VALIDATION OF OSMOTIC FRAGILITY TEST AND DICHLOROPHENOL INDOPHENOL PRECIPITATION TEST FOR SCREENING OF THALASSEMIA AND Hb E

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Abstract. The strategy for screening of thalassemia and Hb E by a combination of osmotic fragility (OF) test and dichlorophenol indophenol precipitation (DCIP) test was validated with 436 unrelated Thai subjects. Hemoglobin (Hb) typing, Hb A_2 quantitation, PCR and DNA sequence analysis were used as confirmatory methods for diagnosis of thalassemia and hemoglobinopathy. The sensitivity and specificity of this strategy was 100% and 79.7%, respectively. The results assessed by two medical scientists were exactly the same with 93.3% accuracy in comparison with the confirmatory methods. A combination of OF and DCIP has been shown to be a reliable, rapid, simple and sensitive strategy for screening thalassemia and Hb E in the Thai population.

INTRODUCTION

Hemoglobin disorders are the most common clinically serious single gene disorders in the world. It is estimated that about 250 million people (4.5% of the world population) carry a potential pathological hemoglobinopathy gene and about 300,000 infants are born with major hemoglobinophathies (Angastiniotis, 1995). Although present management of the disease gives a probable life expectancy beyond the third or fourth decade, the quality of life of patients and their families and the burden that such treatment represents for public health services clearly underline the fundamental aspect of prevention rather than treatment (WHO, 1994; Angastiniotis, 2003).

Prevention of thalassemia by combining 4 strategies through education, carrier screening, counseling and prenatal diagnosis have proved to be effective, acceptable and highly cost-beneficial (WHO, 1994). The aim of carrier screening is to identify carriers of hemoglobin disorders in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality (Galanello, 2003). There are several possible strategies for screening, depending on such factors as prevalence of the disease, heterogeneity of the genetic defects, resources available and social, cultural and religious factors.

In Thailand, the frequencies of α-thalassemia reach 20-30% in Bangkok and northern Thailand, while the frequencies of β-thalassemia vary between 3-9% (Winichagoon *et al*, 1990; Fucharoen *et al*, 1997). The aim of Thai Ministry of Public Health policy is to prevent severe forms of thalassemia, including Hb Bart's hydrops fetalis, homozygous β-thalassemia and β-thalassemia/Hb E. To achieve this, a combination of osmotic fragility (OF) and dichlorophenol indophenol precipitation (DCIP) tests have been used in mass screening. OF test is sensitive enough to detect almost all cases of α^o-thalassemia and β-thalassemia carriers, whereas the blue dye DCIP test can be used as a screening test for Hb E (Katamis *et al*, 1981; Frischer and Bowman, 1975; Fucharoen *et al*, 2004). These screening tests are based on simple technologies that can be transferred to be used in all

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hospitals including those in rural areas in the country.

Presented in this study is the validation of OF and DCIP tests for screening of thalassemia and Hb E. Performance characteristics including sensitivity, specificity, false positive rate, false negative rate and accuracy were studied. Hb typing, Hb A_2 quantitation, multiplex PCR and DNA sequence analysis were used as confirmatory methods.

MATERIALS AND METHODS

Samples

Blood samples anticoagulated with ethylene diamine tetraacetic acid (EDTA) were collected from 436 unrelated Thai subjects.

Osmotic fragility (OF) test

OF test was performed using KKU-OF (PCL Holding, Thailand). An aliquot of 20 μl of EDTA blood was mixed with 2 ml of saline solution in a 13 mm x 75 mm capped plastic tube provided by the manufacturer. The tubes were incubated at room temperature for 15 minutes before the results were determined by visual inspection. Negative sample was clear and positive sample was cloudy. A sample that was slightly cloudy was considered as being positive.

Dichlorophenol indophenol precipitation (DCIP) test

DCIP test was performed using KKU-DCIP-Clear (PCL Holding, Thailand). An aliquot of 20 μl of EDTA blood was mixed with 2 ml of DCIP reagent. The solution was incubated at 37ºC for 15 minutes and then 20 μl of stop solution was immediately added and the solution was mixed before determining the result by visual inspection. Negative sample was clear and positive sample was cloudy. A sample that was slightly cloudy was considered as being positive.

Confirmatory tests

Hb typing and Hb A_2 quantitation were performed using Hb A_2 assay of Hb Gold analyzer (Drew Scientific, United Kingdom). For DNA analysis, DNA was extracted using NucleoSpin Blood kit (Mecherey-Nagel, Germany) and α^o-thalassemia (Southeast Asian and Thai deletions) were detected by using α^o-thalassemia detection kit as previously described (Sangkitporn *et al*, 2002). Samples which have Hb variants were subsequently confirmed by direct DNA sequence analysis in an ABI Prism 310 genetic analyzer (Applied Biosystems, USA) (Nopparatana *et al*, 1994).

RESULTS

Results obtained from 436 individuals were divided into 4 groups based on screening results (Table 1). The $OF^-/DCIP^-$ group composed of 114 normal subjects. Their Hb A_2 levels were $2.3\pm0.3\%$. Of the 30 subjects having $OF^-/DCIP^+$ results, 27 were Hb E heterozygotes and 3 were normal subjects.

Among 86 subjects having $OF^+/DCIP^-$ results, 26 were normal subjects, 13 α^o-thalassemia heterozygotes, 15 β-thalassemia heterozygotes, 19 Hb H disease, 1 EABart's disease, 4 homozygous β-thalassemia, 6 Hb C carriers and 2 Hb Tak carriers. Among 13 α^o-thalassemia heterozygotes, 12 were SEA deletions and one was a Thai deletion.

Of 206 subjects having $OF^+/DCIP^+$ results, 63 were Hb E heterozygotes, 4 α^o-thalassemia heterozygotes, 88 β-thalassemia/Hb E, 37 Hb H disease, 8 homozygous Hb E, 2 EABart's disease, 3 homozygous β-thalassemia and 1 Hb C carrier. Of 63 Hb E carriers, 6 had combined heterozygosity of α^o-thalassemia, SEA deletion type. Percent Hb E in Hb E heterozygotes with α^o-thalassemia (Hb E $21.0\pm0.9\%$, n = 6) and in those without α^o-thalassemia (Hb E $28.6\pm5.4\%$, n = 57) is significantly different (*t*-test, $p<0.05$). For the 3 subjects with EABart's disease, 2 with $OF^+/DCIP^+$ results had a higher Hb E content (Hb E 14.1% and 15.3%) than the one with $OF^+/DCIP^-$ result (Hb E 11.2%). Six of 7 Hb C carriers had $OF^+/DCIP^-$ result whereas both Hb Tak carriers had $OF^+/DCIP^-$ results.

In comparison with the results from the confirmatory tests, OF test alone had 100% sensitivity, 59.0% specificity, 41.0% false positive, 0% false negative and 77.1% accuracy for screening of α^o-thalassemia and β-thalassemia (Table 2). The DCIP test alone had 99.5% sensitivity, 80.6% specificity, 19.4% false positive, 0.5% false negative and 88.8% accuracy for screen-

Table 1
Results of KKU-OF and KKU-DCIP-Clear screening tests in 436 Thai subjects.

Screening results	Subject	Number (n)
OF -/DCIP-	Normal	114
OF -/DCIP+	Hb E heterozygote without α^0-thalassemia	27
	Normal	3
OF +/DCIP-	Normal	26
	α^0-thalassemia carriers	13
	β-thalassemia carriers	15
	Hb H disease	19
	EABart's disease	1
	Homozygous β-thalassemia	4
	Hb C carriers	6
	Hb Tak carriers	2
OF +/DCIP+	Hb E carriers without α^0-thalassemia	57
	Hb E carriers with α^0-thalassemia	6
	α^0-thalassemia carriers	4
	β-thalassemia/Hb E	88
	Hb H disease	37
	Homozygous Hb E	8
	EA Bart's disease	2
	Homozygous β-thalassemia	3
	Hb C carrier	1

Table 2
Performance characteristics of KKU-OF, KKU-DCIP-Clear and combined tests.

		Confirmatory tests	
		Positive	Negative
OF	Positive	192	100
	Negative	0	144
DCIP	Positive	188	48
	Negative	1	199
OF/DCIP	Positive	293	29
	Negative	0	114

ing of Hb E. A combination of OF and DCIP had 100% sensitivity, 79.7% specificity, 20.3% false positive, 0% false negative and 93.3% accuracy for screening of α^0-thalassemia, β-thalassemia and Hb E.

To determine reproducibility of these screening tests, blood samples were screened by 2 medical scientists within 24 hours after blood collection. The same results from both medical scientists were obtained.

DISCUSSION

In Thailand where thalassemia and hemoglobinopathy are common, the implementation of OF and DCIP screening tests for routine service in hospital laboratories has been established since 1990. At the beginning, each laboratory prepared their own reagents and the reliability of test results varied depending on the quality of reagents especially DCIP solution which is very sensitive to pH drift. In 1997, Fucharoen and coworkers developed the KKU-OF and KKU-DCIP-Clear reagents. A combination of these 2 tests have been reported to be an effective preliminary screening for α^0-thalassemia, β-thalassemia and Hb E carriers with 100% sensitivity and 69.8% specificity (Fucharoen *et al*, 2004).

As KKU-OF and KKU-DCIP-Clear are commercially available in Thailand, it is necessary to validate and monitor their performance in screen-

ing the target population. Among 436 subjects, 361 had screening results in accordance with those of the confirmatory tests. The other 75 subjects included 1 EABart's disease subject with false negative DCIP test, 26 normal subjects with false positive OF test, and 3 normal subjects, 4 α^o-thalassemia heterozygotes, 3 homozygous β-thalassemia, 37 Hb H disease subjects and 1 Hb C carrier with false positive DCIP test. These false positive results may be due to mild form of thalassemia and hemoglobinopathy, as well as iron deficiency anemia (Fucharoen *et al*, 1999, 2004). Hb H could be prone to be oxidized by DCIP dye leading to the false positive DCIP test. The EABart's subject with false negative DCIP test had a very low amount of Hb E, but the OF test was still positive. A combination of these 2 screening tests could detect this abnormality. Subjects with α^o-thalassemia, β-thalassemia and/or Hb E with negative results on both tests ($OF^-/DCIP^-$) were absent in this study. Percent Hb E in Hb E carriers without α^o-thalassemia, in Hb E carriers with α^o-thalassemia and in EABart's disease was $28.6\pm5.4\%$ (n = 57), $21.0\pm0.9\%$ (n = 6), and $13.5\pm2.1\%$ (n = 3), respectively. These observations support previous studies, which indicated that, in the presence of α-thalassemia, where α-globin subunits are in limited supply, Hb A will be preferentially formed so Hb E is therefore represented in relatively lower amounts (Honig and Adams, 1986; Sanchaisuriya *et al*, 1997).

KKU-OF and KKU-DCIP-Clear are appropriate screening tests to identify carriers of α^o-thalassemia, β-thalassemia and Hb E in terms of their application, methodology and performance characteristics. These tests require small sample size of only 40 μl of EDTA blood, have rapid turn around time of 30 minutes or less, are easy to use, and require only general equipments such as water bath and automatic pipettes which need minimal maintenance. Reagents cost less than 30 baht (approximately US$ 0.79). All 32 thalassemia carriers and all 162 thalassemia disease subjects, including Hb H disease, β-thalassemia/Hb E, homozygous β-thalassemia, EABart's disease and homozygous Hb E, could be detected by the OF test. DCIP test detected all Hb E subjects except only 1 with EABart's disease with a very low Hb E level. A combination of these 2 screening tests have been shown to be a reliable strategy for screening of α^o-thalassemia, β-thalassemia and Hb E with 100% sensitivity, 79.7% specificity, 93.3% accuracy and there were no false negatives among these important groups. Thus the OF and DCIP tests can be used in a screening strategy provided to a wide population to support the prevention and control of severe thalassemias in Thailand.

ACKNOWLEDGEMENTS

We gratefully acknowledge Miss Sawitree Duangruang and Miss Acharaporn Dumbua for their assistance during the course of this study, which was supported by the Department of Medical Sciences, Ministry of Public Health, Thailand.

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