The Research of Medical Science Review
ISSN: 3007-1208 & 3007-1216 Volume 4, Issue 3, 2026

PREVALENCE AND HEMATOLOGICAL PROFILE OF HEMOGLOBIN D TRAIT AND ITS DIFFERENTIATION FROM B-THALASSEMIA TRAIT USING HPLC: A RETROSPECTIVE STUDY

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DOI: https://doi.org/10.5281/zenodo.19201287

Keywords

Article History
Received: 20 January 2026
Accepted: 05 March 2026
Published: 19 March 2026

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Abstract

Background: *Hemoglobin D (Hb D) is a clinically silent mutation in beta chain of hemoglobin which often remains undiagnosed without proper screening, therefore leading to complications such as anemia, adverse pregnancy outcomes, and risk of transmission to offspring.*

Objective: *This study aimed to evaluate the prevalence and hematological parameters of hemoglobin D to aid in differentiating this mutant hemoglobin from normal individuals and individuals of beta thalassemia trait on clinical basis*

Methods: *A cross-sectional retrospective study was conducted using patient records from the central laboratory of Dr. Ruth K. M. Pfau Civil Hospital. Data was collected from High-Performance Liquid Chromatography (HPLC) and Complete Blood Count (CBC) tests. A total of 911 patients were included in the study. SPSS version 25 was used for statistical analysis.*

Results: *Out of 911 patients, 13 individuals were found to have HbD trait. The findings indicate that mean MCV, MCH and MCHC of the patients with hemoglobin D 72.915±13.4 fL, 21.692±4.8 pg/cell and 29.123±3.2 g/dL, respectively, highlighting mildly reduced MCH and MCHC values, indicating mild hypochromia. Moreover, HPLC results of Hb D individuals revealed significant elevation of Hb F (P=0.022) and a mild decrease in Hb A levels (51.1600%±1.81) as compared to normal individuals (88.1716% ±43.8), approaching statistical significance (P=0.060). However, distinguishing HbD from beta thalassemia is also challenging for clinicians as both present with mild microcytic hypochromic anemia. A key finding of our study indicates normal Hb A2 levels (2.150%±0.49) in patients with HbD, whereas elevated Hb A2 levels (mean 4.371%±0.5) in patients with beta thalassemia trait. This confirms Hb A2 as the most reliable biochemical marker for differentiating Hb D from BTT.*

Conclusion: *This study highlights the importance of HPLC or hemoglobin electrophoresis for correct diagnosis of Hb D. The significant elevation in Hb F and the presence of a Hb D band in HPLC are key biochemical markers that differentiate these carriers from normal individuals. Routine screening, accurate diagnosis via HPLC, and comprehensive genetic counseling are*

essential strategies for preventing transmission, improving patient outcomes and ultimately reducing the burden of major hemoglobin disorders in the population

Introduction

Hemoglobinopathies are among the most common genetic disorders worldwide, with an autosomal recessive pattern of inheritance [1]. They are the most prevalent monogenic disorders, with an approximate carrier rate of 7% among the world population, contributing significantly to childhood morbidity and mortality. According to the World Health Organization (WHO), approximately 300,000–400,000 infants are born with severe inherited disorders of hemoglobin (Hb) each year. Hemoglobin D (HbD) is a common Hb variant distributed worldwide and ranks third in frequency, after HbS and HbC [2]. It results from a glutamic acid (Glu) to glutamine (Gln) substitution at codon 121 of the beta chain, whereas HbS and HbC result from a valine (Val) for glutamic acid (Glu) substitution and a replacement of glutamic acid (Glu) by lysine (Lys) at position 6 of the beta chain, respectively [3]. Conversely, the presence of this Hb is an important parameter for premarital screening, leading to prenatal misdiagnosis [3]. Increased awareness among healthcare professionals regarding the prevalence and manifestations of HbD is essential for accurate diagnosis and management. HbD syndromes may manifest as heterozygous HbD trait, HbD thalassemia, HbSD disease, and homozygous HbD disease [2]. In the heterozygous state, Hb D does not produce any clinical symptoms. Symptoms can be severe when associated with sickle cell haemoglobin and thalassemia [6]. Variants of this haemoglobin are Hb D-Bushman (β16 Gly→Arg), Hb D-Granada (β22 Glu→Val), Hb D-Ouled Rabah (β19 Asn→Lys), Hb D-Los Angeles or Hb D-Punjab (β121 Glu→Gln), Hb D Iran (β22 Glu→Gln), Hb D-Ibadan (β87 Thr→Lys), and Hb D-Neath (β121 Glu→Ala) [4]. Hb D-Punjab (also known as Hb D-Los Angeles) is the most common. The unique feature of these variants is the presence of the same amino acid substitution (Glu → Gln) at position β121 [5]. Undiagnosed HbD may lead to complications such as anemia, poor pregnancy outcomes, and risk of transmission to offspring. Early detection enables better clinical management, informed genetic counselling, and improved maternal and child health outcomes. This study aims to highlight the clinical and hematological profile of HbD patients.

Material and Methods

This was a cross-sectional retrospective study. Data from High-Performance Liquid Chromatography (HPLC) and CBC tests from June 2022 to June 2024 were collected from patients' records of the central lab, Dr. Ruth K. M. Pfau Civil Hospital, Karachi. Ethical clearance was obtained from Dow University of Health Sciences Institutional Review Board. We included patients of all age groups and both genders. Patients with incomplete data and a recent history of blood transfusions within 3 months were excluded from the study. Complete blood count (CBC) was performed on the XN 1000 analyzer, and HPLC was performed on the Array analyzer. The extracted data from patients' records comprised different CBC parameters such as Hemoglobin concentration (Hb), Mean Cell Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), and Hematocrit (Hct), along with the percentage level of different Hemoglobin variants like HbA2, HbF, HbS, HbC, HbD, and HbE. HbA2 value >3.5% was estimated as a cut-off point for beta thalassemia trait. Statistical Package for Social Sciences version 25 (SPSS) was used for statistical analysis of the data.

Results

A total of 911 patients were included in the study. Among them, 490 were males while 421 were females. The male-to-female ratio was 1.16. The mean age of the patients was 11 years, and the mean hemoglobin (Hb) level was 7.62 g/dL.

Table1: Demographics (n=911)

SUBJECT	VALUES
No. of Male	490 (53.8%)
No. of Female	421 (46.2%)
BTT cases	118
BTM cases	22
Hb D cases	13

Table2: Comparison of hematological parameters of BTT and HbD

	BTT	Hb D	P VALUE
Hb Level (g/dL)	9.0±2.3	7.52±3.0	0.037
HCT Mean (%)	33.424±33.4	25.323±9.7	0.151
MCV Mean (fL)	70.332±59.4	72.915±13.46	0.876
MCH Mean (pg/cell)	18.634±3.4	21.692±4.87	0.046
MCHC Mean (g/dL)	28.327±2.9	29.123±3.21	0.362
HB A%	84.44±7.56	51.1600 ±1.81	0.000
HB A2 %	4.371±0.5	2.150±0.49	0.000
HBF %	1.802±5.4	3.608±3.5	0.268

Table3: Comparison of hematological parameters of Normal patients and HbD

	Normal	Hb D	P value
Hb Level (g/dL)	7.486 ±3.19	7.523 ±3.05	0.968
HCT Mean (%)	28.352±19.6	25.323 ±9.7	0.579
MCV Mean (fL)	78.679±51.6	72.915±13.46	0.688
MCH Mean (pg/cell)	24.923±89.5	21.692±4.87	0.897
MCHC Mean (g/dL)	28.090±17.97	29.123±3.21	0.836
HB A %	88.1716 ±43.8	51.1600±1.81	0.060
HB A2 %	2.542±1.1	2.150±0.49	0.629
HB F %	0.851±2.9	3.608±3.5	0.022

Table4: Patients with anemia

ANEMIC PATIENTS		
MALE (<13g/dl)		**FEMALE** (<11g/dl)
No. of cases	459	383
Avg Hb level (g/dl)	7.221	7.071
Min (g/dl)	1.7	1
Max (g/dl)	12.9	10.8

Discussion

Inherited hemoglobin disorders affect approximately 7% of the global population and remain a major public health challenge [7]. These disorders can be classified into two main categories: hemoglobinopathies, which involve structural abnormalities in the globin chains, and thalassemias, which result from decreased production of structurally normal globin chains [8].Pakistan, being the fifth most populous country in the world, exhibits significant genetic heterogeneity with over 18 ethnic groups and more than 60 spoken languages [2]. In this study, we compared hematological profiles of Hb D patients with those of normal patients and patients having beta-thalassemia trait (BTT) to identify parameters useful for differential diagnosis. Among 911 analyzed patients, BTT was the most prevalent (118 cases), followed by beta-thalassemia major (22 cases), Hb O (16 cases), Hb D (13 cases), Hb S (5 cases), and Hb E (5 cases). One patient demonstrated co-inheritance of Hb E and BTT. The cut-off for anemia was Hb less than 13g/dL for males and less than 11g/dL for females. In the anemic subgroup, 459 males and 383 females were identified as anemic. Hemoglobin levels varied widely (1.7–12.9 g/dL in males; 1–10.8 g/dL in females), reflecting the diverse clinical severities of anemia associated with hemoglobinopathies [11].

The mean MCV, MCH and MCHC of the patients with hemoglobin D were 72.915±13.4 fL, 21.692±4.8 pg/cell and 29.123±3.2 g/dL, respectively. These findings are consistent with those of (Tashfeen et al. 2025) and (Sehgal et al. 2022).In general, HbD individuals exhibited mildly reduced MCH and MCHC values, indicating mild hypochromia. The structural alteration caused by the Glu→Gln substitution at β121 likely produces minimal effects on hemoglobin function and red cell morphology, supporting the conclusion that heterozygous Hb D states are largely asymptomatic.

When comparing Hb D individuals to the normal individuals, our results highlight the "silent" nature of the Hb D trait. Our analysis showed no statistically significant differences in the primary CBC indices, including Hb level (P=0.897), MCV (P=0.688) and MCH (P=0.897). These results suggest that Hb D carriers are hematologically indistinguishable from individuals using a standard CBC. In a routine clinical setting, these individuals would likely be cleared as "normal", potentially leading to an underestimation of the variant's prevalence in the population. An HPLC or electrophoresis is therefore essential for detecting the presence of Hb D. Upon analysis of the HPLC results of Hb D individuals with the normal cohort, we found a striking distinction between the two groups. The Hb D group had a significant elevation of Hb F (P=0.022). Furthermore, a mild decrease in Hb A levels was observed in the Hb D individuals (51.1600%±1.81) compared to normal individuals (88.1716% ±43.8), approaching statistical significance (P=0.060). This reduction in Hb A is a logical consequence of the presence of Hb D.

Distinguishing HbD from beta thalassemia trait is a significant challenge for clinicians, as both these conditions present with mild microcytic hypochromic anemia. Our analysis identified key hematological parameters that serve as excellent discriminators between Hb D and BTT. Analysis of CBC indices of both groups yields the finding that despite both groups having low hemoglobin, the Hb D group of our study population had a lower mean hemoglobin (7.52g/dL±3.0) than the beta thalassemia group (9.0g/dL±2.3)(P=0.037). This suggests that in our study population, Hb D presented with a more pronounced clinical phenotype than previously reported. Furthermore, the significant variation in MCH (P=0.046) suggests that while both these conditions are characterized by hypochromia, the degree of hemoglobinization per cell is less severely impacted in Hb D (21.692pg/cell±4.87) than Beta thalassemia trait (18.634pg/cell±3.4). This subtle but statistically significant difference can aid clinicians in flagging samples for hemoglobin analyses like Electrophoresis or High Performance Liquid Chromatography (HPLC). A pivotal finding of our study is the marked difference in Hb A2 fractions between Hb D and beta thalassemia trait. While the Hb D patients had Hb A2 levels within the normal range (2.150%±0.49), the beta thalassemia trait

group consistently elevated Hb A2 levels (mean 4.371%±0.5). This confirms that Hb A2 remains the most reliable biochemical marker in differentiating Hb D from BTT, preventing potential misdiagnosis in routine screenings.

In β-thalassemia trait (BTT), hemoglobin A_2 (HbA_2) levels are characteristically elevated, often above 3.5%, making HbA_2 quantification an essential diagnostic marker for carriers. Heterozygous BTT individuals also present with reduced mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV), reflecting the typical microcytic hypochromic anemia, while HbA is relatively decreased compared with healthy adults. These hematological features have been consistently documented and are used to differentiate BTT from other causes of microcytic anemia.[13][14]

In contrast, individuals with HbD variants, such as HbD-Punjab, who are heterozygous for this abnormal hemoglobin typically exhibit near-normal hematological indices, with HbA_2 and HbA values within or slightly below the normal range, and do not show the significantly elevated HbA_2 characteristic of isolated BTT.[3],[4] High-performance liquid chromatography (HPLC) studies have demonstrated that heterozygous HbD individuals present with a predominant HbD peak (~30–40% of total hemoglobin), while MCH and MCV remain largely normal unless there is co-inheritance of β-thalassemia or another hemoglobinopathy.[15][16]

Thus, BTT is associated with elevated HbA_2, reduced HbA, and decreased MCH, whereas simple HbD trait without co-inheritance generally shows normal HbA_2 and minimal changes in HbA and MCH, reinforcing the diagnostic distinction between these hemoglobinopathies.

Conclusion

This study underscores the importance of specialized hemoglobin analysis in the differential diagnosis of hemoglobinopathies in Pakistan. Hemoglobin D, particularly Hb D-Punjab, is a relatively common but clinically mild hemoglobin variant. Although often asymptomatic, its detection remains crucial in regions like Pakistan where its prevalence is high and coexistence with other hemoglobinopathies is frequent. Hb D carriers often present with a "silent" hematological profile on a standard CBC. The significant elevation in Hb F and the presence of a Hb D band in HPLC are key biochemical markers to differentiate these carriers from normal individuals. When compared to BTT, the significant elevation of HB A2 in BTT helps to distinguish Hb D from BTT.

Our results highlight the necessity of HPLC or Hb electrophoresis along with routine blood indices for accurate screening. Given the high prevalence of these traits in Pakistan, we recommend the implementation of mandatory HPLC or HB electrophoresis in premarital screening programs. Routine screening, accurate diagnosis via HPLC, and comprehensive genetic counseling are essential strategies for preventing transmission, improving patient outcomes and ultimately reducing the burden of major hemoglobin disorders in the population.

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