CASE STUDY

Haemoglobin Reading [α48Leu>Pro; HBA2: c.146T>C]: a comparison of different HbA1c methods in its detection

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ABSTRACT

We report a second occurrence of haemoglobin (Hb) Reading [α48Leu>Pro; *HBA2*: c.146T>C], and like the initial case, this was associated with normal haematological parameters. Mass spectrometry showed the variant α-chain was expressed at 21% of the total α- chain material and this was consistent with normal isopropanol stability. The Hb variant was picked up as an interfering component on HbA1c testing using cation-exchange high performance liquid chromatography (HPLC). Variable results returned from an HbA1c quality control survey highlights the difficulty of detecting Hb Reading, demonstrating how this haemoglobin variant may be under reported.

Key words: Haemoglobin Reading, haemoglobin variant, HPLC, capillary electrophoresis, HbA1c.

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INTRODUCTION

There has only been one previous report of Hb Reading, where it was listed as a personal communication on the Hb variant server (1). It was associated with normal blood count parameters but of unknown pathogenicity in a British subject. In this paper we report a second case of Hb Reading.

Glycated haemoglobin A1c (HbA1c) is widely used to monitor long-term glycaemic control in diabetic patients. HbA1c measurements can be performed using different laboratory analysers. There are several methods of HbA1c measurement which can be divided into two major categories; assays based on molecular charge (HPLC, electrophoresis) or assays based on molecular structure or mass (boronate affinity chromatography, immunoassay) (2). The presence of Hb variants are known to cause interference in HbA1c measurement and can result in aberrant HbA1c values. In general, detection and interference depends upon HbA1c method of assay, and the type of Hb variant (3).

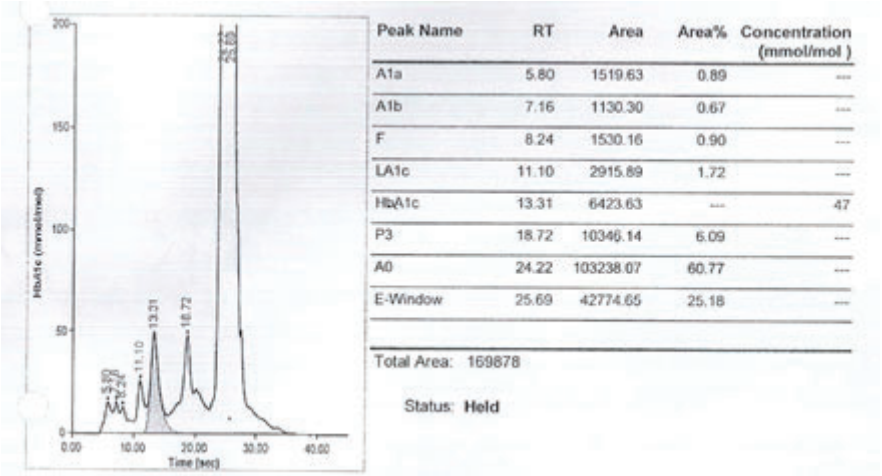
This Hb Reading specimen was sent to laboratories across New Zealand for HbA1c analysis as part of an HbA1c quality control (QC) survey. The aim was twofold: firstly, to see if the Hb variant affected the HbA1c assay with aberrant values, and secondly to compare which HbA1c method/s detected Hb Reading.

CASE STUDY

The subject of this investigation was a 63 year old NZ Maori male. He was at risk of diabetes thus had recurrent HbA1c's with values over previous years ranging between 40 and 47 mmol/mol (reference range <40). Prior to this he had serial fasting glucose measurements which varied between 4.7 and 5.9 mmol/L (normal fasting reference range 3.5-5.4mmol/L). His full blood count was unremarkable: haemoglobin 152 g/L, RBC 5.31 x 10^{12}/L, MCV 89 fl, MCH 29 pg, RDW 12.7%, WBC 8.8 x 10^{9}/L and platelets 262 x 10^{9}/L.

Cation-exchange HPLC for HbA1c testing was undertaken using a Bio-Rad D100 system (Hercules, California, USA) where the Hb variant was picked up as an interfering component. Even though this subject had previous HbA1c tests, this was the first occasion in which an interfering component was detected in his HbA1c testing. Initially, Hb Reading appeared as an abnormal peak of 25.18% eluting in the Hb 'E' window (25.56-27.36) at retention time (RT) 25.69 seconds on the Bio-Rad D100 chromatogram (Figure 1).

A new sample was requested and a week later an abnormal peak of 24.78% eluted in the 'unknown' window (25.33-25.55) at RT 25.49 seconds on the same analyser. The HbA1c values from the two samples were similar at 47 and 46 mmol/mol respectively. The HbA1c (IFCC) result confirmed by affinity method was similar at 45 mmol/mol.



Peak Name	RT	Area	Area%	Concentration (mmol/mol)
A1a	5.80	1519.63	0.89	---
A1b	7.16	1130.30	0.67	---
F	8.24	1530.16	0.90	---
LA1c	11.10	2915.89	1.72	---
HbA1c	13.31	6423.63	---	47
P3	18.72	10346.14	6.09	---
A0	24.22	103238.07	60.77	---
E-Window	25.69	42774.65	25.18	---

Total Area: 169878

Status: **Held**

Figure 1. HbA1c chromatogram with High Performance Liquid Chromatography on a Bio-Rad D100 showing the haemoglobin variant eluting in the 'E'-window.

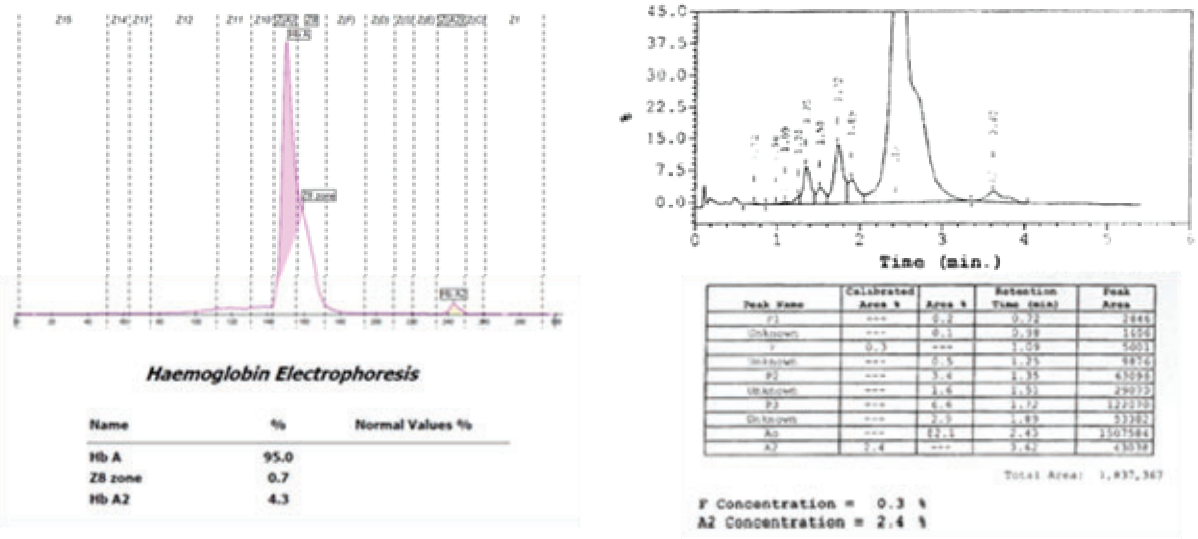


Figure 2. Capillary electrophoresis (A) and Bio-Rad variant II (B) profiles showing the aberrant peak eluting on the downward shoulder of HbA_0 peak.

Capillary electrophoresis was undertaken using a Sebia Capillarys 2 Flex Piercing analyser (Sebia, France). Using the Hb E programme, a slightly elevated HbA_2 of 4.3% (2.2-3.3) and a normal Hb F <1% (<1%) were present. The Hb variant showed as a descending shoulder on the Hb A_0 peak (Figure 2A). Subsequent analysis on a Bio-Rad variant II with a β-Thalassaemia column showed a very obvious shoulder in the descending part of the HbA_0 peak (Figure 2B). The Hb variant did not separate by Hb electrophoresis on cellulose acetate at pH 8.4 and was shown to be stable as the isopropanol flocculation stability test was normal.

Examination of lysate by electrospray mass spectrometry on an Agilent 6230 time-of-flight instrument (4) (Agilent Technologies, Santa Clara, CA,USA) showed a variant a chain with a mass decrease of 16 Da that represented 21% of the total a globin (Figure 3). Further tryptic peptide mapping indicated that the 16 Da decrease in mass was located in peptide α6 (^{41}TYFPHFDLSHGSAQVK^{56}), suggesting possible point mutations of either a42Tyr→Phe, a48Leu→Pro, a49Ser→Ala, or a52Ser→Ala.

DNA sequencing of the coding and non-coding regions of the alpha 1 and 2 globin genes was performed by PCR-based automated fluorescent sequencing (4). Sequencing of the α2 gene showed the presence of a single heterozygous *HBA2*: c.146T>C mutation predicting an α48Leucine→Proline substitution. This mutation (Hb Reading) has been reported only once before as a personal communication (1).

The HbA1c QC survey was processed by 18 laboratories, using a total of 21 analysers comprising of four different HbA1c methods. The methods included: two ion-exchange HPLC methods (D100 (nine) and Variant II Turbo HPLC (one) [Bio-Rad Laboratories, Hercules, California, USA]), two capillary electrophoresis methods (Capillarys 2 Flex Piercing (one), Capillarys 3 Flex Piercing (one) [Sebia, Lisse, France]), one Boronate affinity chromatography method (Primus Premier Hb9210 [Trinity Biotech]) and three immunoassay methods (Cobas 6000 (five), Cobas 8000 (one) [Roche Diagnostics, Indianapolis, IN]), DCA vantage (one) and Dimension Vista 1500 (one) [Siemens Healthineers USA].

The HbA1c results from the QC survey were remarkably consistent between the four different methods employed. The HbA1c values ranged from 39 to 46 mmol/mol (Table 1) with a mean (± SD) of 43 (± 2) mmol/mol. In contrast, from this QC survey data, six out of the 21 analysers detected the aberrant Hb variant. Of the nine Bio-Rad D100's, five detected an aberrant peak, and four of these eluted in the 'E' window. The fifth D100 and the Bio-Rad variant turbo II detected the Hb variant eluting in the 'unknown' window (Table 1).

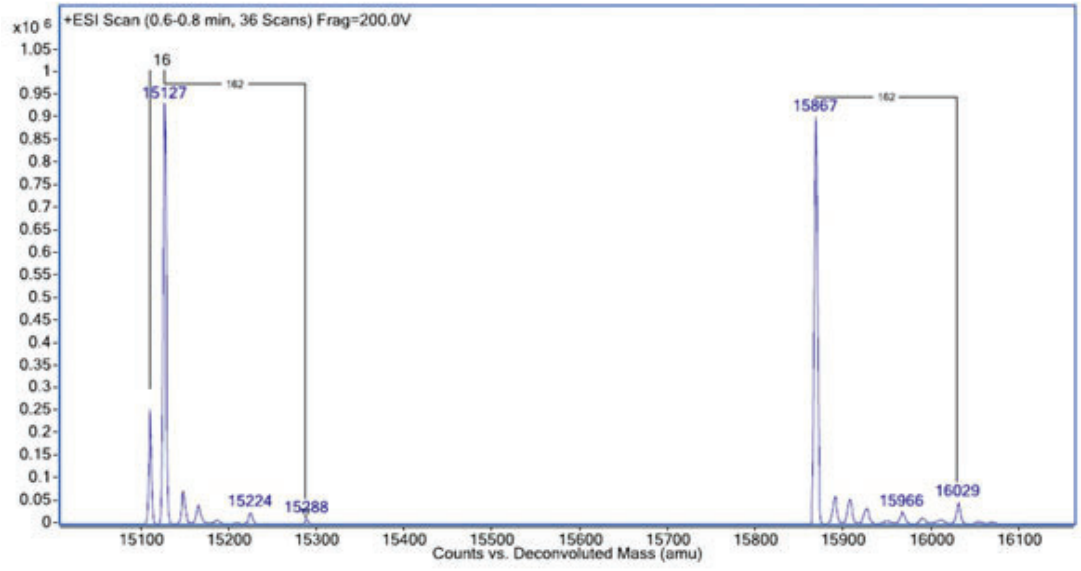


Figure 3. Transformed electrospray mass spectrum of whole lysate from heterozygous carrier of Hb Reading showing normal α and β chains at 15,127 and 15, 867 Da. The new variant a chain at 15,111 Da has a 16 Da decrease in mass and represents 21% of the total a chain material.

Table 1. Summary of HbA1c results and aberrant peak detection on the 21 analysers from the QC HbA1c survey.

HbA1c analyser	Number of analysers	HbA1c results Normal <40 mmol/mol	Hb Reading detected as an aberrant peak	Eluting window of aberrant peak
Bio-Rad D100	9	40,41,43,44,44,45,45,45,46	5	4 in 'E' 1 'unknown'
Bio-Rad Variant II Turbo	1	45	1	1 'unknown'
Sebia Capillarys	2	43,45	0	
Trinity Biotech Primus Hb 9210	1	45	0	
Roche Cobas	6	39,40,42,42,43,44	0	
Siemens DCA Vantage	1	41	0	
Siemens Dimension Vista 1500	1	43	0	

DISCUSSION

Similar to the first documented case of Hb Reading, the present case had no obvious haematological abnormality, a normal full blood count, and normal Hb electrophoresis. Like the first case, this subject also showed the Hb variant as a shoulder on the chromatogram in the descending part of HbA_0. This subject was of NZ Maori descent, unlike the first case that was of British ethnicity. This case verifies that the Hb Reading mutation comprising 21% was on the more highly expressed α2 gene. This cannot be compared to the first case since there was no DNA testing or percentage of variant reported.

There are no previous reports as to whether Hb Reading interferes with HbA1c values, or as to which method of HbA1c (assays based on molecular charge or assays based on molecular structure or mass) can detect the variant. With these aims in mind, the sample containing Hb Reading was sent to 18 laboratories for HbA1c analysis as part of an HbA1c QC survey. Of the 21 analysers across the 18 laboratories, three used assays based on molecular charge: Bio-Rad D100 (HPLC), Bio-Rad Variant II turbo (HPLC) and Sebia Capillary Electrophoresis (Electrophoresis). The other four analysers used assays based on molecular structure or mass: Trinity Biotech Primus Premier Hb9210 (boronate affinity), Roche Cobas, Siemens DCA vantage (immunoassay) and Siemens Dimension Vista 1500 (immunoassay).

Ion-exchange HPLC separates Hb species based on charge differences between HbA1c and other haemoglobins, capillary electrophoresis separates Hb by their electrophoretic mobility according to pH and electro-osmotic flow, boronate affinity assays measure total glycated Hb and immunoassay methods detect structural changes in the N terminal amino acids of Hb using antibodies against the glucose binding sites of β globin chain (5).

Previous studies have documented that the common variants Hb S, Hb C, Hb E, Hb D, as well as other less common or rare variants, can interfere with some HbA1c assay methods (6). It is difficult to study all possible variant haemoglobins with all methods but it is useful to know how the different methods perform with most variants and whether or not the presence of the variant can be detected. In general, Hb variant interference has been shown to be method dependent. If the amino acid substitution causes a change in the net charge of the Hb (as with Haemoglobins S, C, D and E), then it may cause interference with methods such as ion-exchange HPLC or capillary electrophoresis. If there is a substitution at a glycation site, this could alter the rate of glycation and affect certain methods, such as boronate affinity or immunoassays (5).

In this QC study data, there was no significant effect of the method on HbA1c values (Anova test, p=0.09). These findings suggest that Hb Reading does not affect HbA1c values.

However, this is difficult to state categorically without further testing. In contrast, there was significant variation in regards to detection of Hb Reading by the four different HbA1c methods. Previous studies have documented that overall, charge based separation methods have the highest detection rate of Hb variants and derivatives, while assays based on molecular structure or mass have the least detection rate (6). The findings of this QC survey showed similar results. Hb Reading was recognized by two of the assays based on molecular charge, i.e. HPLC (Bio-Rad D100 and Bio-Rad Variant II turbo), through the presence of an abnormal peak, albeit in just over half of the HPLC analysers (6/10). Although the window was different where Hb Reading eluted in, either the 'E' or 'unknown', it was still detected as an aberrant peak. However, this differed in regards to the capillary electrophoresis analysers as none detected the Hb variant. Similar to other studies, all assays based on molecular structure or mass, i.e. Trinity Primus, Roche Cobas, Siemens DCA vantage and Siemens Dimension Vista, did not detect an aberrant Hb. Obviously, detection of this Hb variant by HbA1c analysers is not certain, but is more likely with HPLC.

CONCLUSIONS

The Hb Reading α48Leu>Pro mutation appears to be benign as it is associated with normal red cell indices and normal stability, and the variant α chain is expressed at levels expected for the α2 gene. As shown in this report, Hb Reading does not appear to affect the HbA1c assay, so no apparent difference in HbA1c measurements should be expected. This report highlights the difficulties of detecting Hb Reading with conventional HbA1c methodology. In view of this, the presence of Hb Reading in other subjects is likely to be unrecognised and therefore under reported. However, the detection of Hb variants, especially those affecting HbA1c results are important, given the critical role of HbA1c in the diagnosis and management of diabetes.

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