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ORIGINAL ARTICLE

Unexpected Discovery of Hemoglobin D Iran Families in Punjab, Pakistan by using two different Screening Methods

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ABSTRACT

Background: Hemoglobin D Iran is frequently misdiagnosed as Hb E or Hb D Punjab if only one method of screening is used. The objective of our study was to highlight the importance of using two different screening techniques in diagnosis of a hemoglobin variant, Hb D Iran in our case. Hematological parameters of heterozygous Hb D Iran and compound heterozygous β/Hb D Iran were also compared.
Methods: A descriptive study was carried out on results of 52,379 subjects which were part of thalassemia extended family cascade screening from 36 districts of Punjab from October 2019-March 2021. Cases of Hb D Punjab and Hb E were run on both CE-HPLC (cation exchange-high performance liquid chromatography) and CZE (capillary zone electrophoresis). Resulting Hb D Iran cases were confirmed by ARMS-PCR (Amplification refractory mutation system-polymerase chain reaction).
Results: Forty cases of Hb D Iran were detected out of 160 initially suspected Hb D Punjab cases and 126 Hb E cases. Diagnosis was confirmed by molecular analysis. Statistical significance was found between RBC count, MCV, MCH, Hb F and diagnosis of "heterozygous Hb D Iran" and "compound heterozygous for β/ Hb D Iran".
Conclusion: Hb D Iran can be easily missed and misdiagnosed as Hb E or Hb D Punjab, if two screening methods are not used. This maybe a reason why Hb D Iran remains unreported in our region. CBC and HPLC indices can also be suggestive if a case is of heterozygous D Iran or compound heterozygous β/Hb D Iran.
Keywords: Hb D Iran, Hb E, Hb D Punjab, Cation exchange High performance liquid chromatography, Capillary zone electrophoresis

INTRODUCTION

Hb D Iran is a rare hemoglobinopathy and remains largely unreported due to its asymptomatic phenotype and also because of it being misdiagnosed or missed. It is a clinically insignificant structural hemoglobin variant, arising as a consequence of point mutation in codon 22 of beta globin gene in which glutamine replaces glutamate (GAA>CAA, Glu >Gln). It was first identified in heterozygous form, both in an Iranian patient and in a Pakistani family in the United States[1]. This hemoglobin variant is common in Iran, however its prevalence in Pakistan is generally unknown. Two studies conducted in India revealed only 1 out of 2600 patients with suspected Hb variant as a case of Hb D-Iran (0.038%)[2], while in the second study , HPLC records on 800 patients were analyzed in which Hb D Iran was one of the rarest Hb variant reported[3].

Its only implication is in its diagnostic difficulty if the screening method employed is CE-HPLC (Cation exchange- High performance liquid chromatography). Hb D Iran co-elutes in A_2 window of chromatogram yielding raised A_2 percentage and is often misdiagnosed as Hb E. Similarly, in CZE (Capillary zone electrophoresis) a peak in D zone is often wrongly diagnosed as Hb D Punjab, which is a more prevalent in Pakistan. The need for correct diagnosis is mandatory in cases of compound heterozygous state of β/Hb D Iran. These cases exist as an asymptomatic or in a mild clinical state. They can be wrongly interpreted as homozygous for Hb E or compound heterozygous for β/Hb E. The latter can be mildly symptomatic or manifest as transfusion dependent state. Such cases require intensive workup and PND (prenatal diagnosis) which may otherwise be unnecessary if correctly diagnosed as β/Hb D Iran. Therefore in laboratories utilizing CE- HPLC instruments only, β/Hb D Iran compound heterozygotes are falsely reported as either homozygous Hb E[4] or compound heterozygous for β / Hb E. This is because the elevated Hb A_2 peak is misinterpreted. Hence the need for two methods of detection of Hb variant cannot be stressed upon enough.

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Therefore we aimed to study all cases eluting more than eight percent in the A_2 window on CE-HPLC and those yielding peaks in D zone on CZE and also to determine the frequency of Hb D-Iran existing in heterozygous, homozygous and compound heterozygous state in the subjects under study.

MATERIAL AND METHODS

The study was conducted at Punjab Thalassemia Prevention Project regional labs from October 2019 – March 2021 after permission from IRB. Samples were collected as part of extended family screening of thalassemia major children in Punjab. Written consent was obtained from subjects for hemoglobinopathy screening (CBC and HPLC/CZE) and genetic analysis. Venous blood samples collected in K3 EDTA (ethylene diamine tetra acetic acid) evacuated tube were analyzed for routine complete blood count by Sysmex XP-100. Samples of patients with thalassemic red blood cell indices and their parents were marked for hemoglobinopathy screening. Two methods are employed in our set up; Cation Exchange- High Performance Liquid Chromatography on the Bio-Rad Variant II β-thalassemia short program and Capillary Zone Electrophoresis based on migration of different Hb variants using Sebia Capillary's 2 Flex piercing instrument. Results were analyzed using SPSS version 20.

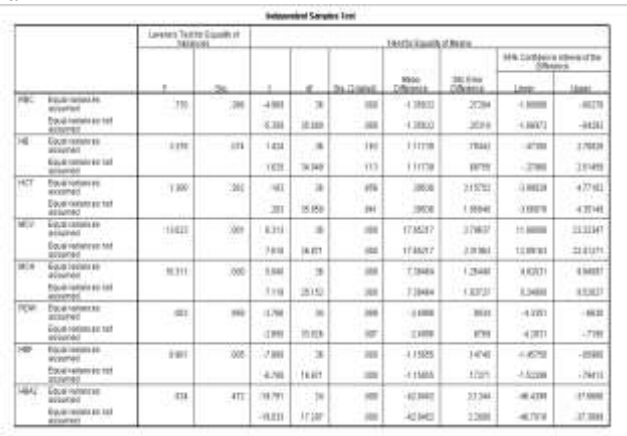
Samples subjected to CE- HPLC initially, yielding Hb A_2 of more than eight percent were taken into account. These were 126 samples which were then analyzed using CZE as a second method to confirm presence of the variant. Those showing migration pattern in D zone were suspected as cases of Hb D Iran and confirmed by ARMS-PCR (Amplification Refractory Mutation System- Polymerase Chain Reaction) T 100 Bio Rad thermal cycler. Molecular characterization was done using forward primer (CAACCTGCCCAGGGCCTCACCACCAACATG) and reverse primer (ACCTCACCCTGTGGAGCCA). Primary denaturation was done at 94-95°C for five minutes, followed by 25-35 cycles of denaturation at 94°C for one minute, 30sec at 55° C for annealing, one minute at 72°C for extension and with a final extension at 72°C for 5 min. The PCR product of 255 bp was electrophoresed by 2.0% agarose gel electrophoresis followed by ethidium bromide staining and visualized by Bio Rad Gel Documentation system.

Samples which were initially analyzed by CZE and yielded peak in D zone (160 cases) were then subjected to CE-HPLC and those yielding Hb A2 >8% were suspected as cases of Hb D Iran and confirmed by PCR. Samples yielding peaks in D window were excluded, as they were cases of Hb D Punjab, the more common variant of Hb D. Samples showing migration pattern in E zone in CZE were also excluded (as cases of Hb E).

RESULTS

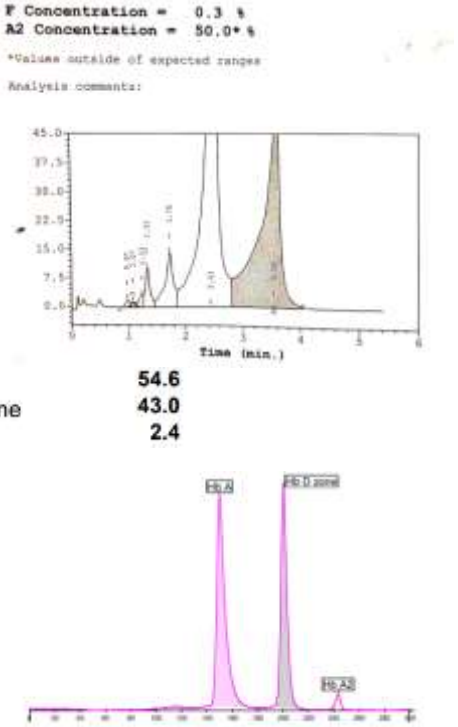
In our study, out of 126 suspected Hb E cases (HPLC initial screening results) 27 were of Hb D Iran. These were confirmed by running these 126 samples on CZE out of which 27 (21.4% of suspected Hb E cases) samples yielded a peak in D zone. Similarly out of 160 suspected Hb D Punjab cases (CZE initial screening results) 13 (8.1%) were of Hb D Iran. These 13 cases were also confirmed by running them on HPLC in which they yield raised Hb A_2 percentage (Figure 1). Total of 40 cases of Hb D Iran were detected on HPLC and CZE and confirmed by ARMS-PCR. There were 19 females (47.5%) and 21 males (52.5%). Twenty three cases (57.5%) were heterozygous for Hb D Iran, and 15 cases (37.5%) were compound heterozygous for β /Hb D Iran and 2 (5%) cases were homozygous for Hb D Iran. A comparison was drawn between heterozygous Hb D Iran and compound heterozygous of β / Hb D Iran, on the basis of CBC findings and HPLC/CZE findings (Figure 2). For heterozygous cases RBC count ranged from 1.89-6.1x10^6/µl whereas the RBC count in compound heterozygous of β / Hb D Iran ranged from 5.49-7.3x10^6/µl. The hemoglobin of heterozygous cases ranged from 4.1-16.0g/dl and that of compound heterozygous ranged from 8.9-13.4 g/dl. Mean cell corpuscular volume was found to be in the range of 53-93.8fl on heterozygous for Hb D Iran and 52.4-63.7 fl in compound heterozygous cases. MCH (Mean corpuscular hemoglobin) was in the range of 13.0-32.4pg in heterozygous Hb D Iran and range of 14.4-18.8pg was found in compound heterozygous cases. RDW-CV (Red cell distribution width-variation coefficient) ranged from 12-24.6% in heterozygous cases and in compound heterozygous cases it ranged from 15-23.2%. On analysis of Hb fractions, Hb A_2 of samples run initially on CE-HPLC was found to be in the range of 38.2-50.4/% in heterozygous Hb D Iran and range of 67.8-92.7% was found in compound heterozygous for β/Hb D Iran cases. Hb F ranged from 0.0-1.2% in heterozygous and 0.9-3.3% Hb F was found in compound heterozygous cases. All suspected cases were run on CE-HPLC as well as CZE to verify the variant. (Figure 3)
T – test was applied and statistical significance was found between RBC count, MCV, MCH, Hb F and diagnosis; which were "heterozygous Hb D Iran" and "compound heterozygous for β/ Hb D Iran."(Table 1)

Table 1: Statistical relationship between CBC and HPPLC indices and diagnosis of hetrozygous hb diran and compound hetrozygous for β/h B D Iran



Suspected cases of Hb D Iran subjected to ARMS-PCR revealed 23 cases of heterozygous Hb D Iran, two cases of homozygous Hb D Iran and 15 cases were of compound heterozygous for β/ Hb D Iran in which the beta thalassemia mutations found were IVS1-5, (seven cases) and Fr 41-42 (eight cases) (Figure 4).

Sample of heterozygous Hb D Iran patient run on both CE-HPLC and CZE respectively



Sample of compound heterozygous β/Hb D Iran patient run on both CE-HPLC and CZE respectively.

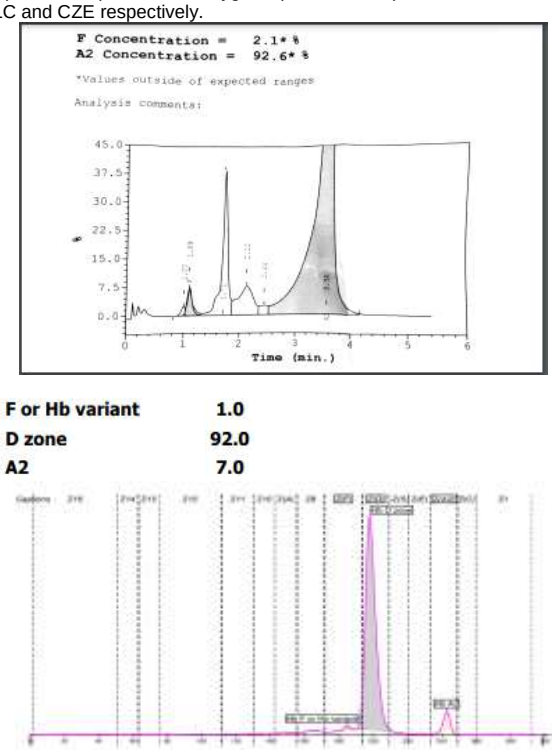
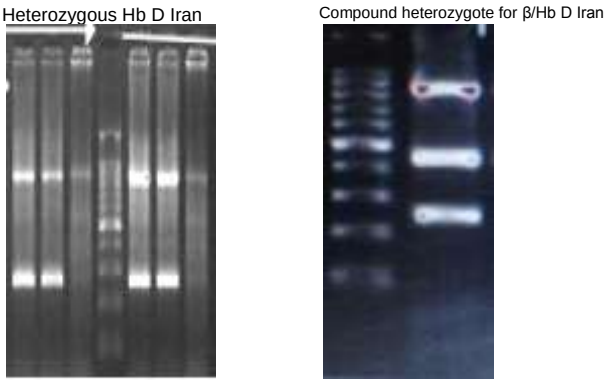


Figure 3: CE-HPLC and CZE chromatograms comparison of heterozygous hb d iran cases with compound hetrozygous for β/HB D IRAN.



DISCUSSION

Itano was first to discover Hb D in a multiethnic family residing in Los Angeles in 1951[5]. Its prevalence is mostly in Punjab region, Northwest India, with a reported probable frequency of 2.0%. There are seven other types of Hb D, according to the Globin Gene Server database resulting from different point mutations in beta globin gene. These include Hb D-Iran, Hb D-Agri, Hb D-Bushman, Hb D-Ouled Rabah, Hb D-Granada, Hb D-Ibadan and Hb D-Neath[5].. The migration pattern of Hb D on alkaline pH is similar to Hb S. In acidic pH, its migration resembles Hb A. Hb D demonstrates normal solubility when in its reduced state and does not exhibit sickling, thus distinguishing it from Hb S. Furthermore, in a study conducted in 1962, Baglioni found five hemoglobin types; namely Hb D-Chicago, Hb D-North Carolina, Hb D-Punjab, Hb D-Portugal and Hb D-Oak Ridge. These variants demonstrate the same chemical composition as Hb D-Los Angeles or Hb D Punjab, which is the most frequent variant of this mutant hemoglobin till date.

Hb D Iran is a structural hemoglobin variant resulting from the substitution of glutamate with glutamine at codon 22 of the beta globin gene. This hemoglobin beta chain variant was first discovered 1973 by Rahber in a family in central Iran[6]. Hb D-Iran has high prevalence in Iran. In an eight year study conducted in Fars, Iran, Hb D was found to be the most common Hb variant after α- and β-thalassemia. One eighty carriers of Hb D-variant were identified in which Hb D-Punjab and Hb D-Iran were found to be almost equally prevalent[17]. In a study conducted on 34,034 healthy adults to determine prevalence of hemoglobinopathies in Tehran, Iran Hb D Iran was found to be most prevalent hemoglobinopathy[8]. Zakererinia *et al*.reported 88 cases (40%) of Hb D Iran out of 220 subjects in Southern Iran [9]. Although scarce data is available regarding its prevalence in our part of the world, there have been few isolated case studies and case series reported in northwestern region of India [10]. In Pakistan, Hb D is reported in approximately one percent of Punjabi population. Hb D Punjab and Hb D Iran exists as two different genotypes of Hb D. They are both clinically silent and have the same electrophoretic mobility and can be distinguished on basis of PCR[11].

According to our study out of 40 confirmed cases of Hb D Iran, 23 cases (57.5 %) were heterozygous for Hb D Iran, 15 cases (37.5%) were of compound heterozygotes of β/ Hb D Iran out of which one was compound heterozygous for β^0 /Hb D Iran and 2 cases (5%) were of homozygous Hb D Iran. The frequency of Hb D Iran heterozygous state was higher (86%) however compound heterozygotes of β/ Hb D Iran was lower (3.4%) in New Delhi as reported by Dass J *et al*[7] There have been very few studies on this hemoglobin variant in Pakistan. Ali A *et al.* reported a single case report of compound heterozygous state of β/ Hb D Iran in a young six months pregnant primigravida of Kashmiri origin in 2019[12].

Thalassemia and related hemoglobinopathies being the most common monogenic disorders are quite frequent in occurrence in Pakistan. This is due to diverse ethnic backgrounds and high consanguinity rate. These facts are also supported by our study in which families with Hb D Iran had ancestral lineage from Iran and their pedigrees showed high rate of consanguinity. Consanguineous marriages was also probably the reason why this variant was concentrated in only few pockets of Punjab. Prevalence of hemoglobinopathies is variable in different geographic locations. In Punjab province most common Hb mutations are beta thalassemia followed by Hb D Punjab and Hb E in descending order [13]. No known data regarding prevalence of cases pertaining to Hb D Iran in Pakistan has been published, to the best of our knowledge.

A number of reasons contribute to the apparent low prevalence of Hb D Iran. First and foremost, Hb D Iran in heterozygous and homozygous form is clinically silent except for cases of compound heterozygotes of β/Hb D Iran which present with mild to moderate anemia, fatigue and weakness [7]. Majority of homozygous and heterozygous cases are detected retrospectively as a part of extended family screening. Hence most of the Hb D Iran cases are missed because of its asymptomatic phenotype.

Secondly and perhaps more importantly, practice of a single technique to diagnose a hemoglobin variant leads to misdiagnosis/missing of such cases. Hb D Iran has the same migration pattern as Hb S on alkaline electrophoresis. Hb D Iran is heat stable and does not affect oxygen equilibrium, intracellular 2,3 -DPG or the Bohr affect. Due to same migration pattern, Hb D Iran it can be misdiagnosed as Hb S [6]. This can result in unnecessary invasive investigation like CVS for PND if spouse is also wrongly labelled as carrier of Hb S. Additional investigations like a sickling test and CE-HPLC need to be performed. CE-HPLC distinguishes Hb S (retention time 4.30-4.70 minutes) from Hb D Iran (retention time 3.30-3.9). Hb D Iran elutes in A_2 window on CE-HPLC. In Pakistan the most common Hb variant eluting in A_2 window is Hb E, which is clinically significant if it exists in compound heterozygous state with B thalassemia and necessitates extended family screening, genetic counselling, PND and termination of pregnancy. Needless to say, this is a huge economic burden on our already financially restrained government setups and it is also coupled with emotional and mental trauma of the inflicted family. Therefore it is of utmost importance to employ at least two different techniques based on different principles to confirm a hemoglobin variant and avoid misdiagnosis. In our setup we commonly use CE-HPLC as well as CZE as a screening tool. In case of a hemoglobin variant, we countercheck it with the second method and genetic analysis. Hb D Iran co elutes with Hb A_2 and Hb E on HPLC at retention time of 3.30-3.90 minutes. On CZE it yields a peak in D zone. ARMS- PCR shows substitution of glutamate with glutamine at codon 22 of the beta globin gene and confirms presence of Hb D Iran. Samples yielding a peak in D zone on CZE are counter checked on CE-HPLC. If the peak falls between retention time of 3.90 - 4.30 minutes diagnosis of Hb D Punjab is made and confirmed by ARMS-PCR. If it co elutes in A_2 window in CE-HPLC it is most likely Hb D Iran. It is also important to differentiate Hb D-Iran from Hb D-Punjab essentially in the cases of compound heterozygous Hb D Punjab/ Hb S. This is because Hb D Punjab promotes sickling in compound heterozygous Hb D-Punjab /Hb S state. Conversely, Hb D-Iran/ Hb S is a clinically benign syndrome [7]. Therefore use of two different techniques using different principles is absolutely mandatory to give a confirm diagnosis of a hemoglobin variant.

On evaluating the red blood cell parameters and results of hemoglobin fractions obtained from HPLC and CZE we observed that compared to heterozygous Hb D Iran, compound heterozygous for β/ Hb D Iran had higher mean red blood cell count (6.20 vs 4.84 x 10^6/ul, P <0.001), lower mean MCV(58.2 vs 75.8 fl, P<0.001), lower mean MCH (17.4 vs 24.57, P< 0.001), higher mean Hb F (1.59 vs 0.4, P < 0.001) (Table 1) . Therefore on the basis of our analysis we can safely say that CBC indices

and HPLC/CZE results yielding high RBC count, low MCV, MCH and raised Hb F suspicion of compound heterozygous form of Hb D Iran can be kept high and confirmed by DNA analysis.

CONCLUSION

The purpose of our study was to report that hemoglobin variants require use of two methods of diagnosis. Cases of Hb D Iran cases remain largely unreported in our country owing to its asymptomatic phenotype, and also misdiagnosed as Hb D Punjab or Hb E when either only CZE or only HPLC is used. This leads to unnecessary pre natal diagnosis if misdiagnosed as Hb E. We believe much higher prevalence of Hb D Iran exists in Pakistan owing to its multiple ethnicity and high consanguinity rate. Larger sample size is recommended to reflect the true prevalence of this rare hemoglobin variant.

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Ethical declaration: Punjab Thalassemia Prevention Project is an initiative of Health department, Govt. of Punjab, Pakistan. It is an independent organization. The data presented in this article under title "Unexpected discovery of Hemoglobin D Iran families in Punjab, Pakistan by using two different screening methods" is from day to day routine work. Informed written consent was obtained from individuals for screening and further molecular genetic testing for extended screening.

Conflict of interest: The authors declare no conflict of interest

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