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An Epidemiological Study of Thalassemia, its Quick Diagnosis and Influence of Malaria in Chittagong Area of Bangladesh

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Abstract: Molecular biology techniques were used for quick diagnosis of thalassemia in the present study. The incidence of thalassemia markers of both sexes, aged between 0.5-44 years, living in different areas of Chittagong, Bangladesh was studied. Information was collected from the subjects by questionnaire to find out the relationship of the thalassemia with sex, age, present illness, post illness, family relationships, personal history etc. Serum sample from the individuals were tested for routine hematological examination test and using molecular biology technique. Out of 45 subjects tested, a total of 29 cases (64.44%) were found to be thalassemia positive. Analysis of thalassemia markers in different sex groups showed that the incidence of thalassemia is greater in male (65.51%) than female (34.48%). The study also showed that thalassemia major were lower (34.48%) than the thalassemia minor (65.51%). Most of them are dependent on blood transfusion for their lives. It is concluded that the disease could be diagnosed quickly by electrophoresis and most important way to prevent the disease is to detect thalassemia carriers, parental diagnosis and pre-marriage counseling.

Key words: Thalassemia, hereditary disease, malaria, transfusion, blood, hemoglobin

INTRODUCTION

Thalassemia is one of the most common hereditary diseases prevalent in this part of the world. If this disease continues to pass from one generation to another, it may take the form of a disaster. Unfortunately in our country there is no way except blood transfusion to save the thalassemic patients. As a result, the only way to prevent the disease is carrier detection and awareness among the people about the disease. Chittagong is an area where there is a possibility of thalassemia. As far as we are concerned, no work on the prevalence of thalassemia in Chittagong region has been carried out until now. Consequently this work has been planned to carry out the prevalence of the disease in this area.

The term "thalassemia" refers to a group of blood diseases characterized by decreased synthesis of one of the two types of polypeptide chains (α or β) which form the normal adult human hemoglobin molecule (HbA, $\alpha_2\beta_2$), resulting in decreased filling of the red blood cells with hemoglobin and thus anemia. Depending on the involved genes, the defect is identified as α-thalassemia or β-thalassemia. Hemoglobin is a heterogeneous molecule. Hemoglobin is a tetramaric protein made up of heme and globin. There molecular weight is 64,450. It is a globular molecule made up of 4 subunits. Each subunit contains a heme moiety conjugated to a polypeptide. The thalassemia are a heterogeneous group of Mandelian disorders, all characterized by the lack of or decreased synthesis of either α or β globin chain of hemoglobin. The thalassemia can be classified according to the reduction in globin chain synthesis of the β chain.

Thalassemia is considered till as one of the commonest inhered Hemolytic Anemia in Bangladesh, though proper epidemiologic survey has not been carried out yet nationally to assess the gene frequency of this disorder. Thalassemia brings much morbidity, early mortality, a great deal of misery and despair for a family both financially and emotionally. Though the exact date regarding the incidence and prevalence of thalassemia in our country is not known but it seems to be increasing. A conservative world health report estimates that 3% are carriers of β thalassemia and 4% are carrier of Hb E in Bangladesh which means that there are 3.6 millions carriers of β thalassemia and 4.8 million carriers of Hb E and affected birth per thousand of thalassemia is .0106 and .300 of Hb β thalassemia. More than two thousand thalassemic children are born every year in Bangladesh. At present blood transfusion is the only form of treatment in the vast majority of patients[1]. Partial control of the disease by carrier detection and prenatal diagnosis will only be feasible in our country if it is possible to obtain

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The techniques for diagnosis of thalassemia have undergone radical changes since they were first introduced in 1981[2] and they used mid trimester fetal blood sampling to study globin chain synthesis and hence detect reduced β globin output. Recent technique like chronic villous sampling to obtain fetal DNA permits diagnosis in the first trimester and is safer with a 4% risk to the fetus as compared to the 5.4% risk in the case of fetal blood sampling. Since majority of the β thalassemia are caused by single nucleotide substitutions, these can be detected by the standard method of southern blot analysis with complimentary oligonucleotide probes[3]. In cases where the mutations cannot be definitely characterized, more advanced techniques like polymorphic restriction site analysis of the β globin gene cluster can be used[3].

Gross structural changes such as deletions or inversions such as those seen in δβ thalassemia and HPFH can be diagnosed by restriction end nuclease analysis followed by hybridization with the appropriate globin gene probe[3,4]. Recently employed Polymer Chain Reaction (PCR) followed by direct sequencing of the amplified product with either the earlier used radioactive or the currently used non radioactive probes labeled with horse reddish peroxides is the latest technique being employed world wide. PCR when combined with denaturing gradient gel electrophoresis rapidly localizes several β thalassemia mutations to specific region of the genome which then can be amplified and sequenced[5]. Loosekoot *et al.*[5] claim this technique to be superior than the PCR combined with oligonucleotide probes which they claim may miss 20% of the β thalassemia mutations.

A study[6] showed that variation in hemoglobin concentration and deformed RBC fails to nourish the malarial parasite. As a result thalassemic patients do not become infected with malaria. In this study a quick diagnostic method and a relationship between the malaria were studied. Since the research area is prone to malaria it is important to establish the relationship. A study in Papua New Guinea showed that α^+-thalassemia protects children against disease caused by other infections as well as malaria[7].

MATERIALS AND METHODS

The study was conducted from March 2002 to December 2003 in the Chittagong city of Bangladesh. The samples were collected from different hospitals and analyzed in the Laboratory of the Department of Microbiology, Chittagong University, Bangladesh.

Hematology: Blood was collected by venous puncture. The cubital vein was cleaned with alcohol and a rubber tourniquet was applied round the arm. Three milliliter blood was drawn from antecubital vein using disposable plastic syringe and collected in an EDTA (anticoagulants) bulbs. The blood was used for hemoglobin estimation, Differential Count (DC), TC, WBC, ESR estimation, Packed Cell Volume (PCV) determination, reticulocyte counts and osmotic fragility test by following standard procedures for hematological tests.

Agarose gel electrophoresis: The hemolysate was subjected to agarose gel electrophoresis in plate. Each slot was filled with 10 μL of hemolysate. The gel was run at constant 200 volts at 5-8 mA for 90 min. After electrophoresis, the gel was immersed in 7.5% trichloroacetic acid for 5-7 min for fixation. After fixation the gel was covered with moist filter paper and kept in an incubator at 37°C for 3-4 h. The gel was immersed in Ponceau stain for 5 min for staining. Destaining was done in 5% acetic acid with three consecutive washing.

Quantitative estimation of HbF: In a small test tube 1.6 mL N/12 NaOH was taken and kept at 20°C for several minutes. To it 0.1 mL of hemolysate was added and the tube was rinsed six times. The tube was then shaken gently for ten seconds. Exactly 1 min after adding the hemolysate, the tube was inverted six times. It was filtered through whatman filter paper No. 44 and the filtrate was crystal clear. The optical density was determined against water as a blank at 540 nm. * The dilution of the HbF solution was 1:51 and that of the untreated hemoglobin control was 1:251. The ratio is therefore 0.203.

* HbF (%) = OD of test / OD of control x 20.3

RESULTS AND DISCUSSION

Hemoglobin electrophoresis: When normal blood sample is electrophoresed, two strong bands (HbF and HbA) were observed along with a thin band (HbA2) which is present at times. In the thalassemia patients, all the three bands were observed only different in their thickness. Depending upon the band the thalassemia was divided into 2 types thalassemia major and thalassemia minor. Those with HbF+HbA/HbA2 were thalassemia major and those with HbF/HbA+HbA2 were thalassemia minor. The incidence and sex distribution of different types of thalassemia is given in Table 1.

Quantitative estimation of HbF: The normal value of HbF is <2%. The ideal values of thalassemia major are 10-25% and thalassemias minor are 2%. Present study found HbF value was 0-16%.

Table 1: The incidence and sex distribution of different types of hereditary thalassemia

Type	Male	Female	Total
Thalassemia major	8(27.58%)	2(12.5%)	10
Thalassemia minor	11(37.9%)	8(50%)	19
Normal	10(34.48%)	6(37.5%)	16
Total	29	16	45

Table 2: Study of thalassemia major

Investigation	Ideal value	Minimum	Maximum
Hemoglobin	M(13-18), W(11.5-16.5).	3.1 g dL^{-1}	11.3 g dL^{-1}
PCV	M(.31-.34), W(.37-.47)	14	40
RBC count	M(4.5-6.5), W(3.8-5.8).	2.1	5.4
Reticulocyte count	0.2-2.0%	1.5	10
ESR	M(0-10), W(0-20)	15	60
Osmotic fragility	---	Decreased	Decreased
Electrophoresis	---	HbF	HbF+HbA
HbF quantity	10-25%	Positive	Strongly positive
Age incidence	---	½ year	38 year.

Table 3: Study of thalassemia minor cases

Investigation	Ideal value	Minimum	Maximum
Hemoglobin	M (13-18), W (11.5-16.5).	2.9 g dL^{-1}	13.5 g dL^{-1}
PCV	M (.31-.34), W(.37-.47)	11	38
RBC count	M (4.5-6.5), W (3.8-5.8).	1.4	5
Reticulocyte count	0.2-2.0%	1.0	16
ESR	M (0-10), W(0-20)	8	160
Osmotic fragility	---	Decreased	Decreased
Electrophoresis	---	HbA	HbF+HbA +HbA2
HbF quantity	10-25%	Negative	Negative
Age incidence	---	0.5	44

Growth retardation was observed apparently in all the cases. Twenty four cases are transfusion dependent and two were not given blood transfusion at all. All the cases showed strongly positive Fetal Hemoglobin (HbF). Osmotic fragility is decreased by NESTROFT in all cases. MCV and MCH were also decreased significantly.

Biochemical investigations: In all the cases of β thalassemia major, the electrophoretic pattern showed HbA and HbF. Quantity estimation of HbF by alkali denaturation technique was also done. In all cases HbF was positive as shown in Table 2.

β thalassemia major group: Osmotic fragility by NESTROFT procedure was found to be positive in all the cases of β thalassemia.

Malaria relationship: None of the thalassemic patient have infected with malaria reported in the study. However, a number of thalassemic patients were found to have malaria just prior to develop thalassemia.

A total of 45 cases were studied in this experiment. Two individual families, one with 6 members and the other with 3 members, were also included in the study. The rest of the samples were collected by random sampling from different individuals.

Out of the 45 cases, 29 were thalassemia positive. Among the 29, thalassemia major accounted for 10 and thalassemia minor accounted for the rest 19 (Table 1). In most of the thalassemia major cases, the syndrome begins to appear within the first year of their birth. After the first year, the symptom gradually increases as the age increases. These results correlate with the observations of Kattamis[8]. In a comparative study[9], it was observed that β thalassemia major appeared usually within the first year of life. In the present study, we have studied the thalassemia disease in people aged between 6 month and 44 years.

In thalassemia major, there is always a severe degree of anemia with extremely low Hb level, MCV and MCH, which could be judged by the inspection of the peripheral blood[10]. In the present study, the incidence of the thalassemia major group with respect to Hb levels, RBC counts, MCV, PCV, MCH and MCHC all of which were higher compared to the controls. This has also been observed by Pootrakul[11].

The most striking hemoglobin finding is gross abnormality in the morphological appearance of the red cells as shown in Fig. 1 and 2. This was definitely seen in all the 10 cases of thalassemia major where RBC morphology showed a severe degree of anisopoikilocytosis with all predominantly microcytes, microspherocytes and occasional macrocytes. Variation in shape included target cells, tear-drop cells, elliptocytes and schistocytes. Marked degree of hypochromia was observed. Mean value of Reticulocyte count in the thalassemia major group were increased. The RBC of thalassemia major contain increased amount of HbF and thus remain the diagnostic feature of the forms of this condition[10]. It is to be emphasized that a technique like alkali denaturation used to measure HbF levels tend to

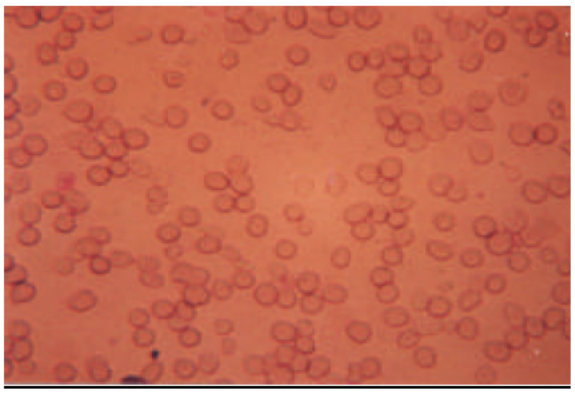

Fig. 1: A normal blood film showing characteristic red blood cells. Cells are smooth and well bordered

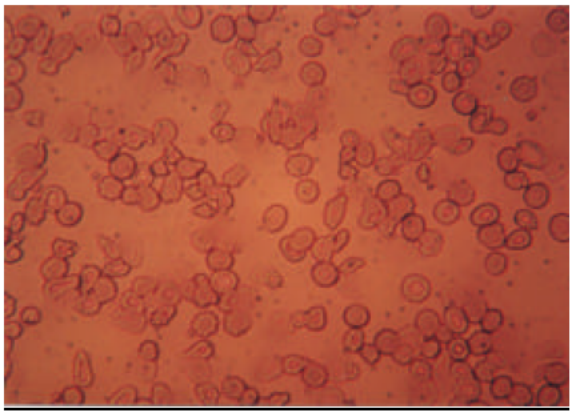

Fig. 2: A blood film of a patient with thalassemia showing the abnormal pale red blood cells, many of which are distorted indicating the presence of thalassemia

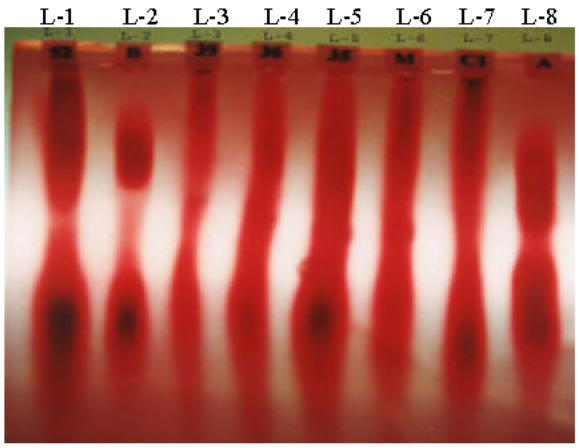

Fig. 3: Agarose gel electrophoresis of blood. Electrophoresis was performed by 2% agarose at constant 200 volts at 5-8 mA for 90 mins. Lane-2, 7 and 8 contain cord blood, lane-1 showing normal blood and other are positive thalassemia blood sample

give low values at higher HbF level. Mean HbF levels in thalassemia major group were highly increased with a wide range between 10 and 25% (Table 3).

Electrophoresis is one of the easy, quick and efficient diagnostics methodologies for thalassemic patient. In thalassemia major, after electrophoresis of the blood sample, three bands were observed. Of those, 2 were strong and the other was weak. The two bands were HbF and HbA. In normal blood, both the bands (HbF and HbA) were equally distinct but in thalassemic patients, the bands were not equal in their thickness. In the thalassemic patients, HbF was thicker and HbA was thinner. The patients with strong HbF bands have been observed to have a weak HbA2 band instead of HbA band. This might be due to absence of HbA in this type of patients. This difference in thickness of the bands may be attributed to the difference in their concentration.

In all the cases of β thalassemia major the NESTROFT test was positive. This was in agreement with the report of Kattamis[12].

It is clear from this study that thalassemia minor have significant degree of anemia when compared to control and this was in agreement with castaldi[13].

In heterozygote thalassemia, reticulocyte counts are usually increased[14]. This appeared to be similar in the present study with mean reticulocyte count of 2% which was slightly higher than normal but lower than that of thalassemia major. It is to be noted that males had higher level of indices than in females in the present study as well as in other series. No explanation can be given for this finding. Probably it is the individual variation in human life.

Increase in HbA2 levels remains the diagnostic parameter for β thalassemia trait condition[10]. The mean value for HbA2 levels heterozygote is 5%[15] with a range between 3.5% and 7%[10]. HbF levels in heterozygous β thalassemia are only slightly elevated[10]. In present study few cases showed mildly positive to HbF which shown a similar trend with the other workers.

In thalassemia minor, two bands (HbF/HbA2 and HbA) were observed. Of these HbF band was thinner than the HbA band as shown in Fig. 3. The difference in the thickness of bands may be due to the difference in their concentration.

In the present study NESTROFT was positively seen in all cases. A decreased MCV of less than 75fl and increased HbA2 levels of more than 3.5% was diagnostic of heterozygous β thalassemia in 3 cases. These results are in agreement with the reports of Mehta[16] and Kattamis[12].

It should be noted that iron deficiency can give a false positive NESTROFT but other parameters like hemoglobin level, peripheral study, red cell indices and HbA2 level clearly distinguish it from the β thalassemia minor cases having lower levels of all these, along with a more severe RBC morphology on the peripheral smear. We can confirm it by doing the serum iron level. In the present study, none of β thalassemia minor cases had evidence of iron deficiency, although iron studies would be required to confirm the same. However, the possibility of false positive NESTROFT due to iron deficiency was ruled out by near normal RBC indices and peripheral blood smear in this case. We also examined some random

blood sampling to find out the carrier because of small study design but the all sample showed normal blood picture.

A major finding found in this study is that a number of patients have developed β thalassemia just after being recovered from malaria. This is actually in contrast with the report of Allen[7] where he mentioned that α^+- Thalassemia protects children against disease caused by other infections as well as Malaria. However this may be due to some genetic changes/ modifications which allow malaria/ thalassemia to develop or decrease. This should be a vital area of research which may open up a new frontier for the cure of any diseases.

Finally it may be concluded that pre-marriage counseling is an ideal way to wreck this disease despite modern molecular biology and biotechnology being developed to prevent the Thalassemia disease which means that it is necessary to identify whether couples are bearing this disease or not. It is not difficult to detect the thalassemia carrier. This can be possible by the minimum laboratory cost. But problem is not only money; but also the lack of awareness in both public and physicians. All physicians in our country should involve in this matter and marriage counselor should have a role to prevent the disease by involving the community for pre marital examination. Though prenatal diagnosis is possible in thalassemia, it is not yet done in our country and it is very costly too. Mass consciousness is required to prevent this disease. For this, government has to come forward to eradicate the disease.

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