Pak-Euro Journal of Medical and Life Sciences
ISSN: 2707-4471 (Print), 2707-448X (Online)
www.readersinsight.net/pjmls

Research Article

Quantitative determination of HbA2 level in β-thalassemia trait by using Capillary Electrophoresis in Balochistan.

Naseeb Ullah[1], Shafi Muhammad Khosa[2], Syed Muhammad Ishaque[2], Muhammad Ali Khan[1*], Mohsin Ali[1], Ashiq Hussain[3], Abdul Majid[4], Muhammad Nauman[5] and Muhmmad Mehdi[2].

[1]Centre for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, Pakistan
[2]Department of Pathology, Bolan University of Medical and Health Sciences, Quetta, Pakistan.
[3]Department of Microbiology, Bolan University of Medical and Health Sciences, Quetta, Pakistan.
[4]Department of Social Work, University of Balochistan, Quetta, Pakistan.
[5]Department of Pathology, Liaquat National Hospital, Karachi, Pakistan.

*Corresponding author: drkhanpishin@yahoo.com

Abstract

In Pakistan, β-thalassemia is considered as one of most typical genetic abnormality. Different countries like Italy, France, and Greece have recognised thalassemia as preventive disorder of society. In Pakistan, since 1994 different efforts were made to control thalassemic epidemic. Inspite of this, there is no significant decline in occurrence of childbirth with transfusion dependent β-thalassemia incidents. In order to obtain fruitful results of general thalassemia eradication program nation wide,it is vital to evaluate β-thalassemia trait (BTT) by specific, low price, and accurate mechanism of diagnosis. So, this current research was carried out to decide the levels of HbA2 in samples by the use of Capillary Zone Electrophore and Cellulose Acetate Hemoglobin Electrophoresis.
This comparative research was done in the four groups: i) Normal Individuals having no sign or symptom of thalassemia ii) Patients having BTT i.e β-thalassemia trait iii) Patients having IDA i.e Iron Deficiency Anemia iv) Parents of β-thalassemia major child with iron deficiency anemia (BTT and IDA). It means that both parents are carrier of β-thalassemia not diagnosed yet.
The level of HbA2 were estimated by the Cellulose Acetate Hemoglobin Electrophoresis and Capillary Zone Electrophoresis independently and then compared to design established results.
Result: Both these methods have found to be correct for the patients having β-thalassemia trait (BTT) but in combined condition, cases of β-thalassemia trait (BTT) were failed to be analyzed by both these systems.
Conclusion: As this technique is very easy, easily reachable and affordable. So, it is highly recommended for the diagnosis in the poor province like Balochistan in Pakistan. In thalassemia prevention, it plays a vital role.

Keywords: *β-thalassemia trait (BTT), Iron Deficinecy Anemia (IDA), Cormorbid Disorder (BTT+IDA), HbA2 level, Capillary Zone Electrophoresis, Cellulose Acetate Hemoglobin Electrophoresis.*

ARTICLE INFORMATION

Received: 21.07.2019
Revised: 20.08.2019
Accepted: 30.09.2019

DOI: 10.31580/pjmls.v2i3.1138

INTRODUCTION

In the red blood cells (RBCs), hemoglobin is oxygen carrier entity of the cell. It is a polypeptide molecule consisting of globule like shape and comprises of two pairs of globin webs i.e. alpha and beta(1). There are many disorders of prevalent hereditary hemoglobin worldwide known as hemoglobinopathies (2-4).

In the 2006 report published by the world health organization (WHO), approximately 7% of world population is considered as the carrier of any type of disease (5). It is further stated that in the same year, 3-5 million newly born children have dangerous level of hemoglobin syndromes and above 50 thousands having β-thalassemia majors (6). It is estimated that after 20 years, thalassemia will be one of the world wide health difficulty for the people and ninty thousand newborn will be effected from birth (7-9).

Thalassemia in our province Balochistan is death lasting disease which can't be controlled or diagnosed easily. It results to anemia and blood forming ability of the cells (1). There are approximately 1800 cases of recorded thalassemia cases in Quetta. There are two functional thalassemia centres in district Quetta operating in two famous hospitals of the city i.e Quetta's Civil Hospital and Bolan University of Medical and Life Scicence Quetta. The blood of more than 60 children were given to these centres daily for the diagnostic practices of

thalassemia. Among the β-thalassemia is considered as one of the most occurring inherited disorder. It is due to incorrect production of α and β chains of hemoglobin and subsequently recognized as α and β thalassemia.

Different diverse mutation in the β globin named as heterozygote or homozygote mutation results into severe cases of β-thalassemia. It requires lifetime checking and β-thalassemia trait is necessary for the diagnosis of thalassemia (19). The parameter designed to diagnose β-thalassemia is one and only hemoglobin A2 (HbA2), a class of hemoglobin occurring in the type of blood cells called RBCs of the individuals. Minor components of it are two α and two β chains. 2.5% of hemoglobin is of this hemoglobin A2 (HbA2) (12).

Balochistan being the underdeveloped province of Pakistan, health facilities are very rare and very difficult to approach. Present research work was done to have enough knowldedge in diagnosing β-thalassemia for ensuring the better plan to prevent this disease or disorder in the populaiton of Balochistan.

MATERIALS AND METHODS

Study Design and Selection of Participants

This was a crossectional study carried out at different hemotoligcal centres of Bolan University of Medical and Health Sciences and Civil Hospital Quetta, Balochistan in collabartion with CASVAB, UoB. The total test population comprised of 200 individuals that were further distributed into following four significant groups:

i. Normal individuals having no signs and syptoms of β-thalassemia trait (BTT) and Iron deficiency anemia (IDA).
ii. Patients with β-thalassemia trait (BTT).
iii. Patients with Iron deficiency anemia (IDA).
iv. Parents of β-thalassemia major child with iron deficiency anemia (It means that both parents are carrier of β-thalassemia not diagnosed yet).

Among these samples, 150 were tested population and 50 were control subjects, selected on the basis of inclusion criteria, received by the hematology unit, thalassemia enter, BUMHS and Civil Hospital Quetta.

In order to obtain the sample from each subject, 7 ml of venous whole blood was collected with the help of disposible syrings in such a way that 3ml of it was inserted in CBC tubes and 2ml in gel tubes. CBC tubes whole blood is used for measurement of completed blood count parameters and gel tubes whole blood were used for estimation of serum ferritin hormone level. These tubes comprises of commercially availabe anticougulant ehtylene diamine tetraacetate (EDTA). Remaining 2ml whole blood was centrifuge at 4000 rpm for 5 minutes and serum is isolated for iron deficiency test.

Equipments and Kits

i. Complete blood count tests were performed on th Sysmex KX-21, made in Japan (Tokyo). It is an computerized mechnaical analyzer working efficiently. The CBC parameters includes Hb, RBC, MCV, MCH, MCHC and platelles etc. 16 samples, standard and lyse tubes were run at the same time by capillary electrophoresis. It uses about 40 to 50µl whole blood and give accurate result with in hour.

ii. Capillaries (Sebia, Inc, Norcross, Ga) were used to evaluate capillary zone electrophoresis (Fig 1 to Fig 4).

iii. Semi automatd machine, made in Italy (Rome) with brand number Interlab Roma Microtech Series 648iso, was used to accomplish cellulose acetate hemoglobin electrophoresis (Fig 5 to Fig 6).

iii. Enzyme linked immunoassay (ELISA) kits were provider and manufactured by Rosh E411. It is used to measure the serum ferritin level, which further helps in dignosis of iron deficiency anemia. It uses about 100 to 150µl serum and gives resutls within 40 to 60 minutes.

All these machine have instrument tract, samples of subjects were placed on it. The results were automatically processed by the machines and shown on the screen.

The alkaline buffer used in electrophoresis have pH 9.4, provided by manufacturer (Sebia). The endosmosis doesn't effectes the pH of solution. 415 nnm waveelengh was used to measure hemoglobin. The presence oof HbA helps to interpret structural variants by appearance of zone demarcations. In the case of any mistake, test of sample was repeated with 1:1 mixture of normal control subjects.

Data Analysis

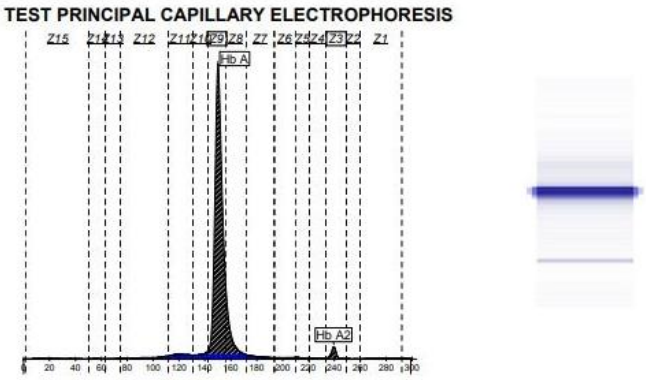
Statistical analysis were done by using SPSS statistical software verrsion 16 (Chicago, IL, USA). The data was evaluated on the basis of mean ± SD to show logical resuts of study. Due to smaller sample size *p* value < 0.05 was consideredon statisticaly significant.

RESULTS

BTT containing samples have mild anemia according to Complete Blood Count, while other patients with IDA have different levels of anemia from moderate to severe. Red Blood Cell distribution and its count were different in other cases of BTT and IDA (Table No 1).

Table I. Testified four groups showing parameters of CBC.

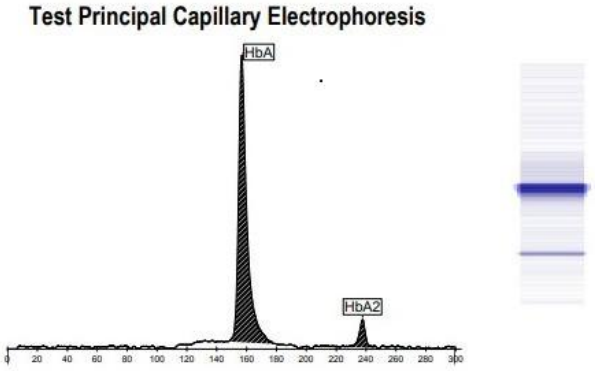
Groups	Hb (g/dl)	RBC (x106/ul)	MCV (fl)	MCH(pg)	MCHC(%)	RDW (%)	Ferritin(ng/ml)
Normal Subjects	12.0±1.1	4.6±0.4	86.7±6.7	26.8±2.9	30.8±1.7	13.3±2.5	94.1±14.0
Patients with β-thalassemia trait (BTT)	11.4±1.1	5.7±0.4	70.4±5.3	19.2±2.1	26.8±2.3	15.9±1. 6	112.0±11.0
Patients with Iron Deficency Anemia (IDA)	7.4±1.1	3.2±0.7	67.8±8.1	20.1±1.7	26.0±2.5	20.3±4.1	112.6±11.7
Patients with BTT and IDA	9.1±1.4	3.9±0.4	71.6±9.9	21.3±2.9	27.9±2.1	15.9±1.9	116.9±11.6
***p* values**							
Normal subjects vs Patients with BTT	0.001	0.001	0.001	0.001	0.001`	0.502	0.091
Normal subjects vs Patients with IDA	0.001	0.001	0.001	0.001	0.001	0.001	< 0.001
Normal Subjects vs Patients with IDA + BTT	0.001	0.001	0.001	0.001	0.001	0.086	< 0.001



Hemoglobin Electrophoresis

Fractions	%	Ref. %
Hb A	**97.7**	96.8 - 97.8
Hb A2	**2.3**	2.2 - 3.2

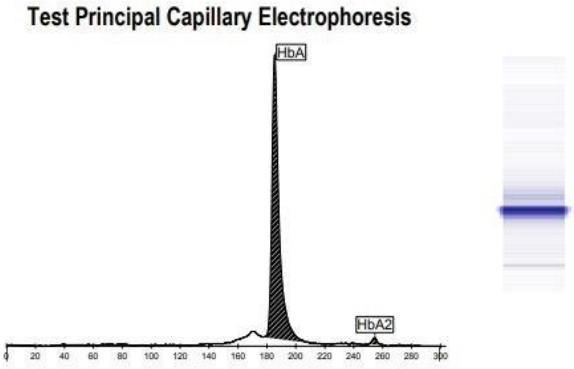
Fig. 1. Result of normal subject from capillary zone elctrophoresis



Hemoglobin Electrophoresis

Fractions	%	Ref. %	Ref. g/dl
HbA	**93.1**		
HbA2	**6.9**		

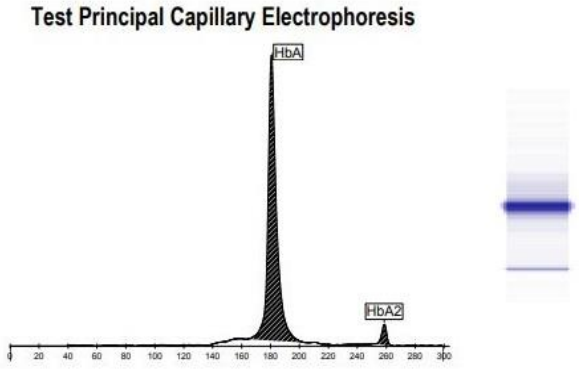
Fig. 2. Results of patients of group ii from capillary zone electrophoresis



Hemoglobin Electrophoresis

Fractions	%	Ref. %	Ref. g/dl
HbA	**98.3**		
HbA2	**1.7**		

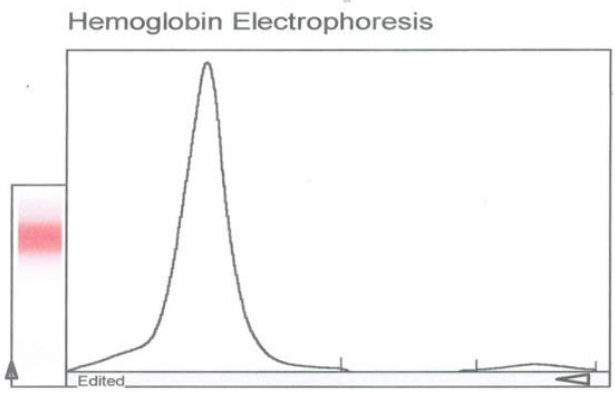
Fig. 3. Result of patient of group iii from capillary zone elctrophoresis



Hemoglobin Electrophoresis

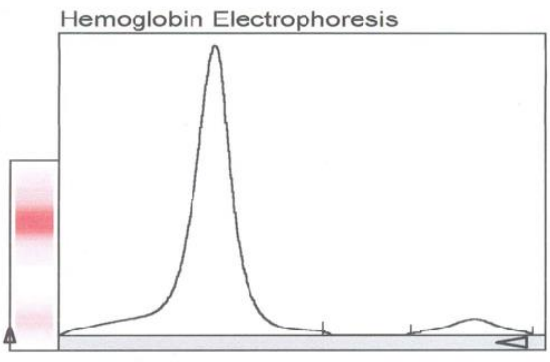
Fractions	%	Ref. %	Ref. g/dl
HbA	**95.9**		
HbA2	**4.1**		

Fig. 4. Results of patients of group iv from capillary zone electrophoresis



Fractions	%	% Normal
HbA1	96.7	96.00 - 98.00
HbF	0.2	< 2.00
HbA2	3.1	1.50 - 3.50

Fig. 5. Result of normal subject from cellulose acetate electrophoresis



Fractions		%	% Normal
HbA1	↓	93.0	95.50 - 98.50
HbF		0.2	< 2.00
HbA2	↑	6.8	1.50 - 3.50

Fig. 6. Results of patients of group ii from cellulose acetate electrophoresis

In the combined cases of BTT and IDA, no CBC parameters have been detected. Capillary zone electrophoresis and cellulose acetate electrophoresis both have shown the greater level of HbA2 in patients having both β-thalassemia trait and iron deficiency anemia.

The results were received by comparing it with control subjects having no abnormality.In combined situation of subjects having both BTT and IDA were found inadequate in using either of these techniques (Table II).

Table II. Comparative Analysis of Capillary Zone electrophoresis and Cellulose Acetate Hemoglobin Electrophorei

Groups	Capillary Zone Electrophoresis			Cellulose Acetate Hemoglobin Electrophoresis		
	HbA	HbA2	HbF	HbA	HbA2	HbF
Normal Subjects	95.6±0.7	2.8±0.1	0.4±0.2	95.5±0.2	2.7±0.1	0.3±0.1
Patients with β-thalassemia trait (BTT)	94.0±0.6	4.3±0.4	0.4±0.2	92.9±1.0	5.5±0.6	0.5±0.2
Patients with Iron Deficency Anemia (IDA)	96.4±0.3	1.8±0.4	0.5±0.2	96.3±0.4	1.5±0.6	0.5±0.2
Patients with BTT and IDA	95.6±0.4	2.6±0.3	0.4±0.2	96.4±0.7	2.9±0.6	0.4±0.2
***p* values**						
Normal Subjects vs Patients with BTT	0.001	0.001	0.001	0.001	0.001`	0.014
Normal Subjects vs Patients with IDA	0.032	0.001	0.077	0.015	0.001	0.016
Normal Subjects vs Patients with IDA + BTT	0.980	0.863	0.639	0.789	0.941	0.162

While the final results of this study demonstrates that normal BTT and classical BTT cases were easily and constantly diagnosed by the use of either Cellulose Acetate Hemoglobin Electrophoresis or Capillary Zone Electrophoresis. Neither of these techniques can detect BTT in combined form in the patients.

DISCUSSION

In Balohistan Pakistan, the two typical cases of microcylic hypochromic anemias are the beta thalassemia and IDA have been recorded to the date [1]. Beta thalassemia effected cases are increasing day by day to the existing cases of Pakistan's population [2]. Pakistan with increase rate of growth of 3% with existing population of 160 million, were categorized into five major ethical groups i.e. Punjabi, Pathan, Sindhi, Baloch and Urdu spokers. These groups are further partitioned into classes or 'Biradaris' [3]. Marriage are often carried out in the close relatives like cousins, a strong tradition of the Pakistan's population seen from centuries in the region of sub-continent [4-5].

Many reports have revealed that thalassemia has reached to the terrifying situation in the Balochistan. Its symptoms are very difficult to be diagnosed by the typical medical practices. The consequeses of thalassemia are complications in the blood forming potential and mild form of anemia (6-10).There are approximately 1800 cases of recorded thalassemia in Quetta. There are two functional thalassemia centres in district Quetta operating in two famous hospitals of the city i.e Quetta's Civil Hospital and Bolan Medical Complex. The blood of more than 60 children were given to these centres dialy for the diagnostic practices of thalassemia.

Cousin marriages are the vital cause of Beta thalassemia due to genetic disroders inherited from either of the effected parents (10-15). It is an important social and religious factor. To prevent these genetic diseases, it is very highly mandatory to develop the awareness programs in the general populations of Pakistan (16). Beta thalassemia the most dangerous disease causing many deaths in the population is the due to beta thalassemia gene present in both parents in heterozygous state.. In 1994, Government of Pakistan has taken steps in the form of an extensive redication programe to minimize the spread of β-thalassemia. This program was basically for the awareness of Beta thalassemia minor, especially in the initial diagnosis stages.

Furthermore, due to these programs and improved diagnostic techniques, a deduction has been observed in beta thalassemia major cases (9).

Various parameters for the detection of BTT by the Complete Blood Count have been shown in Table No I. In order to detect beta thalassemia in patients, HbA2 tests are confirmed by capillary and cellulose acetate electrophoresis. In this research, level of Hemoglobin A2 less than 3.5 % is considered as the normal cases and above 3.5% is considered as those having BTT, according to Table No II.

Classical Cellulose Acetate Hemoglobin Electrophoresis techniques in relation to semi-automated method is less favorable because it has less mistake chances than classical one. Capillary Zone Electrophoresis has been considered for the diagnosis because of its efficacy and less time duration and precise counting of Hemoglobin (11).

As for as detection of BTT is concerned, both these techniques Capillary Zone Electrophoresis and Cellulose Acet ate Hemoglobin are very operative and favorable. However as shown in table, both these techniques have shown no significance of statistics. So, there is no doubt that both these methods are equally suitable for the diagnosis of BTT in the patients (18).

According to the table No 1, serum ferritin and appearance of blood and microcytic hypochromic anemia were used to diagnose in IDA group. Low levels of Hemoglobin A2 were seen in the IDA group of patients. So, according to table No 3, neither of these techniques is effective for diagnosis of IDA. The general population of Pakistan exhibit approximately 7% and 42% of Beta thalassemia and IDA respectively (14, 18).

The main factor in the diagnosis of combined condition, if marriage has been done between and effected individual of BTT and combined condition, the chances of child having beta thalassemia major is very increased on birth. Such marriage should be control to reduce the spread of Beta thalassemia gene in public of Pakistan, being the vital cause of disease. On the other hand in Pakistan, it is alsoeffecting the prevention program of thalassemia. Female children have greater probability of developing the IDA condition. However,there are reported undignosed cases of BTT and IDA. The reason behind it is abnormal level of IDA and sightly normal level of HbA2. Abnormal level of IDA drages HbA2 to normal

condition. It is also being recommended that combined cases should be identified at any cost to reduce the increase of Beta thalassemia in population [14].

In the current research, both these techniques of Capillary Zone Electrophoresis and Classical Acetate Hemoglobin Electrophoresis have shown no significance in the diagnosis of BTT as shown in table No 2 (p=0.006). Table No 2 demonstrates the mean levels of HbA2 were also 2.74 and 3.04 respectively. Both these techniques may mislead the physicians and health consultants, as combine cases of BTT and IDA are not being detected by the both these methods. So, if an individual with the normal result of Hb electrophoresis should not be considered normal until it have probability of any iron deficiency from mild to moderate level and this iron deficiency should be diagnosed at any cost (14, 19).

According to current research, Cellulose Acetate Hemoglobin Electrophoresis and Capillary Zone Electrophoresis both are effective and time efficient for levels of HbA2 in both cases of BTT and simultaneously in combined condition. In Pakistan generally, and in Balochistan particularly Capillary Zone Electrophoresis is costly procedure for the low economical population of poor province.Health facilities are also very misera ble in this province of country.

CONCLUSION AND RECOMMENDATIONS

β-thalassemia is now-a-days considered as one of the most commone genetic abnormality worldwide. Different countries have already admitted it a preventable disease and they have also designed various effected compaines nationally, to reduce the onsets of affected families. Although there are many factors of its propagation, but different studies reveals the role of iron deficiency anemia in the spread of β-thalassemia especially in Pakistan. Iron deficiency anemia and beta thalassemia minor are the common disorders in Pakistan. Iron deficiency anemia causes reduction in HbA_2 levels while pathophysiological effect of beta thalassemia minor results in elevated HbA_2 levels. As a results, the patients with β-thalassemia trait (BTT) and iron deficiency anemia (IDA) shows normal level of hemoglobin A2. These patients are socially recognized as normal individuls due to poor facilities of detection, absence of accurate knowledge among working physicans and pathologists. Moreover, these people also avoid proper screening of β-thalassemia due to expensive costs of diagnosis. In Pakistan, marriage sometimes held between undiagnosed β-thalassemia minors, that will give birth to children having β-thalassemia major. It is also another hurdle in the proper elimination of thalassemia from our region.

Basically, this study was intended to assess the most exact and cheap for thalassemia prevention programme. Two methods, caplliary zone electrophoresis and cellulose acetate hemoglobin electrophoresis were used in this study to estimate HbA_2 levels. This study reaveld that semi automated cellulose acetate Hb-electrophoresis is s reliable method from screening of BTT in population.In low income countries like Pakistan,wher typical health care infrastructure does not facilitate poor and nee dy people properly,capillary zone electrophoresisia a expensive procedure as compare to semi automated cellulose acetate hemoglobin. These people are also unable to afford to screen β-thalassemia through capillary zone electrophoresis. This research work suggestes that semi automated cellulose acetated electrophoresis is aslo equal helpful in dignosing of β-thalassemia trait like capillay zone electrophoresis. It shuold be used for pathologist to prevent β-thalassemia abnormalitis nationaly. It also highlights significance of semi automated cellulose acetate hemoglobin electrophoresis in less developed country like Pakistan, where β-thalassemia cases areincreasing day byday.Following different recommendations are suggested according to facts beings noted:

1. Awareness about thalassemia should be spread through printing and audio visual media among urban and rural population of Pakistan.
2. Legislation related to thalassemia status before marriage should be implemented at grass root level through governm ent sector.
3. In the communities where inter-linkage marriages are very common because of certain customs;the thalassemia status of couple should be detected before marriage to use the boy 's only screening formula.
4. In countries like Pakistan no caseof microcytic/hypochromic anemia with a high index of suspicion for beta thalassemia trait should be declared as normal on Hb-electrophoresis or HPLC alone. In such cases, the possibility of co-existing iron deficiency should be ruled out by serum iron profile dete rmination.
5. Awareness about co-morbid disorder i.e. beta thalassemia trait with iron deficiency anemia should be created among working pathologists and general physicians.
6. Introduction of simple, easily available and cost effective methods like single tube osmotic fragility test, CBC and cellulose acetate hemoglobin electrophoresis should be intr oducedfor screening and diagnosis of BTT.Celluloseacetate hemoglobin electrophoresis facility should be made availabl e at least to the every district of each province.

ACKNOWLEDGEMENTS

I am grateful to express my deepest appreciations to my supervisor Dr. Muhammad Ali Khan, Co-supervisor, Dr. Shafi Muhammad Khosa, and Director CASVAB Prof Dr. Muhammad Masood Tariq Kiani from CASVAB, UoB whose experience, guidance, support and understanding made it possible for me to write a research article on the topic I have chosen for the study. I am very thankful to Mr. Mohsin Ali M.Phil Scholar, for helpin me in article write up, Mr. Zainuddin lab technician Hematology section and Dr. Muhammad Haneef Mengal (Head of Pathology Department) in Bolan University of Medical and Health Sciences Quetta, in sample collection.

REFERENCES

1. Ahmed S, Petrou M, Saleem M. Molecular genetics of betathalassaemia in Pakistan: a basis for prenatal diagnosis. *Br JHaemato*l1996;94:476-82.
2. Ahmed S, Saleem M, Modell B, Petrou M. Screening extended families for genetic hemoglobin disorders in Pakistan. *N Eng J Med* 2002 347:1162-1168.
3. Arif, F., J. Fayyaz and. Hamid A. Awareness among parents of children with thalassemia major. *J. Pak. Med. Assoc*2008 58: 621-624.
4. Camaschella C, Cappellini Thalassemia intermedia Haematologic 1995;80:58-68.
5. Cavazzana-Calvo M, Payen E, Negre O. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia *Nature* 2010;467:318- 322.
6. Cotton F, Changying L, Fontaine B, et al. Evaluation of a capillary electrophoresis method for routine determination of hemoglobins A2 and F. *Clin Chem.* 1999;45:237-243.
7. Craver RD, Abermanis JG, Warrier RP, et al. Hemoglobin A2 levels in healthy persons, sickle cell disease, sickle cell trait, and β-thalassemia by capillary isoelectric focusing. *Am J Clin Pathol.* 1996;107:88-91

8. Daniel YA, Turner C, Haynes RM, et al. Quantification of hemoglobin A2 by tandem mass spectrometry. *Clin Chem.* 2007;53:1448-1454.

9. Danjou, F, Anni F, Galanello R. Beta-thalassemia: from genotype to phenotype. *Haematologica*2011;96:1573-1575.

10. Dogaru M, Coriu D, Higgins T. Comparison of two analytical methods (electrophoresis and HPLC) to detect thalassemias andhemoglobinopathies. *RevistaRomână de Medicină de Laborator*2007;9:39-48.

11. Dussiot M, Maciel T, Fricot A, ChartierC Negre, O, Veiga J, GraptonD,Paubelle E, Payen E, Beuzard Y, Leboulch P, Ribeil J, Arlet J, Coté F, Courtois G, Ginzburg Y, Daniel T, Chopra R, Sung V, Hermine O & Moura I. An activin receptor IIA ligand trap corrects ineffective erythropoiesis in β-thalassemia. *Nature Medicine* 2014;2: 398–407.

12. Galanello R and Origa R. Beta-thalassemia. *Orphanet Journal of Rare Diseases*2010;5:11-15.

13. Gulbis B, Fontaine B, Vertongen F, et al. The place of capillary electrophoresis techniques in screening for haemoglobinopathies. *Ann Clin Biochem.* 2003;40:659-662.

14. Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MM. Influence of iron deficiency anaemia on haemoglobin A2 levels: possible consequences for beta-thalassaemia screening. *Scand J Clin Lab Invest* 1999;59:65-70.

15. Nazir G, Naz S, Ali S, et al. Anaemia: the neglected female health problem in developing countries. *J Ayub Med Coll Abbottabad*2011;23:8-11.

16. Olivieri, NF. The β-thalassemias. *N Engl J Med.* 1999;341:99-109.

17. Primary health care approaches for prevention and control of congenital and genetic disorders: report of a WHO meeting. Geneva, Switzerland: World Health Organization, 2000. (Accessed January 6, 2015, at http://www.who.int/iris/handle/10665/66571)

18. Stephens AD, Angastiniotis M, Baysal E. International Council for the Standardisation of Haematology (ICSH). ICSHrecommendations for the measurement of haemoglobin A2. *Int J Lab Hematol*2012;34:1-13.

19. Usman M, Moinuddin M, Ahmed SA. Role of iron deficiency anemia in the propagation of beta thalssemia gene. Korean J Hematol2011;46:41-4.

20. Usman M, Moinuddin M, Usman S. Cap +1 mutation; an unsuspected cause of beta thalassaemia transmission in Pakistan.Turk J Hematol2009;26:167-70.

21. Vichinsky EP. Changing patterns of thalassemia worldwide. *Annals of the New York Academy of Sciences* 2005;1054:18-22.

22. Wang J, Zhou S, Huang W. CE-based analysis of hemoglobin and its applications in clinical analysis. *Electrophoresis.* 2006;27:3108-3124.

23. Weatherall D.The Thalassemias: the role of molecular genetics in an evolving global health problem. *American journal of human genetics*2004;74: 385-392.

24. Weatherall D. J. and Clegg J. B. Molecular genetics of human hemoglobin. Ann. Rev Genet 1976;10:157-78.