ABSTRACT
Objective: To determine the frequency of β-thalassemia carriers in healthy population from Muzaffarabad Azad Kashmir. Methods: In this cross sectional population based study, people from Muzaffarabad were motivated to get themselves tested for beta thalassemia trait. People found healthy on basis of a questionnaire and clinical examination were included in the study. Complete blood counts and peripheral smear review were performed on EDTA samples. Hemoglobin (Hb) electrophoresis was performed. Subjects with mean corpuscular volume (MCV), <76 fl, and/or mean corpuscular Hb (MCH), < 27 pg were subjected to hemoglobin A2 (HbA2) level estimation by elution method. Hemoglobin A2 of more than 3.5% was considered diagnostic for beta-thalassemia trait. Azad Kashmir is a territory with considerable ethnic heterogeneity. This is basically a tribal society with a high proposition of consanguineous marriages. We created awareness among the general population. Results: Found that gene is present in almost all tribes studied. Conclusion: - Using strict criteria for healthy population, we conclude that incidence of thalassemia trait is 5.6 % in our study population. β-thalassemia trait is present in almost all ethnic groups. To control thalassemia major, screening program for thalassemia carriers must be initiated in the area. Keywords: Thalassemia, Hemoglobin A2, Mean corpuscular Hb (MCH)

INTRODUCTION
Hemoglobinopathies are defined as disorders with abnormal hemoglobin and constitute a group of qualitative and quantitative disorders of hemoglobin while thalassemia is the term used to describe disorders with significant decrease in the rate of synthesis of one or more globin chains.\(^1\) Hereditary disorders of hemoglobin have a worldwide distribution. The frequency of abnormal hemoglobin’s varies considerably with geographical location and racial groups. It is estimated that approximately 7 % of the world population carry a gene for clinically important hemoglobin disorder.\(^2\) Hemoglobinopathies are a major public health problem in Saudi Arabia. Important hemoglobinopathies in Saudi Arabia and other middle east countries are alpha thalassemia, beta thalassemia and sickle cell anemia. In a premarital screening program for thalassemia and sickle gene, incidence of these two genes was reported to be 7.75 %.\(^3\) Similar figures are reported from other gulf countries. In a community based survey of under-five years age children in Sultanate of Oman, incidence of thalassemia and sickle gene was reported as 8.27 %.\(^4\) β-thalassemia major is the most common form of thalassemia worldwide. Approximately 1.5 % of world population carries genes for beta thalassemia. It is common in Greeks, Turks, Cypriots, Italians and to a lesser extent in Indian subcontinent.\(^5\) In various countries there is considerable variation in the incidence of β-thalassemia in small geographical regions.\(^6\) Among hemoglobinopathies, β- thalassemia is a major problem in Pakistan with a carrier rate of 5-8 %. First cousin marriages are 37.1 % and total consanguineous marriages are 50.3 %. Using Hardy Weinberg equation, each year about 6,000 to 7,500 homozygous β-thalassemia are born in Pakistan. Regional incidence varies considerably depending on the gene prevalence and birth rate in
the area.\textsuperscript{7-9} Large number is added annually to the already existing homozygote population. Most of the available studies about frequency of beta thalassemia are in small groups of hospital-based patients and/or population groups. We also understand that the incidence is higher in some groups.\textsuperscript{10} Overall there is a stated lack of information about the true gene frequency in many parts of the world.\textsuperscript{11} Similarly incidence in various ethnic groups in Pakistan is not well known. More accurate assessment of gene frequency of \(\beta\)TT in the population provides solid data about the health burden posed by the disease and is of great help to plan control programs for \(\beta\)-thalassemia in the area.\textsuperscript{12} With efforts, many countries are able to considerably reduce the birth rates of homozygous \(\beta\)-thalassemia in different parts of the world. In some smaller countries no newborns with the disease are reported. This was possible due to control programs including screening population surveys for heterozygous \(\beta\)-thalassemia, antenatal diagnosis along with increasing awareness in the medical profession, and in the population by large-scale education and counseling. Classical example is Sardinia that has substantially reduced the birth of homozygous thalassaemias from 1:250 to as low as 1:4000 births. In Cyprus no affected infants were born during year 2000-2007.\textsuperscript{13} Sardinia has also achieved very good progress in prevention.\textsuperscript{14} Saudi has achieved 70\% reduction in births of thalassaemias during year 2004 to 2009.\textsuperscript{15} Azad Kashmir is a territory with considerable ethnic heterogeneity. Information about the prevalence of thalassaemia is patchy but it is known that disease is relatively common in Azad Kashmir. This is basically a tribal society with a high proposition of consanguineous marriages. Although it is possible that hemoglobinopathies may be concentrated in some geographical locations,\textsuperscript{16} study of every region is an impossible proposition due to lack of infrastructural facilities, expertise, and resources. The present study was undertaken to find out the incidence of beta thalassemia gene in the area. At the same time, data on ethnic variation, if any is generated.

Blood Complete picture is very important primary investigation in the identification of \(\beta\)-thalassemia carriers.\textsuperscript{17} It is recommended that screening for beta thalassemia should consist of a complete blood count,\textsuperscript{18} high performance liquid chromatography of hemoglobin or hemoglobin electrophoresis. The investigations should include Hb \(A_2\) quantitation. If microcytosis (mean cellular volume < 76 fL) and/or hypochromia (mean cellular hemoglobin < 27 pg) are picked up on CBC and hemoglobin electrophoresis or high performance liquid chromatography is normal, a brilliant cresyl blue stained blood smear must be examined to identify hemoglobin H bodies. Simultaneously serum ferritin should be performed to exclude iron deficiency anemia.\textsuperscript{19}

**METHODOLOGY**

This cross sectional population based study was carried out, with approval and support of AJK health department, at Combined Military Hospital Muzaffarabad. After obtaining verbal consent, each individual was given a number and a detailed questionnaire was filled. Questionnaire included details of age, sex, and cast, family history of hemoglobinopathies and blood transfusion history. Questionnaire was developed on basis of literature. Clinical examination included evidence of anemia, splenomegaly, lymphadenopathy. All healthy people were included. People with family history of hemoglobinopathies and history of blood transfusion or showing evidence of anemia, splenomegaly, lymphadenopathy were excluded. A random sample of five hundred persons was selected from residents of municipal area on door to door basis. Three milliliter blood was collected under standardized conditions in potassium EDTA for complete blood count (CBC)\textsuperscript{20} and hemoglobin electrophoresis. Smear prepared immediately and blood poured in EDTA bulb. Sample was gently mixed soon after collection. EDTA sample was processed on Sysmex K-1000 hematology analyzer. Equipment was calibrated by the vendors. Weekly and monthly maintenance performed as per recommendations. Quality control was performed by running three controls daily morning and then after every 50 samples. Analyzer performs hemoglobin estimation by modified Drabkin’s method. Instrument measures the number and size of particles suspended in an electronically conductive fluid. Particles are forced to flow through a small aperture having an emerged electrode on either side. With passage of particle through aperture, there is a change in resistance between electrodes that produces a voltage pulse of short duration. The magnitude of this pulse is proportional to the particle size. Series of pulses are
then electronically computed and red cell indices are derived. Dried smears were stained with Leishman’s stain in a batch of ten slides each. Mainstay of the project was cellulose acetate electrophoresis using Tris-EDTA – Borate buffer at a pH of 8.9. Hemolysate was prepared from packed cells obtained after removing plasma from centrifuged blood and then washing by centrifugation in 3-5 volumes of saline three times. Packed cells were mixed with equal volume of distilled water and shaken vigorously for two minutes. To this mixture, half the amount of carbon tetrachloride was added, and mixed for five minutes. It was then centrifuged for 20 minutes at 3000 rpm. Top layer of lysate was removed and one drop of 2 % potassium cyanide was added to the lysate. Hemoglobin content of lysate was adjusted between 3 and 4 g/dl as instructed by the manufacturer of the electrophoresis strips.

Whole process of electrophoresis was carried out as per procedure manual of the electrophoresis apparatus and kit insert of the electrophoresis strip. Tris-EDTA – Borate buffer at a pH of 8.9 was used and same was used for Hb A2 quantitation. Elevation of Hb A2 presents the best practical approach for the diagnosis of beta thalassemia. Hemoglobin A2 estimation was done where red cell morphology or indices indicated the need to do so. Elution technique using cellulose acetate and tris-EDTA buffer pH 8.9 was used because of non-availability of column chromatography. Quality control was established by Hb A2 reference preparation.

Red cell parameters /indices including hemoglobin level, red blood cell counts, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, were assessed. Stained peripheral smear for all the people were examined for abnormal findings.

Iron deficiency was excluded on complete blood picture and peripheral smear findings. Parents and siblings of children found to have β-thalassemia were called for investigation for βTT and followed up for counseling as required. Simple percentages were used for statistical purposes.

RESULTS
A total of 500 cases were studied. 292 male and 208 males comprised this study with an overall male to female ratio of 2.43: 1.73. Age and sex wise distribution of sample is shown in Table 1;

<table>
<thead>
<tr>
<th>Age</th>
<th>Male No</th>
<th>Male %</th>
<th>Female No</th>
<th>Female %</th>
<th>Total No</th>
<th>Total %</th>
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<tbody>
<tr>
<td>Less than 5 years</td>
<td>78</td>
<td>15.6</td>
<td>29</td>
<td>5.8</td>
<td>107</td>
<td>21.4</td>
</tr>
<tr>
<td>More than 5 to 15 years</td>
<td>45</td>
<td>9.0</td>
<td>31</td>
<td>6.2</td>
<td>76</td>
<td>15.2</td>
</tr>
<tr>
<td>More than 15 to 35 years</td>
<td>118</td>
<td>23.6</td>
<td>116</td>
<td>23.2</td>
<td>234</td>
<td>46.8</td>
</tr>
<tr>
<td>More than 35 to 50 years</td>
<td>35</td>
<td>7.0</td>
<td>23</td>
<td>4.6</td>
<td>58</td>
<td>11.6</td>
</tr>
<tr>
<td>Above 50 years</td>
<td>16</td>
<td>3.2</td>
<td>9</td>
<td>1.8</td>
<td>25</td>
<td>5.0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>292</td>
<td></td>
<td>208</td>
<td></td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Age and sex wise distribution of sample

All the people studied were residents of Muzaffarabad. 93.8 % had their origin from Muzaffarabad, 3.4 % from Poonch, 1 % from NWFP, 1 % from Afghanistan and 0.8 % from Punjab and Baluchistan.
Table 2: Race wise distribution of sample

<table>
<thead>
<tr>
<th>CAST</th>
<th>#</th>
<th>%</th>
<th>CAST</th>
<th>#</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raja</td>
<td>110</td>
<td>22.0</td>
<td>Pathan</td>
<td>31</td>
<td>6.2</td>
</tr>
<tr>
<td>Awan</td>
<td>101</td>
<td>20.2</td>
<td>Qureshi</td>
<td>29</td>
<td>5.8</td>
</tr>
<tr>
<td>Khawaja</td>
<td>59</td>
<td>11.8</td>
<td>Abbasi</td>
<td>21</td>
<td>4.2</td>
</tr>
<tr>
<td>Syed</td>
<td>52</td>
<td>10.4</td>
<td>Sheikh</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Choudhary</td>
<td>47</td>
<td>9.4</td>
<td>Qazi</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Mughal</td>
<td>43</td>
<td>8.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# = Number of people from each race.

Figure 2: Cast wise distribution of sample

Out of 500 people studies, a total of 28 cases were diagnosed as having β-thalassemia trait
Age wise distribution of β-thalassemia carriers is depicted in Table 3:

Table 3: β-thalassemia carriers age wise

<table>
<thead>
<tr>
<th>Age in years</th>
<th>β-thal Carriers</th>
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<tbody>
<tr>
<td>Upto 5 yrs</td>
<td>7</td>
</tr>
<tr>
<td>&gt;5 to 15 yrs</td>
<td>1</td>
</tr>
<tr>
<td>&gt;15 to 35 yrs</td>
<td>15</td>
</tr>
<tr>
<td>&gt;35 to 50 yrs</td>
<td>4</td>
</tr>
<tr>
<td>Above 50 yrs</td>
<td>1</td>
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</table>

Table 4: Race wise incidence of β-thalassemia trait

<table>
<thead>
<tr>
<th>CAST</th>
<th>#</th>
<th>Number of carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raja</td>
<td>110</td>
<td>6</td>
</tr>
<tr>
<td>Pathan</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>Awan</td>
<td>101</td>
<td>5</td>
</tr>
<tr>
<td>Qureshi</td>
<td>29</td>
<td>2</td>
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<tr>
<td>Khawaja</td>
<td>59</td>
<td>2</td>
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<tr>
<td>Abbasi</td>
<td>21</td>
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<tr>
<td>Syed</td>
<td>52</td>
<td>4</td>
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<tr>
<td>Sheikh</td>
<td>5</td>
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</tr>
<tr>
<td>Choudhary</td>
<td>47</td>
<td>2</td>
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<tr>
<td>Qazi</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mughal</td>
<td>43</td>
<td>1</td>
</tr>
</tbody>
</table>

# = Number of people from each case.

Cellulose Acetate Electrophoreis (CAE) Hb A2 was quantitated in cases where RBC counts or RBC morphology or A2 prominence on CAE indicated to do so. In total cases studied it had mean, standard deviation, variance, maximum and minimum of 3.90 %, 1.2 %, 1.5 %, 6.3 % and 1 % respectively. Range of Hb A2 in β-thalassemia carriers was 3.9 % to 6.3 % with mean, standard deviation and variance, 4.9 %, 0.5 %, 0.3 % respectively. Absence of Hb A2 was not noted in any case.

DISCUSSION & RECOMMENDATIONS

A cross-sectional population based study that was designed to find out and compare the frequency of β-thalassemia was carried out at CMH Muzaffarabad. Hematological problems are well known in Pakistan and hemoglobinopathies further contribute to it. β-thalassemia is the commonest inherited disorder in Pakistan. Around 9000 new thalassemia patients are added every year to the existing thalassemia patients. There are 9.8 million carriers of this disease who transmit disease to their next generation.

Hereditary disorders of hemoglobin production are not uncommon in Northern areas of Pakistan with a more common prevalence of β-thalassemia. To plan an effective control it is important to have firsthand information about the gene frequency. The current study confirmed that beta thalassemia gene is prevalent in the area. The overall rate of β-thalassemia carriers was 5.6 %.

Strength of our study was that we approached general population on door to door basis. Questionnaire was set up to exclude possible
confounding factors. We looked into various tribe/casts staying in the area. We did not have the sources for iron profiling to exclude iron deficiency but excluded iron deficiency by meticulous study of red cell indices and hematologist review of peripheral blood smears.31 In a study in Rawalpindi and Islamabad a carrier frequency of 4 % is reported. 32 In Karachi a prevalence rate of 5.5 % for β-thalassemia is reported in a survey in general population.33

Another study on 500 people of northern areas of Pakistan revealed an overall prevalence of 5.4 %. Gene was detected in all ethnic groups. Pathans had higher prevalence rates (7.96%) in comparison with Punjabis (3.26%). 34

Our findings are closer to most of these findings and indicate that gene frequency is significantly high in our population also. A very high frequency of β-thalassemia trait has been found in referred cases for hemoglobin electrophoresis to various hospitals in Pakistan like in Dera Ismail Kahn this frequency was 18.5 %. 35

Families of thalassemia patients also show very high gene frequency. Studies show that in Pakistan carrier rate in these families vary between 31-68 % in comparison to 5 % in general population. In a study conducted in Lahore, 52 % of samples from families of patients suffering from beta thalassemia major were carriers of gene on hemoglobin electrophoresis.36

In Faisalabad incidence was 44.4 % in a large Pakistani Family.37 In certain communities, prevalence may be very high such as a prevalence of 17% has been reported in certain communities in India.38

While in neighboring countries a bit lower prevalence of β-thalassaemia carriers have been reported. In Indian Punjab it is reported as 3.5%. Similarly prevalence rate for Saudi Arabia 3.4%, Southern China 2.54% and Hong Kong 3.4% have been documented.39-41 Consanguineous marriages have contributed to the increased incidence of this disease.42-43

Thalassemia is an autosomal recessive disorder associated with a reduced or absence of synthesis of globin chains of hemoglobin molecule causing severe anemia in homozygotes.44 If a β-thalassemia carrier marries another carrier, in every pregnancy there is 25 % chance that baby will be thalassemia major, 50 % thalassemia trait and 25 % chance that baby will be normal.45

According to word health organization, if for any disease, the birth rate of affected infants exceeds 0.1/1000 an effective screening program should be initiated.46 Those who suffer need comprehensive care, safe blood, iron chelating drugs and bone marrow transplantation for selective patients. Patients suffer misery, financial hardships, complications of thalassemia and treatment related iron overload.47-50 Azad Kashmir is ethnically diverse territory with approximate population of 4 million with per capita income of 847 US dollars.51 Azad Kashmir has its own health department with four medical colleges. With gradual control of malnutrition and communicable disease β-thalassemia major patients who died young are now surviving enough to seek medical attention.52-53 CMH Muzaffarabad is providing support to forty eight beta thalassemia major patients. In countries with optimum health care facilities, affected children receive regular transfusions, iron chelation therapy, regular monitoring of growth patterns, endocrine functions, bone, heart and liver iron status.52-54 Selected patients undergo bone marrow transplantation. Bone marrow transplantation results in extended disease free survival in majority of patients.55 In Iran average annual treatment cost of optimally treated patients is estimated as 6500 USD.56

In Azad Kashmir, treatment options are difficult. Blood transfusion arrangement is an uphill task. Chelation is expensive and affordable to just a few.57 dedicated expertises to manage beta thalassemia patients are not available. Diagnostic facilities to monitor the treatment are lacking. Overall management of these patients is suboptimal. Carrier detection is nonexistent. Situation leaves us with only strategy of prevention. Therefore prevention can be the only way forward. Dictum prevention is better than cure holds true for thalassemia also. Cost of prevention of one case of thalassemia (100 USD)58 is much less than one year optimum treatment ( 6500 USD) of a thalassemia major patient. We need to see a marked reduction in the birth of new thalassemia patients like many other nations have succeeded. Effectiveness of screening program observed in Sardinian59 is evidenced by reduction and similarly a reduction is observed in a two decades in Iran60. If we start prevention today, we will be able to focus and provide better care to the existing thalassemia patients otherwise the
burden of disease is continuing and quality of care of thalassemia will continue to deteriorate. People with thalassemia trait are well and usually only detected through routine blood testing. Preventive strategies for thalassemia could be

- Correct information about the disease. (People still believe in unnatural forces, unseen powers causing disease)
- Awareness programs highlighting the importance of screening.
- Screening and counseling of targeted families.
- Premarital screening and prenatal screening. These may incorporate; population screening, genetic counseling, prenatal diagnosis and option of terminating affected pregnancies. These could be the mainstay of a successful program. However screening strategies vary depending upon religious, social and cultural needs of the particular society. HPLC would be an ideal investigation to diagnose beta thalassemia trait. In our study, due to non-availability of HPLC, HbA2 estimation by elution was used. Public knowledge about disorder is low and preventive strategies are scarce. Even the families having affected children know very little about the disease. Ideally whole population should be screened. Keeping in view the poor socioeconomic status and very high rate of consanguineous marriages, extended family screening may be an appropriate strategy. Therapeutic facilities are unaffordable so public awareness and motivation are key to the prevention of thalassemia. Carrier detection is non-existing in Muzaffarabad. Sophisticated antenatal testing utilizing CVS and amniotic fluid analysis is invasive and expensive and patients need referrals to outside territory.

To plan effective control, it is important to have firsthand information about the gene frequency. We planned a descriptive study that was destined to know the frequency of β-thalassemia trait. Aim was to determine the frequency of at risk couples for β-thalassemia minor. These results may guide to those involved in planning the health care provision and this information may be incorporated in the decision making process.

Best approach would be the mass education through media, training of general practitioners, organizing seminars, symposia involving social workers, religious leaders, and political figures. Particularly focusing on pediatricians and obstetricians and provision of laboratory facilities for supporting the screening program is expected to be rewarding.

CONCLUSION

Using strict criteria for healthy population, we conclude that incidence of thalassemia trait is 5.6% in our study population. β-thalassemia trait is present in almost all ethnic groups. To control thalassemia major, screening program for thalassemia carriers must be initiated in the area.

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