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Efficacy evaluation of new hemoglobin E screening test in community hospitals

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The objective of the research was to evaluate of the efficacy of new HbE screening test (E-Sure, Mitr Medical, Thailand) compared to the modified 2,6 dichlorophenol indophenols precipitation test (KKU-DCIP) and results were confirmed by capillary electrophoresis. Blood samples were collected from patients who came for physical examinations at Kutchap Hospital and hospitals with inter-laboratory cooperation from October 2013 to February 2014. The samples were screened for those with mean corpuscular volume (MCV) lower than normal (MCV < 80 fl), resulting in 326 samples. After screening and confirming for HbE carriers, 226 cases of HbE carriers were found, including 234 HbE trait and 32 homozygous HbE carriers. When the efficacy of KKU-DCIP screening method was compared to that of the HbE screening test (E-Sure) in community hospitals, it was found that the KKU-DCIP test had sensitivity, specificity, positive predictive, negative predictive and efficiency values of 95.1, 91.6, 98.1, 80.9 and 94.5%, respectively. Whereas, HbE screening test, which had sensitivity, specificity, positive predictive, negative predictive and efficiency values of 99.2, 96.7, 99.2, 96.7 and 98.8%, respectively. The HbE screening test is higher efficacy, as well as being more convenient and easier to interpret, which is suitable for HbE carrier screening in community hospital laboratories.

Key word: Thalassemia, hemoglobin E, screening method, hemoglobin E carrier.

INTRODUCTION

Thalassemia is a genetic abnormality that causes abnormal hemoglobin production by less globin chain protein. The red blood cells are abnormal in size and shape, which prone to destruction. Many abnormal thalassemia gene types are found in Thailand. The convergence of these genes causes abnormality and varying levels of pathological severity, ranging from showing no symptoms at all to the highest severity, which causes fetal death or death at birth (Wasi, 1981; Fucharoen and Winichagoon, 1992). The Public Health Department announced a national policy to promote the prevention and control of thalassemia and abnormal hemoglobin, considering it a basic right of pregnant women and their husbands to receive screening services and confirmation of whether or not the couple is at risk of having a child with severe thalassemia. Every positive case would receive the service to prenatal diagnose before birth giving. So, each service center must organize the service system according to standard.

HbE, this is structurally abnormal hemoglobin where

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the β-globlin chain ($\alpha_2\beta_2^E$) is induced by G-A substitution at codon 26 of the β-globlin gene, causing the amino acids changing the location from glutamate to lysine (Weatherall and Clegg, 2001a; Weatherall and Clegg, 2001b). Generally, heterozygous-type HbE carriers do not have anemia, whereas, their mean corpuscular volume (MCV) value and mean corpuscular hemoglobin (MCH) value are slightly lower (Fucharoen and Winichagoon, 2000). However, if alleles are formed between HbE and β-thalassemia or HbE/β-thalassemia (Vichinsky, 2007; Weatherall and Clegg, 2001b), the patient will have a chronic hemolytic anemia by red blood cell destruction and must receive frequent blood transfusions over lifetime, which may subsequently lead to iron overload. The excess iron may accumulate in various organs, which may cause complications and premature death (Fucharoen et al., 2000). HbE carriers are widely found in Thailand and Southeast Asia (Vichinsky, 2007) and β-thalassemia carriers are also found widely in this region. Thus, the chance of HbE/β-thalassemia may occur very high. The World Health Organization (WHO) estimates that there will be 100,000 HbE/β-thalassemia people in the next twenty years and will also happening in India, Sri Lanka, Malaysia, and South China. This health concerning has also spread to America and Europe as well by resulting from migration and intermarriage with Asian peoples (Vichinsky, 2007; Weatherall and Clegg, 2001a).

HbE/β-thalassemia is classified as thalassemia intermedia, a group of patients with intermediate symptoms including: intermediate pallor with no need frequent blood transfusions, mild jaundice with a possibility they may have gallstones, osteoporosis/low bone mass in the case of iron overload. Some of them can be thalassemia major, which have severe symptoms including: prominent pallor; must receive blood transfusions from childhood, hypersplenism and spleenomegaly (at approximately 6-10 years of age) and need to remove the spleen or spleenoectomy; and iron overload will occur and must be constantly treated with medication. If the patient does not receive appropriate treatment and medication, severe symptoms will occur, such as, characteristic changes of the facial bones and skull, short stature, low body weight, retarded growth, osteoporosis/low bone mass and other complications may occur. Premature death may occur to infections or heart failure (Fucharoen et al., 2000).

HbE screening to prevent the incidence of HbE/β-thalassemia is an important issue to which the government sector should pay attention. Detecting and measuring of HbE can be done through many techniques, such as cellulose acetate electrophoresis (CAE), weak cation-exchange high performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) (Clarke and Higgins, 2000; Winichagoon et al., 2008). These techniques are capable of efficiently detecting and measuring HbE; however, these techniques are not capable of mass population screening. For example, cellulose acetate electrophoresis (CAE) has limitations due to it requiring many steps, from electrophoresis on cellulose acetate sheets, to dying and measuring the intensity of color. Furthermore, this test can only be conducted on 16 samples at a time. The HPLC and CZE methods are more suitable, such as a high throughput, and the HPLC method can present the ratio of hemoglobin A2 and F, which are clearly seen, produce accurate measurement and require a short running time. β-thalassemia carriers have hemoglobin A_2 quantity between 4.0 to 10%. HbE carriers have the EA hemoglobin characteristic and HbE quantity between 25 to 35%. HbE/β-thalassemia has the EF characteristic of hemoglobin, and HbE quantity is between 40 to 60%. Homozygous HbE has the EE hemoglobin characteristic and HbE quantity between 85 to 100%. These techniques require an automatic analyzer, which is expensive. For routine work, the HbE screening method that is easiest performed is the 2,6 dichlorophenol indophenols (DCIP) precipitation test, the basis of which is that the DCIP color will cause unstable hemoglobin, such as hemoglobin E and hemoglobin H to precipitate, which can be accomplished for a mass population. If this method is used together with the 0.36% NaCl osmotic fragility test, the results of the hemoglobin E screening will be easier to interpret, especially in the densely populated or rural areas (Winichagoon et al., 2002; Wiwanitkit et al., 2002). Nevertheless, the DCIP test has issues with reading the results, namely that the dark blue color may make it difficult to read the precipitation and may requiring decolorization (Fucharoen et al., 2004). Furthermore, observing the precipitation may require usage of a light box and solid line written to read the results (Chapple et al., 2006). DCIP testing also requires appropriate temperature and accurate incubation time to avoid false positive results. Additionally, the DCIP color is an oxidizing agent which may deteriorate if stored for a long time (Hughes, 1983; VanderJagt et al., 1986). However, the reading of the results of this method can be improved by a clearing reagent called modified DCIP test (KKU-DCIP), which is widely used at the present (Kor-anantakul et al., 1998).

The new HbE screening test or E-Sure test (Mitr Medical, Thailand) is based on ion exchange chromatography. After washing by other buffer systems at a different pH it is passed through the column, only hemoglobin E will elute from the column, and is seen as red or pinkish solution in the test tube. This method is quick, specific, highly accurate and simple to perform and does not require any highly skilled technician. It can be used in the rural areas or in a community laboratory with limited resources (Tatu and Kasinrerk, 2012; Sanguansermsri et al., 1998). For the abovementioned reasons, the researcher was interested in the evaluation of the efficacy of the new HbE screening test (E-Sure) compared to the modified DCIP test (KKU-DCIP), which

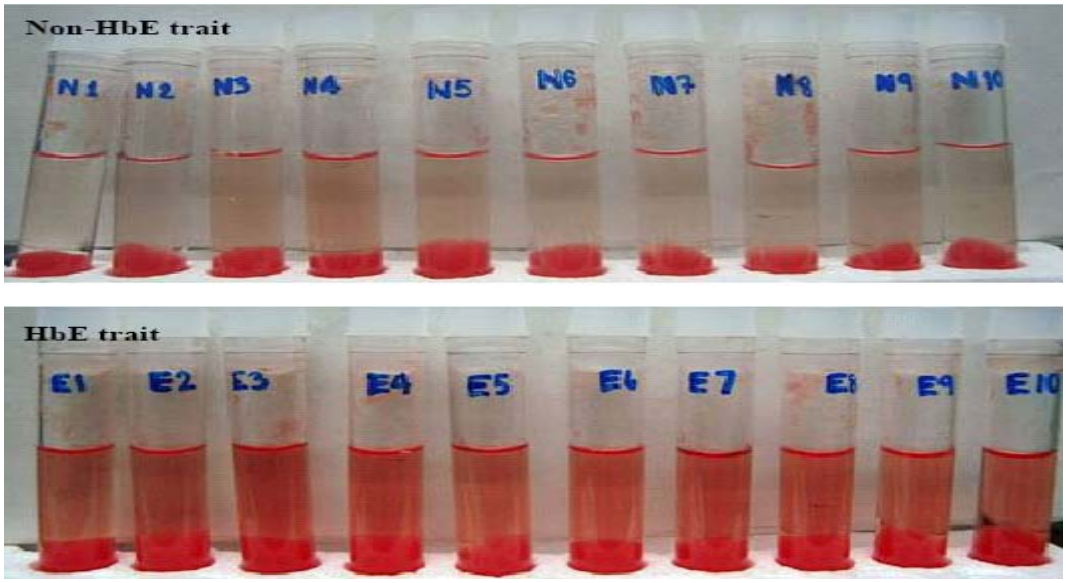


Figure 1. Presenting the results of hemoglobin E carrier testing. The uncolored solution in the top row read as negative results, which interpreted as Non-HbE trait (top row). The reddish-pink colored solution in the bottom row read as positive results and interpreted as HbE trait (Tatu and Kasinrerk, 2012).

is at present the conventional method in community hospitals. The results are confirmed by capillary electrophoresis, which can confirm β-thalassemia carriers as well. The results of this study may be used as a guideline in selecting screening methods for HbE carriers to prevent HbE/β-thalassemia risk at the community level.

MATERIALS AND METHODS

Sample collection and selection for HbE screening test

Ethylenediaminetetraacetic acid (EDTA) blood samples were collected from patients who came for check-ups at Kutchap Hospital and other hospitals with inter laboratory cooperation namely Nong Wuasaw hospital, Nong Saeng hospital and Pen hospital in Udon Thani Province, Thailand. This took place during the normal services of the hospital from October 2013 to February 2014. The research program had to pass the approval of the hospital directors and the Board of Human Research Ethics Committees of the hospital and all subjects gave written consent. The subjects were record the name, surname, AN, HN, ward, time and date of sample collection and clearly label the specimen tubes, as well as correctly giving the details of the specimen for testing in the request form. Before screening for HbE, a complete blood count (CBC) was required as a routine laboratory test. The automatic analyzer was Celltac E MEK-7222 (Nihon Kohden, Japan), and the criterion of sample selection for HbE screening was lower corpuscular volume (MCV< 80 fl).

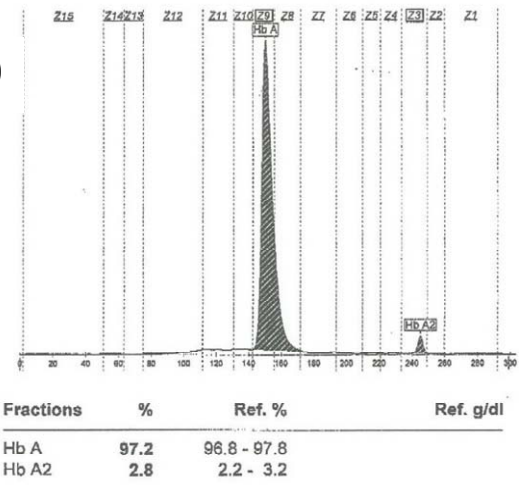
Evaluation of HbE screening tests and HbE confirmation

The conventional HbE screening was performed by using the modified 2,6 dichlorophenol indophenols (DCIP) precipitation test (KKU-DCIP) method, which was based on that the DCIP will cause unstable hemoglobin to precipitate. This was conducted by adding 2-3 ml blood to test tubes, which were contained the anti-coagulant, EDTA and centrifuged at 100 g for 10 min. The plasma was removed and 20 µl of red blood cells was added to the test tube containing 5 ml of DCIP reagent (KKU-DCIP Clear Reagent Kit). The blood and reagent were mixed by gently turning the test tubes and then warmed to 37°C for 1 h. The results were then accurately read. Positive result was turbid solution and clear solution was negative result.

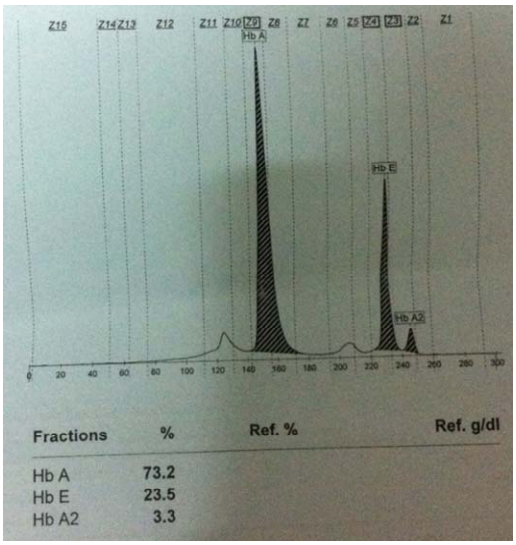
The new HbE screening test or E-Sure test is based on ion exchange chromatography according by characteristics, charge, and structure of each type of hemoglobin, which has different anions and cations. When the hemoglobin is placed in a particular buffer solution, the hemoglobin becomes electrically charged and then hemoglobin solution is passed through the column (diethyl aminoethyl [DEAE]) each hemoglobin molecules will be captured by the column. After washing by another buffer system at different a pH, it is passed through the column, only hemoglobin E will elute from the column, and is seen as red solution in the test tube. Color intensity is dependent on the quantity of Hb E. It is notable that homozygous HbE (EE) gives a dark red color to the solution and heterozygous HbE (EA) gives a pinkish red color (Figure 1). The procedures of E-Sure were done by 1) open micro-column cap and then, put the micro-column in 13 × 100 mm test tube 2) pipette 2 ml of buffer A to micro-column 3) pipette 20 µl of whole blood to buffer A in micro-column 4) adding the stick of test kit to micro-column, press the stick to elute buffer A into test tube until it will be finished and throw away buffer A from test tube) 5) pipette 4 ml of buffer B to micro-column 6) adding the stick of test kit and press the stick to elute buffer B into test tube and 7) read and interpret to results as describe on above.

Hemoglobin typing was used to distinguish normal, thalassemia and other types of hemoglobin abnormalities especially between β-thalassemia, heterozygous HbE (EA) and homozygous HbE (EE) and also to diagnose patients and carriers. The capillary electrophoresis was performed by an automatic analyzer, Capillarys II hemoglobin (Sebia electrophoresis, USA), which gave a high electrical charge to capillary tubes inside of the vessel. Both ends

(A)



(B)



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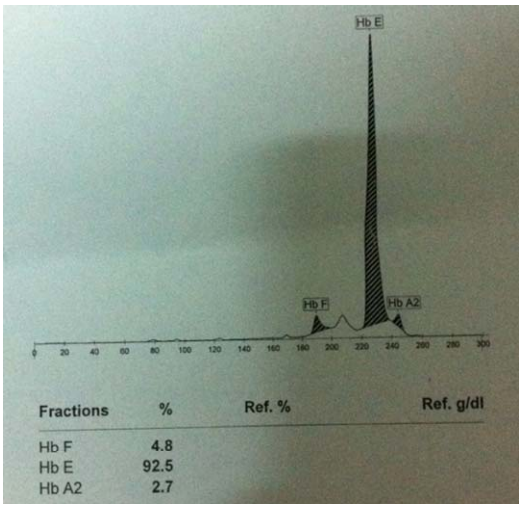

Figure 2. Electropherogram of hemoglobin typing by capillary electrophoresis. The results read as follows: A) A_2A; B) EA; C) EE, which were interpreted as normal, Heterozygous HbE and Homozygous HbE, respectively.

were immersed in electrolyte solution and caused the ions inside the sample to run from the positive to negative pole according to the strength of the electroosmotic flow (EOF). The zones of the separated solution after the electrical charge will pass the detection window and the changing of the signal from the detector (deuterium lamp) is sent to the data processor. This result is called an electropherogram. A report using a normal person's values as a reference is: Hb A = 79.4-93.4, Hb A_2 < 4.0, Hb F = 0.0-1.2. When each sample has been confirmed by hemoglobin typing, the new HbE screening (E-Sure) test is evaluated for sensitivity, specificity, positive predictive value, negative predictive value and efficiency (accuracy) of the analysis compared to modified DCIP (KKU-DCIP) method. The calculation of sensitivity, specificity, positive predictive value, negative predictive value and efficiency of tests were done by following formulae (TP = number of true positive; TN = number of true negative; FP = number of false positive and FN = number of false negative):

%Sensitivity = [TP/ TP+FN] x 100
%Specificity = [TN/ TN+FP] x 100
%PPV = [TP/ TP+FP] x 100
%NPV = [TP/ TP+FP] x 100
%Efficiency = [TP+TN/ TP+FP+TN+FN] x 100

RESULTS

326 HbE carriers (MCV < 80 fl) were screened by the modified 2,6 dichlorophenol indophenols precipitation test (KKU-DCIP) and HbE screening test (E-Sure) test and confirmed with Hb typing by capillary electrophoresis to find false positives and false negatives from both screening methods. The hemoglobin electropherograms were presented as the A_2A, EA and EE and interpreted to normal, heterozygous HbE and homozygous HbE, respectively (Figure 2). After screening and confirming for HbE carriers, 226 cases of HbE carriers were found, including 234 HbE trait and 32 homozygous HbE carriers (Table 1). When the efficacy of the KKU-DCIP screening method was compared to that of the HbE screening test (E-Sure) in community hospitals, it was found that the KKU-DCIP test had sensitivity, specificity, positive predictive, negative predictive and efficiency values of 95.1, 91.6, 98.1, 80.9 and 94.5%, respectively. Whereas, HbE screening test, which had sensitivity, specificity, positive predictive, negative predictive and efficiency values of 99.2, 96.7, 99.2, 96.7 and 98.8%, respectively (Table 2).

DISCUSSION

For the EDTA blood samples with low MCV (< 80 fl) values were presented anemic condition. MCV is one of the regular blood indices from CBC when applied together with screening test. There can increase the sensitivity of screening test to diagnose hemoglobin E carriers (Yeo et al., 1994; Nadarajan et al., 2010; Sharma et al., 2013) and other thalassemia (Alkindi et al., 2011; Italia et al., 2014), such as, alpha-thalassemia carriers (Pornprasert et al., 2013). However, the many studies of

Table 1. The numbers of normal and HbE carriers after confirmation (N = 326).

Hb typing	Interpretation	Number of cases
A_2A	Normal or Non clinical significant	60
EA	Heterozygous HbE	234
EE	Homozygous Hb E	32

Table 2. Two-by-two table showing diagnostic indices of KKU-DCIP and E-Sure screening tests to identify the hemoglobin E trait among low MCV samples (N = 326).

Test	Test result	Hb trait status		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
		Normal	HbE trait					
KKU-DCIP	Negative	55	13	95.1	91.6	98.1	80.9	94.5
	Positive	5	253					
E-Sure	Negative	58	2	99.2	96.7	99.2	96.7	98.8
	Positive	2	264					

screening test for efficacy did not use the MCV value as the “cut off” before comparing between each screening methods. MCV can be used generally to screen thalassemia, which has long been widely practiced in eastern countries (Cao et al., 2002). At present, many hospitals use automatic red blood cell analyzer to test CBC, which also gives MCV and Mean Corpuscular Hemoglobin (MCH) values as it is convenient for laboratory work. Furthermore, red cell distribution width value, RDW of blood indices can screen thalassemia carriers and hemoglobin E carriers as well (Sharma et al., 2013). Nevertheless, using MCV and MCH values to screen thalassemia carriers or thalassemia were limited as far as results interpretation and distinguishing them from other conditions that were lower MCV and MCH, e.g., iron deficiency anemia and chronic blood loss. Thalassemia screening by MCV values lower than 80 fl and/or MCH 27 pg were lower in sensitivity and specificity than screening by the one tube osmotic fragility test, OF and DCIP, because the Thai population has a number of HbE carriers with values MCV $\geq$ 80 fl and MCH $\geq$ 27 pg (Sanchaisuriya et al., 2005). In this study, 60 samples were interpreted to normal, but had low MCV values and interpreted to be a non- hemoglobin E trait but possibly a heterozygous alpha thalassemia-1 trait with hemoglobin typing as A_2A (normal). It was necessary to run further confirmatory tests by multiplex polymerase chain reaction (PCR) (Chang et al., 1991; Tongsong et al., 2000; Panyasai et al., 2002; Tungwiwat et al., 2006). Furthermore, other anemic conditions, such as chronic blood loss and iron deficiency anemia can also give low MCV results.

Wanapirak et al. (2009) compared the efficacy of the CMU-E method (Chiang Mai University-HbE, Thailand) and KKU-DCIP. The CMU-E method uses microcolumn chromatography, which is similar to E-Sure. The study results showed that CMU-E method produced less false positives because the results were easier to read and interpret than KKU-DCIP method, which must read positive results from murky solution, even though clearing reagent is used. Sometimes, false positive may be produced arising from the time to warm the solution and blood at 37°C, which is too long, and may also give positive results for other unstable Hb, such as, HbH disease.

HbE screening by the E-Sure test was newly developed by Tatu and Kasinrerk (2012) as a method with high sensitivity and specificity; however, this method has never been tested in community hospitals. This study used blood indices of MCV (< 80 fl) before using hemoglobin E E-Sure screening test kits, compared to the widely used method, KKU-DCIP. Community hospitals with limited highly skilled laboratory technician can also use the latter method; however, the reading and interpreting of results by E-Sure tests is easier and gives less false positives. The sensitivity, specificity, positive predictive, negative predictive and efficiency values are all higher than that of the KKU-DCIP method. However, results from hemoglobin E screening by the E-Sure method still presented 2 false positives and 2 false negatives. This may have occurred from reusing columns too many times, or from the reading and interpretation of the technician performing the test.

The capital cost for E-Sure screening is lower (0.78-0.94 USD/test) than the KKU-DCIP method (1.56-1.88 USD/test). If the E-Sure method would be used in place of the KKU-DCIP method, the capital costs for hospital labs would be reduced, especially community hospitals with limited budgets. Furthermore, hospitals with Quality Assurance (QA) policies, such as Hospital Accreditation (HA), ISO 15189 and Laboratory Accreditation (LA), among others, using the E-Sure method in place of the

KKU-DCIP method could report results faster. The E-Sure test is takes 5 to 10 min turnaround time; whereas, the KKU-DCIP method takes an approximately 30 min turnaround time. This could increase the quality of health services. The government sector and relevant agencies support SMEs that conduct business involving biomedical material and health science appliances to develop the biomedical material and industry. E-Sure tests were developed under the support of the National Innovation Agency (NIA), Thailand. The researcher perceives that there are benefits of this study to provide information to support the quality of products currently on the market, and also support Thai innovations.

Conclusion

The new HbE screening test, E-Sure test is a screening test with higher efficacy, as well as being more convenient and easier to interpret than the KKU-DCIP test. Furthermore, no additional special appliances are needed to conduct the test. This method is suitable for hemoglobin E carrier screening in community hospital laboratories.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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