

IMPROVED PROTOCOL OF ISOPROPANOL PRECIPITATION TEST FOR HEMOGLOBIN E SCREEN

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Abstract

Isopropanol precipitation test is the test established to screen for hemoglobin E (HbE). This test was tedious, requiring several steps to complete the protocol. This study was aimed to improve the protocol of isopropanol precipitation test so that it can simply and accurately screen for HbE. The incubation time, percentage of isopropanol, hemolysate preparing method, and volume of working isopropanol were re-optimized. The effect of hematocrit as well as the stability of the reagent and of the EDTA blood were also determined. The improved isopropanol precipitation test was found to work well with adding 50 mL hemolysate; prepared by mixing whole EDTA blood in 0.05% Triton-X 100, into 500 mL of 19% (v/v) isopropanol in 0.1 M Tris-HCl, pH 7.4 and incubated at 37°C for 20 min. This improved test was as effective as the traditional dichlorophenol indolphenol precipitation test and stable for at least 2 months at both 4°C and 30°C. The EDTA blood could be stored at 4°C for one month with no effect on test results. We concluded that this improved isopropanol precipitation test was effective and, coupling with the very low investing cost, could be the new choice for HbE screen.

Keywords: Dichlorophenol indolphenol precipitation test, Hemoglobin E, HbE screen, HbE trait, Isopropanol precipitation test

Introduction

HbE is an abnormal hemoglobin commonly found in SEA countries. In Thailand, this abnormal hemoglobin is observed across the country with the estimated prevalence of 13%, increasing to 50-60% at the Thai-Laos-Khmer border (Fucharoen *et al.*, 1998). Compound

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heterozygosity of the HbE gene (β^E) with the β -thalassemia gene (β^{Thal}) results in HbE/ β -thalassemia (β^E/β^{Thal}), a severe type of thalassemia syndrome. The β^E gene in this HbE/ β -thalassemia comes from one of the parents. Thus, detecting HbE in the parents is a crucial way to prevent the birth of a HbE/ β -thalassemia offspring.

In most laboratories, Hb typing either by cellulose acetate electrophoresis (CAE), cation-exchange high performance liquid chromatography (HPLC), cation-exchange low pressure liquid chromatography (LPLC), and capillary zone electrophoresis (CZE) are the standard techniques for detecting HbE in blood. However, these techniques require sophisticated and advanced machines which are expensive and not suitable for mass population-based screening, especially in budget-limited laboratories. Thus, initial screening using simple laboratory tests is necessary. The most widely used HbE screening test is the dichlorophenol indolphenol precipitation (DCIP) test.

The DCIP test has been shown to be sensitive and specific for detecting HbE in HbE trait whose Hbs A and E are found in blood (Winichagoon *et al.*, 2002; Wiwanitkit *et al.*, 2002; Fucharoen *et al.*, 2004; Soogarun *et al.*, 2004; Sanchaisuriya *et al.*, 2005; Chapple *et al.*, 2006; Wanapirak *et al.*, 2009). However, due to its oxidizing property, dichlorophenol indolphenol is easily reduced, leading to a loss of its oxidizing property. Therefore, other HbE screening tests were established to solve this problem. These included HbE tube test (Tatu and Kasinrerk, 2012) and microcolumn-based HbE screen test (Wanapirak *et al.*, 2009).

Isopropanol precipitation test is a classic test invented for HbE screening in 1980 by Ali and coworkers (Ali *et al.*, 1980). However, there has been no substantial utilization of this test for HbE screening since its first publication, although its protocol was documented in many hematology text books. This study thus was aimed to re-establish and improve the protocol of the isopropanol precipitation test so that it becomes simple and

easy test for HbE screen.

Materials and Methods

Original Isopropanol Precipitation Test (OIPT)

The original isopropanol precipitation was performed according to the procedure described previously (Ali *et al.*, 1980). Briefly, 200 μ L of diluted hemolysate was mixed with 2 mL of 17% isopropanol (PROPAN-2-OL; Analytical grade, RCI Labscan Limited, Samutsakorn, Thailand) in 0.1 M Tris-HCl buffer, pH 7.4. Thereafter, the mixture was incubated in a 37°C water bath and checked for flocculation of HbE inside the tube at 5, 20, 30, 40, and 50 min.

Improvement of Isopropanol Precipitation Test (IPT)

The protocol of IPT was improved, including incubation time, percentage of isopropanol, hemolysate preparing method, and working volume of the test kit. The effect of hematocrit on test results was also determined as well as the stability of the test kit and of the EDTA blood.

Validation of the IIPT

The effectiveness of the IIPT was evaluated in 110 unknown blood samples. The IIPT results obtained were compared with the conventional DCIP test results (THALCON™, Qualified Group, Bangkok, Thailand). The cellulose acetate electrophoresis of hemoglobin at pH 8.6 was performed as the reference method (Weatherall and Clegg, 2001).

Calculation of Effectiveness of the IIPT

Sensitivity and specificity were calculated to determine effectiveness of the IIPT. Sensitivity of the IIPT test was calculated by the equation; Sensitivity (%) = [n of True positive/(n of True positive + n of False negative)] $\times$ 100. Specificity was calculated by the equation; Specificity (%) = [n of True negative/(n of True negative + n of False negative)] $\times$ 100.

Results

The Original Isopropanol Precipitation Test (OIPT)

The OIPT was performed on 2 blood samples; HbE trait with Hb type AE and normal individual with Hb type A₂A. The OIPT was able to discriminate between AE and A₂A. However, the incubation time in 37°C was 50 min, not 30 min as established in the original protocol (Figure 1).

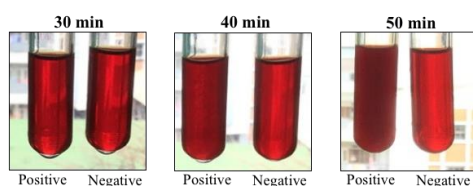


Figure 1. The OIPT results on blood samples having Hb types of AE (Positive) and A₂A (Negative). The flocculation in the “Positive” sample was clearly seen at 50-min incubation, while the solution remained clear in the “Negative” sample

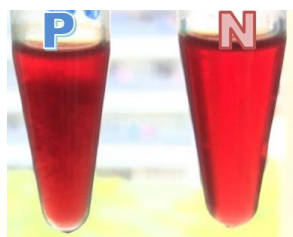


Figure 2. The Improved Isopropanol Precipitation Test; IIPT. “P” stands for “Positive case” having Hb types AE and “N” for “Negative case” having Hb types A₂A. Notice that flocculation exists inside the “P” tube, but not in “N” tube

Improvement of the Protocol

The protocol of the isopropanol precipitation test was improved so that it was simpler than the original test. In this improved protocol, 50 μ L of hemolysate (prepared by mixing whole EDTA blood at 1:1 ratio with

0.05% Triton-X 100) was added to 500 μ L of 19% isopropanol in Tris-HCl buffer pH 7.4, and incubated for 20 min at 37°C before visualizing for flocculation. The clear 1.5-mL microcentrifuge tube was used as a container of the reagent. This improved test was then named “Improved Isopropanol Precipitation Test; IIPT”. This IIPT was able to clearly discriminate HbE positive samples (AE) from those not having HbE (A₂A) as shown in Figure 2.

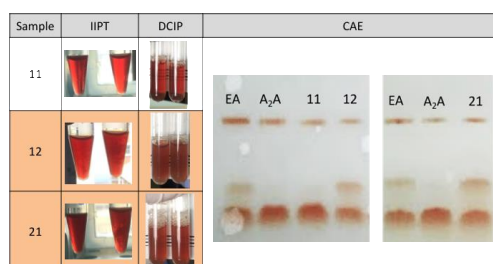


Figure 3. Example of results obtained from IIPT, DCIP and CAE. Sample number 11 had Hb type A₂A and was negative for both IIPT and DCIP as seen by clear solution. Samples number 12, 21 had Hb type AE and were positive for both IIPT and DCIP as seen by turbid solution

Validation of the IIPT and the Impact of Anemia on Test Results

The effectiveness of the IIPT in screening HbE trait (AE) and the impact of anemia on test results were assessed simultaneously in 110 unknown EDTA blood samples (Figure 3). True positive and false positive results were found in 5 and 2 blood samples, respectively. True negative results were seen in 104 blood samples. No false negative was observed in this cohort. Effectiveness of the tests was then assessed as the sensitivity and specificity of the test. Sensitivity, specificity, positive predictive value, and negative predictive value of IIPT and DCIP test were both 100%, 98.1%, 71.4%, and 100%, respectively. In addition, the IIPT was able to successfully perform in blood samples having hematocrit values of 11-70%.

Storage Effect on the IIPT

Stability of the IIPT reagents at both 4°C and at ambient temperature of about 30°C was assessed using AE and A₂A blood samples. It was found that the MIPT reagent was stable in both conditions for at least 2 months.

Storage Effect on Hemoglobins in EDTA Blood

The stability of hemoglobins in EDTA blood samples used for the test was evaluated using the known blood samples (AE and A₂A). These blood samples were kept in 4°C and 25-30°C and tested everyday by the IIPT. It was found that the 4°C-storage could preserve hemoglobins up to 34 days, but only 3 days for blood stored at 25-30°C.

False Positive of IIPT in HbH Disease

The IIPT was performed in the known cases of HbH disease, whose Hb types by CAE at pH 8.6 comprised Hbs A₂AH. The positive result was shown, being the same as that for HbE carriers (Figure 4).

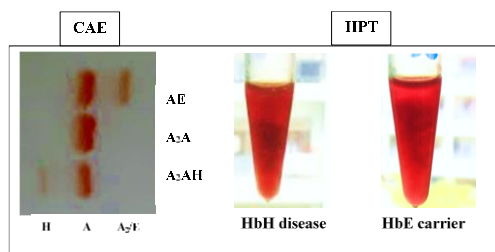


Figure 4. False positive IIPT in HbH disease (A₂AH on CAE). The flocculation is produced at the same appearance as that of HbE carrier (AE on CAE). Normal individual has normal Hb type of A₂A. Note that HbA₂ band is so pale that it is invisible in the picture

Discussion

Isopropanol (isopropyl alcohol, propan-2-ol, 2-propanol; C₃H₈O) is a polar alcohol used for preparing disinfectant and for DNA, protein precipitation (Marney *et al.*, 2008; Green, and

Sambrook, 2017; Martinez *et al.*, 2017). For hemoglobin analysis, it has been used to screen for unstable hemoglobins such as HbE (Ali *et al.*, 1980; Bender *et al.*, 1981). It was postulated that this alcohol pulled water molecules away from DNA and protein, hence, rendering precipitation of these macromolecules. This technique is, however, attractive by its ease in production, simplicity, and durability. Therefore, this reagent should be suitable for population-based screening of HbE in regions endemic of this abnormal hemoglobin. However, this technique has never been practically adapted for this particular purpose, although its protocol has been well documented in the textbooks (Wild and Bain, 2011).

Screening for HbE carriers is still necessary for Thailand and many developing countries in which this abnormal hemoglobin is endemic (Wiwanitkit, 2013). Beside the HbE tube test and chromatography-based test mentioned earlier, screening of HbE can be performed by the more sophisticated techniques such as isoelectric focusing (Charoenkwan *et al.*, 2010) and mass spectrometry (Yu *et al.*, 2017). However, the later two methods are too expensive for budget-limited countries to perform. Then, the DCIP test is still in common use. The DCIP test itself has several drawbacks as stated and the HbE tube test recently invented in our laboratory needs complicated steps of production that might be difficult to control the quality of the test kit. This report has shown that the isopropanol precipitation test could still be effectively performed to screen for HbE, and, improvement of the protocol was particularly successful.

This improved isopropanol precipitation test or IIPT has been proven to be as effective as the conventional DCIP test which is widely performed at present. Compared to the Hb typing results obtained by the standard CAE, both IIPT and DCIP showed identical results and effectiveness. This result therefore emphasized the potential of the IIPT in HbE screening.

Isopropanol is an alcohol, and easy to evaporate. This could lead to a decrease of its quantity in the MIPT reagent, leading to false

negative screening results. We were greatly concerned about this point and so tested for the durability of this 19% IIP reagent. The 2-month stability of this reagent at both 4°C and at ambient temperature of Thailand (around 30°C) would indicate that this reagent was stable. It was thus suitable for routine use, particularly in small laboratories. Longer storage should not affect the test performance as long as the lid of the container is tightly closed or the 19% IIP reagent is kept as stock solution, also tightly capped, in 4°C without aliquoting. This was to minimize rate of evaporation of isopropanol.

Stability of hemoglobins in blood samples was also concerned as prolonged storage would lead to falsely positive results due to denaturation of hemoglobins in blood samples. Storing blood sample in cold was found to preserve hemoglobins well. This result indicated that if blood samples are to be kept before testing, keeping in cold at 4-8°C, which is the temperature in all laboratory refrigerators, is enough.

Concerning the cost of investment, the DCIP kit costs approximately 30 THB, but less than 5 THB is the approximate cost of the IIP kit.

Conclusions

We concluded that this IIP could be the new choice for population-based screening of HbE trait in Thailand and developing countries where HbE is the national burden. However, for future improvement, spectrophotometric measurement for turbidity by should be set up to generate numerical data of light transmission. The numerical data of light transmission can then be used to establish the cutoff values for differentiating HbE and non-HbE containing blood, as well as statistical analysis.

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