ORIGINAL ARTICLE

Evaluation of Dichlorophenolindophenol (DCIP) Test for Haemoglobin E (Hb E) in Normal Red Cell Indices Individuals

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ABSTRACT

Introduction: The current cut-off value for thalassaemia screening is based on a mean corpuscular haemoglobin (MCH) level of less than 27 pg. Samples with MCH < 27 pg require additional confirmatory testing, while samples with MCH > 27 pg are considered normal. However, there is a possibility of missing some types of haemoglobinopathy such as haemoglobin (Hb) E trait, by using this cut-off value. This study aimed to evaluate the dichloroindophenol (DCIP) test for Hb E carriers within healthy individuals. **Methods:** In total, 200 leftover blood samples with normal full blood count (FBC) results and MCH ≥ 27 pg were collected from students who were involved in the thalassaemia screening program. Blood samples were screened for Hb E by the DCIP test and subjected to haemoglobin analysis using capillary electrophoresis (CE) and gel electrophoresis. **Results:** Of the 200 leftover blood samples with normal FBC indices, 10 were positive for the DCIP test of which 8 had Hb E trait and 2 had Hb Constant Spring. The DCIP test showed 100% sensitivity and 98.96% specificity with a 100% negative predictive value (NPV) and an 80.0 % positive predictive value (PPV). **Conclusion:** The DCIP test was an excellent screening tool that, when combined with FBC parameters can produce better results, particularly in areas with a high Hb E incidence.

Keywords: Thalassaemia, Hb E trait, Dichlorophenolindophenol (DCIP) test, Screening test

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INTRODUCTION

Thalassemia is a group of haemoglobin synthesis disorders in which one or more haemoglobin globin chains are absent or produced insufficiently. It is the commonest inherited haemoglobin disorder that can be prevented in the community. Thalassaemia prevention includes public education with cost-effective screening strategies aiming to prevent and control the emergence of new cases. Every year, an estimated 300,000 children are born with thalassemia or haemoglobinopathies.

Malaysia aimed to reduce thalassemia by 2035 with a 95% reduction target through effective screening methods. The current screening strategy for thalassaemia is based on the cut-off value red cell indices of a mean corpuscular volume (MCV) of less than 80 fl and a mean corpuscular haemoglobin (MCH) less than 27 pg. Blood samples with MCH levels less than 27 pg will be subjected to further confirmatory tests, while samples with MCH above 27 pg are considered normal. However, there have been reported cases of thalassaemia carriers with a completely normal full blood count with normal red cell indices (1). The use of an MCH level of < 27 pg to determine carriers of haemoglobinopathies like Hb E may be deceiving especially in areas where thalassaemia is prevalent. Meng et al. found Perlis has quite a prevalence of Hb E carriers at 19.3% of its 227,000 population in 2010 (2). Hb E is common not only in northern Malaysia but also in Sabah (3).

Hb E may cause significant morbidity if a carrier were married to a beta thalassaemia person and produced an offspring with Hb E-β thalassaemia, which is also known as a compound heterozygous state. It constitutes about 50% of the clinically severe β thalassaemia disorders, which usually require regular blood transfusion and lead to iron overloading, low quality of life and ultimately premature death (4). The Malaysian thalassaemia registry reported that Hb E-β thalassaemia forms the largest group of thalassaemia patients with 2744 (34.37%), with the number increasing each year (5).

The dichlorophenolindophenol (DCIP) test has been used to screen Hb E in Thailand, especially in under-resourced areas. It could detect the existence of Hb E when a normal full blood count fails to do so. In this test, the 2,6 dichlorophenol indophenols will precipitate when combined with unstable haemoglobin such as

Hb E and Hb H, resulting in the cloudiness of positive samples. The test's ability to detect the presence of Hb E in a person with normal full blood count indices will enhance the sensitivity of Hb E screening (6).

MATERIALS AND METHODS

Study population

Form four students were briefed at their respective schools at Kedah before blood sampling day and informed consent from parents and students was obtained. Full blood count was performed at the selected primary health care centres involved in the thalassaemia screening program. The discarded blood samples from males and females with MCH > 27 pg and MCV > 80fl were included in this study, as they were regarded as normal samples. Meanwhile, blood samples with MCH < 27 pg and MCV <80 fl were excluded.

Analytical methods

We obtained 3 mls of peripheral blood from 200 students anticoagulated with ethylenediaminetetraacetic acid (EDTA). The blood samples were put on ice and transported to Hospital Sultanah Bahiyah, Kedah within four hours. The samples were subjected to the DCIP test using a modified 2,6 dichlorophenol indophenol precipitation test kit (KKU-DCIP, Thailand). Capillary electrophoresis using Capillarys 2 flex-piercing (Sebia, France) and agarose gel electrophoresis (AGE) using Sebia Hydrasys (Cedex, France) at alkaline pH were performed to confirm the findings.

The DCIP test was conducted by adding 20 µL of the whole blood to 2 ml of the DCIP solution and warmed at 37°C for 15 minutes. The clearing reagents were added, and the samples were left at room temperature for at least five minutes before visual interpretation. Positive samples were cloudy whereas negative samples remained clear. The samples tested with DCIP were run together with positive and negative controls (Figure 1). The minimal cloudiness of the samples was regarded as positive.

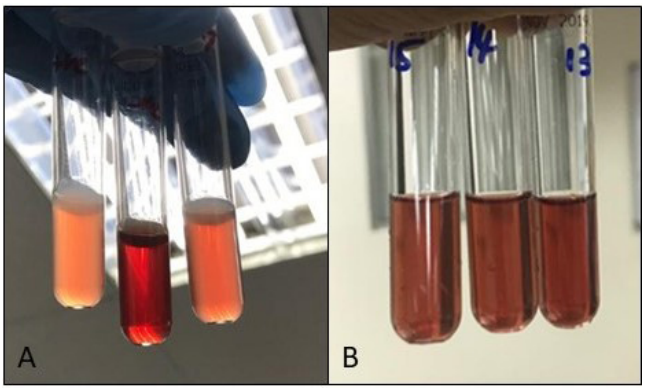

Fig 1 : (A) DCIP test showed (left) positive control, (middle) negative control and (right) positive sample. (B) Negative samples were clear

The results of the DCIP test were evaluated using standard methods at our laboratory. Capillary electrophoresis (CE) was performed using an automated analyser. At high voltage in an alkaline buffer, haemoglobin fractions were separated by their electrophoretic mobility. The fractions were detected at an absorbance of 415 nm. The fraction patterns were divided into specific zone. Each zone represents specific haemoglobin typing with possible variants migrating within the zones.

The haemolysate from washed the red blood cells was subjected to agarose gel electrophoresis. The haemoglobins were separated on alkaline gels via electrophoresis, and the fractions were visualised by staining with amido black.

Statistical analysis

The DCIP test was assessed for sensitivity, specificity, PPV and NPV. The analysis was compared to the CE and AGE methods.

Ethics

The study was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-18-3774-45217), and the Ethics Committee for Research Involving Human Subjects, University Putra Malaysia (JKEUPM-2020-389).

RESULTS

Two hundred blood samples were collected from primary health care centres at Kedah. All samples showed normal Hb, MCV, MCH values, and other haematological indices. All samples were from 16-year-old school students involved in the thalassaemia screening programme of whom 119 (59.5%) were male and 81(40.5%) were female. The participants were predominantly Malays (189, 94.5%) followed by Siamese (5, 2.5%), Chinese (4, 2%) and Indian (2, 1%). Ten (5%) out of the200 samples were positive for DCIP, while 190 (95%) were negative. All samples were subjected to CE and gel electrophoresis for confirmation of DCIP test (Figures 2, 3). A total of 190 samples were found to be normal, 8 were positive for heterozygous Hb E and 2 were positive for Hb Constant Spring. The proportion of heterozygous Hb E with normal FBC was 4%.

The DCIP test was found to have 100% sensitivity and 98.96% specificity for Hb E, whereas the positive predictive value (PPV) was 80% and the negative predictive value (NPV) was 100% (Table I).

DISCUSSION

The screening method is the most important phase of the prevention strategy. A full blood count with red cell indices is commonly used to screen thalassaemia

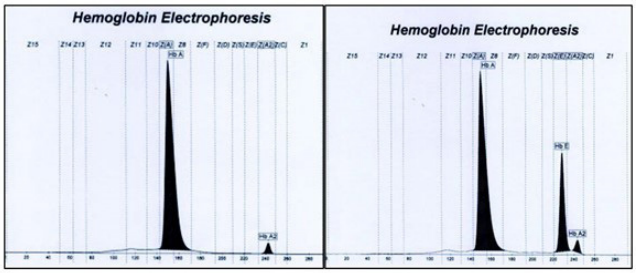

Fig 2: Capillary electrophoresis in a normal individual (left) and Hb E carrier (right)

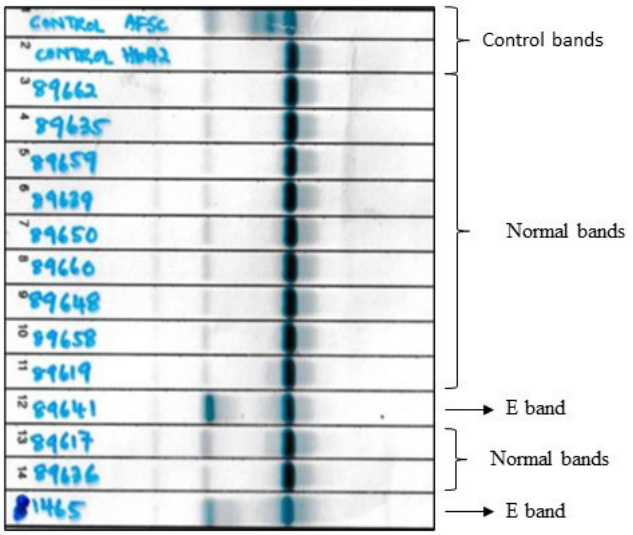

Fig 3: Agarose gel electrophoresis showed the control bands with the presence of normal band and E band

carriers. Individuals with low MCV (< 80 fl) and MCH (<27pg) will need further investigation to determine the types of thalassaemia using electrophoresis, high-performance liquid chromatography (HPLC) or DNA analysis.

Meanwhile, individuals with normal red cell indices will be regarded as normal and eliminated from the screening pool. Unfortunately, as shown in this report, these cut-off points do not completely rule out the existence of haemoglobinopathy. Among the 200 students who were initially thought to be normal, we discovered 4% of them were Hb E carriers and 1% Hb Constant Spring carriers. If these cut-off values are continuously being applied, many Hb E carriers will be missed (1,7). Hb E carriers should not be overlooked during thalassaemia screening, particularly in areas where the prevalence is high. In this study, analysis of the full blood count results including the red cell indices for all 200 samples was within the normal range. However, the mean Hb level, Hct, MCV, MCH and RDW values for Hb E carriers were slightly lowered compared to those of the normal group but still within normal ranges (data not shown). Khondaker et al. also demonstrated that using MCH < 27 pg and MCV <80 fl as cut-off parameters in detecting Hb E showed no diagnostic value (8). A study in Kuantan also found Hb E carriers and α thalassaemia carriers in individuals who had MCV > 80 fl (9). Ittarat et al. also showed the same result (10).

It is a significant problem when thalassaemia screening using MCV and MCH cut-off values consistently produces false-negative results, particularly in a population where Hb E is prevalent. Missed identification of Hb E carriers will cause people to be unaware of their status. This will result in the absence of counselling and public education as well as offspring with varying degrees of thalassaemia heterogeneity. Regardless of red cell indices, Hb E carrier identification should be performed in appropriate clinical situations (7).

We obtained excellent DCIP test results for Hb E carriers with normal red cell indices (MCH > 27pg). This screening test was100% sensitive and 98.6% specific with PPV 80% and NPV 100%. Hence, false negatives and positives were uncommon. We found two cases giving rise to false-positive Hb Constant Spring, but it leads to the completion of a conclusive evaluation, which would be a benefit rather than a drawback. We found 8 of Hb E trait and 2 of Hb Constant Spring carriers; thus, standard screening procedures have a high rate of false positives and miss those with Hb E trait.

Studies have also shown that the DCIP test was effective in detecting HbE carriers (11, 12, 13, 14). We validated the usefulness of this test kit utility as a screening tool. To our knowledge, the DCIP test has never been incorporated in any clinical services in Malaysia. While the test may be qualitative and more time consuming compared to automated full blood count, it can be easily performed by a person with minimum technical skill at a low cost. Cost per test was roughly RM 10.00. Many studies have recommended DCIP to be incorporated in the thalassaemia screening strategy especially in high prevalence areas (6, 14, 15).

CONCLUSION

An effective screening strategy should be implemented to enhance the failure of undetectable thalassaemia carriers for further counselling and awareness. Therefore, before an individual with MCH > 27pg is labelled normal, their sample should undergo the DCIP test especially in areas where thalassemia is prevalent. The limitation of this study is the confirmation of the results using the molecular method was not performed because of financial constraints.

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