

Abnormal Hemoglobins (HbD and HbQ^{India}) and β -Thalassaemia among the Indian Sindhis

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Abstract

The Sindhi is one of the largest linguistic communities, migrated about 65 years back from the Sind province of West Pakistan to India. Though they share so many common practices with the Punjabis (especially those from Multan) and Gujarati Lohanas restricted endogamy and occupational specificity among this community is prominent [1]. Linguistically the community has been classified under the Indo-European linguistic sub-division and racially proximity has been postulated with the Mediterranean and Alpo-Dinarics. Earlier investigation among the Sindhis of Nagpur city has revealed the existence of a number of subgroups with marked territorial identity and these subgroups were found to be heterogeneous with respect to selected sero-genetic loci [2].

Introduction

The Sindhi is a high risk community for β thalassaemia gene in India [3,4] with a carrier frequency ranging from 5 to 12% with a distinct regional variability. Along with β thalassaemia, other abnormal hemoglobin variants are also reported among Sindhi. As such, HbD, HbQ, HbJ, HbL and Hb M show higher rate of occurrence among the populations inhabiting North-west India [3,5] of which, HbD (β -121, Glu-Gln, CD121, GAA \rightarrow CAA) is reported to be preponderant among the Sindhi and Punjabis [5,6-10]. Reported from India in mid 50s with a frequency of above 0.5, HbD presents no clinical severity and the carriers remain healthy normal throughout life. Similarly, HbQ^{India} (α -64 Asp-His, CD64 AAG \rightarrow GAG), an alpha chain variant [11] is also reported among the Sindhi [12,13]. Both these variants are reported in association with β thalassaemia from various parts of India [12,14]. Interestingly these compound heterozygote conditions are clinically silent and do not result thalassaemic phenotype. Nevertheless, HbD- β Thalassaemia and HbQ- β Thalassaemia compound heterozygotes are occasionally associated with mild anemia and show trivial clinical severity of pallor, hepatomegaly. But being benign in expression, the individuals with these genotypes often remain undiagnosed and may have Thalassaemic offspring with moderate to severe clinical presentation when married to another β Thalassaemia trait individual. Hence, detection of the HbD and HbQ variants in the populations with reported prevalence of thalassaemia gene is essential in successful prevention of the clinically severe Thalassaemic births.

As far as the detection of these variants is concerned in alkaline electrophoresis, both of them show similar mobility and hence become difficult to distinguish during routine screening practices. To be precise, HbQ^{India} has a similar but slightly faster mobility towards the anode as HbD [3], in alkaline electrophoresis. The position of the HbQ^{India} band is between HbS/HbD and HbF with a closer migration towards HbS/HbD. Careful application helps better identification, which can be confirmed later with quantitation, HPLC, mutation analysis through DNA analysis and family studies.

Looking at the higher prevalence of β thalassaemia and reported HbD and HbQ variants among Sindhi of India, the present study was undertaken on the Sindhi of Nagpur city, Maharashtra, Central India as a programme to detect β Thalassaemia gene and other abnormal hemoglobins under Research Fellowship programme of the Anthropological Survey of India, Nagpur. The project with an aim to study the intra ethnic heterogeneity of the hemoglobinopathies in

Sindhis was a preliminary effort in central India. Simultaneously, an effort is being made to evaluate the electrophoresis findings for HbD and HbQ variants against DNA based mutation confirmation methods. This will help in adopting hemoglobin electrophoresis and HPLC based quantitation as

Secondary confirmation of these abnormal hemoglobinopathies. The present communication briefs the findings in this focus.

Material and Methods

1498 unrelated Sindhi individuals are included in the study for β thalassaemia carrier identification and abnormal hemoglobins after obtaining written consent. The necessary institutional ethical clearance was obtained before the approval of the project. NESTROFT was performed on fresh finger prick blood and 3 to 5 mL of intravenous blood sample was collected in EDTA (Di-Sodium salt) vacutainers (BD Inc, USA) from each individual. CBC was done within 12 hours of collection using automated haematology analyzer (PC 607, ERMA, Japan). Cellulose Acetate membrane hemoglobin Electrophoresis in Tris-EDTA-Boric Acid buffer (pH 8.6), Quantitation of HbF by one minute denaturation process at 540 nm using UV-VIS spectrophotometer (Spectronix Inc, Ahmedabad, India) was performed as per the standard methods recommended [15]. Based on the decreased MCV and MCH (below 80 fL and below 27 pg respectively), HbA₂ values more than 4.0% [16] and algorithm based on haematological parameters [17], β thalassaemia carrier diagnosis was done, which was later confirmed by family studies. Sickling test was performed with a fresh finger prick blood for all subjects showing hemoglobin band of similar mobility with that of HbS. Subjects with a negative sickling test and having a hemoglobin band at the position of

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HbS in electrophoresis were suspected as carrying an HbD zone variant. Similarly suspicion of HbQ^{India} was based on specific electrophoretic mobility and quantitation of the abnormal hemoglobin band separated in the same zone [3,18,19]. The second tire confirmation for HbA₂ estimate, Chromatographic separation of HbD group hemoglobins and HbQ^{India} hemoglobins were done by HPLC using Variant Classic™ as per the manufacturer's instruction (Bio-Rad Inc, CA, USA). DNA was extracted by phenol chloroform method using standard protocol from vaccutained venous blood freshly collected for all the suspected HbQ^{India} cases. Mutation detection by PCRARMS [20] was done for samples with suspected HbQ^{India} only for which the primers are used as mentioned earlier [19]. Briefly a set of mutation specific primers was used to amplify the α -1 globin gene for DNA containing the CD64 G→C mutation specific to HbQ^{India}, along with internal control primers chosen from the α 1 gene. PCR was done with 100-150 ng genomic DNA, 0.2 mM of each primer, 0.5 U Taq polymerase (Sigma-Aldrich, USA) in a ARMS reaction master mix containing 0.75M betaine, 5% DMSO, 200 mM of each dNTP, 0.9 mM MgCl₂, 750mM Tris/HCl at pH 9.0, 200 mM (NH₄)₂SO₄, 0.1% (wt/vol) Tween 20. The 25 mL PCR reaction was carried out with initial denaturation of four minutes at 95°C followed by 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 and a final extension for five minutes at 72°C. The products were examined by ethidium bromide staining and 2% agarose gel electrophoresis. A product of 370 bp specific to the mutant primer was considered to confirm the presence of HbQ^{India} allele.

Observation and Discussion

Altogether, 1498 Sindhi subjects were tested for hemoglobinopathies. 31 cases of HbD abnormal hemoglobins were detected of which three cases of HbD- β Thalassaemia were found. Seven cases of HbQ^{India} were identified (suspected by hemoglobin electrophoresis and confirmed by CEHPLC and mutation analysis), of which one case was HbQ^{India}- β Thalassaemia compound heterozygote (Table 1).

The incidence of HbD and HbQ^{India} is found to be 2.07% (1.87 and 0.20% respectively for HbD heterozygote and HbD- β thalassaemia) and 0.47% (0.4 and 0.07% respectively for HbQ^{India} heterozygote and HbQ^{India} - β thalassaemia) respectively. The frequency of β thalassaemia trait is 14.49% among the Sindhis subgroups ranging from 9.25 to 20.47% indicating a clear wide variability in occurrence. The frequency is highest among the Sakhru subgroup. The distribution of HbD and β thalassaemia is heterogeneous across the subgroups (P<0.05). HbQ^{India} shows no significance heterogeneity, the occurrence is mostly confined to only Ladkana subgroup. Similarly, HbD gene is found to be restricted

peculiarly to the Ghotki subgroup. The accretion of the occurrence of all HbD traits among the Ghotki (11/31) is in consonance to their high preference to their preferential practice of subgroup endogamy [21].

The RBC indices and other hematological parameters for the abnormal hemoglobins and β thalassaemia traits identified are given in table 2. No significant difference in the mean values of HCT and MCHC among HbD, HbQ^{India} and β thalassaemia trait were observed. HbD and HbQ^{India} show within-normal range of RBC values in comparison to the elevated mean value in β thalassaemia trait. HbD shows comparatively higher mean hemoglobin percent than HbQ^{India} and β thalassaemia trait. Also the mean MCV and MCH values are higher in HbD and HbQ^{India} traits than the β thalassaemia trait indicating that HbD and HbQ^{India} are comparatively normocytic and normochromic. Though both Hb D and HbQ^{India} trait had presented similar in their osmotic

Fragility character, we found that about one fourth of all HbD trait cases with reduced osmotic fragility had hemoglobin level below 11.5 gm/dl and association of IDA was indicated by the algorithm based on their haematological indices. Similar observation of HbD trait with IDA was earlier reported by [4]. As anticipated, about 98% of all β thalassaemia traits cases show higher rate of persistence of red cells in hypotonic buffered saline solution. The mean HbF quantity was also higher among the β thalassaemia traits (1.23%) in comparison to that of HbD traits (0.61%). Interestingly, the HbQ^{India} traits had slightly elevated (although statistically insignificant, P>0.05) mean HbA₂ (3.5 \pm 0.49) than that of the HbD traits (2.66 \pm 1.14).

Mean quantity of HbD protein is 28.07% (13.4 - 42.08) as estimated only for the HbAD cases. The distribution of the quantity shows that nearly half of the HbAD samples fall within the range of 23 to 33%. In contrast, the mean quantity of HbQ^{India} is found to be significantly lower (9.86%) with a range of 7.82 to 11.7%. It is prominent that the lowest quantity of HbD quantity does not overlap with that of the highest quantity of HbQ^{India}. Differentiation of HbD band from HbQ^{India} is very difficult and requires cautious observation and skill especially in alkaline electrophoresis. However, the difference in the quantity of the two proteins definitely can be used to indicate the cases. HbD as reported earlier, is found in higher quantity than HbQ^{India} [4,18] which was confirmed by anion exchange HPLC. The findings of the present study agree to the earlier reports. All the cases with suspected HbQ^{India} in alkaline hemoglobin electrophoresis and quantified were cross checked for the characteristic chromatogram and quantity by CE-HPLC (Figure 1A).

Occurrence of HbD- β thalassaemia is conditionally rare [3]. The presence of a single band at the position of HbD often creates diagnostic

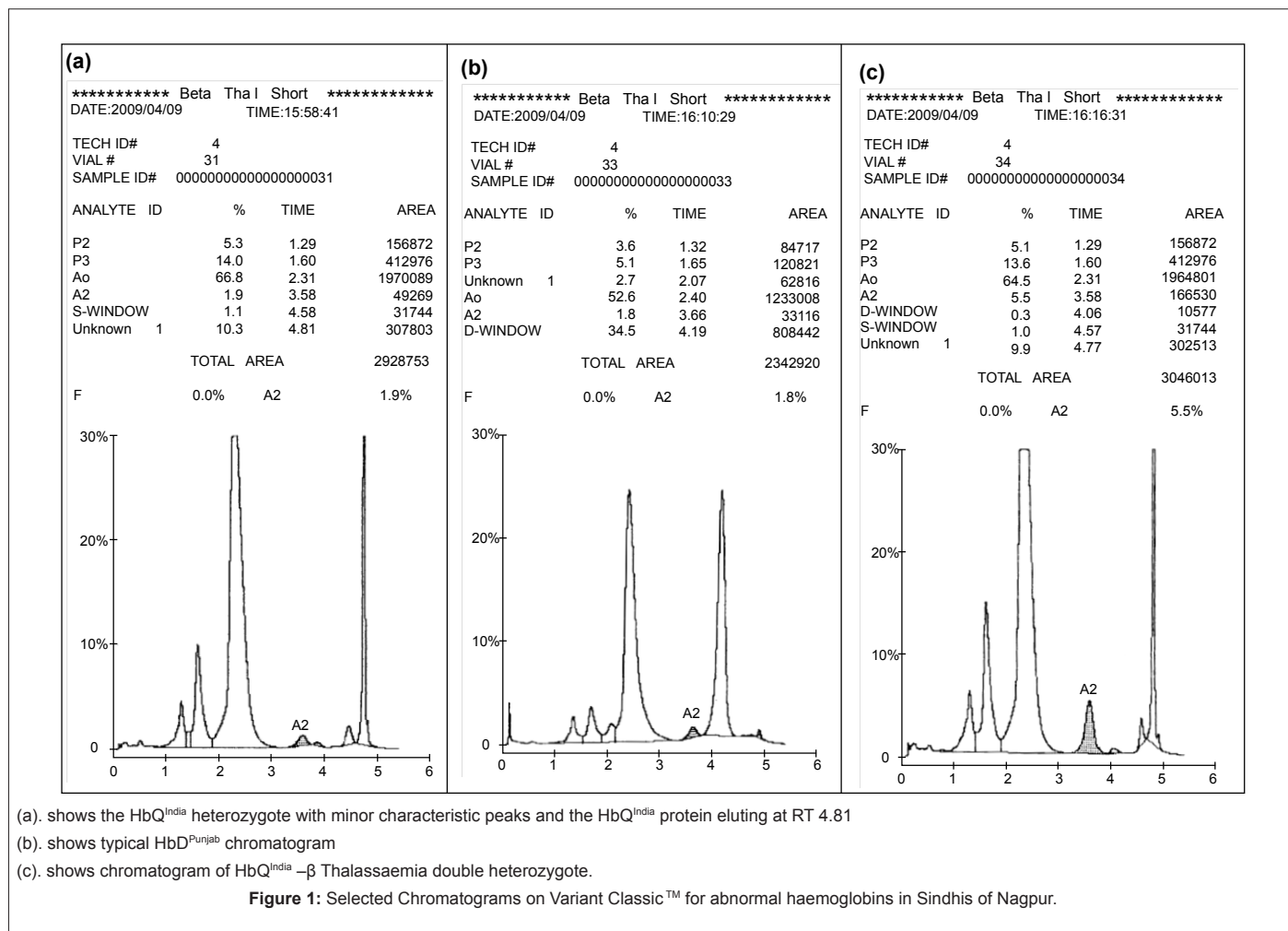
Sindhi Sub groups	β -Thalassaemia		HbD				HbQ		
	N	No.of β -thal trait (%)	N	No. HbD trait (%)	No. HbD homozygote	No. HbD- β thal (%)	N	No. HbQ trait (%)	No. HbQ- β thal (%)
Sahiti	227	21(9.25)	227	6 (2.64)	-	-	227	1 (0.44)	-
Ghotki	261	47(18.01)	261	10 (3.83)	-	1 (0.79)	261	-	-
Ladkana	477	72(15.09)	477	7 (0.79)	-	1 (0.79)	477	5 (1.05)	1 (0.21)
Sakhru	127	26(20.47)	127	1 (0.79)	-	1 (0.79)	127	-	-
Mixed Others*	180	25(13.89)	180	2 (1.11)	-	-	180	-	-
Unspecified	226	26(11.50)	226	2 (0.88)	-	-	226	-	-
Total	1498	217(14.49)	1498	28 (1.87)	-	3 (0.20)	1498	6 (0.40)	1 (0.07)

N= number tested

*1 case of HPPFH heterozygote in Chhapru Sindhis

χ^2 test of homogeneity: β -thal 12.85 (df 5), p < 0.05 significant
HbD 40.91 (df 15), p < 0.001 significant
HbQ 9.84 (df 10), p > 0.05 insignificant

Table 1: Distribution of the abnormal haemoglobins and β Thalassaemia status among the Sindhi subgroups of Nagpur city.



	Hematological indicators						% HbA2 Mean \pm SE	Osmotic Fragility		% Hb F	Abnormal haemoglobin Quantity in % Mean \pm SE
	Mean \pm SE values							Reduced (%)	Normal (%)		
	RBC	HGB	HCT	MCV	MCH	MCHC					
HbD ⁺ N = 28	4.49 \pm 0.21	12.06 \pm 0.63	34.28 \pm 1.79	73.34 \pm 3.54	25.87 \pm 1.30	33.96 \pm 1.60	2.66 \pm 1.14	8 (33.30)	16 (66.70)	0.61	28.07 \pm 1.67
HbQ ⁺ N = 6	4.49 \pm 0.21	11.30 \pm 1.30	34.22 \pm 2.33	76.58 \pm 6.33	25.24 \pm 2.64	32.80 \pm 1.13	3.50 \pm 0.49	2 (33.33)	4 (66.67)	ND	9.86 \pm 0.60
β thal N = 217	5.76 \pm 0.08	11.20 \pm 0.15	34.76 \pm 0.53	60.81 \pm 0.71	19.80 \pm 0.34	33.12 \pm 1.08	5.99 \pm 0.09	213 (98.20)	4 (1.80)	1.23	--
Individual cases of double heterozygotes for abnormal hemoglobin and β thalassaemia											
HbD- β thal ^s											
Case G1	5.05	11.90	35.20	69.70	23.60	33.80	4.50	Reduced		0.40	81.30
Case L2	4.04	12.40	30.50	73.00	27.70	38.00	7.80	Reduced		ND	88.00
Case S3	5.75	8.90	27.50	48.00	15.50	32.40	5.90	Reduced		2.00	92.00
HbQ- β thal ^s											
Case L4	4.12	9.40	32.40	77.10	22.40	29.00	5.50	Reduced		0.80	9.90

Figures in the parentheses () represent, percentage, ND: Not Detected, P: Present
 * Haematology could not be performed on one sample with HbAD, osmotic fragility could be performed on only 24 HbD subjects
 ** Hematology could be performed on only 5 samples out of total 6 HbQ^{India} heterozygote cases.
 # Indicates the presence of the abnormal hemoglobin
 \$ Samples are drawn from pooled population and are not unrelated subjects.
 HbF quantified by 1 minute denaturation method
 Students' 't' test between the quantity of HbD and HbQ^{India} = 10.26. P < 0.05

Table 2: Distribution of indicative parameters of abnormal haemoglobins and β -Thalassaemia among the Sindhi subgroups^s of Nagpur city.

confusions between HbD- β thalassaemia and HbD homozygotes. The similar spectrum of benign clinical presentation and hematological parameters make it difficult to differentiate between the genotypes. Advanced techniques like automated HPLC and DNA based mutation analysis are recommended for accurate detection of these abnormal conditions especially where both β Thalassaemia and HbD genes are found in higher frequencies. As many laboratories do not have HPLC, IEF or DNA based technologies at their reach, a careful observation of the quantity of HbD, HbA₂ and a follow up family study are definitely the useful tools for the diagnostic inferences to differentiate HbD- β thalassaemia from HbD homozygote in the population level.

In the present study, out of the three cases of HbD- β thalassaemia, one (S3) showed a single concentrated band at the position of HbS/HbD (92%) with elevated HbA₂ level (5.97%). The minimum presence of HbA band could not be quantified. The case was a 25 years old female (S3) and was severely anaemic (hemoglobin=8.9 gm/dl) with hepatosplenomegaly. The sickling test was found negative. Family screening revealed the mother of the subject to be a carrier for β thalassaemia and the father to be HbAD. Probable cause of the case with the single HbD band and no HbA may be attributed to the interaction of a β^+ type Thalassaemia mutation. The other two cases (G1 and L2) showed trace HbA and prominent HbD bands along with visible level of HbA₂. The quantity of HbD in these two samples are 81.3% and 88.0% respectively and had reduced MCV level, normal HGB and borderline or low MCH levels. RBC was increased except in only one case. All the three cases showed reduced osmotic fragility. The parents of all these cases were followed up to confirm the genotypes.

The only case HbQ^{India}- β thalassaemia is found among the Ladkana subgroup. The subject was severely anaemic (hemoglobin = 9.4 gm/dl), showed reduced MCV, MCH, MCHC and osmotic fragility, slightly smaller red cell volume but normal RBC count. The quantity of HbA₂ is estimated to be 5.5% and the HbQ^{India} concentration is 9.9% (Figure 1 C). The family study reveals the father of the subject to be a carrier of HbQ^{India} and the mother to be a carrier for β thalassaemia trait.

After initial suspicion from the hemoglobin electrophoresis pattern and quantity, all the HbQ^{India} cases were confirmed for the mutation by PCR-ARMS using a mutation specific primer (Figure 2). The presence of the mutation in all the 7 subjects indicate that the electrophoretic separation of HbQ^{India} with a quantitative measurement of the abnormal protein can be highly sensitive and specific where facilities like HPLC or DNA based technologies are not available or affordable.

Though the double heterozygotes for HbQ^{India}- β thalassaemia trait and HbD- β thalassaemia trait are nearly asymptomatic and occasionally anaemic, still in purview of evolutionary context and studies pertaining to the inter and intra ethnic variations; these hemoglobin variants definitely have important role [3]. Moreover, clinically severe form of genotypes with fatal patho-physiological episodes may result when HbD- β thalassaemia trait or HbQ^{India}- β thalassaemia trait subjects marry to either β thalassaemia trait or HbS trait subjects. On the other hand HbD and HbQ^{India} are found to be peculiarly localized to the populations of North-western Indian origin and more or less sporadic in the rest of the country, hence they need to be studied taking into consideration the ethno-geographic identity, related environmental and epidemiological factors.

Although the rapid technical advances have taken place in the diagnosis of beta thalassaemia, still hematological parameters are found to be suitable screening tests in Indian subcontinent populations, where a high prevalence of the disorder is reported. Hematological

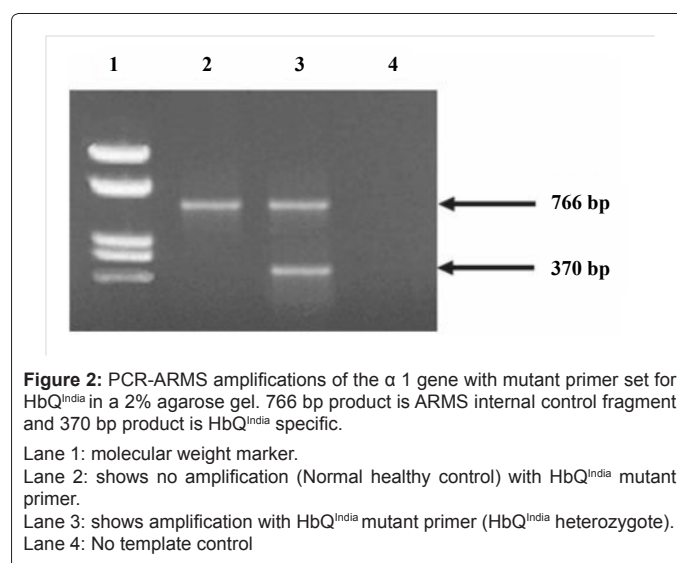


Figure 2: PCR-ARMS amplifications of the α 1 gene with mutant primer set for HbQ^{India} in a 2% agarose gel. 766 bp product is ARMS internal control fragment and 370 bp product is HbQ^{India} specific.

Lane 1: molecular weight marker.
Lane 2: shows no amplification (Normal healthy control) with HbQ^{India} mutant primer.
Lane 3: shows amplification with HbQ^{India} mutant primer (HbQ^{India} heterozygote).
Lane 4: No template control

presentation has been reported to vary significantly among the normal and the β thalassaemia carriers [22,23] as well as among different ethnic groups [24]. However, little is known about the variability among different types of β Thalassaemia across the ethnic divisions within and among populations in India [25]. In addition, suppressed production of β globin chain, leading to relative imbalance of the α/β globin chains in the red cells, are also found to have contributed to the differential variability in hematological parameters among individual and groups of subjects [26,27]. Importance of hematological presentation and red cell indices are however, not explored for their role in differentiating between individuals with structural hemoglobin variants. The present study shows no clear variability of hematological presentation among the abnormal hemoglobin variants in their heterozygote state. Although HbA₂% varied significantly between the HbD carriers the HbQ^{India} carriers, but neither the hematological presentation (complete blood count) nor the red cell osmotic fragility test seems to be appropriate to screen these abnormalities. However, the only distinct difference ($p < 0.05$) between these two groups of abnormal hemoglobins remains in their respective quantitation of the abnormal fractions. As the relative electrophoretic mobility of HbD and HbQ^{India} are practically indistinguishable during routine screening in alkaline medium, hence we suggest that the finding of a feeble band at the zone for HbS/HbD may be well considered as HbQ^{India}. Here mention can be made that the HbD fraction (both in heterozygote and in homozygote state) remains reportedly unaffected by the presence of a thalassaemia [28, 29].

We have found a 100% sensitivity and specificity of identification of such a variant band in the zone to be HbQ^{India} which is hitherto the first presentation in Sindhi population to have been confirmed against PCR based mutation study. Hence we recommend that in absence of gold standard techniques like DNA based mutation analysis or cost intensive HPLC, simple and robust techniques like alkaline hemoglobin electrophoresis and a cautious quantisation with family follow up can be the suitable option for detection of abnormal hemoglobinopathies, especially in an ethnically diverse country like India.

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