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Hemoglobin variants uncovered during routine HbA1c testing: Diagnostic pitfalls and clinical significance

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Abstract

Background: Hemoglobin A1c (HbA1c) is a standard marker for long-term glycemic control in diabetes mellitus. However, the incidental detection of hemoglobin variants during HbA1c testing poses diagnostic challenges, especially in multiethnic populations with high prevalence of hemoglobinopathies.

Objective: This study aimed to determine the incidence, types, and clinical implications of hemoglobin variants identified during routine HbA1c testing using ion-exchange high-performance liquid chromatography (HPLC) in a tertiary care center in South India.

Methods: A prospective observational study was conducted over 24 months, analyzing 10,200 unique HbA1c samples. Chromatograms were reviewed for abnormal peaks; samples with unidentified peaks exceeding 6% were further evaluated using the extended HbA2/F mode. Variants were categorized based on retention times. HbA1c values were reported only if variant interference was excluded.

Results: Hemoglobin variants were detected in 85 cases (0.83%). The most frequent variants were HbD heterozygous (27.1%), HbE heterozygous (25.9%), and HbS heterozygous (22.4%). A male predominance was observed across most variants. HbD heterozygous showed the widest HbA1c range (4.5%-13.8%). High HbF was identified in 12.9% of variant cases. No beta-thalassemia trait cases were detected due to method limitations.

Conclusion: The detection of hemoglobin variants during HbA1c testing highlights the need for chromatogram review to avoid misinterpretation of glycemic status. Identifying such variants provides an opportunity for earlier diagnosis of hemoglobinopathies and genetic counseling. Laboratories should establish protocols for variant detection and reporting to ensure accurate clinical decision-making.

Keywords: HbA1c, hemoglobin variants, ion-exchange HPLC, diabetes mellitus, hemoglobinopathies, chromatogram interpretation, glycemic control, India

Introduction

Hemoglobin A1c (HbA1c) serves as a reliable indicator of average plasma glucose levels over the preceding 8 to 12 weeks. An HbA1c threshold of 6.5% or higher is diagnostic for diabetes mellitus (DM), as recommended by international guidelines ^[1]. The National Glycohemoglobin Standardization Program (NGSP) has validated several methodologies for HbA1c measurement, including iso-electric focusing, ion-exchange high-performance liquid chromatography (HPLC), boronate-affinity chromatography, immunoassay, enzymatic assay, and capillary electrophoresis ^[2]. Among these, ion-exchange HPLC and capillary electrophoresis offer the advantage of generating chromatograms with distinct hemoglobin fraction peaks based on retention times. This graphical output allows not only HbA1c quantification but also the incidental detection of hemoglobin variants, which may have significant clinical implications.

Many hemoglobinopathies are geographically linked, with higher prevalence in Southeast Asia, the Indian subcontinent, the Mediterranean, and parts of Africa. In India, hemoglobin variants such as HbE, HbS, HbD, and high HbF are frequently encountered, especially in specific ethnic groups and regions ^[15, 16]. Traditionally, these disorders are detected through targeted screening using hematological indices, HPLC or electrophoresis, and confirmed by molecular methods. However, with the increasing use of automated HPLC systems for HbA1c measurement in diabetes care, hemoglobin variants may be incidentally uncovered in patients undergoing routine monitoring ^[5, 6].

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Detecting these variants during HbA1c testing provides an opportunity for earlier diagnosis, which could otherwise be missed in asymptomatic carriers.

The incidental detection of hemoglobin variants during HbA1c testing poses both diagnostic and clinical challenges. Certain variants may interfere with HbA1c quantification, leading to falsely low or high values depending on the analytical method used [8]. Misinterpretation of such results can affect diabetes diagnosis and management. Therefore, laboratories must carefully review chromatograms and be equipped to identify variant patterns. This awareness is especially important in multiethnic populations and regions with evolving epidemiology of hemoglobinopathies due to migration, where unexpected variants may emerge outside their traditional geographic zones [9-11]. Detection of such variants during routine HbA1c testing presents an opportunity for early diagnosis of hemoglobinopathies, particularly in regions with high prevalence of genetic hemoglobin disorders. We conducted a study to determine the incidence and distribution of hemoglobin variants detected during routine HbA1c testing in a tertiary care hospital in South India, and to classify these variants using chromatographic parameters.

Aim

To determine the incidence and spectrum of hemoglobin variants detected incidentally during routine HbA1c testing using ion-exchange HPLC in a tertiary care center in South India.

Objectives

1. To classify hemoglobin variants based on chromatographic retention times and peak patterns observed during HbA1c testing.
2. To evaluate the potential impact of hemoglobin variants on the reliability of HbA1c measurement and its implications for clinical management.

Methods

We performed a prospective observational study analyzing chromatograms from all HbA1c tests conducted over a continuous 24-months period (July 2018-June 2020) at a Department of Biochemistry and Department of General Medicine, in a tertiary care center in South India. The study included 10,200 unique HbA1c samples, excluding follow-up and duplicate samples from previously tested individuals. HbA1c quantification was carried out using ion-exchange HPLC on the Bio-Rad D10 dual HbA2/F/A1c platform (Bio-Rad Laboratories Inc., California, USA), which has a reportable HbA1c range of 3.7-18.4%. This analyzer offers two operational modes:

- A short program (3-minute HbA1c mode) for routine HbA1c measurement.
- An extended program (6.5-minute HbA2/F mode) for quantifying HbA2 and HbF and detecting hemoglobin variants.

All HbA1c chromatograms obtained from the 3-minute runs were prospectively reviewed. Samples displaying unidentified peaks exceeding 6% of the total area were subsequently reanalyzed in the extended 6.5-minute mode to classify the hemoglobin variant, based on retention time and peak characteristics. Variant identification was cross-referenced with known retention times from the manufacturer's guidelines.

For patients with detected variants, HbA1c results were reported only after confirming that the variant did not interfere with HbA1c quantification. As per manufacturer's instructions, HbA1c values were not reported in cases with HbF or P3 peaks exceeding 10%, or in homozygous or compound heterozygous variants.

Additionally, we compared HbA1c values obtained from the short (HbA1c) and extended (HbA2/F) modes to assess analytical agreement. This comparison was evaluated using intra-class correlation coefficient (ICC).

Results

Table 1: Hemoglobin Variants Detected in Patients Tested for HbA1c

Hemoglobin Variant	No. of Variants (n = 85)	% Among Variants	% Among Total HbA1c Runs
HbD heterozygous	23	27.1%	0.23%
HbE heterozygous	22	25.9%	0.22%
HbS heterozygous	19	22.4%	0.19%
High HbF	11	12.9%	0.11%
HbE homozygous	5	5.9%	0.05%
HbQ heterozygous	2	2.4%	0.02%
HbJ heterozygous	2	2.4%	0.02%
Compound heterozygous for HbD-β thalassemia	1	1.2%	0.01%

Table 2: Gender Distribution Across Hemoglobin Variants

Hemoglobin Variant	Male (n = 48)	Female (n = 37)	% Male	% Female
HbD heterozygous	14	9	60.9%	39.1%
HbE heterozygous	12	10	54.5%	45.5%
HbS heterozygous	11	8	57.9%	42.1%
High HbF	6	5	54.5%	45.5%
HbE homozygous	3	2	60.0%	40.0%
HbQ heterozygous	1	1	50.0%	50.0%
HbJ heterozygous	1	1	50.0%	50.0%
Compound heterozygous for HbD-β thalassemia	0	1	0%	100%

Table 3: Age Distribution by Hemoglobin Variant

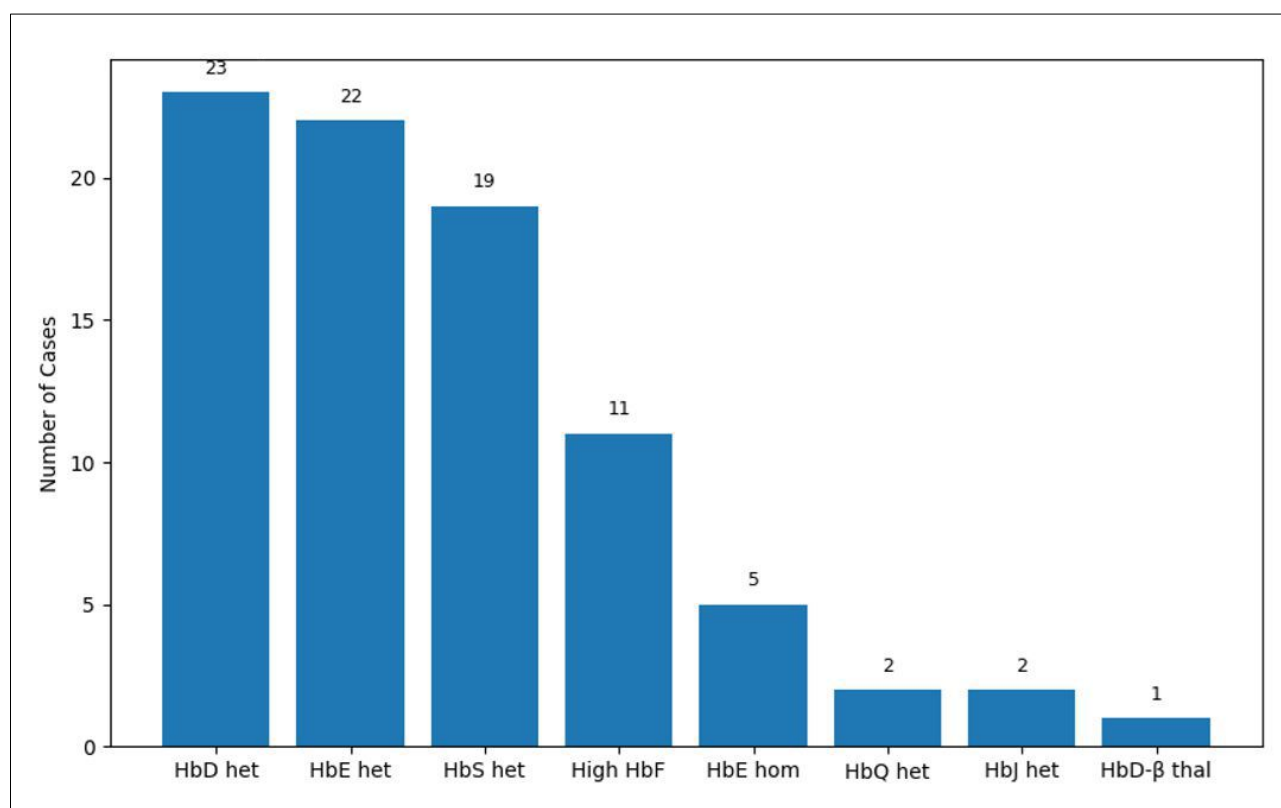
Hemoglobin Variant	Min Age (years)	Max Age (years)	Mean Age (years)
HbD heterozygous	30	74	52.5
HbE heterozygous	32	70	50.8
HbS heterozygous	28	65	48.3
High HbF	29	68	49.9
HbE homozygous	34	60	46.2
HbQ heterozygous	38	55	46.5
HbJ heterozygous	40	58	49.0
Compound heterozygous for HbD- β thalassemia	35	35	35.0

Table 4: HbA1c Range Across Hemoglobin Variants

Hemoglobin Variant	Min HbA1c (%)	Max HbA1c (%)	Mean HbA1c (%)
HbD heterozygous	4.5	13.8	7.8
HbE heterozygous	4.7	12.5	7.4
HbS heterozygous	4.3	11.9	7.1
High HbF	4.9	10.8	6.8
HbE homozygous	4.6	9.9	6.7
HbQ heterozygous	5.1	8.7	6.5
HbJ heterozygous	4.8	8.9	6.6
Compound heterozygous for HbD- β thalassemia	5.0	7.5	6.3

The analysis of 85 hemoglobin variant cases among 10,200 HbA1c samples revealed that HbD heterozygous was the most frequently detected variant (27.1%), followed closely by HbE heterozygous (25.9%) and HbS heterozygous (22.4%). Gender distribution across variants showed a male predominance, particularly in HbD (60.9% male) and HbE (54.5% male), while compound heterozygosity for HbD- β thalassemia occurred exclusively in a female patient. The age distribution indicated that most variants were identified in middle-aged adults, with mean ages ranging from 46.2 to

52.5 years, except for compound heterozygous cases presenting at 35 years. HbA1c levels varied substantially across variants, with HbD heterozygous showing the widest range (4.5% to 13.8%, mean 7.8%), suggesting possible assay interference or variable glycemic control in this group. Overall, these findings highlight the importance of recognizing variant hemoglobins during HbA1c interpretation, given their differential prevalence across age, sex, and glycemic ranges.

**Fig 1:** Frequency of Hemoglobin Variants Detected (n=85)

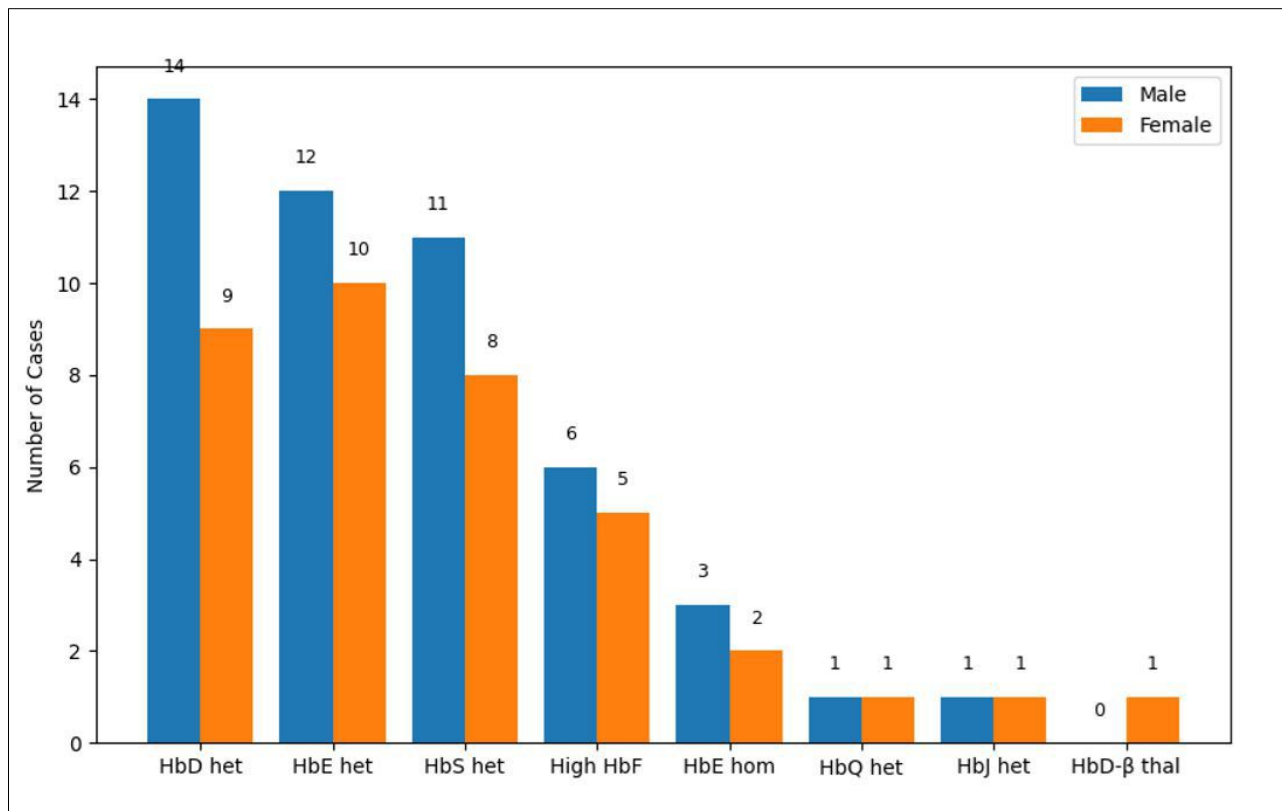


Fig 2: Gender Distribution Across Hemoglobin Variants

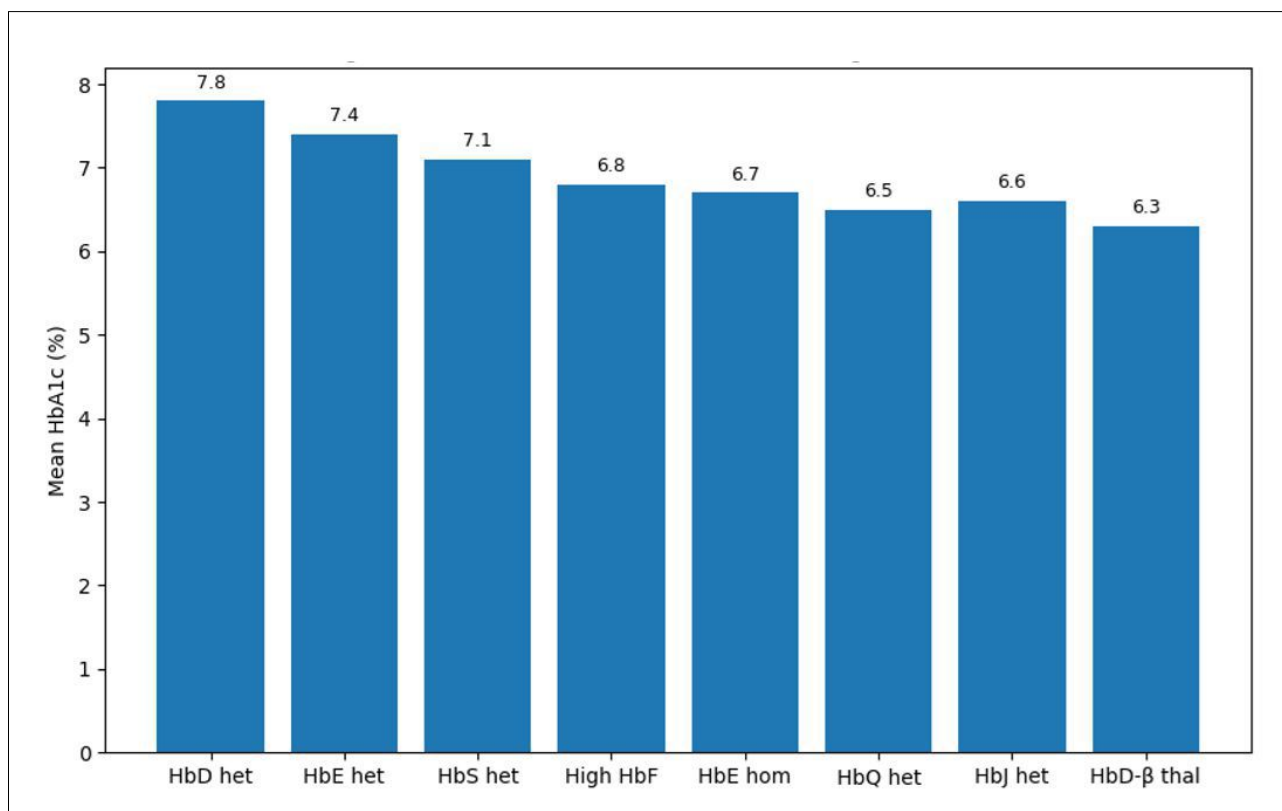


Fig 3: Mean HbA1c Levels Across Hemoglobin Variants

Discussion

Hemoglobinopathies are highly prevalent across Southeast Asia, Mediterranean regions, and Africa [15]. In India, the most frequently encountered hemoglobin disorders include beta-thalassemia, HbE, high HbF (suggestive of hereditary persistence of fetal hemoglobin [HPFH] or delta-beta thalassemia trait), HbS, and HbD. Traditionally, these

conditions are identified through screening initiatives in at-risk populations, utilizing red blood cell indices, high-performance liquid chromatography (HPLC), or electrophoretic techniques, with confirmatory diagnosis via molecular genetic analysis [15, 16]. Interestingly, hemoglobin variants can also be incidentally detected during routine HbA1c testing in diabetic individuals [5, 6]. Instruments like

the Bio-Rad D10 HPLC analyzer automatically generate chromatograms for every HbA1c assay, necessitating careful review to identify variant windows before releasing HbA1c results [6].

While several case reports have documented incidental detection of hemoglobin variants during HbA1c testing in India—including cases of HbQ (7), HPFH [8], HbD Iran with beta-thalassemia [9], HbJ Meerut [10], HbE disease [11], Hb Hope [12, 13], and delta-beta thalassemia trait [14]—our study represents one of the largest single-center cohorts, analyzing 10,200 HbA1c samples and detecting hemoglobin variants in 85 patients (0.83%). The predominant variants identified were HbD heterozygous (27.1%), HbE heterozygous (25.9%), and HbS heterozygous (22.4%), aligning with the known distribution of hemoglobinopathies across India [15-18]. High HbF was the fourth most common variant (12.9%), consistent with patterns reported in northern and eastern Indian populations [19, 20].

An interesting observation in our study was the presence of HbE heterozygosity (25.9%) in Karnataka, a state where earlier studies reported negligible HbE prevalence [16]. This apparent shift in epidemiological distribution may be attributable to increased interstate migration over the past two decades, potentially introducing hemoglobinopathies into regions previously considered low-prevalence zones [15]. Moreover, more than half of the patients with HbS trait in our series were of non-Indian origin, reflecting the influence of medical tourism from African and West Asian countries, further diversifying the local hemoglobinopathy spectrum.

Despite beta-thalassemia trait being the most prevalent hemoglobinopathy in India [20], it was not detected in our cohort due to the limitations of the HbA1c testing mode, which cannot differentiate beta-thalassemia trait in the chromatogram. Clinicians should remain vigilant for beta-thalassemia trait in patients presenting with a discordantly low HbA1c despite high fasting or postprandial glucose values. These individuals often exhibit a normal HbA1c chromatogram but show microcytosis (low MCV), a Mentzer's index <13, and normal iron studies—warranting further diagnostic evaluation.

A key clinical implication of our findings is the unreliability of HbA1c measurements in patients with homozygous or compound heterozygous hemoglobin variants, attributable to markedly reduced or absent HbA levels, which compromises glycation-based assays. Using HbA1c in such individuals risks misdiagnosis and mismanagement of diabetes [21]. Therefore, for patients with homozygous or compound heterozygous variants, alternative glycemic monitoring methods like fructosamine, glycated serum albumin, or capillary glucose monitoring are recommended. In contrast, in heterozygous states, assay interference depends on the specific analytical method. The Bio-Rad D10, as used in our study, has been shown not to interfere with HbA1c quantification in heterozygous states for most variants, except in cases with high HbF or where the P3 peak exceeds 10% [21]. Even so, it is critical to inform patients about their carrier status, as genetic counseling can help prevent homozygous or compound heterozygous outcomes in future generations. Unfortunately, due to resource limitations, confirmatory genetic testing was not performed in our study.

In summary, our findings underscore the added diagnostic value of HbA1c testing as an opportunistic screening tool for hemoglobin variants, highlighting the need for routine

chromatogram review to ensure that hemoglobinopathies are not overlooked, and that reported HbA1c values are valid and clinically actionable.

Conclusion

This study highlights the added diagnostic value of reviewing chromatograms during routine HbA1c testing for the incidental detection of hemoglobin variants. Among 10,200 samples, hemoglobin variants were identified in 0.83% of cases, with HbD, HbE, and HbS heterozygous states being the most prevalent. The detection of such variants has important clinical implications, as some may interfere with HbA1c measurement, leading to inaccurate assessment of glycemic control and potential mismanagement of diabetes. Laboratories should implement protocols for variant detection, interpretation, and reporting to ensure accurate clinical decisions. Furthermore, identifying hemoglobin variants provides an opportunity for early diagnosis, genetic counseling, and prevention of homozygous or compound heterozygous states in future generations. Routine chromatogram review should therefore be integrated as a standard practice in laboratories using HPLC-based HbA1c analysis, particularly in regions with a high prevalence of hemoglobinopathies.

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