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 hemoglobinopathies (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-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
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 A2 quantitation, multiplex PCR and DNA sequence analysis were used as confirmatory methods.

MATERIALS AND METHODS

Samples
Blood samples anticoagulated with ethylenediamine 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 37°C 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 A2 quantitation were performed using Hb A2 assay of Hb Gold analyzer (Drew Scientific, United Kingdom). For DNA analysis, DNA was extracted using NucleoSpin Blood kit (Mecherey-Nagel, Germany) and α-thalassemia (Southeast Asian and Thai deletions) were detected by using α-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 A2 levels were 2.3±0.3%. 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-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-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-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-thalassemia, SEA deletion type. Percent Hb E in Hb E heterozygotes with α-thalassemia (Hb E 21.0±0.9%, n = 6) and in those without α-thalassemia (Hb E 28.6±5.4%, 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-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-
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 α²-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 co-workers 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 α²-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 αº-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 αº-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 αº-thalassemia, in Hb E carriers with αº-thalassemia and in EABart's disease was 28.6±5.4% (n = 57), 21.0±0.9% (n = 6), and 13.5±2.1% (n = 3), respectively. These observations support previous studies, which indicated that, in the presence of β-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 αº-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 αº-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.

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REFERENCES


