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BRIEF COMMUNICATION

HbA2 levels in normal, β-thalassaemia and haemoglobin E carriers by capillary electrophoresis

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Abstract

Objective: The capillary electrophoresis (CE) is a new system that utilizes the principle of electrokinetic separation of molecules in eight electrolyte buffer-filled silica capillaries. In this study, we established the normal ranges of haemoglobin A_2 (HbA_2) and haemoglobin F (HbF) levels for normal individuals using this system and also the HbA_2 level in β thalassaemia and haemoglobin E (HbE) individuals. *Materials and Methods*: 154 samples from normal individuals, 218 samples from β thalassaemia heterozygotes and 91 samples from HbE heterozygotes were subjected to high performance liquid chromatography (HPLC) and CE analysis. *Results:* The normal ranges for HbA_2 and HbF by CE were 2.75% (SD 0.26%) and 0.03% (SD 0.24%) respectively, which were significantly lower than that of HPLC 2.88% (SD 0.25%) and 0.58% (SD 0.61%) ($p < 0.001$). The HbA_2 level for HbE heterozygotes was 3.58% (SD 0.44%), which was significantly higher than normal ($p < 0.001$) but lower than that of β-thalassaemia heterozygotes ($p<0.001$) and the true HbE level was 24.28% (SD 3.38%). *Conclusion*: The CE system provided a fully automated and high throughput system for haemoglobin analysis. We established the normal ranges for HbA_2 and HbF levels by CE. We also determined the ranges for HbA_2 in beta thalassaemia and HbE heterozygotes using this system.

Keywords: capillary electrophoresis, HbA_2, β thalassaemia, haemoglobin E, normal range

INTRODUCTION

Thalassaemia is one of the common genetic abnormalities in Malaysia and 4.5% of Malaysians are carriers of β thalassaemia.[1] Rapid screening is vital to cater for the high number of cases. A combination of full blood count, haemoglobin electrophoresis and liquid chromatography have been the mainstay of tests used to make thalassaemia diagnosis. HPLC has long been used to make a presumptive diagnosis of β thalassaemia heterozygotes based on its ability to precisely quantify HbA_2 level. Recently, we acquired a relatively new technology of capillary electrophoresis (CE), to supplement the tests used for thalassaemia diagnosis in our Haematology Unit, Universiti Kebangsaan Malaysia Medical Centre (UKMMC).

The CE is a relatively new system that utilizes the principle of electrokinetic separation of molecules in electrolyte buffer-filled silica capillary. The narrow capillaries, with internal diameter of less than 100 um, filled with positively charged electrolyte buffer set at a very high voltage and tight temperature control are able to perform eight simultaneous analysis. This allows a fast turnover time as well as giving an excellent resolution and reproducibility. To date, the system has been utilized for various haemoglobinopathy detections. The most reported was its use in the neonatal sickle cell screening. The authors concluded that the system allowed a reliable, fast and fully automated analysis for neonatal sickle cell screening test, even on dried blood samples.[2,3]

The CE system has also been used to detect and

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quantify Hb Bart's in cord blood. In a report by Munkongdee *et al*. in 2010, quantification of Hb Bart's in cord blood was reported to be useful to diagnose alpha thalassaemia in newborns. The level of Hb Bart's also increased according to the numbers of the defective alpha globin genes. They also concluded that Hb Bart's levels of 0.2% by CE could be used as a cut-off point for alpha thalassaemia diagnosis in newborns.[4]

Liao *et al*. have also reported successful detection of the non-deletional, unstable Hb Constant Spring (Hb CS) by CE. Hb CS in its heterozygous state is difficult to detect due to very low levels and its unstable property, however the CE system efficiently detected all HbCS cases screened. Using this system, the authors then extended their studies to determine the prevalence of Hb CS to be 0.3% in the Guangdong province, South China.[5, 6]

In this study, we established the normal ranges of HbA_2 and HbF levels for normal individuals using this system. We also determined the HbA_2 level in β thalassaemia heterozygotes by CE and compared it with high performance liquid chromatography (HPLC). Lastly, we also established the ranges of HbA_2 and HbE levels in HbE heterozygous patients.

MATERIALS AND METHODS

Specimens

A total of 463 EDTA-anticoagulated routine blood samples from individuals aged above one-year-old, received by the Haematology unit, Department of Laboratory Diagnostic Services, UKMMC were analysed. One hundred and fifty-four samples were from normal individuals based on their normal haematology indices (haemoglobin >12g/dl, mean cell volume > 80fl and mean cell haemoglobin >26pg) and normal haemoglobin fractions as determined by high performance liquid chromatography (HPLC). Two hundred and eighteen samples were from β thalassaemia heterozygotes and the remaining ninety-one were HbE heterozygotes as demonstrated by their hypochromic microcytic red cell indices, normal or raised red cell count and normal or mildly raised red cell distribution width together with raised HbA_2 as determined by HPLC.[7]

High Performance Liquid Chromatography

This study utilized The Bio-Rad VARIANT (Bio-Rad, Hercules, CA) HPLC machine. Preparation of haemolysate was performed using 5µl EDTA blood sample mixed with one ml of haemolysing reagent which was left for five minutes. The haemolysate was then arranged in the instrument rack of the device, after which the subsequent processes were automated.

Capillary Electrophoresis (CE)

CE was performed using the Capillarys® (Sebia, Inc., Norcross, Ga). The blood sample was centrifuged at 5000 rpm for 5 minutes and the plasma was removed. The remaining erythrocyte pellet was vortexed for 5 seconds. The samples were placed on the instrument rack and the samples were automatically processed by the machine.

RESULTS

HbA_2 and HbF ranges in normal adult population

CE results showed that the normal ranges for HbA_2 and HbF were 2.75% (SD 0.26%) and 0.03% (SD 0.24%) respectively. These levels were significantly lower than that of HPLC 2.88% (SD 0.25%) and 0.58% (SD 0.61%) ($p < 0.001$ for both).

HbA_2 range in β thalassaemia

For β thalassaemia heterozygotes, the HbA_2 level by CE was slightly higher than that of HPLC (5.23% (SD_0.63%) vs. 5.14% (SD 0.55%), $p<0.001$).

HbA_2 and HbE ranges in HbE heterozygotes

The HbA_2 level for HbE heterozygotes was 3.58% (SD 0.44%), which was significantly higher than that of the normal range ($p < 0.001$) but lower than that of β thalassaemia heterozygotes ($p<0.001$). Peculiar just to CE, the HbE level in HbE heterozygotes was determined to be 24.28% (SD 3.38%).

DISCUSSION

The earliest determination of a raised HbA_2 for β thalassaemia carrier detection can be traced back to 1950s .[8] In the following years, various methods have been used to aid the detection of thalassaemia carrier status, with more refined and accurate levels being possible with the advent of improved technologies. The current most widely methods used to determine HbA_2 are cellulose acetate electrophoresis, high-performance liquid chromatography and isoelectric focusing (IEF).

More recently, a capillary electrophoresis technology that separates haemoglobin fractions

by negative-charged narrow diameter silica capillaries under high voltage was invented. The most notable advantage of this system is its ability for full automation, without the need to even prepare haemolysate from the whole blood samples. The device gives a very high throughput since seven to eight samples can be analysed simultaneously in a ten minute-run. The device is also able to separate all common normal and abnormal haemoglobin fractions in pre-determined calibrated zones at a high degree of confidence.[9] In terms of precision, the Capillarys® has been shown to have an acceptable coefficient of variance for the haemoglobins separations.[10]

The level of HbA_2 varies between different methods and also by pre-analytical variables. In high performance liquid chromatography (HPLC) the presence of haemoglobin variants such as HbS, HbE and Hb Lepore results in elevation of HbA_2 level because of co-elution. In this study, we found that the levels of HbA_2 by CE in a normal population were in the range of 2.25-3.25% with a mean of 2.75%. However, by HPLC, the range was 2.38-3.38, with a mean of 2.88%. This conferred the levels of HbA_2 by CE to be significantly lower range than that of HPLC. These findings were in agreement with a previous study by Higgins *et al*, who reported the upper limit of the HBA2 reference range to be 3.1% by CE as compared to 3.6% by HPLC.[11] Similarly, Van Delft also reported a slightly lower level of HbA_2 by CE compared to HPLC.[9]

Unlike HbA_2, an accurate determination of HbF level is less critical for the diagnosis of β thalassaemia carriers. HbF levels can either be normal or raised in β thalassaemia heterozygotes. However, determination of a raised level of HbF beyond the early infancy period is still required to suggest certain diagnoses such as δβ-thalassaemia and hereditary persistence of haemoglobin F (HPFH). The proportion of HbF in relation to other haemoglobin components could also help to determine thalassaemia phenotypes in compound heterozygous cases. Our study showed a significantly lower HbF level by CE than that of HPLC measured from the normal population. This could be due to the presence of HbA1c fractions that could overlap with HbF in HPLC analysis. Van Delft *et al*. have clearly shown that the levels of HbF with and without overlapping with HbA1c were different and this limitation was not seen with CE.[9] This was because the glycated HbA fractions were not separated from HbA in CE, thus circumventing the problem of overlap with other haemoglobins. However, Keren *et al*. in 2008 reported that normal HbF level by CE was identical to that of HPLC with a mean of 0.9% and SD of 5.6% and 5.4% respectively.[12] They, however, compared the CE result with Primus Resolution HPLC method, instead of VARIANT.

HbE is the most frequent haemoglobin variant especially in South East Asia with a reported prevalence of 7-30% in Malaysia.[13] An interesting property unique to CE is its ability to separate HbE from HbA_2, a feature that was not seen in many of the widely used methods before. HbE migrates with HbC, HbO and HbA_2 in alkaline electrophoresis and HPLC. Even at acid electrophoresis, HbE is still inseparable from HbA_2. Although this seldom causes any diagnostic problem, the ability to separate HbE from HbA_2 does give additional information such as the actual levels of HbA_2 and HbE in HbE heterozygous and homozygous cases. Mais *et al*. 2009 specifically measured the range of HbA_2 in 52 samples from HbE heterozygotes in Michigan, USA. They found the HbA_2 level in HbE heterozygotes was found to be $3.4\pm0.4\%$, a significantly higher than $2.6\%\pm0.4\%$ of the control group.[14] We also found that a raised level of HbA_2 of 3.58% (SD 0.44%) in HbE heterozygous, as compared to 2.75% (SD 0.25%) of the normal population. The raised HbA_2 level is expected since HbE is formed from a β globin chain variant (β^E) which is synthesized at a slower rate than the normal β^A chain producing a mild thalassaemic effect and a compensatory increased δ globin chain synthesis, hence the raised HbA_2 level.

This study also observed that the HbE level was 24.28% (SD 3.38%) by CE which was much lower than that of HPLC. This finding was anticipated because by HPLC, the HbE and HbA_2 coeluted at the same retention time, while CE measured the actual level of HbE in the sample.

CONCLUSION

The capillary electrophoresis system is a reliable and fast technique for thalassaemia diagnosis. This study determined the normal upper levels of HbA_2 and HbF for this device in our laboratory to help with thalassaemia diagnosis. Furthermore, the HBA_2 levels by capillary electrophoresis for HbE and β thalassaemia heterozygotes were also determined. Lastly, unique to the CE system, the actual HbE level was also able to be ascertained.

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