Hb Q-India: an uncommon variant diagnosed in three Punjabi patients with diabetes is identified by a novel DNA analysis test

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Aims: An abnormality in the glycated haemoglobin peak (Hb A1c) on Diastat (Bio-Rad) cation exchange low pressure liquid chromatography (LPLC) was found in three Punjabi patients with diabetes. The aims of this study were to identify the variant by chromatography and electrophoresis and to determine whether a DNA analysis test could be designed for confirmation that could be generally applied for the identification of any unusual abnormal haemoglobin.

Methods: The presence of an Hb variant was confirmed by cellulose acetate electrophoresis at pH 8.6. The variant was characterised further by high performance liquid chromatography (HPLC; Bio-Rad Variant) and isoelectric focusing (IEF) electrophoresis. A novel DNA analysis test based on the amplification refractory mutation system (ARMS) and the polymerase chain reaction (PCR) was developed to confirm the presence of the mutation for the uncommon variant.

Results: Comparison of the HPLC retention time and IEF band position determined the presence of the variant Hb Q-India in all three cases. Hb Q-India is caused by the mutation CAC → CAC at codon 64 of the α-1 globin gene and is clinically silent. ARMS-PCR specific primers were designed and used successfully to confirm the presence of the mutation for Hb Q-India.

Conclusions: The results show that the ARMS-PCR technique, developed previously for the diagnosis of βthalassaemia mutations, can also be adapted to provide a simple, rapid, and inexpensive approach for the identification of abnormal haemoglobins.

Materials and Methods

The estimation of glycated haemoglobin is carried out on large numbers of patients with diabetes in our teaching hospital in Punjab, North India. The estimation is performed on an HPLC Diastat instrument according to the manufacturer’s instructions (Bio-Rad, Hemel Hempstead, UK). A printout gives the retention time of the various fractions and also measures each fraction as a percentage of the whole of the major peaks, including glycosylated haemoglobin. Some variants such as Hb S and Hb D are also revealed as abnormal bands. Where such bands are shown, cellulose acetate electrophoresis using barbitone buffer at pH 8.6 is carried out.

Samples with unusual findings were sent to the national haemoglobinopathy reference laboratory, institute of molecular medicine, John Radcliffe Hospital, Oxford, UK for further investigation of the abnormal Hb. Analysis there was carried out using HPLC (Bio-Rad Variant) and isoelectric focusing (IEF). The HPLC retention time for the abnormal peak was determined, together with the IEF position of the abnormal band in relation to the Hb A band. A comparison was then made of the retention times and IEF positions of the variants with those of known variants previously identified by DNA sequence analysis.

Abbreviations: A1c, glycated; ARMS, amplification refractory mutation system; CMC&H, Christian Medical College and Hospital; Hb, haemoglobin; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LPLC, low performance liquid chromatography; PCR, polymerase chain reaction.
One of the three samples included in our study was examined further by DNA analysis for the presence of the Hb Q-India mutation using a novel diagnostic test based on the amplification refractory mutation system (ARMS). A mutation specific primer was designed to amplify only DNA containing the mutation for Hb Q-India in the α-1 globin gene. A mismatch was designed at the 3′ terminus of the primer to target the G → C mutation of Hb Q-India. PCR was carried out in a 25 µl reaction containing 0.75M betaine, 5% DMSO, 200 µM of each dNTP, 0.9mM MgCl₂, 750mM Tris/HCl at pH 9.0, 200mM (NH₄)₂SO₄, 0.1% (wt/vol) Tween 20, 0.9µM genomic DNA, 0.2 µM of each primer, 0.5 U Platinum Taq (Invitrogen, Paisley, UK). The PCR conditions were four minutes at 95°C for the initial denaturation step, then 30 cycles of denaturation for one minute at 95°C, annealing for one minute at 60°C, and extension for one minute at 72°C. After a final extension period of five minutes, the products were examined by ethidium bromide staining and agarose gel electrophoresis.

Routine haematological examinations were carried out by standard methods.

RESULTS
Case 1
A 55 year old man, recently diagnosed with diabetes mellitus and on diet control, presented in the diabetic clinic (Christian Medical College and Hospital, Ludhiana (CMC&H), India) for a routine evaluation. Blood was drawn for glycated haemoglobin (Hb A1c) estimation. The Hb A1c assay was carried out on a Diastat machine. The results showed an abnormal haemoglobin variant peak with a retention time of 4.42 minutes.

Case 2
A 55 year woman, a known diabetic and hypertensive for 10 years, presented in the medical outpatients department (CMC&H) with complaints of faecal incontinence for the past eight months. A general physical examination was normal. A routine glycated haemoglobin assay was done. The assay was carried out on the Diastat machine, which showed an abnormal haemoglobin variant peak with a retention time of 4.42 minutes.

Case 3
A 42 year old woman, a known diabetic and hypertensive for the past five years, presented in the diabetic clinic (CMC&H) for a routine checkup. A general physical examination revealed mild pallor but no icterus or cyanosis. A routine anaemia investigation was performed, which included haemoglobin electrophoresis. Haemoglobin electrophoresis run on...
The low red blood cell indices seen in case 1 result from carrying Hb Q-India (fig 3). The DNA from case 1 gave a positive signal with the Hb Q-India specific ARMS primer, confirming that the patient ing. The DNA sequencing studies have shown the Hb Q-India mutation to be AAG → GAG in codon 64 of the α gene. This variant haemoglobin occurs normally in the heterozygous form and is not associated with a thalassaemia phenotype. In case 1, the patient was also found to have co-inherited α-thalassaemia by gap PCR studies (hence the increased expression of Hb Q-India seen in this case), whereas in case 3 the patient was found to have a normal α genotype and the low mean cell volume and mean cell haemoglobin values resulted from iron deficiency. Furthermore, it is probable that this variant does not cause haematological disorders because the residue involved, α64 (E 13), is on the surface of the haemoglobin tetramer and charge changes at these positions do not affect the properties of the haemoglobin molecule.

Identification of the Hb variant

Samples from the three patients were subjected to HPLC using the Bio-Rad Variant analyser. All three showed an identical variant peak with a retention time of 4.46 minutes.

Table 1: Results of HPLC (Bio-Rad variant) on the 3 cases

<table>
<thead>
<tr>
<th>Case</th>
<th>%Hb A2</th>
<th>%Hb F</th>
<th>%Hb X</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>1.3±0.5</td>
<td>0.5</td>
<td>23.6</td>
<td>4.76</td>
</tr>
<tr>
<td>Case 2</td>
<td>1.2±0.5</td>
<td>1.7</td>
<td>14.2</td>
<td>4.78</td>
</tr>
<tr>
<td>Case 3</td>
<td>1.0±1.2</td>
<td>1.2</td>
<td>15.4</td>
<td>4.76</td>
</tr>
</tbody>
</table>

Hb, haemoglobin; HPLC, high performance liquid chromatography.

Identification of the Hb variant by screening is carried out on those found to have an abnormal band and further steps are taken to identify the variant.

A small number of Hb variants can be characterised by comparing their HPLC retention times with reference chromatograms in a library provided by the manufacturer, and also by comparing their IEF positions with those on a published chart of normal Hb variants. In general, HPLC and IEF provide reliable and reproducible data, enabling the retention time and IEF position to be used to identify variants indirectly. However, several variants are known to have identical retention times and the same applies to IEF positions. Thus, the data are limited in suggesting a candidate variant when only one technique is used. A more accurate identification is obtained when both HPLC and IEF are performed and the HPLC retention time and the IEF position match the data of a known variant, as happened in this case with Hb Q-India. However, the number of variants for which there is both a known HPLC retention time and a published IEF position is very small, and thus identification of most of the uncommon variants by this method is not possible.

In routine glycosylated haemoglobin Diastat analysis, the presence of Hb Q-India could lead to a patient being falsely designated as having the clinically relevant variant Hb S.

The definitive method for the identification of Hb variants is characterisation by DNA sequencing of the α globin and

<table>
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<th>Table 2: Blood count data</th>
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<tr>
<td>Age</td>
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<tr>
<td>-----</td>
</tr>
<tr>
<td>Case 1</td>
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<tr>
<td>Case 2</td>
</tr>
</tbody>
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Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; RBC, red blood cell count.
β-globin genes. This is an expensive technique and not practical for the routine identification of uncommon variants. Because most α-chain and β-chain variants result from a single point mutation, a simple, rapid, and inexpensive method of diagnosing point mutations is required for the definitive characterisation of the uncommon haemoglobin variants. Such a method has been developed for the routine diagnosis of β-thalassaemia genes in Asian Indians by ARMS-PCR, and this technique has since been applied successfully in developing countries to screen for β-thalassaemia mutations in other ethnic groups, using a panel of primers specific to the local spectrum of mutations. We have now adapted this technique to enable the quick identification of the α-chain variant Hb Q-India and the successful result presented here demonstrates that in principle the technique can be used to identify any uncommon variant of the α-globin or β-globin genes for which the mutation is known. For the many variants that have not had their causative point mutation confirmed by DNA sequence analysis, an ARMS primer would have to be designed to detect the presumed mutation predicted by the genetic code and the amino acid change. However, all new Hb variants are now characterised by DNA sequence analysis, and the number of variants having their mutation confirmed by DNA sequencing is growing, enabling a panel of ARMS primers to be developed that would be specific to the local spectrum of Hb variants. Thus, the identification of variants by a simple mutation detection test by ARMS-PCR following preliminary characterisation by HPLC or electrophoresis is a feasible approach, and the results presented here prove that this technique can form the basis of a new routine diagnostic strategy for the uncommon abnormal haemoglobins.

Take home messages
- The amplification refractory mutation system polymerase chain reaction (ARMS-PCR) technique, which was developed for the diagnosis of β-thalassaemia mutations, can be adapted for the identification of abnormal haemoglobins.
- The ARMS-PCR technique provides a simple, rapid, and inexpensive approach for the identification of abnormal haemoglobins, which would be suitable for use in the developing world.

REFERENCES