trait	low or normal		low or normal		> 15	A-E/A_2
Hb E disease	low or normal		low or normal		> 75	A-E/A_2
δβ thal trait *	low or normal		low or normal		>1.5-<3.5	A-F-A_2
α thalassemia **	< 79	< 77	< 27	< 26	< 3.5	A-A_2
Other thalassemia#	< 79	< 77	< 27	< 26	< 3.5	A-A_2
Iron deficiency ##	< 79	< 77	< 27	< 26	< 3.5	A-A_2

Note: * = Hb F is increased (5-20%)

      ** = Serum ferritin level is normal and there is history of microcytic hypochromic in the family

      # = Serum ferritin level is normal and there is no history of microcytic hypochromic in the family

      ## = Serum ferritin level is reduced.

DATA COLLECTION

Firstly, the volunteers were asked to read the inform consent (see appendix 2). Then, after they agreed to participate in this study subsequently they were also asked to sign the inform consent form and filled up another form i.e. status of the study (see appendix 1). From the last form, the administrative data were gathered.

Then, 10 mL. blood samples were drawn from median cubital vein by aseptic technique. EDTA blood samples were collected in one vacutainer tube and heparinized-blood samples were collected in different vacutainer tube. Hemoglobin level, RBC indexes, hemoglobin F, hemoglobin A_2 and blood smear were examined from this sample. Whereas, the modified O.F.T. and the previous O.F.T. tests were done from heparinized-blood samples. Plasma samples for bilirubin and ferritin level determination were obtained from heparinized-blood. Data collection was performed in Dr. Sardjito General Hospital, Yogyakarta, Indonesia.

DATA ANALYSIS

The dichotomous data of both the modified O.F.T. and the previous O.F.T. were validated with a standard screening procedure and analyzed separately by using two by two tables (see table 3.5.) in order to determine their performance.

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Table 3.5.   A validation of a test to the gold standard

		Hemoglobinopathies	
		Present	Absent
	Positive	a	b
Test	Negative	c	d

Sensitivity was defined as the proportion of people with the disease who had a positive test for the disease. It was calculated by this formula :

$$\text{Sensitivity} = \frac{a}{a + c}$$

Specificity was the proportion of people without the disease who had a negative test. It was calculated by this formula :

$$\text{Specificity} = \frac{d}{b + d}$$

Positive predictive value (PPV) was the probability of disease in a patient with a positive (abnormal) test result. It was calculated by the following formula :

$$PPV = \frac{a}{a + b}$$

Negative predictive value (NPV) was the probability of not having the disease when the test result was negative (normal). It was calculated by this formula :

$$NPV = \frac{d}{c + d}$$

Accuracy was the proportion of all the test results, both positive and negative, that were correct. It was calculated by this formula :

$$Accuracy = \frac{a + d}{a + b + c + d}$$

Another alternative to describe the performance of a diagnostic test is the likelihood ratio. The likelihood ratio was defined as the probability of the test result in the presence of disease divided by the probability of the result in people without disease. Likelihood ratios express how many times more (or less) likely a test result is to be found in diseased, as compared to nondiseased, people. It can be calculated by the formula below :

Likelihood ratio for positive test (LR +)

$$LR + = \frac{a}{a + c} / \frac{b}{b + d}$$

Likelihood ratio for negative test (LR -)

$$LR - = \frac{c}{a + c} / \frac{d}{b + d}$$

Pre-test probability (prevalence) can be converted to pre-test odds by using the formula below :

$$Odds = \frac{\text{probability of event}}{1 - \text{probability of event}}$$

Likelihood ratio can then be used to convert pre-test odds to post-test odds by the following formula :
Pre-test odds X Likelihood Ratio = Post-test odds (Fletcher R.H. *et al*.,1988)

The comparison between the modified and the previous O.F.T. was done by comparing all of the characteristics of these two tests. Ideally, the comparison between two laboratory tests should be done by drawing the receiver operator characteristic (ROC) curve. Unfortunately, this curve can not be drawn from a single point of each test because these two tests use a single saline concentration i.e.

0.36 %.

ETHICAL CONSIDERATIONS

1.   Volunteers were asked to participate in this study.

2.   The blood samples were taken from median cubital vein by aseptic technique.   Therefore, drawing 10 ml blood from the subject of the study did not cause any health problem in adult people.

3. People who participate in this study were informed of all of test results individually with some explanations if needed.

LIMITATIONS AND OBSTACLES

The screening procedure used as a gold standard to validate the modified O.F.T and the previous O.F.T. may not be able to detect silent carrier cases.   It also might not be able to detect other hemoglobinopathies such as sickle cell disease.

The consequence of the use of imperfect gold standard is that the new test may seem worse even when it is actually better (Fletcher R.H., *et al.*, 1988). It can occurs when two of inaccurate tests are compared. The biased result might be

produced from this comparison.

There were 4 cases of microcytosis that might be a thalassemia trait. For confirmation, it needed the further investigation of microcytic history among their each family (see appendix 3). Unfortunately, it had not been done yet due to geographical constraint and time limitation. Another obstacle was reagent supply for Hb A_2 determination. It was imported reagent so that sometimes it came delayed.

EXPECTED BENEFITS AND APPLICATIONS

1. If the modified OFT visualized by button formation of unlysed erythrocyte had a good performance, this test could be used in the field or in clinical laboratories that had limited facilities.

2. Both the equipment and reagents needed in this test could be packed in a small container so that it is easy to carry them. This would be very useful during studies in the field.

3. The modified O.F.T. is efficient because in one microplate we can run 32 samples if we use two wells (0.45 and 0.1 %) as a control for each test.

4. The modified O.F.T. is relatively fast because in two hour we can run 32 samples.

5.   The modified O.F.T. is cheap, simple and therefore, it would be useful to monitor the prevalence of hemoglobinopathies in community.