นิพนธ์ต้นฉบับ

Screening model for thalassemia carriers.

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Thalassemia is a heterogenous group of inherited anemias caused by genetic disorders of hemoglobin synthesis. A high prevalence of this disease is found in some areas of Thailand. Prevention and control are necessary in order to decrease the amount of the disease and the numbers of carriers. The objectives of this study were to determine the reliability of each combination of screening test, as well as the agreement between two observers, and to obtain an appropriate combination for use in the field.

One hundred and ten subjects from Chulalongkorn Hospital of normal β -thal trait, and HbE trait were included in the study. The tests were blood indicies, blood smear, Osmotic Fragility (OF) and Dichlorophenol Indophenol Precipitation (DCIP). They were compared to standard method. It was found that the combination of blood indicies, OF and DCIP was reliable and produced a sensitivity 100%, specificity 83.3%, accuracy 90.0%, false positive 16.7%, False negative 0.0% and corrected observed agreement (Kappa) of 88.1%

All of the combinations of screening methods were tested in another data set of 68 patients from Chulalongkorn Hospital. The above combination was determined to be an appropriate method for use in outlying areas, along with genetic counselling in order to achieve control and prevention of this disease.

Key words : *Thalassemia carriers, Screening medthods.*

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ธาลัสซีเมีย เป็นโรคซีดทางพันธุกรรมโรคหนึ่ง ซึ่งเกิดจากความผิดปกติของยีน พบอุบัติการสูงมากในบางพื้นที่ของประเทศไทย จึงจำเป็นต้องป้องกันและควบคุมเพื่อลดจำนวนของโรคและพาหะ การวิจัยนี้มีวัตถุประสงค์ เพื่อหาความถูกต้อง และเชื่อถือได้ของรูปแบบวิธีการต่าง ๆ ในการตรวจกรองตลอดจนความสอดคล้องระหว่างผู้ใช้รูปแบบดังกล่าวในการตรวจและหารูปแบบที่เหมาะสมที่สุด ที่จะนำไปใช้ตรวจกรองโรคในภาคสนาม ประชากรที่ศึกษา 110 คน จากคนปกติและพาหะของ β-thal และพาหะของ HbE ณ. โรงพยาบาล จุฬาลงกรณ์นำมาตรวจรูปแบบต่าง ๆ ซึ่งประกอบด้วย Blood indicies, Blood smear, Osmotic Fragility (OF) และ Dichlorophenol Indophenol Precipitation (DCIP) เปรียบเทียบกับวิธีมาตรฐาน พบว่ารูปแบบที่ประกอบด้วย Blood indicies, OF และ DCIP มีความเชื่อถือได้โดยมีความไว 100% ความจำเพาะ 83.3% ความถูกต้อง 90.9% ผลบวกลวง 16.7% ผลลบลวง 0.0% มีความสอดคล้องระหว่างผู้ทำการวินิจฉัย (Kappa) สูงถึง 88.1% รูปแบบต่างๆ นี้นำมาทดสอบซ้ำกับกลุ่มตัวอย่างในคนไข้ที่มาตรวจที่โรงพยาบาลจุฬาลงกรณ์อีกจำนวน 68 คน พบว่ารูปแบบดังที่กล่าวข้างต้น น่าจะเป็นรูปแบบที่เหมาะสมที่จะนำไปใช้ในระดับชุมชนได้ เพื่อให้คำปรึกษาทางด้านพันธุศาสตร์อันจะเป็นการควบคุมและป้องกันโรคนี้ให้ได้ผลดี

Thalassemia syndromes are a heterogeneous group of inherited anemias caused by genetic disorders of hemoglobin synthesis and it is characterized by a diminishing or absence of one or more of the globin subunits of the hemoglobin molecule. The thalassemia can be subdivided, based on genetic principle, into 5 groups; α-, β-, ∂β-α, γ∂ and γ∂β-thalassemia.[1] Alpha and β-thalassemia are more important because of the fact that Hb A ($\alpha_2\beta_2$) are the major Hb(97%) in adults. In Southeast Asia there is high frequency of Hb E (β^{26} glu - lys).[2] The combination of Hb E with β-thalassemia gives rise to anemia with moderate to severe clinical manifestations. In Thailand the incidence of α-thalassemia is 20% in Bangkok and 30% in Northern Thailand.[3,4] The frequency of total β-thalassemia varies from 3 - 9 % and β^0 - thalassmia is perdominate over β^+ - thalassemia. The frequency of Hb E averages 15 % but is about 40 - 50 % in the northeastern region especially in Surin province.[2,5] The clinical syndromes associated with thalassemia arise from the combined consequences of inadequate hemoglobin accumulation and an unbalanced accumulation of globin subunits. The former causes hypochromia and microcytosis, the latter leads to ineffective erythropoiesis and hemolytic anemia. In β-thalassemia the clinical manifestations are diverse, ranging from asymtomatic hypochromic and microcytosis in heterozygotes (traits) to profound anemia, which leads to massive bone marrow expansion in the severe form. This exerts numerous adverse effects upon growth development and the function of critical organ systems. Children with β-thalassemia develop characteristic thalassemia faces, thin long bone, growth retardation, splenomegaly, heart failure and susceptibility to infection. Treatment with red blood cell transfusions can prolong life but complications of chronic transfusions therapy, including iron overload, usually prove fatal before age 30.[6] There are several reports that the cost of thalassemia diagnosis and treatment, including general medical care, blood transfusions, iron chelation, splenectomy, are very expensive and can cost about 200,000 baht per person per year.[7] Thus thalassemia can cause major social and economical problems. The control and prevention of this disease by diminishing the numbers of the thalassemic newborn can be accomplished by mass screening to look for the thalassemia carriers and then counselling them before their marriage or pregnancy. It is accepted that there are limitations to the screening tests, nevertheless, all of the screening tests give valuable information by excluding normal subjects and therefore further investigations for a precise diagnosis are conducted or a small proportion of suspected subjects. Dichlorophenol indophenol (DCIP) precipitation test is useful in detecting the presence of HbE and HbH.[8,9] One tube osmotic fragility test (OF) is effective in detection of β-thalassemia trait.[10] Screening strategy based on electronic measurement of blood indices also used for screening of β-thal trait.[11] However, Maccioni L and Cao A recommended that in a population in which both α-and β-thalassemia are prevalent, the screening method should combine the one tube osmotic fragility test with electronic measurement of Rbc indices which are nearly normal in α-and β-thal heterozygotes.[12] Therefore the combination of the screening tests may increase the effectiveness of screening. Rbc morphology, reticulocyte count and inclusion body[13] may be not significant criteria in detection of traits, but possible useful methods in detection of thalassemic disease which can be found during mass screening.

Objectives of the Study

1. To determine the reliability of each combination of screening test which can diagnose the carriers (β-thal trait and Hb E trait)

2. To determine errors in the diagnosis between two persons.

3. Finally, to obtain one appropriate combination of screening tests for mass detection of thalassemic carriers.

Materials and Methods

Training data set

Blood samples were obtained from normal adults and the parents of β-thal / Hb E and homozygous β-thalassemia patients. A testing data set of blood specimens from patients suspected to be abnormal (thalassemia or hemoglobinopathies) were included. Three milliters of EDTA blood samples were collected from each donor and kept at 4°c in a refrigeretor in order to perform the test within 1-2 days. All samples were processed as follows :-

- **Blood indicies** composed of : Rbc count, Hb, Hct, MCV, MCH, MCHC and automatically calculated by an electronic cell counter such as Coulter-Counter.

- **Blood Smear** composed of:

Rbc morphology obtained by examining a thin smear of blood, stained with Wright's stain, under a microscope.

Reticulocyte count (Retic) and inclusion body test (IB): performed by using 1% brilliant cresyl blue.[13]

- **One tube osmotic fragility test (OF)** : tested by using 0.36% buffered saline solution.[10]

- **Dichlorophenol indophenol precipitation test(DCIP)** : tested in two by using a spectrophotometer to detect absorbancy change after various time intervals after incubation at 37°c.[8]

A. 30, 60, 90, 120 min

B. 60, 120 min

C. 90, 120 min or

D. by visual inspection to grade the precipitation after 1-hour incubation at 37°c.[9]

- **Hemoglobin typing** composed of:

1. Starch gel electrophoresis[14] to separate the different types of hemoglobin

2. Cellulose acetate electrophoresis[15] to quantitate Hb E and others without any staining procedure

3. Hb A_2-microcolumn[16] for confirmation of the high percentage of Hb A2 (using Beta-thal Hb A_2 Quick column™ Kit Cat. no. 5341 Helena laboratories) and

4. Alkaline denaturation test[17] for determination of Hb F.

In order to identify the appropriate screening methods, the tests were combined as follows:-

Method I Blood indices and blood smear,
II Blood indices, blood smear, OF and DCIP
III A Blood indices, OF and DCIP (30, 60, 90 120 min)
III B Blood indices, OF and DCIP (60,120 min)
III C Blood indices, OF and DCIP (90, 120 min)
III D Blood indices, OF and DCIP (60 min)
IV Blood smear,Of and DCIP
V OF and DCIP

Analysis

Diagnostic values, including senitivity, specificity, positive and negative predictive values, false positive and false negative results as well as accuracy, were calculated to determine the reliability of the

screening methods. The gold standard included blood indices, blood smear and hemoglobin typing. The screening tests were the five combinations mentioned above. Verification of the screening tests was made by two medical technologists using the blind technique. Kappa statistics were also used to determine the agreement between two observers.

In testing the data set, the DCIP was added by visual inspection (Method III D) and this did not require the spectrophotometry.

Procedure

After collecting the blood specimens from the normal subjects and the parents, the blood indices, Rbc morphology, reticulocyte count and inclusion body test were performed. The remainder of the blood specimens was divided into three parts for testing of OF, DCIP (A,B,C) and Hb typing.

Result

Training data set

Within the 110 samples there were 48 normal adults, 27 adults with β-thal trait and 35 adults with Hb E trait.

The statistical, reliability parameters shown in Table I provide sensitivity, specificity, accuracy, false positive, false negative, positive predictive values (PPV) and negative predictive values (NPV). The data reveals that all combinations provided high sensitivity, accuracy and specificity but method III (A,B,C), which was composed of blood indicies, OF and DCIP, gave the best percentages of sensitivity (95.23, 98.39,100.00) and accuracy (93.61, 89.58,83.33). In addition, method III (A,B,C) gave low false negative report of 4.77, 1.61 and 0.00 respectively. The erroneous diagnosis made by the two medical technologists are shown in table II. There was no significant difference in the diagnosis of thalassemia carriers by the two medical technologists and the diagnostic assessment indicated a high degree of agreement(Kappa).

Testing data set

Within the second data set of 68 samples, there were nine normal subjects, seven with β-thal trait, 14 with Hb E trait and 38 abnormal cases such as β-thal, β-thal/Hb E homozygous Hb E, Hb H disease with and without Hb constant spring. All the samples were run through the same combination of tests as the previous group. They were also give other tests,such as DCIP test by visual inspection. The statistical reliability parameters of sensitivity, accuracy and specificity were close to the other methods, except for the specificity of method I as shown in Table III. The percentages of false negative of method III(A, B, C,D) were equal at 6.78 but false positives of method III A were lower than with the others (11.11) and gave high sensitivity (93.22, 92.64, 88.89). Conclusively, method III should be recommended as the test for mass detection of thalassemia carriers in field work and method III D is the most appropriate procedure because it is less time consuming and it avoids the use of the spectrophotometer.

Table 1. Reliability of various thalassemia diagnosis.
(Blood received from normal and thalassemic patients'parents).

Methods \ Diagnostic Values		Sensitivity (%)	Specificity (%)	Accuracy (%)	False+ve (%)	False-ve (%)	+ve PV (%)	-ve PV (%)
I.	Blood indicies							
	Retic and IB	93.54	89.54	91.81	10.42	6.45	92.06	91.49
	Rbc morphology							
II.	Blood indices							
	Rbc morphology	91.80	83.67	88.18	16.33	8.20	87.50	89.13
	OF,DCIP							
III.	A Blood indices							
	OF,DCIP (A)	95.23	93.61	92.54	6.39	4.77	95.24	93.62
	(30,60,90,120)							
III.	B Blood indices							
	OF,DCIP (B)	98.39	89.58	94.55	10.42	1.61	92.42	97.73
	(60,120)							
III.	C Blood indices							
	OF,DCIP(C)	100.00	83.33	90.91	16.67	0.00	86.11	100.00
	(90,120)							
IV.	Rbc morphology	91.94	81.25	87.27	18.75	8.06	86.36	88.64
	OF,DCIP (A)							
V.	OF,DCIP (A)	81.96	89.58	84.54	10.42	18.04	90.91	78.18

Table 2. Agreement between two medical techologists for thalassemia diagnosis in training data set.

Methods \ Diagnostic values	Observed agreement (%)	Corrected observed agreement (Kappa) (%)
I.	90.00	79.95
II.	98.45	90.56
III. A	95.45	90.68
III. B	90.00	78.76
III. C	94.55	88.09
IV.	90.00	78.26
V.	92.73	85.46

Table 3. Reliability of various thalassemia diagnosis.
(Blood received from normal and thalassemic patients)

Methods \ Diagnostic Values	Sensitivity (%)	Specificity (%)	Accuracy (%)	False+ve (%)	False-ve (%)	+ve PV (%)	-ve PV (%)
I.	96.61	33.33	88.23	66.67	3.39	90.47	60.00
II.	93.22	88.89	92.64	11.11	6.78	98.21	66.67
III. A	93.22	88.89	92.64	11.11	6.78	98.21	66.67
III. B	93.22	77.78	91.18	22.22	6.78	96.49	63.64
III. C	93.22	77.78	91.18	22.22	6.78	96.49	63.64
III. D	93.22	77.78	91.18	22.22	6.78	96.49	63.64
IV.	86.44	77.78	85.29	22.22	13.56	96.23	46.67
V.	93.22	88.89	92.64	11.11	11.78	98.21	66.67

+ve PV = positive predictive value

-ve PV = negative predictive value

Discussion

Because of the high incidence of thalassemia in Thailand, it can be estimated that about 625 fetuses will be born with Thalassemia major (homozygous β-thalassemia) and about 3,25 0f etuses with β-thalassemia / HbE disease per year, based on a total birth rate of about 1 million per year.[18] To diminish the number of thalassemic newborn, thalassemia carrier screening requires a simple and economical method. One tube osmotic fragility test (OF) is effective in detecting β-thalassemia trait.[10] The dichlorophenol indophenol precipitation test (DCIP) is useful in detecting the presence of HbE and HbH.[8,9] These two rapid screening tests are simple, cheap and easy to apply. Additionally, is no requirement for well-equipped laboratories and technical expertise. However, a recent trend in clinical laboratories is to utilize automated tests because of their precise measurement and high reliability. For this reason, automated blood cell counters such as the Coulter-counter are common in laboratories. Blood indicies, such as MCV and MCH, automatically determined by blood cell counter are also useful in detecting thalassemia carriers. The combination of blood indicies with the former two carrier screening tests would increase the effectiveness of screening (Table I). Method III seems to be the best screening test. In the detection of thalassemia carriers, when Rbc morphology combined with OF and DCIP engenders a little decrease in effectiveness (Table I, II method II, and IV), it may be due to a less specific characteristic of Rbc morphology in thalassemia carriers. Method III B and III C (Table I, II) can be modified to increase the simplicity. The sensitivity is more acceptable than the specificity.

There was no significant difference of performance in the diagnosing of β-thalassemia and Hb E carriers by the two medical technologists (Table II). The results of their diagnosis showed high observed agreement and corrected observed agreement. The testing data sets are shown in table III. These specimens were composed of nine normal subjects, 21 persons with carrier states, and 38 persons with abnormality (thalassemia, or hemoglobinopathies). Because of the large sample size of abnormal) subjects which are markedly abnormal regardless of Rbc morphology, size, osmotic fragility ect., most of the methods resulted in the same velues of sensitivity at about 93.22% but the specificity was decreased. This may be because of the interference of the disease of each patient. Most of the subjects in these groups are suspected to have a type of blood disease. Iron deficiency anemia is one of the blood diseases which results from a reduced output and disorder of red blood cell maturation. Method I in Table III is the test that considers only the abnormal red blood cell count and will give more positive results which leads to decreased the specificity to 33.33%.

The blood indicies OF and DCIP at 60 min (Method III D) is an additional method. It seems to be the best method for use in the field because it's statistical values are close to the values of method III A, B, C and it is an economical and easy to use technique, which dose not require the use of spectrophotometer, and it is also less time consuming. A further study will be conducted in the field to confirm that method III D is the appropriate technology for screening thalassemia disease and detecting carriers. It is expected that the prevention and control of thalassemia in Thailand will be sucessful under the appropriate technology.

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