Measurement of HbA_{1c} and HbA_{2} by Capillarys 2 Flex Piercing HbA_{1c} programme for simultaneous management of diabetes and screening for thalassemia

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Abstract

Introduction: Thalassemia could interfere with some assays for haemoglobin A_{1c} (HbA_{1c}) measurement, therefore, it is useful to be able to screen for thalassemia while measuring HbA_{1c}. We used Capillarys 2 Flex Piercing (Capillarys 2FP) HbA_{1c} programme to simultaneously measure HbA_{1c} and screen for thalassemia.

Materials and methods: Samples from 498 normal controls and 175 thalassemia patients were analysed by Capillarys 2FP HbA_{1c} programme (Sebia, France). For method comparison, HbA1c was quantified by Premier Hb9210 (Trinity Biotech, Ireland) in 98 thalassaemia patients samples. For verification, HbA1c from eight thalassaemia patients was confirmed by IFCC reference method.

Results: Among 98 thalassaemia samples, Capillarys 2FP did not provide an HbA_{1c} result in three samples with HbH due to the overlapping of HbBart’s with HbA_{1c} fraction; for the remaining 95 thalassaemia samples, Bland-Altman plot showed 0.00 ± 0.35% absolute bias between two systems, and a significant positive bias above 7% was observed only in two HbH samples. The HbA_{1c} values obtained by Capillarys 2FP were consistent with the IFCC targets (relative bias below ± 6%) in all of the eight samples tested by both methods. For screening samples with alpha (α-) thalassaemia silent/trait or beta (β-) thalassemia trait, the optimal HbA_{2} cut-off values were ≤ 2.2% and > 2.8%, respectively.

Conclusions: Our results demonstrated the Capillarys 2FP HbA_{1c} system could report an accurate HbA_{1c} value in thalassemia silent/trait, and HbA_{2} value (≤ 2.2% for α-thalassaemia silent/trait and > 2.8% for β-thalassemia trait) and abnormal bands (HbH and/or HbBart’s for HbH disease, HbF for β-thalassemia) may provide valuable information for screening.

Key words: haemoglobin A_{1c}; thalassemia; capillary electrophoresis (CE); haemoglobin A_{2}

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Introduction

Haemoglobin A_{1c} (HbA_{1c}), the major form of all glycated haemoglobin species, is produced by the non-enzymatic addition of glucose residues to valine moieties at the N-terminal end of the haemoglobin (Hb) β-chain (1). HbA_{1c} concentrations are used as an index for long-term glycaemic control, and have recently been recommended for the diagnosis of diabetes mellitus (2-4). Various methods have been developed to measure HbA_{1c}, however, their accuracy can be adversely affected by the presence of haemoglobinopathies (5,6). In addition, HbA_{1c} provides an estimate of blood glucose concentrations over a normal erythrocyte lifespan, and any conditions altering erythrocyte survival (e.g., haemolytic anaemia, iron deficiency anaemia or haemoglobinopathies) may lead to misinterpretation of the HbA_{1c} result and hence misdiagnosis and mistreatment (7).

Thalassemia is the most common monogenetic disease caused by defects in the synthesis of one or more of the haemoglobin chains (8,9). Alpha (α-) thalassemia is caused by reduced or absent synthesis of α globin chains, including four main forms: α-thalassemia silent, α-thalassemia trait (minor), HbH (intermedia) and HbBart’s hydrops fetalis syndrome (major). Beta (β-) thalassemia is...
caused by reduced or absent synthesis of β globin chains, including three main forms: β-thalassemia trait (minor), β-thalassemia intermedia and β-thalassemia major. Several studies have suggested that HbA1c measurement may be affected by thalassemia, which is probably due to different methods used, and the genotypes of samples (10-16). In addition, the imbalance of globin chains in thalassemia can lead to haemolysis and impaired erythropoiesis. A decrease of erythrocyte lifespan has been reported in β-thalassemia (17). Therefore, it is useful to be able to screen for thalassemia while measuring HbA1c. Detection of thalassaemia is especially important for the identification of couples at risk of having a child with thalassemia major.

Recently, Sebia (Evry Cedex, France) introduced a capillary electrophoresis (CE) method for the determination of HbA1c using Capillarys 2 Flex Piercing (Capillaries 2FP). Our previous study indicated that HbA1c concentrations measured by Capillaries 2FP system in normal samples, without haemoglobinopathies, showed high correlation and concordance with those obtained using a boronate affinity high-performance liquid chromatography (HPLC) system, i.e. Premier Hb9210 (Trinity Biotech Plc, Bray, Ireland) (5). Further accuracy verification demonstrated a great consistency with International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference method (HPLC/CE) values (5). However, the effect of thalassemia on the CE method is unclear. The HbA1c programme on Capillaries 2FP could provide a rapid and reliable separation of HbA2, and HbA2 results are systemically lower compared to those obtained with the haemoglobin programme, with an average bias of 0.29% (10). HbA2 plays an important role in screening programme for thalassemia: its decrease might reveal α-thalassemia while its increase might indicate β-thalassemia (18). Therefore, in this study, we evaluated the effect of thalassemia on HbA1c measurement using Capillaries 2FP by comparison with the Premier Hb9210 and IFCC reference methods, and, furthermore, assessed the HbA1c programme on Capillaries 2FP for thalassemia screening.

Materials and methods

Subjects
The Research and Ethics committees of our institution approved this study. All subjects were informed on the study contents and provided written consents for participation. Blood samples were obtained from the Clinical Laboratory of the Second Affiliated Hospital of Guangzhou University of Chinese Medicine between June 2014 and August 2014. These samples included 498 healthy adults (normal controls) and 175 patients with thalassemia. The 175 thalassemia patients included 51 with α-thalassemia silent, 57 with α-thalassemia trait, 7 with HbH and 60 with β-thalassemia trait. Whole blood samples were collected in EDTA-containing tubes (2.0 mL, BD Franklin Lakes NJ, USA), divided into small aliquots and stored at -80°C before analysis as previously described (19,20).

Normal control samples were selected from subjects undergoing routine laboratory check-up examinations. Complete blood count (CBC) parameters were measured with BC-6800 (Mindray Medical Electronics Co., Shenzhen, China). Inclusion criteria were as follows: Hb concentrations > 130 g/L (in men) or > 115 g/L (in women), mean corpuscular volume (MCV) > 80 fL, HbF < 5% without Hb disorders (Hb phenotype analysis was performed by Bio-Rad Variant II system (Bio-Rad, Japan) using the beta thalassemia programme). Thalassemia samples were collected from routine laboratory test samples for thalassemia. Gap-polymerase chain reaction (PCR) was used to identify the α-thalassemia deletion mutations, and PCR reverse-blot hybridization was used to detect β-thalassemia point mutations (YANENG Bioscience Co. Ltd., Shenzhen, China) (21,22).

Method comparison
The HbA1c values of 98 thalassemia patients (29 α-thalassemia silent, 41 α-thalassemia traits, 7 HbH and 21 β-thalassemia traits), were quantified by Capillaries 2FP and Premier Hb9210 systems according to the manufacturers’ instructions. The analysers were calibrated once prior to any sample analysis. Because glycated and non-glycated haemoglobins are separated regardless of haemoglobin species,
thalassemia is unlikely to interfere with the IFCC reference method and boronate affinity chromatography method (23). The boronate affinity HPLC method Premier Hb9210 was used as the comparative method. For verification, eight thalassemia samples were sent to Shanghai Center for Clinical Laboratory and confirmed by the IFCC reference method HPLC/CE.

**Thalassemia screening**

A total of 498 normal controls and 168 thalassemia samples (51 α-thalassemia silent, 57 α-thalassemia traits, and 60 β-thalassemia traits) were analysed by Capillarys 2FP HbA1c programme.

**Statistical analysis**

For method comparison, the differences between two methods (Capillarys 2FP vs Premier Hb9210) were presented in a Bland-Altman plot. The relative bias was calculated by the Capillarys 2FP value against the comparative method (Premier Hb9210) value, and > ± 7% was considered clinically significant (National Glycohemoglobin Standardization Programme (NGSP) criterion) (24). For verification testing, the relative bias was calculated by the Capillarys 2FP value against the IFCC reference method value, and the proficiency testing acceptance limit of ± 6% provided by the College of American Pathologists (CAP) was set as the accuracy limit (25).

Measurement data of HbA2 were normally distributed and the independent sample t-test was performed to detect statistical difference between any two groups. Receiver operating characteristic curve (ROC) was applied to determine the best cut-off for screening α- and β-thalassemia. A P value of < 0.05 was considered statistically significant. Statistical analysis was performed using the MedCalc version 14.8.1 (MedCalc Software, Ostend, Belgium).

**Results**

**Method comparison**

Among the 98 thalassemia samples, HbA1c was not detected by Capillarys 2FP in three samples with HbH (Figure 1). For the remaining 95 thalassemias with detectable HbA1c, the Bland-Altman plot showed that the absolute bias of HbA1c was 0.00 ± 0.35% between Capillarys 2FP and Premier Hb9210 systems, and 3.4% of values were discordant (i.e., differences outside the range mean ± 2SD) (Figure 2). A significant positive bias above 7% was found only in two HbH samples.

To validate these results, the samples of eight thalassemias including two α-thalassemia silent, two α-thalassemia trait, two HbH and two β-thalassemia trait, were analysed by IFCC reference method HPLC/CE for HbA1c. The results showed that the HbA1c concentrations obtained by Capillarys 2FP were consistent with the results...
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obtained by IFCC HPLC/CE reference method, with relative bias below ±6% (Table 1).

Thalassemia screening

The HbA2 concentration in α-thalassaemia silent/trait (0.9 - 2.6%) was significantly lower than normal controls (1.7 - 2.8%) (P < 0.001, Figure 3A). ROC analysis showed an area under curve (AUC) of 0.81 (95% confidence interval, 0.78 - 0.84) and the optimal cut-off value was ≤ 2.2% with a sensitivity of 76.9% and specificity of 73.7% (Figure 3B).

The HbA2 concentration in β-thalassaemia trait (4.0 - 5.6%) was significantly higher than normal controls (1.7 - 2.8%) (P < 0.001, Figure 3A). ROC analysis demonstrated an AUC of 1.00 (95% confidence interval, 0.99 - 1.00) and the optimal cut-off value was > 2.8% with sensitivity 100% and specificity 100% (Figure 3C).

Discussion

Our results indicate that the presence of α-thalassaemia silent/trait and β-thalassemia trait had no significant effect on the accuracy of HbA1c measurement by Capillaries 2FP. Previous studies have also revealed agreement between Capillaries 2FP and Trinity Biotech Ultra2 (boronate affinity chromatography method) or Adams Arkray HA-8160 (ion-exchange HPLC method) systems for β-thalassemia samples (14,26). However, HbA1c concentrations in some HbH samples became immeasurable as HbH was separated as the first fraction and the HbBart’s fraction overlapped with HbA1c. Thus, Capillaries 2FP could provide valuable information (HbH and/or HbBart’s) for immeasurable samples. For some measurable HbH samples

![Figure 2. Bland-Altman plot showing the differences in haemoglobin A1c results between Capillaries 2 Flex Piercing (Capillaries 2FP) and Premier Hb9210 systems in thalassaemia samples (NGSP units, %). Solid line (mean) – mean difference. Dotted lines - 95% confidence interval of mean difference. Dashed lines (± 1.96 SD) - ± 1.96 standard deviation of mean difference.](image)

| Genotype         | IFCC reference method | Capillaries 2FP | Relative bias (%) |
|------------------|-----------------------|----------------|------------------|
|                  | IFCC units, mmol/mol | NGSP units, % | IFCC units, mmol/mol | NGSP units, % |                  |
| 1 -α3.7/aa       | 39                    | 5.7            | 41               | 5.9            | 3.5              |
| 2 -α4.2/aa       | 40                    | 5.8            | 42               | 6.0            | 3.4              |
| 3 --SEA/aa       | 30                    | 4.9            | 28               | 4.7            | -4.1             |
| 4 --SEA/aa       | 29                    | 4.8            | 29               | 4.8            | 0.0              |
| 5 -α3.7/-SEA     | 21                    | 4.1            | 23               | 4.3            | 4.9              |
| 6 -α2.7/-SEA     | 31                    | 5.0            | 32               | 5.1            | 2.0              |
| 7 CD41-42        | 31                    | 5.0            | 30               | 4.9            | -2.0             |
| 8 CD41-42        | 38                    | 5.6            | 40               | 5.8            | 3.6              |

HbA1c - haemoglobin A1c, IFCC - International Federation of Clinical Chemistry and Laboratory Medicine. Capillaries 2FP - Capillaries 2 Flex Piercing. NGSP - National Glycohemoglobin Standardization Programme. The relative bias was calculated in NGSP units, as the Capillaries 2FP value against the IFCC reference method value. The proficiency testing acceptance limit ±6% of CAP was set as the accuracy limit (25).
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The results were consistent with that by IFCC reference method. The limitations of our study are the insufficient number of HbH samples and IFCC reference method confirmed samples. Another limitation of our study is the lack of β-thalassemia intermedia/major samples. The β-thalassemia is characterized by variable increases in HbF and unusually high HbA2 concentrations. Our previous study found that an increased proportion of HbF could affect the HbA1c results using Capillarys 2FP (5). Thus, the β-thalassemia intermedia/major sample with highly elevated HbF (> 10%) could interfere the HbA1c measurement by Capillarys 2FP. Capillarys 2FP could report an accurate HbA1c value in β-thalassemia trait and provide valuable information (elevated HbA2 and/or HbF) for β-thalassemia intermedia/major. Further investigation is warranted using a larger number of thalassemia intermedia/major patient samples.

HbA2 is well separated and quantified by the HbA1c programme on Capillarys 2FP, while most other HbA1c methods are not able to separate HbA2 from HbA0. In the present study, we found the optimal HbA2 cut-off values for screening samples with α-thalassaemia silent/trait or β-thalassemia trait were ≤ 2.2% and > 2.8%, respectively, which was consistent with the previous report for β-thalassemia (10). In addition, abnormal bands could also provide valuable information for thalassemia screening (HbH and/or HbBart’s for HbH disease, HbF for β-thalassemia). The HbA2 concentrations obtained with the HbA1c programme needs to be corrected to obtain the real HbA2 concentrations, which warrants further investigation. Although the HbA2 concentration cannot be reported, laboratories must be cautious when thalassemia is suspected based on screening during HbA1c measurement and communicate with clinicians to note whether the patient is anaemic. Further confirmatory analysis for thalassemia and other indicators to diagnose diabetes are recommended.

In summary, our data, although limited, demonstrated that Capillarys 2FP HbA1c system could report accurate HbA1c results in thalassemia silent/trait, and HbA2 concentrations (≤ 2.2% for α-thalassaemia silent/trait and > 2.8% for β-thalassemia trait) and abnormal bands (HbH and/or HbBart’s for HbH disease, HbF for

Figure 3. The values of haemoglobin A2 (HbA2) were analyzed by Capillarys 2 Flex Piercing for normal controls and thalassaemia. (A) The box-plots show the frequency of HbA2 in normal control, α-thalassaemia silent/trait and β-thalassaemia trait. The horizontal line in each box represents the median values. The upper and lower limits of each box correspond to the 25th and 75th percentile values. The highest and lowest horizontal bars represent the minimum and maximum values. (B) For screening α-thalassaemia silent/trait, the receiver operating characteristic curve (ROC) shows an area under curve (AUC) of 0.80 (95% confidence interval, 0.78 - 0.84) and cutoff value ≤ 2.2% (sensitivity 76.9%, specificity 73.7%). (C) For screening β-thalassaemia trait, ROC shows AUC of 1.00 (95% confidence interval, 0.99 - 1.00) and cutoff value > 2.8% (sensitivity 100%, specificity 100%).
β-thalassemia) may provide valuable information for screening.

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**Potential conflict of interest**

None declared.

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