

# Prevalence of Beta-Thalassemia Trait Among Students of the University College of Science and Technology-Palestine

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**Abstract:** Thalassemias are a group of recessively inherited genetic disorders mostly common in the Mediterranean, the equatorial and near equatorial regions of Africa and Asia. Large number of mutations cause abnormal globin gene expression and result in complete absence or reduction of globin chain synthesis which lead to thalassemia.  $\beta$ -thalassemia is the result of deficient or absent synthesis of  $\beta$ -globin chains, leading to excess  $\alpha$  chains. This study was conducted in order to determine the prevalence of  $\beta$ -thalassemia trait among students of University College of Science and Technology (UCST) in Khan Younis, Gaza Strip-Palestine. Allele Specific PCR (ASPCR) was used to determine the intervening sequence IVSI-6 (T→C) and IVSI-110 (G→A) mutations. The study population consisted of 348 subjects recruited from the UCST (144 males: 41% and 204 females: 59%). Blood samples were collected in EDTA tube for CBC. Mentzer index was calculated for all samples. Blood film was done and stained using Giemza stain. DNA was isolated from 12 samples that had normal RBCs and low MCV and whose Mentzer index was >13. These samples were subjected to Allele Specific PCR in order to detect IVSI-6 (T→C) and IVSI-110 (G→A) mutations. The hemoglobin level in females was found to be about 20% less than the level recorded in males ( $11.40 \pm 1.01$  vs  $14.30 \pm 0.79$  g/dl). The results also revealed that there were significant differences in all measured CBC parameters and indices between males and females except that for WBC, RBC, MCHC and PLT. IVSI-6 (T→C) mutation was detected only in two samples and both were heterozygous. IVSI-110 (G→A) mutation was not detected in this study. The present results showed that the case of  $\beta$ -thalassemia carrier have normal RBC, MCH, Hb and normal Mentzer index which could be missed in routine screening test.

**Keywords:**  $\beta$ -thalassemia Trait, Allele Specific PCR (ASPCR), Mentzer Index

## 1. Introduction

The term thalassemia is derived from the Greek, *thalassa* (sea) and *haima* (blood). Thalassemia refers to a group of inherited blood disorders. They occur most commonly among Asian people especially Southeast Asian, Southern Asian and people of Mediterranean ancestry [1] which was recognized in 1925 for the first time by Tomase Cooley and Lee [2]. The fundamental abnormality in thalassemia is impaired production of the globin chain. Thalassemia can be classified into alpha and beta thalassemias by their clinical manifestations and their genetic background.  $\beta$ -thalassemia is

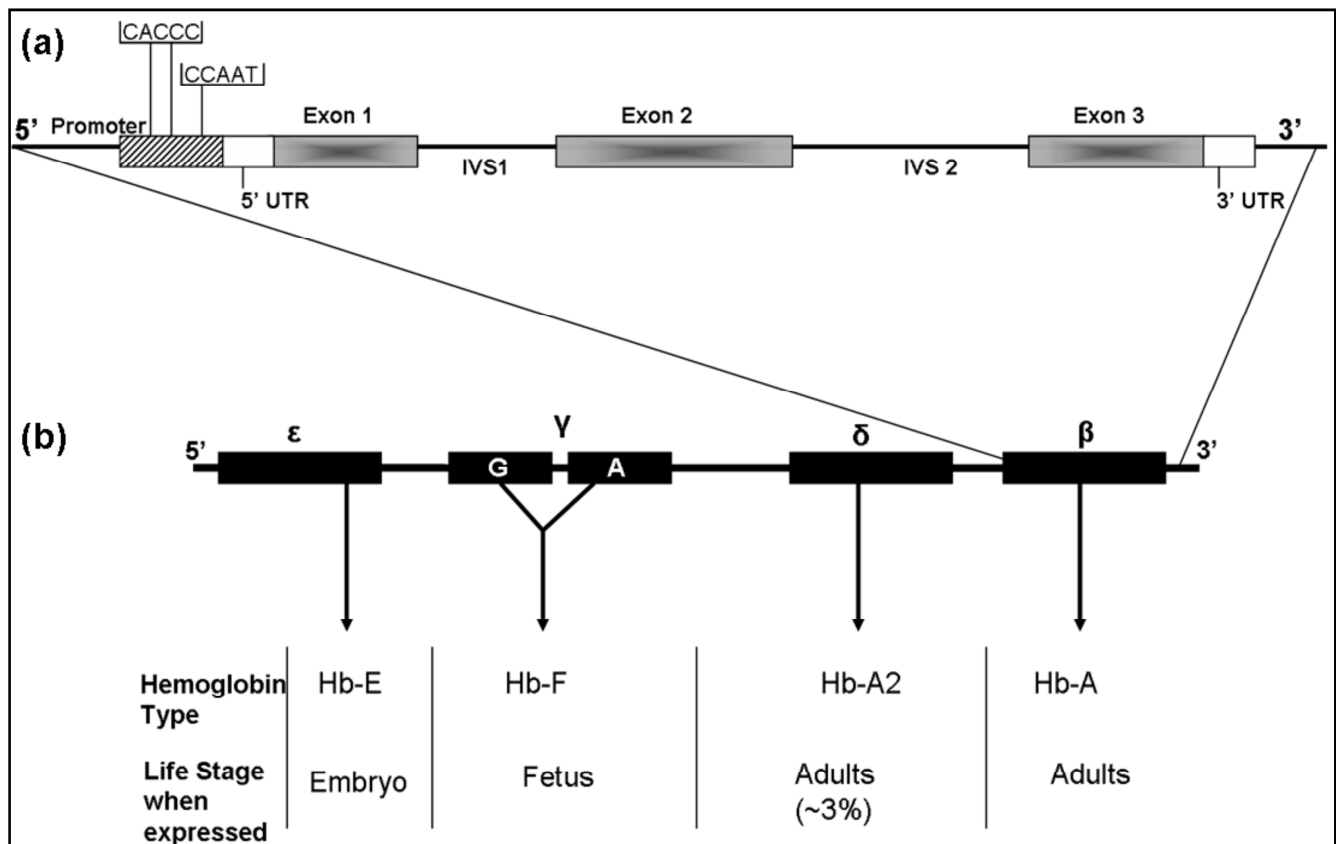
a common genetic disorder caused by mutations in one or more of the  $\beta$ -globin gene loci that result in reduced  $\beta$ -globin production [3].

The human  $\beta$ -globin gene is part of a multigene family that is expressed in a tissue-specific and developmentally specific manner [4]. The  $\beta$ -globin chain is encoded by the *HBB* gene, which spans 1.6 kb on the short-arm of chromosome 11 (11p15.4) [5]. The genomic sequence of *HBB* contains three exons, two Intervening Sequences (IVS1 and IVS2) and the 5' and 3' untranslated regions (UTRs), as shown in Figure 1. The *HBB* gene is regulated by a 5' promoter region that contains the classical TATA,

CAAT and duplicated CACCC boxes (Figure 1a). Upstream of the  $\beta$ -globin cluster is another regulatory element for *HBB*, namely the locus control region ( $\beta$ LCR).

The expression of individual globin genes is governed by

direct physical interaction between the  $\beta$ LCR and globin promoters, mediated by the binding of tissue-restricted and ubiquitous transcription factors [6].



**Figure 1.** Schematic representations of  $\beta$ -globin locus: (a) the  $\beta$ -globin gene with 3 exons and the two IVS (b) the  $\beta$ -globin locus that resides on chromosome 11p15.4 [7].

Recently, more than 200 different mutations have been detected affecting the diverse levels of  $\beta$ -globin gene expression and cause  $\beta$ -thalassemia. These mutations are not uniformly distributed, but have a geographical specificity and racial origin, as each is characterized by the presence of few common mutations and variable numbers of rare ones [5]. Mutations such as nucleotide substitutions and/or frame shifts of the insertion/deletion type have been reported to interfere with the transcription of the  $\beta$ -globin gene, splicing procedures and translation of  $\beta$ -globin gene mRNA, resulting in either absence or reduction of synthesis of  $\beta$ -globin chains [6].

In addition to the direct effects of reduced  $\beta$ -globin synthesis, many of the symptoms of this disorder appear to be consequences of the resulting cytotoxic buildup of free  $\alpha$ -globin. Free  $\alpha$ -globin is highly unstable and readily precipitates, damaging membrane structures and triggering the apoptotic cell death of erythroid precursors. It has been suggested that such an excess of  $\alpha$ -chains is the source of oxidative stress [8, 9]. Clinically,  $\beta$ -thalassemia are divided into  $\beta$ -thalassemia major which is homozygous state and  $\beta$ -thalassemia trait or minor which corresponds to heterozygous state [10].

The significant clinical manifestations and complications commonly associated with  $\beta$ -thalassemia major are not seen in  $\beta$ -thalassemia minor. That is, most affected individuals are asymptomatic or characterized clinically by mild anemia with persistent microcytosis that usually goes unnoticed [11].

## 2. Methodology

Blood samples were collected from 348 subjects recruited from students of the University College of Science and Technology Khan Younis, Gaza Strip (UCST). Each sample was collected in EDTA tube. The EDTA samples were kept at 4°C and were used within 24 hours for Complete Blood Count (CBC), DNA extraction and subsequent PCR analysis. CBC was performed using Orphee Automatic Hematology Analyzer. Mentzer index was calculated for all samples by dividing MCV over RBC count. A blood film was prepared from fresh blood sample collected in EDTA tube to evaluate abnormalities in shape and/or size of samples with Mentzer index (MI) < 13.

Genomic DNA was extracted from blood samples that have low MCV, normal RBCs and MI below 13 using Wizard DNA extraction kit (Promega, USA) following the manufacturer's

instructions from fresh EDTA whole blood cells.

PCR was performed using the primers described by Newton *et al.* [12]. The nucleotide substitution in the primers was focused on nucleotide position of IVSI-6 and IVSI-110. Four allele specific PCR reactions were carried out for each sample. Primer nucleotide sequences, the primer combination for each reaction and amplification product length are listed in Table 1. For each reaction, 10 µl PCR master mix (Promega, USA), 2 µl deionized water, 2 µl (100 ng) DNA template and 0.5 µl of each allele specific primers (5 pmol) in one micro-tube were mixed. PCR was performed in a thermal cycler (Biometra, Germany). In the first reaction, IVSI-6 (T→C) primer pair was used to amplify the 449 bp fragment to detect the normal alleles. The IVSI-6 (T→C) mutant primer pair in the second reaction was selected to amplify the 286 bp fragment for mutant allele detection. In the third

reaction, the IVSI-110 (G→A) primer pair was selected to amplify the 419 bp product to detect normal DNA sequence at this point. Finally in the fourth reaction, the mutant IVSI-110 (G→A) primer pair was applied to produce the 419 bp fragment to detect the point mutation at this position. In each PCR, specific primers with the sequences:

forward: 5'-GAGTCAAGGCTGAGAGATGCAGGA-3' and reverse: 5'-CAATGTATCATGCCTCTTGCACC-3') were used as internal positive control that produces a 861 bp fragment.

The cycling conditions were: an initial denaturation for 3 min at 95°C, followed by 35 cycles of 60s at 94°C, 60s at 60°C, 90s at 72°C and an additional 3 min at 72°C for final extension. Upon completion of PCR, the products were analyzed by electrophoresis on 3% ethidium bromide stained agarose gel.

**Table 1.** PCR primers sequence used for  $\beta$ -globin gene mutation.

| PCR reaction | Primer pair  | Fragment size (bp) | Mutation name   |
|--------------|--|--------------------|-----------------|
| 1            | Forward: 5'AGTTGGTGGTGAGGCCCTGGGCAGGTTGGT3'<br>Revers: 5'CCCTTCCTATGACATGAACTTAA3' | 449                | Normal IVSI-6   |
| 2            | Forward: 5'TCTCCTTAAACCTGTCTTGTAACCTTCATG3'<br>Reverse: 5'ACCTCACCTGTGGAGCCAC3'    | 286                | Mutant IVSI-6   |
| 3            | Forward: 'ACCAGCAGCCTAAGGGTGGGAAAATACACC3'<br>Reverse: 5'ACCTCACCTGTGGAGCCAC3'     | 419                | Normal IVSI-110 |
| 4            | Forward: 'ACCAGCAGCCTAAGGGTGGGAAAATAGAGT3'<br>Reverse: 5'ACCTCACCTGTGGAGCCAC3'     | 419                | Mutant IVSI-110 |

#### Data Analysis

The data were entered, stored and analyzed by personal computer using the Statistical Package for Social Sciences (SPSS) version 16.0. Independent-Samples T-test and Chi Square Test were used to compare between the two groups of the study (i.e., males and females). P-value < 0.05 was considered statistically significant.

### 3. Results

The results of our work are presented as cross tabulation tables to illustrate the number and frequencies of the subjects according to the different factors and characteristics. The comparisons between the mean values of the hematological parameters of the two groups were determined using the T-test analysis and Chi Square.

#### 3.1. Study Population

The study population consisted of 348 subjects (144 males, 204 females). The percentage of males was 41.40% while that of females was 58.60%.

#### 3.2. General Characteristics of the Study Groups

There was no significant difference in the mean age of the males (20.71±1.60 years) nor in the females (20.33±2.29 years) of the present work (P-value = 0.091). Most of the study population were reside in Khan Younis with 72.90% (n=105) for males and 72.10% (n=147) for female (Table 2).

**Table 2.** General characteristics of the study groups.

| Character         | Males n=144 |         | Females n=204 |         | P-value                  |
|-------------------|-------------|---------|---------------|---------|--------------------------|
| Age (years)       | Mean ±SD    |         | Mean ±SD      |         | Independent T-test 0.091 |
|                   | number      | percent | number        | percent |                          |
| Khan Younis       | 105         | 72.90   | 147           | 72.10   | Chi Square Test 0.002    |
| Rafah             | 28          | 19.40   | 56            | 27.50   |                          |
| Middle Prefecture | 11          | 7.70    | 1             | 0.50    |                          |
| Gaza city         | 0           | 0.00    | 0             | 0.00    |                          |

#### 3.3. Hematological Characteristics of the Study Groups

**Table 3.** Hematological characteristics of the study groups.

| Parameters               | Male n=144   | Female n=204 | P-value |
|--------------------------|--------------|--------------|---------|
|                          | Mean ± SD    | Mean ± SD    |         |
| WBC X10 <sup>9</sup> /L  | 6.44±1.75    | 7.37±1.80    | 0.925   |
| RBC X10 <sup>12</sup> /L | 4.45±0.33    | 4.22±0.39    | 0.063   |
| Hb (g/dL)                | 14.30±0.79   | 11.40±1.01   | 0.020   |
| HCT (%)                  | 38.70±2.20   | 36.30±3.20   | 0.031   |
| MCV (fl)                 | 87.10±5.50   | 85.70±7.40   | 0.009   |
| MCH (pg)                 | 28.70±2.08   | 27.00±2.50   | 0.019   |
| MCHC (g/dL)              | 32.70±2.60   | 31.30±1.30   | 0.418   |
| PLT X10 <sup>9</sup> /L  | 257.67±64.50 | 297.11±71.15 | 0.149   |
| RDW %                    | 12.14±0.90   | 11.62±1.35   | 0.001   |

WBCs: white blood cells, RBCs: red blood cells, Hb: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLT: platelets count.

The hematological characteristics of the study groups are shown in Table 3. All CBC parameters and indices measured in the present work revealed significant differences between males and females except that for WBC, RBC, MCHC and PLT. The hemoglobin level in females is found to be about 20% less than that recorded for males,  $11.40 \pm 1.01$  vs  $14.30 \pm 0.79$  g/dL, respectively. The MCV and MCH values in females were  $85.30 \pm 9.10$  fl and  $27.00 \pm 2.50$  pg compared to  $87.10 \pm 5.50$  fl and  $28.70 \pm 2.08$  pg in males. The P-values were 0.009 and 0.019 respectively.

### 3.4. Reference Range of Hematological Parameters

A reference range was obtained following standard guidelines, showing the 2.5-97.5 percentile intervals and median values for each of the hematological parameters determined for the 144 males and 204 females subjects (Table 4).

**Table 4.** Specific median value and reference interval of the hematological parameters.

| Parameter | Unit        | Males  |  | Females |  |
|-----------|-------------|--------|--|---------|--|
|           |             | Median | Reference interval (2.5 – 97.5 percentile) | Median  | Reference interval (2.5 – 97.5 percentile) |
| WBC       | $10^9/L$    | 6.90   | 3.80-11.20                                 | 6.90    | 3.80-11.20                                 |
| LYM       | %           | 35.01  | 20.27-55.48                                | 35.01   | 20.27-55.48                                |
| MON       | %           | 7.90   | 4.40-12.13                                 | 7.90    | 4.40-12.13                                 |
| GRAN      | %           | 57.10  | 35.00-74.43                                | 57.10   | 35.00-74.43                                |
| RBC       | $10^{12}/L$ | 4.42   | 3.90-5.20                                  | 4.19    | 3.46-5.07                                  |
| Hb        | g/dl        | 14.30  | 12.70-15.90                                | 11.40   | 9.20-13.20                                 |
| HCT       | %           | 38.70  | 35.00-43.20                                | 36.50   | 30.10-43.00                                |
| MCV       | fl          | 87.50  | 66.06-95.60                                | 87.50   | 66.06-95.60                                |
| MCH       | Pg          | 28.10  | 21.10-31.23                                | 28.10   | 21.10-31.23                                |
| MCHC      | %           | 32.10  | 28.70-34.60                                | 32.10   | 28.70-34.60                                |
| PLT       | $10^9/L$    | 278.00 | 159.72-453.75                              | 278.00  | 159.72-453.75                              |

### 3.5. RBCs Morphology

We evaluated the morphology of blood cells in 12 subjects with normal RBCs count and low MCV. Hypochromic and microcytosis were observed in 11 of the 12 subjects (91.67%). Normal shape and cell size were noticed in only one subject (8.33%). The results related to microcytic hypochromic mean of Hb, RBCs, MCV and MCH were  $10.90 \pm 0.90$ ,  $3.65 \pm 0.41$ ,  $75.60 \pm 6.60$  and  $24.00 \pm 2.20$  respectively.

### 3.6. Mentzer Index of the Study Groups

The best index that showed the distinction between iron deficiency anemia and minor thalassemia was Mentzer (MI). The mean of MI of all samples was 20.63 and 19.72 for males and females respectively, P-value = 0.022.

### 3.7. PCR Results

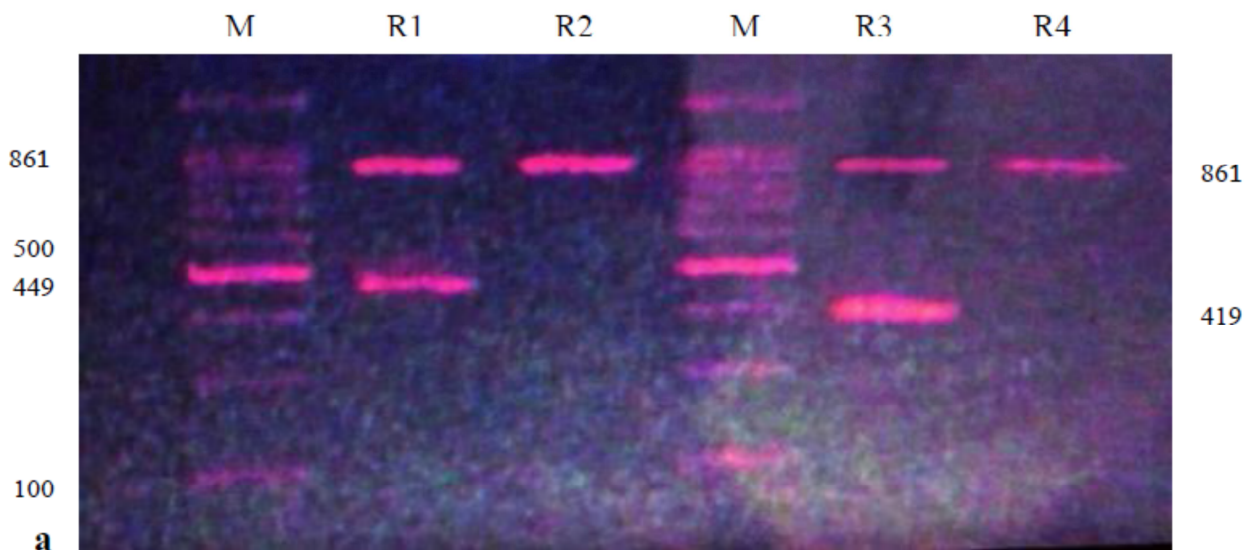
Detection of  $\beta$  globin gene mutation by Allele Specific PCR

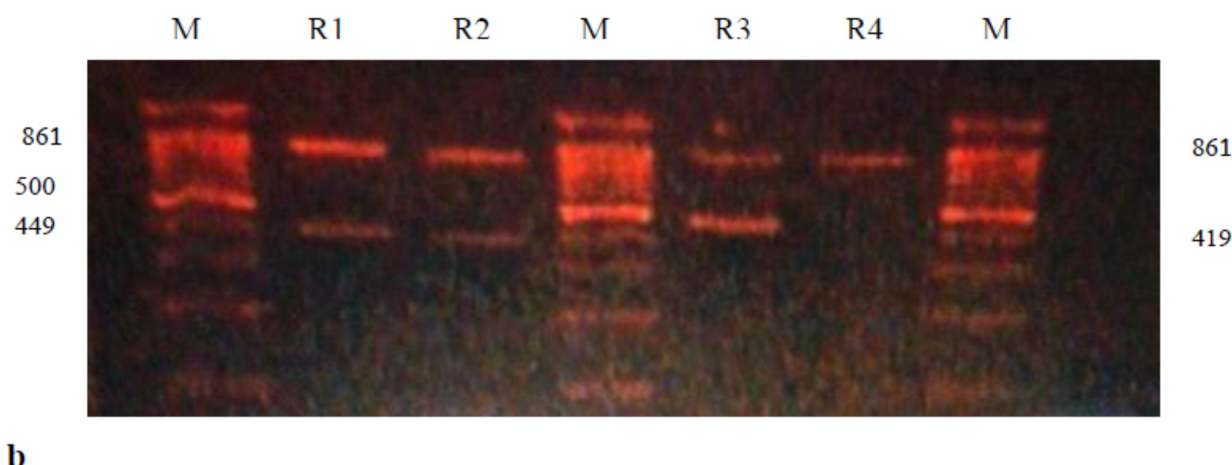
Table 1 showed products of Allele Specific PCR assay. Only 12 samples were used for PCR which have normal RBCs count with high MCV for detection of mutation at IVSI-6 and IVSI-110 position. IVSI-6 (T→C) mutation was observed only in two samples, while IVSI-110 (G→A) mutation was not observed in this study (Table 5).

**Table 5.** PCR products according to the type of mutation.

| Type of mutation | PCR Reactions |     | Genotype          |
|------------------|---------------|-----|-------------------|
|                  | R1            | R2  |                   |
| IVSI-6           | 449           | 286 | Heterozygous      |
|                  | 449           | -   | Homozygous normal |
| IVSI-110         | R3            | R4  | Homozygous normal |
|                  | 419           | -   |                   |

We determined IVSI-6 mutation only in one allele of two subjects from all molecular investigated samples. All other subjects were homozygous normal for IVSI-110 (Figure 2).





**Figure 2.** The electrophoresis pattern of recognized genotypes using the ASPCR method. Lane M indicates the 100 bp ladder. The R1 - R4 lanes show the PCR reaction number.

- a) homozygous normal for both IVSI-6 (T→C) and IVSI-110 (G→A)  
 b) heterozygous for IVSI-6 (T→C) and homozygous normal for IVSI-110 (G→A)

## 4. Discussion

Palestine is one of the Mediterranean basin countries in which thalassemias are prevalent, however, few studies have been conducted on the disease. Sirdah *et al.* (1998) showed that the overall prevalence of  $\beta$ -thalassemia in the Gaza Strip area was 4.3%. Occurrence of hereditary hemochromatosis among  $\beta$ -thalassemia intermediate and  $\beta$ -thalassemia minor subjects in Gaza Strip was assessed by Harara, 2006 [14]. In addition, immunological assessment of  $\beta$ -thalassemic major children aged 5 to 12 years old attending Abd El-Aziz El-Rantisy hospital in Gaza Strip was performed only recently [15].

However, no previous study was conducted for detection of the prevalence of  $\beta$ -thalassemia minor. Therefore, this study is the first in this regard and will be useful in evaluation, control and prevention of  $\beta$ -thalassemia.

### 4.1. Hematological Characteristics of the Study Groups

The population screened in the present work showed a significantly altered hemogram specially with HCT, Hb and related indices (MCV and MCH) and RDW between the two groups. Comparing our hematological findings shown in Table 4 with other studies, our calculated means are close to those reported by Zacharias Habib (2009) in Egypt [16]. The means reported in that study were: male 5.07, 87.82 and female 4.47,

84.4 for RBCs and MCV respectively and ours were: male 4.43, 87.5 and female: 4.19, 87.5 respectively. The mean of HCT for female was in good agreement with our female's HCT mean. However, the mean of HCT in the present study was lower than that had been reported in the Egyptian study funding for males. The Hb mean for both males and females was lower (15.35 for males and 13.00 for females).

Our results revealed that the mean of Hb, MCV, MCHC, RDW and PLT in both groups similar to the results reported by Al-Sweedan *et al.*, 2012 in Jordan [17]. Their reported WBC's mean for the two genders was slightly less.

### 4.2. Hematological Reference Range of the Study Groups

The results of this study can be regarded as normal reference values in the University College of Science and Technology being part of the Palestinian population. This study covered the entire age range 17 to 28 years in males and females and showed the value of hematological parameters and red cell indices. The reference range found in this study matched that reported in the Palestinian Clinical Laboratory Tests Guide (2005) by the Palestinian National Authority Ministry of Health except for Hb, MCV, MCH MCHC and RDW (Table 6).

**Table 6.** Hematological parameters comparison between current study and that reported by Palestinian Ministry of Health.

| Parameter              | Current study |               | Ministry of Health report |               |
|------------------------|---------------|---------------|---------------------------|---------------|
|                        | Males         | Females       | Males                     | Females       |
| WBC $\times 10^9/L$    | 3.80-11.20    | 3.80-11.20    | 4.60-11.00                | 4.60-11.00    |
| RBC $\times 10^{12}/L$ | 3.90-5.20     | 3.46-5.07     | 4.69-6.13                 | 4.10-5.50     |
| Hb (g/dL)              | 12.70-15.90   | 9.20-13.20    | 13.50-17.50               | 12.00-16.00   |
| HCT (%)                | 35.00-43.20   | 30.10-43.00   | 43.50-53.70               | 37.00 -48.00  |
| MCV (fl)               | 66.06-95.60   | 66.06-95.60   | 80.00-100.00              | 80.00-100.00  |
| MCH (pg)               | 21.10-31.23   | 21.10-31.23   | 27.00-31.20               | 27.00-31.00   |
| MCHC (g/dL)            | 28.70-34.60   | 28.70-34.60   | 31.00-35.00               | 31.00-35.00   |
| PLT $\times 10^9/L$    | 159.72-453.75 | 159.72-453.75 | 150.00-450.00             | 150.00-450.00 |
| RDW %                  | 10.50-14.20   | 9.80-15.20    | 11.50-14.50               | 11.50-14.50   |

#### 4.3. $\beta$ -globin Gene Mutation by Allele Specific PCR

The Allele Specific PCR (ASPCR) is an amplification strategy in which a polymerase chain reaction primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue. The main advantage of ASPCR is that the amplification step and the diagnostic steps are combined, in that the presence of an amplified product indicates the presence of a particular allele and vice versa. It is a very time-efficient method [18]. The system is simple and reliable. It clearly distinguishes heterozygotes at a locus from homozygotes for either allele [12].

The molecular screening protocol we followed, which included two common Middle Eastern HBB mutations, enabled us to identify the causative mutations in  $\beta$ -globin gene of the  $\beta$ -thalassemic carrier included in this work. Our study showed that the IVSI-6 (T→C) which is the mutation that reduces the efficiency of splicing at the 5' site was observed in two samples only. This mutation has the lowest frequency and concomitant to what had been reported in neighboring countries. IVSI-6 (T→C) is the lowest prevalent mutation in Egypt (13.60%), Lebanon (15%), the pre 1948 Occupied Palestine areas (14.70%), Syria (4%), Jordan (6.60 %), Kuwait (7.30%) and Saudi Arabia (7%) [19]. El-Gawhary et al., (2007) reported that IVSI-6 is more frequent than IVSI-110, but their study covered Fayoum in Upper Egypt, Cairo, Alexandria and Tanta in Lower Egypt and the Nile Delta. The proportion of IVS-I-6 (T→C) was 36.30% and that of IVSI-110 (G→A) was 25.8% [20].

The IVS-I-110 (G→A) mutation that causes aberrant splicing of pre-mRNA and deficient beta-globin chain synthesis is the most prevalent mutation that reported in neighboring countries, in Egypt (33%), Lebanon (33%), Syria (24%), Jordan (22%), and Saudi Arabia (22%) [19] and in the Palestinians living at West Bank where the IVS-I-110 (G→A) mutation showed a prevalence of 17.6% [21]. This mutation was not in our study and this may be attributed to the prevention methods that have been used to reduce the disease.

## 5. Conclusions

This study showed that there is no statistically significance for WBCs, RBCs, PLTs and MCHC between male and female with high mean value of WBCs and PLTs in female.

The hematological characteristics Hb, MCV, MCH and RDW measured in the present work revealed significant differences between males and females with P-value 0.02, 0.009, 0.019 and 0.001 respectively. The hemoglobin level in females was found to be about 20% less than the level reported in males.

The present study estimated the hematological parameters reference range for the students of the University College of Science and Technology-Khan Younis, Palestine. The IVSI-110 mutation was not found in our population while IVSI-6 mutation was detected only in 2 of the 12 isolated DNA samples and both were heterozygous. The results suggests

regular screening for  $\beta$ -thalassemia carrier in habitants of the Gaza Strip in order to discover the prevalence of the different types of  $\beta$ -globin mutation among them.

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