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Hemoglobin E Prevalence among People Residing in Malaria Areas

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ABSTRACT

Background: Malaria can infect erythrocytes and hence cause different pathogenesis episodes leading to death mostly in pregnant women and children under the age of 5 years. The selective pressure of these parasites leads to the production of new human genetic diseases. The most prevalent genetic alterations in the human genome are thalassemia and hemoglobinopathies (Hb E, Hb S), which are recognized throughout the world, including Saudi Arabia.

Methods: From May 2018 to August 2019, 13972 Saudi citizens from King Fahd Central Hospital and premarital facilities in the Saudi Arabian province of Jazan participated in this study. This study aims to compare the prevalence of Hb E and other hemoglobinopathies in positive versus negative cases of malaria. So, CBC, malaria test, Hb-electrophoresis, and molecular study were investigated.

Result: For thalassemias and Hb disorders, 36% with abnormal Hb (47% of them) carried Hb S in their blood, 37% with α-thalassemia, 11% for β-thalassemia and 4% of Hb E. Significant variations in CBC parameters were observed in Hb E patients. There was significant decrease in MCV, MCH and MCHC and slightly increase in WBCs, RBCs, RDW and PLT as compared to controls.

Key words: Consanguineous marriage, Hb A, Hb E, Hemoglobinopathies, Jazan, Malaria.

INTRODUCTION

Malaria is a disease in tropical countries caused by a bite of an infected female Anopheles mosquito. Humans can be infected by only a specific type of *Plasmodium* species including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale,* or *P. knowlesi*. The most severe type is *P. falciparum* with a high mortality rate (Garcia, 2010). All the clinical symptoms of malaria that result from *P. falciparum* infection of human red blood cells (RBCs), which intensifies anemia and malaria in the cerebral stage, are caused by merozoites. Therefore, the clinical manifestations of malarial infections are associated with the rupture of infected RBC during the blood stage of the parasite's life cycle (Maier *et al*., 2009).

Since hemoglobin (Hb) is an important component in RBCs, malaria parasites feed on Hb for survival and reproduction. Any synthetic or structural molecular defects in Hb called hemoglobinopathies that result from a mutation in one or more Hb genes. The mutated genes coded either the proteins that build up the Hb molecule or that are used for globin chain synthesis or regulating synthesis. The production of these mutations leads to qualitative or quantitative defects in Hb synthesis. The normal rate of Hb synthesis or semi-normal is known as a qualitative defect that is characterized by structure alternation of amino acid sequence in globin chains of Hb molecules. On the contrary, any reduction in the rate of globin chain synthesis of Hb in the absence of an alteration in its amino acids sequence absolutely will lead to quantitative defects called Thalassemias. Anemia is produced in response to the shortage synthesis of Hb amount and enhances the appearance of un-mutated other Hb to replace anemia. According to these variations, the hematological doctors classified them into two main categories, structural (qualitative) defect, and synthetic (quantitative) defect, thalassemia. However, many doctors mention hemoglobinopathies for structural defects only (Weatherall, 2010).

Thalassemia is an inherited monogenic Hb disorder that is classified into two major categories, according to a defect

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in Hb synthesis (thalassemia α/β), or in Hb structure such as (Hb S, C and E) variants of Hb (Weatherall, 2010). This monogenic disorder in Hb results in around 7% of carriers worldwide (Old *et al*., 2005). Structural variants of Hb resulted from a single replacement of amino acid sequences in the chain of α or β. However, these variants are considered mild for any disturbance in Hb functions and stability leads to disorders with clinical features (Voskaridou *et al*., 2001).

Until now, Sickle Cell Disease, or the heterozygous form combines with the gene of Hb C to produce (Hb S/C) disorder, which is characterized to be milder with remarkable public health problems. The commonest universal hemoglobin-variant is (Hb E). Both homozygous and heterozygous forms are milder. However, when the heterozygous form combines with β-thala to output Hb E/β-thala, it becomes highly common with very serious health problems in many regions of Asian countries (Weatherall *et al*., 2006).

It has been reported that SCA, Thala, Hb E, and other variants are common in regions that suffered from malaria in the past. Moreover, social factors play important roles that lead to the wide spread of hemoglobinopathies through the population structure activities such as ethnical intermixing (Thein, 2005). Therefore, to understand the correlation between hemoglobinopathies and malaria infection, this proposal aims to study the prevalence of one of the hemoglobinopathies which is Hb E among people in the residing area and link this at phenotypic and genotypic levels.

MATERIALS AND METHODS

Identification and screening of the patients who are eligible for our study were done at King Fahd Central Hospital, Jazan in Saudi Arabia for hematological studies such as CBC, Hb Electrophoresis and Malaria Screening. Genetics Study carried out at the National Center for Disease Prevention and Control. The consent form was signed by patients and controls with IRB No: CAMS 050-3839. The study sample size was set to them. Data obtained from patients and their parents, and guardians and available in clinical history included information about age, malarial infection history and relative marriage and degree of consanguineous relative marriage or from the pre-marriage clinic.

This case-control study was performed over ten ten-month period from June 2018 to March 2019 in patients who carried HbE in their erythrocytes' Hb or malaria parasites in their blood. All study participants provided written informed permission that was included in the study. The study was approved by the ethical committee of the College of Applied Medical Sciences (CAMS, KSU) with IRB No: CAMS 050-3839.

The sample number of the present study was 13972. The samples were collected with two Ethylene Diamine Tetra-acetic Acid (EDTA) vacutainer tubes, 5ml in each tube. The first tube for the hematological study involved an automated complete blood count (CBC), malaria test screening (microscopic and rapid test) and Hb electrophoresis screening. The second one is used for Sanger sequencing-based molecular analysis.

Automated complete blood cell count (SYSMEX, XN-1000), hematological analyzer machine used in diagnostic and clinical laboratories for the identification, quantitative and proportion of EDTA whole blood samples *via* electrical resistance, dye application and laser light scatter. Principle flow cytometric technique used to measure the size, shape, and concentration of blood cells (RBCs, WBCs and PLT Counts) and blood indices, Hb%, Hematocrit or Packed Cell Volume (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Cell Distribution Width (RDW), Mean Platelets Volume (MPV) and Differential of WBCs.

The malaria diagnosis can be obtained in 2 ways: (1) identification of malarial parasites by using microscopic technique. (2) detection of Antigens/products of parasites in peripheral blood.

Direct observation of parasites through stained thick or thin peripheral blood smear or quantitative buffy coat test was performed. A simple technique was carried out by using a light microscope (thick and thin smear), slides were stained with Romanosky (Giemsa's) stain and examined for intra-erythrocyte parasites. A thick smear is 20-40% more sensitive than the thin one of detected Plasmodium (10-50 trophozoites/µl). A thin smear was used for the morphological study and identification of the parasite involving mixed infection, parasitemia, schizont and hemozoin in monocytes. Immunochromatographic test by capturing Ags of the parasite in peripheral blood through monoclonal or polyclonal specific Abs for parasite Ags. The test was used to target (1) Histidine-rich protein 2 of P. falciparum, which is a soluble protein for asexual stage and gametocytes. (2) Pan-malarial *Plasmodium* aldolase is an enzyme of glycolytic pathway for mixed infection Pf/Pv. (3) Parasite lactate dehydrogenase is a soluble enzyme for glycolytic in both sexual and asexual stages. Rapid diagnostic tests such as dipstick, strip, pad, well, or cassette. Done by applying 2-50 µl of blood, mix with buffer containing hemolyze and specific Abs labeled with a visual detectable marker.

The fully automated VARIANT II Hb testing system is designed for high volume, featuring positive specimen identification, LIS interface and complete, ready-to-use test kits for precise, accurate results. The machine is compatible with a barcode, barcode reader and closed or diluted sample tubes. Chromatographically, an EDTA whole blood specimen is injected into the analysis process. It came with 2 modules, the VARIANT II Chromatographic Station (VCS) and the VARIANT II Sampling Station (VSS). Clinical Data Management (CDM) software computer program used to control the Variant II System. The VARIANT II used the principle of High-Performance Liquid Chromatography (HPLC).

Capillary 2 Sebia Flex Piercing capillary Hb E is an automated Hb electrophoresis that enables to identification, detection and well-elution (separation) of Hb Fractions and

variants. By automation processing, achieves all sequences to release accurate, precise, and quantified of the maximum numbers of Hemoglobinopathies (Hb S, Hb C, Hb E, Hb D, Hb F and Hb A,) also Hb H and Bart's.

Principle assay is carried out on the capillary electrophoresis; Hb fractions are well-separate in silica capillary in the presence of a great voltage in alkaline buffer solution by electro-osmatic flow and electrophoretic movement. Also, blood from the Hb E patient was extracted and purified by using the GeneJet Genomic DNA Purification Kit with catalog number: K0721 from Thermo Scientific. This kit is rapid and efficient purification with more quality gDNA.

ARMS is a fast and simple technique used in the detection of single-point mutation and small deletion. Originally, ARMS was used for allele-specific amplification to determine the specific genotype in one-step PCR. Standard ARMS-PCR utilizes 2 reactions complementary with 3 primers. One primer for specific whole sequence DNA in 2 reactions. The specificity of alleles is given through the dissimilarity in the bases of the 3′ terminal of different primers compared to either a whole sequence of DNA or a mutant one. In allele-specific primer, the normal primer will refractory to PCR on mutant sequencing of DNA. Allele-specific Hb E primer t-ARMS PCR was then discriminated by gel electrophoresis.

Data was recorded into a database system using a double data entry format and analyzed with Microsoft Excel 2010 software. Normally distributed continuous variables were summarized as mean and compared using the student's t-test with a P-value of less than 0.05 being statistically significant.

RESULTS AND DISCUSSION

Based on the results of hematological and electrophoresis tests conducted on 13972 Saudi participants in the Jazan region from May 2018 to August 2019, 8843 participants had normal hemoglobin (63.3%) as shown in Table (1) and 5129 participants had abnormal hemoglobin (36.7%) (Table 2). Particularly, 37.3% of these abnormal Hb reported with α-thalassemia, 11.2% with β-thalassemia and Hb E were 230 cases which reported 4.5% of total Abnormal Hb. Other abnormal Hbs (Hb H represented 0.2%, while Hb C, Hb D, Hb O Arab, and Hb HPFH) represented 0.0% (Table 3). It is reported that erythrocyte defense against malaria parasite

Table 1: Prevalence of hemoglobinopathies among people in malaria area in jazan.

	Hospital	Pre-marriage	Total	%
Normal	3660	5183	8843	63.30
SCT	749	799	1548	11.08
SCD	815	38	853	6.11
β-Thala	463	109	572	4.09
α-Thala	1038	873	1911	13.68
Hb E	66	164	230	1.65
Hb H	9	1	10	0.072
Hb C	1	0	1	0.007
Hb D	1	1	2	0.014
Hb O Arab	1	0	1	0.007
Hb HPFH	1	0	1	0.007

CT: Sickle cell trait, SCD: Sickle cell disease.

Table 2: Summary of abnormal HB E finding.

Prevalence of abnormal hb from total collected samples		
Hb	Total	Percentage
Normal	8843	(63.30%)
Abnormal	5129	(36.70%)
Prevalence of malaria infection in hemoglobinopathies patients		
Malaria Infection	Total	Percentage
Malaria -ve	5060	(98.60%)
Malaria +ve	69	(01.40%)
Prevalence of abnormal hb in malaria +ve samples		
Types	Total	Percentage
Hb S	18	(26.10%)
β/S	08	(11.60%)
β-thala	11	(15.90%)
α-thala	29	(42.00%)
Hb E	03	(04.40%)

invasion and development may interact to explain the high occurrence of Hb mutations in malaria locations (Chotivanich *et al*., 2002). The polymorphism balance in the defense mechanism is caused by hemoglobinopathies and thalassemia. Saudi Arabia experiences severe health issues such as Hb abnormalities, just like other nations with endemic malaria (Al-Suliman, 2006; Alsaeed *et al*., 2018).

Malaria screening performed on this abnormal Hb group revealed that 69 (1.4%) of the samples tested positive for the disease, whereas 5060 (98.7%) tested negative (Table 2). In this set of positive results, there were 26.1% Hb S, 11.6% thala/s, 15.9% β-thalassemia, 24% α-thalassemia and 4.4% Hb E. The mean age of the 230 Hb E participants was 27.4 years, with a range of 1-66 years, comprised 117 women (50.9%) and 113 men (49.1%). 55 people (23.9%) were single, while 175 (76.1%) were married. Patients' reports on consanguineous marriage (41.7%), family history of hemoglobin E (64.8%) and history of malaria (21.3%) (Table 4). The electrophoresis report of Hb E patients showed 76.5% (176 patients) were heterozygote Hb A/E whereas 6.5% were homozygote Hb E/E. The combined Hb E was reported as 9.1% Hb S/E, 17.4% α-thala/E and 28.3% with β-thala/E (Table 4).

Hb E is an autosomal recessive β-gene, where a single point mutation at position 26 caused a disease (Chotivanich *et al*., 2002).

There was a significant change between WBCs, PLT, and RDW in Hb E patients and control (Table 5). As shown in Table (6), the difference between the PLT means of Hb E patients and controls was only marginally significant. RDW mean for patients was 16.3%, compared to 13.9% for controls, with a high significance detected by RBCs, Hb, Hct, MCV, MCH and MCHC as being extremely significant.

There was no Hb E found in healthy people, the mean Hb E level reported in patients was high (28.61±17.79%), while the mean Hb S level was 04.96±16.40%. Patients' Hb A, average was 03.56±0.93%, while controls' average was 02.96±0.15%, with a highly significant difference ($P<0.0001$) (Table 7).

Consanguineous marriage, which accounts for up to 50% of marriages in Saudi Arabia, is one of the major factors contributing to the spread of genetic abnormalities and the risk of inheritance among some families (Alsalem *et al*., 2022). Hemoglobinopathies have a significant impact on patients, their families and the government due to an increase in mortality and the associated financial, medical, psychological and social burdens (Zaini, 2016). Therefore, early detection and accurate diagnosis are crucial for controlling and preventing many illnesses (Ashley-Koch *et al*., 2000). Greater than in the western, northern and central regions, the Jazan region has the highest prevalence of Hb E and other hemoglobinopathies (Gosadi *et al*., 2021;

Table 3: Hemoglobinopathies prevalence in hospitals and pre-married centers.

Sources	SCT	SCD	β-Thala	α-Thala	Hb E	Hb H	HPFH	Hb C	Hb D	Hb O Arab	Total
Hospitals	749	815	463	1038	66	9	1	1	1	1	6804
PMSCs	799	38	109	873	164	1	0	0	1	0	7168
Total	1548	853	572	1911	230	10	1	1	2	1	13972
Percentage	30.1%	16.6%	11.2%	37.3%	4.5%	0.2%	0.0%	0.0%	0.0%	0.0%	100%

Table 4: Clinical characteristics and zygosity of HB E patients.

Data	Gender		
	Male	Female	All
No of patients	113 (49.1%)	117 (50.9%)	230 (100%)
Age	2-66 Years	1-64 Years	1-66 Years
Age (Mean)	28.7 Years	26.1 Years	27.4 Years
Married	89 (38.7%)	86 (37.4%)	175 (76.1%)
Unmarried	24 (10.4%)	31 (13.5%)	55 (23.9%)
consanguinity	42 (18.3%)	54 (23.5%)	96 (41.7%)
Hb E family fistory	70 (30.4%)	79 (34.3%)	149 (64.8%)
Malaria history	33 (14.3%)	16 (7%)	49 (21.3%)
Positive malaria result	3 (1.3%)	0 (0%)	3 (1.3%)
		Zygosity	
A/E	63 (27.4%)	66 (28.7%)	129 (56.1%)
E/E	02 (0.8%)	0.0 (0%)	02 (0.8%)
S/E	09 (3.9%)	10 (4.3%)	19 (8.3%)
β-thala/E	26 (11.3%)	19 (8.3%)	45 (19.6%)
α-thala/E	11 (4.8%)	23 (10%)	34 (14.8%)
HPFH/E	01 (0.4%)	0.0 (0%)	01 (0.4%)

Alenazi *et al*., 2015). Memish and his colleagues (2011) published an increased number of at-risk marriage groups in 2011 and refused to consult on marriages that attempted to avoid or control the prevalence of diseases in future generations among any age group or community. SCA and β-thalassemia are more common in Jazan than in the northern, western, and central regions, but they are still less common than in the eastern region (Akhter *et al*., 2021). Jazan has the highest incidence and prevalence of Hb E (Alenazi *et al*., 2015; Memish and Saeedi, 2011). Based on data from the current study, there are attempts to clarify differences between regions in hemoglobin diseases including Hb E. Some factors play an important role in increasing the prevalence and incidence of these diseases (Olivieri *et al*., 2011).

Here, 50.9% of the participants in the study were female patients. When compared to the control mean of 6.1 × 10y / L for WBCs and 299.2 × 10y /L for PLT (Table 8). RDW mean for patients was 16.4%, compared to 14.1%. Patients' average Hb E concentration was 25.16±13.95%, while healthy volunteers had no Hb E. Additionally, patients' Hb S means were 5.19±16.95% without being detected in healthy participants. Hb A and Hb A, in the patient's blood were significantly different from the control (Table 9).

Utilizing ARMS-PCR and an allele-specific Hb E primer with a 462 bps, a genotyping investigation was conducted (Table 10). The bands on the electrophoresis of 2% agarose gel reflected the entire β-globin gene with 691 bps. 276 bps for the normal Hb E allele and 462 bps for the mutant Hb E allele (Fig 1). According to Sanger

Table 5: Clinical characteristics and zygosity of HB patients.

			Complete blood count		
WBCs × 10^9/L	6.83	2.3	5.96	1.77	0.0005
RBCs × 10^{12}/L	5.24	0.85	5.05	0.57	0.008
Hb g/dL	12.33	2.5	14.31	1.56	<0.0001
Hct %	38.07	7.18	42.14	4.21	<0.0001
MCV fL	72.37	8.21	83.69	4.6	<0.0001
MCH pg	23.3	3.01	28.42	1.76	<0.0001
MCHC g/dL	32.21	2.78	34.01	1.31	<0.0001
RDW %	16.33	3.85	14.01	0.98	<0.0001
PLT × 10^9/L	314.72	86.52	288.8	64.64	0.003
			Hb Sub-types		
Hb A %	63.25	24.84	97.02	0.13	<0.0001
Hb F %	1.97	6.8	0.02	0.08	0.005
Hb S %	5.05	16.62	-	-	-
Hb E %	26.83	15.97	-	-	-
Hb A_2 %	3.46	0.84	2.97	0.13	<0.0001

Table 6: Clinical findings in male HB patients and controls.

Parameter	Hb E patients	Control	Tow-tailed P-value
		Complete blood count	
WBCs × 10y /L	06.98±02.51	05.88±01.94	0.0025
RBCs × 10^{12}/L	05.47±00.93	05.34±00.48	0.0006
Hb g/dL	13.37±02.78	15.24±01.11	<0.0001
Hct %	40.34±07.99	44.50±03.29	<0.0001
MCV fL	73.21±08.74	83.48±04.77	<0.0001
MCH pg	24.00±03.20	28.63±01.82	<0.0001
MCHC g/dL	32.87±03.08	34.35±01.36	<0.0001
RDW %	16.26±04.54	13.99±00.98	0.00012
PLT × 10^9 /L	298.9 ±76.66	282.4±58.59	0.0125
		Hb Sub-types	
Hb A %	60.61±26.77	97.04±00.15	<0.0001
Hb F %	03.29±09.30	00.01±00.05	0.007
Hb S %	04.96±16.40	-	-
Hb E %	28.61±17.79	-	-
Hb A_2 %	03.56±00.93	02.96±00.15	<0.0001

Values are means±SE.

sequencing, the mutation replaced glutamic acid with lysine (GAG→AAG).

Through the study; 5 forms of Hb E zygosity were recorded, Hb A/E (76%) with no signs and symptoms. The nature of thalassemic features of Hb E is due to the replacement of glutamic acid by lysine on codon 26 of the β-globin chain (Balgir, 2007). This mutant reduced the production of variant β-globin and changed the normal migration by a cryptic splice site activation (Fucharoen and Weatherall, 2012).

The result showed 56% heterozygous of Hb E, 1% homozygous and 43% Hb E compound with other Hb variants. The microcytosis and hypochromic morphology of Hb E erythrocytes reflect the reduction of MCV and MCH values in patients, which were reported as 72 fL and 23 pg respectively. That provides the thalassemic features of Hb E disorder with microcytic hypochromic anemia.

This study demonstrated that the Jazan region of the Kingdom of Saudi Arabia had a higher prevalence of hemoglobinopathies (particularly Hb E) than other regions. In Hb E patients, we observed a considerable rise in Hb A, which exacerbates the disease and causes complications. Moreover, in certain families with an increase in the rate of consanguineous marriage, we also found significant incidences of Hb E. In some regions where malaria infection was formerly endemic, we also observed a rise in the prevalence of Hb E. This also shows the need for thorough studies, such as the clinical epidemiological status of Hb E prevalence in various Saudi Arabian regions.

Table 7: Clinical findings in male hb e patients and controls with P-value<0.005.

Parameter	HB E patients	Control	Tow-tailed P-value
		Hb Sub-types	
Hb A %	60.61±26.77	97.04±0.15	<0.0001
Hb F %	03.29 ±09.30	0.01±0.05	0.007
Hb S %	04.96±16.40	- -	-
Hb E %	28.61±17.79	- -	-
Hb A_2 %	03.56±0.93	02.96±0.15	<0.0001

Values are means±SE.

Table 8: Clinical findings in female HB E patients and controls.

Parameter	HB E patients	Control	Tow-tailed P-value
		Complete blood count	
WBCs × 10^9/L	6.67±2.09	06.10±1.45	0.049
RBCs × 10^{12}/L	05.01±0.69	04.57±0.33	<0.0001
Hb g/dL	11.29±1.59	12.79±0.80	<0.0001
Hct %	35.79±5.43	38.29±2.25	<0.0001
MCV fL	71.56±7.66	84.03±4.36	<0.0001
MCH pg	22.62±2.66	28.08±1.62	<0.0001
MCHC g/dL	31.56±2.28	33.45±1.01	<0.0001
RDW %	16.40± 3.10	14.05±1.01	<0.0001
PLT × 10^9/L	330.2±93.02	299.2±73.09	0.018
		Hb Sub-types	
Hb A %	65.7±22.76	96.99±0.09	<0.0001
Hb F %	00.7 0± 1.40	00.04±0.11	0.006
Hb S %	05.19±16.95	-	-
Hb E %	25.16±13.95	-	-
Hb A_2 %	03.36±00.75	02.97±0.09	<0.0001

Table 9: Clinical findings in female Hb E patients and controls, with P-value<0.005.

Parameter	Hb E patients	Control	Tow-tailed P-value
		Hb Sub-types	
Hb A %	65.70±22.76	96.99±0.09	<0.0001
Hb F %	00.70±1.40	00.04±0.11	0.006
Hb S %	05.19±16.95	- -	-
Hb E %	25.16±13.95	- -	-
Hb A_2 %	03.36±0.75	02.97±0.09	<0.0001

Table 10: Hb E primers used in genotypes study.

Parameter	Hb E Patients	Control	Tow-tailed P-value
Hb E mutation	HbE-OF	CCC TTC CTA TGA CAT GAA CTT AAC CAT A	691
	HbE-OR	GGC TGT CAT CAC TTA GAC CTC AC	
	HbE-IF (Normal)	ACCAACCTGCCCAGGGCaTC	276
	HbE-IF (Mutant)	GTGAACGTGGATGAAGTTGGTGtT	462

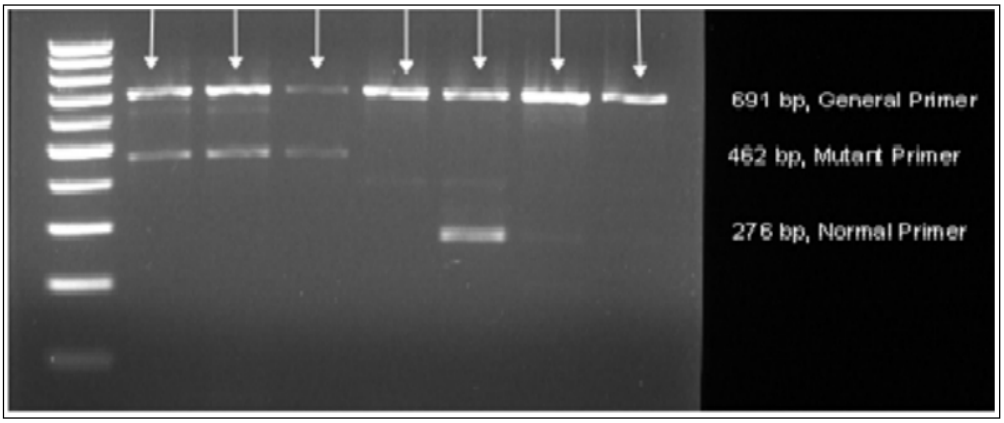

Fig 1: "2%" Agarose gel electrophoresis of β-globin gene and Hb E, the whole β-globin gene appeared in 6911 bps, Hb E band appeared in 462 bps.

CONCLUSION

Jazan had a higher prevalence of hemoglobinopathies (particularly Hb E). There was a significant difference in CBC parameters between Hb E and controls. In Hb E patients, we observed a considerable rise in Hb A, , which exacerbates the disease and causes complications. This shows the necessity for extensive research on Hb E prevalence in various countries and thorough clinical epidemiological research on Hb E.

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Data availability statement

All the datasets generated or analyzed during this study are included in this published article.

Conflict of interest

The authors declare no conflict of interest.

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