Effect of HbE heterozygosity on the measurement of HbA1c

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Summary

**Aim:** Measurement of HbA1c provides an excellent measure of glycaemic control for diabetic patients. However, haemoglobin (Hb) variants are known to interfere with HbA1c analysis. In our laboratory HbA1c measurement is performed by Variant II turbo 2.0. The aim of this study is to investigate the influence of HbE trait on HbA1c analysis.

**Methods:** Haemoglobin variants were identified by HbA1c analysis in 93 of 3522 samples sent to our laboratory in a period of 1 month. Haemoglobin analysis identified HbE trait in 81 of 93 samples. To determine the influence of HbE trait on HbA1c analysis by Variant II Turbo 2.0, boronate affinity high performance liquid chromatography (HPLC) method (Primus PDQ) was used as the comparison method. Two stage linear regression analysis, Bland Altman plot and Deming regression analysis were performed to analyse whether the presence of HbE trait produced a statistically significant difference in the results. The total allowable error for HbA1c by the Royal Australasian College of Pathologists (RCPA) external quality assurance is 5%. Hence clinically significant difference is more than 5% at the medical decision level of 6% and 9%.

**Results:** Statistically and clinically significant higher results were observed in Variant II Turbo 2.0 due to the presence of HbE trait. A positive bias of ~10% was observed at the medical decision levels.

**Conclusion:** Laboratories should be cautious when evaluating HbA1c results in the presence of haemoglobin variants.

**Key words:** Boronate affinity method, diabetes mellitus, HbE heterozygous, HbA1c, ion exchange method.

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INTRODUCTION

It is estimated that there are approximately 366 million people worldwide who are suffering from diabetes mellitus (DM) and by 2030, 552 million people will be suffering from this condition.1 The chronic complications which affect many organ systems are responsible for the majority of morbidity and mortality associated with DM. In diabetic patients, glycated haemoglobin, measured as HbA1c, provides a good indication of overall glycaemic control and is a predictor of long-term complications. Glycated haemoglobin is formed as a result of irreversible non-enzymatic glycation of one or both N-terminal valines of the haemoglobin β chain. We know that intensive therapy aimed at keeping HbA1c levels as near to normal as possible can reduce the development and/or progression of long-term complications.2

Treatment goals for HbA1c have been established, and more recently the International Expert Committee has recommended HbA1c measurement for the diagnosis of diabetes.3,4 Hence, accurate and precise measurement of HbA1c is important. Methods that are commonly used to measure HbA1c are high-performance liquid chromatography, immunooagglutination, boronate affinity, electrophoresis and enzymatic assays. However, it has been noted that HbA1c measurement can be affected by a variety of genetic, haematological and illness-related factors.5 The effect of variant haemoglobins (Hb) on HbA1c measurement varies depending on the specific variant and the HbA1c method used. As in other parts of South-East Asia, α thalassaemia, β thalassaemia, haemoglobin E (HbE) and Haemoglobin Constant Spring are prevalent in this country and one in four individuals are estimated to be a carrier of an abnormal gene.6

HbA1c is measured in our laboratory using the Bio-Rad Variant II Turbo 2.0 system. The effect of HbE in HbA1c measurement on Bio-Rad Variant II Turbo has been widely reported. However, to date there are not many reports on the effect of HbE in HbA1c measurement by the Bio-Rad Variant II Turbo 2.0.

The objective of this study is to evaluate the effect of HbE on HbA1c assay that is used in our laboratory.

MATERIALS AND METHODS

In the month of January 2012, 3522 whole blood samples were received by our laboratory for HbA1c analysis. HbA1c was measured using ion-exchange HPLC by Bio Rad Variant II Turbo 2.0 (Bio-Rad laboratories, Hercules, CA, USA). Ninety-three of the 3522 samples showed the presence of a variant window on chromatograms of HbA1c. In order to study the effect of these variants on HbA1c measurement, these samples were analysed on boronate affinity high performance liquid chromatography (HPLC) analyser (Primus PDQ; Trinity Biotech, USA) within 6 hours of collection.

These samples were then sent to the Haematology unit for Hb analysis and subsequent identification of these variants. These samples were screened by HPLC Bio-Rad Variant II (Bio-Rad Laboratories) utilising β thalassaemia short program and the variants were confirmed by electrophoresis which was performed on Sebia Hydrazo agarose gel electrophoresis (Sebia Electrophoresis, USA). HbE trait was diagnosed in 81 of 93 samples. To study the correlation between Bio-Rad Variant II Turbo 2.0 and the comparative method, 84 homozygous HbA were included in the study. Boronate affinity HPLC was used as the comparison method since this has been shown to be not affected by HbE.7 This study was approved by the Ethics Committee of our hospital.

Statistical analysis

An overall test of coincidence of two stage least-squares linear regression lines was performed using SPSS Software version 18 (SPSS, USA) to determine
whether the presence of HbE caused a statistically significant difference ($p < 0.05$) in results relative to the comparative method. Bland–Altman analysis and Deming regression analysis was performed using Analyse-it method evaluation software (Analyse-it Software, UK) to determine whether the presence of these variant traits produced a clinically significant effect on HbA1c results. Bias attributable to the presence of Hb variant was studied using total allowable error by the Royal College of Pathologists of Australasia (RCPA) at medical decision points of 6% and 9%.

RESULTS

Correlation of the HbA1c values measured by the Bio-Rad Variant II Turbo 2.0 ion-exchange and Primus PDQ boronate affinity methods

The HbA1c values in the homozygous A measured by the ion-exchange HPLC method ranged from 4.3 to 13.6% and the mean ± 2 standard deviation (SD) was $7.3 ± 3.98\%$. For the same samples analysed by boronate affinity HPLC method HbA1c values ranged from 4.4 to 13.6% and the mean ± 2SD was $7.3 ± 3.97\%$. There was statistically no significant difference in the HbA1c values measured by these two methods.

Bland–Altman plot and Deming regression analysis showed that these two methods correlate well (Fig. 1A,B).

The effect of HbE on HbA1c values by the Bio-Rad Variant II Turbo 2.0

The two stage least-squares linear regression showed F-statistics $=1.18$ and $p=0.007$. This indicated that the presence of haemoglobin variant caused a statistically significant difference ($p < 0.05$) in results between the routinely used method and the comparative method. Deming regression analysis and Bland Altman plot clearly indicated that HbA1c values measured by Bio-Rad Variant II turbo 2.0 were higher than the comparative method and the bias was approximately 10% (Fig. 2A,B). The presence of HbE trait resulted in a clinically significant positive bias throughout the range of HbA1c values (Table 1).

DISCUSSION

HbE is a $\beta$ chain variant with a mutation in the $\beta$ globin gene where lysine is substituted for glutamine at position 26 of the $\beta$ globin chain. According to the manufacturer’s package insert for Bio-Rad Variant II Turbo 2.0, HbE heterozygotes have no effect on the analysis of HbA1C.$^8$ The Bio-Rad Variant II Turbo showed HbE trait as a variant-window that was shown as a split A0 peak on the chromatogram. Therefore, the falsely
high HbA1c level may be due to the decreased Hb A0 area percentage. Little et al.9 investigated the effects of HbE and HbD traits on the glycated Hb level by 23 commercial methods in which the boronate affinity method was used as a reference method. They found interference by HbE on Bio-Rad Variant II Turbo analyser where HbA1c levels were falsely increased and values were unacceptable. Bio-Rad Variant II Turbo 2.0 differs from Variant II Turbo in separating the HbE trait as a separate variant peak and not appearing as a split peak of HbA0. Hence, we would expect no interference by this trait. However, a mild overlap of A0 and variant peaks cannot be entirely excluded which can affect the calculation of HbA1c.

Lin et al.10 confirmed in their recent publication that Variant II Turbo 2.0 did not show any clinically significant interference of HbA1c with concurrent haemoglobin C, D, E, and S traits. Contrary to their findings, we observed positive bias for HbA1c values for samples with HbE trait that was statistically and clinically significant. Lin et al.10 collected whole blood samples in EDTA tubes and the samples were frozen at −70 °C in small aliquots before HbA1c analysis at four different sites. In our study we analysed the sample within 24 h of sample collection in the same laboratory, which was the only difference between the two studies. We are unsure whether this could contribute to the difference in the results that was observed between these studies. However, HbA1c results have been shown to be unaffected when samples were stored at −70 °C.11 Hence, more studies with a larger number of HbE trait samples would be needed to confirm this. Even though the manufacturer has stated that HbE trait has no interference in this method, laboratories should be cautious when evaluating results from patients with haemoglobin variants.

### Table 1

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>HbA1c (MDP) %</th>
<th>Bias</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
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<tr>
<td>Homozygous A</td>
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<td>−0.01</td>
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<td></td>
<td>9</td>
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Medical decision point (MDP) analysis calculated by Deming regression.

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References