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Detection of alpha-thalassaemia in newborn cord blood from North Okkalapa General Hospital

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Thalassaemia is a heterogenerous group of disorders with a genetically determined reduction in the rate of synthesis of one or more types of normal haemoglobin polypeptide chain. A total of 308 cord blood samples from North Okkalapa General Hospital were screened for the presence of thalassaemia and haemoglobinopathies. The tests included haemoglobin electrophoresis, Hb A_2 estimation, and H inclusion bodies examination. Hb F alone band was found in 256 cases (83.12%), Hb EF band revealed in 25 cases (8.12%), Hb FH band was seen in 23 cases (7.47%) and Hb EFH band revealed in 4 cases (1.3%). Haemoglobin H was seen in 27 cases (8.77%) in Hb FH and Hb EFH bands. Among them, 21 cases had Hb H inclusion percent < 10% of RBC and 3 cases had Hb H inclusion percent > 30%. Twenty-four cases showed Hb A_2 < 2%. According to this study, on cord blood electrophoresis, α-thalassaemia prevalence is comparable to those previously reported (10%).

INTRODUCTION

The thalassaemias, the commonest monogenic diseases, are a family of inherited disorders of haemoglobin (Hb) synthesis characterized by a reduced output of one or more of the globin chains of adult Hb. Most of the important forms of thalassaemias are inherited in a Mendelian fashion; carrier parents who are symptomless have a one in four chance of having a severely affected child [1].

The thalassaemias are distributed across the Mediterranean region, the Middle East, the Indian subcontinent, and throughout South East Asia, including Myanmar. In many of these countries gene frequencies for the different thalassaemias and structural Hb variants are high. As social conditions improve in developing countries and childhood mortality due to infection and malnutrition declines, children with thalassaemia who would previously have died early in life are now surviving long enough to require treatment. The reason for very high frequency of thalassaemia is that carriers are protected from the consequences of infection with malaria parasite [1].

Thalassaemias are generally classified according to the particular globin chain that is ineffectively produced, as two main groups, α and β. The α-thalassaemias are disorders in which there is defective synthesis of α chains, i.e. Hb A, Hb A_2 and Hb F. The deficiency of α chains leads to an excess of γ chains in the fetus and of β chains in the adult [2].

Cord blood is composed of Hb A 20-25% and Hb F 70-90% of total Hbs [3]. In α-thalassaemia, α chains are shared by fetal and adult Hb; and hence the disease is manifest in both fetal and in adult life. Furthermore, excess γ and β chains do not precipitate immediately in the bone marrow

like α chain but produce the physiologically useless and unstable tetramers: γ_4 (Hb Bart's) and β_4 (Hb H). Since the α genes are duplicated, the genetics of thalassaemia is more complicated than that of β-thalassaemia. The genetic makeup of normal individuals can be written as αα/αα. Loss of both α genes on a chromosome is called α^0-thalassaemia, and is represented −/αα. Loss of one of the linked pairs of a globin genes is called α^+-thalassaemia, −α/αα. The homozygous state for α^0-thalassaemia produces intrauterine death with a profoundly anaemic and hydropic fetus: the Hb Bart's hydrops fetalis syndrome. Compound heterozygotes for α^0- and α^+-thalassaemia have a milder illness characterized by anaemia and splenomegaly which is called Hb H disease. Carriers for α^0-thalassaemia and homozygotes for α-thalassaemia have mild hypochromic anaemia, while carriers for α^+-thalassaemia have no haematological abnormalities [1].

The carrier state for all the important thalassaemias can be identified and methods for their prenatal diagnosis are well established. The first brief report of Hb H disease in Myanmar was published by Aung Than Ba Tu *et al.* in 1968 [4]. Their observations indicated that both α- and β-thalassaemias are prevalent in Myanmar. Hb Bart's in the cord blood for the detection of the incidence of α-thalassaemia was reported [5].

Haemoglobin H inclusion bodies (precipitated beta chain tetramers) are found in α-thalassaemia. Haemoglobin H has red cells which on exposure to brilliant cresyl blue developed multiple blue green spherical inclusions like the pitted pattern on a golf ball. Sometimes, haemoglobin H inclusion is not demonstrated. Definitive diagnosis can only be given by precise methods in molecular biology [6].

In Myanmar, there is high incidence of the important thalassaemias; i.e. α-thalassaemia 10%, Hb E 28%, β-thalassaemia 0.54-3.8% and 1-4.9 births per 1,000 infants with a major haemoglobinopathy [7]. Therefore, α-thalassaemia is an important health problem for Myanmar and it is essential to detect and identify the carrier state of thalassaemia and abnormal haemoglobin. Therefore, the present study is undertaken with the aims:

1. To detect the α-thalassaemia in newborn babies
2. To determine the haemoglobin bands among newborn cord blood
3. To estimate the content of haemoglobin A_2
4. To identify the haemoglobin H inclusion bodies

MATERIALS AND METHODS

A cross-sectional study was conducted to detect alpha-thalassaemia in newborn cord blood from North Okkalapa General Hospital during 2004-2006. A total of 308 newborn babies were included in this study. After getting an informed consent from the mother, thorough history taking was done. Diagnosis of α-thalassaemia trait was based on detection of haemoglobin H inclusion body and confirmed by finding Hb A_2 concentration less than 2% of total haemoglobin concentration (HbA_2 concentration 2-4% is normal) [6].

Haemoglobin H inclusion

For Hb H inclusion, equal volumes of fresh blood or blood collected into EDTA and 10 g/l brilliant cresyl blue (BCB) were mixed together in a small test tube. The preparation was kept at 37 ˚C for 20 min, and films made at intervals during this time. The films were allowed to dry and examined without counterstaining. Hb H precipitates as multiple pale-staining greenish blue, almost spherical bodies of varying size which can be clearly differentiated from the darker staining reticulo-filamentous material of reticulocytes. The number of cells containing inclusions varies according to the type of α-thalassaemia.

Haemoglobin A_2 determination

Hb A_2 was determined by cellulose acetate electrophoresis followed by elution method. The cellulose acetate strips were soaked in the buffer containing tris (hydroxymethyl) aminomethane, ethylenediaminetetraacetic acid, boric acid (TEB buffer, pH 8.9) for 5-10 min and gently blotted dry. They were then stretched across the bridge of the electrophoresis tank connected to the TEB buffer chamber by a double-layered filter paper with 10µl of hemolysated blood (Hb concentration 8-10 g/dl) applied via a microcapillary pipette and allowed to soak in.

Electrophoresis was carried out at 220 V (approx: 5mA) for about $1^1/_2$ hours, after which the strips were removed and the Hb A and Hb A_2 zones were cut into small strips and eluted in 15 ml and 1.5 ml TEB buffer respectively. When elution was complete (approx: 30 mins) the tubes were inverted to mix the contents, and the optical density (OD) was determined at 415 nm. The percentage Hb A_2 was then calculated as:

$$\%HbA_2 = \frac{OD^{415}\ HbA_2}{OD^{415}HbA2 + (OD^{415}HbA \times 10)} \times 100$$

Iso-Electric Focusing (IEF)

Typical 1mm polyacrylamide gel was prepared with following gel composition.

Distilled Water	3 ml
Acrylamide	3 ml
Sucrose	11 ml
Pharmolyte	300 µl (pH 6.7 – 7.7)
Ampholine	300 µl (pH 7.0 - 9.0)
Ampholine	150 µl (pH 3.5 -10.0)
Ammonium Persulfate	20 µl
NNN´N´ tetramethylethylenediamine	50 µl

Ten microliter of whole blood was mixed with 50 µl of saponin in microtitre plate wells. The three filter paper strips were placed accordingly (1 in centre and 2 in periphery of the gel plates). The central strip was soaked homogeneously with 0.2 mole of phosphoric acid solution and the peripheral strips were soaked homogeneously with 0.2 mole of NaOH solution. Prefocusing was done for 20–30 minutes with 300 V.

Pieces of filter papers (5 mm length) were placed into each well of samples and then soaked with blood. The soaked paper was placed on the gel plates. Then again, continued focusing was done with 400 V for 2 hours. The separation was carried out at a constant pH [6].

RESULTS

Detection of haemoglobin H inclusion bodies

Out of total 308 cases, Hb H inclusions appeared as golf balls were detected in 27 cases (8.77%) of which 18 were males and 9 were females. Among 27 cases (8.77%), 21 cases (6.82%) showed H inclusion bodies less than 10% and they were suggested as α-thalassaemia trait. Three cases (0.97%) showed H inclusion bodies 25-30% and they were diagnosed as thalassaemia intermedia. The remaining 3 cases (0.97%) showed H inclusion bodies more than 30% and were Hb H disease (Table 1).

Table 1. H inclusion bodies in newborn cord blood

	H negative (normal)	Positive(H)			Total
		H<10% α-thalassaemia trait	H 25-30% α-thalassaemia intermedia	H>30% Hb H disease	
Male	130	9	3	3	145
Female	151	12	0	0	163
Total	281	21	3	3	308

H = Haemoglobin H inclusion bodies
H<10% = H less than 10% of red blood cell
H 25-30% = H 25-30% of red blood cell
H>30% = H more than 30% of red blood cell

Iso-electric focusing (IEF)

Hb F alone was seen in 256 cases (83.12%) of which 120 were males and 136 were females. Hb EF band was revealed in 25 cases (8.12%) i.e. 9 males and 16 females and Hb FH was seen in 23 cases (7.47%) i.e. 12 males and 11 females. Hb EFH band was detected in 4 cases (1.3%) i.e. 3 males and 1 female (Fig 1 & Table 2).

Table 2. Various types of haemoglobin bands detected by iso-electric focusing using polyacrylamide gel in newborn cord blood

	F alone	EF	FH	EFH	Total
Male	120	9	12	3	144
Female	136	16	11	1	164
Total	256	25	23	4	308

F = haemoglobin F band
EF = haemoglobin E and F bands
FH = haemoglobin F and H bands
EFH = haemoglobin E, F and H bands

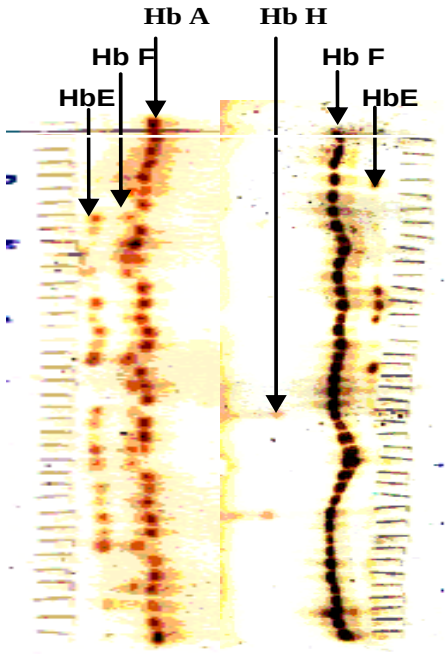

Fig. 1. Haemoglobin bands detected by iso-electric focusing

Table 3. Haemoglobin A_2 level in newborn cord blood

	Hb A_2 <2%	Hb A_2 2-4%	Hb A_2 4-9%	Hb A_2 >9%	Total
Male	12	122	2	9	145
Female	12	143	0	8	163
Total	24	265	2	17	308

Haemoglobin A_2 level

In 308 cases, normal Hb A_2 level (2-4%) was seen in 265 cases (86.04%) of which 122 were males and 143 females. Hb A_2 less than 2% was seen in 24 cases (7.79%) where 12 males and 12 females were found. Hb A_2 level 4-9% was detected in 2 cases (0.65%) both of which were males. Hb A_2 more than 9% was seen in 17 cases (5.52%) i.e. 9 males and 8 females (Table 3).

DISCUSSION

There are about 55 million people in South-East Asia carrying a thalassaemia gene or a Hb E gene. Control and prevention programs at any level are essential since this disease burden is expanding and at large. It has been predicted and speculated that thalassaemias/haemoglobinopathy are likely to impose an increasing health problem for many developing countries during the early part of the new millennium [8].

In this study, we demonstrated the prevalence of α-thalassaemia by screening of 308 samples of newborn cord blood by haemoglobin electrophoresis using iso-electric focusing by polyacrylamide gel, brilliant cresyl blue for examination of H inclusion bodies and cellulose acetate membrane for quantitative estimations of Hb A_2.

In α-thalassaemia, red cell haemoglobin H inclusion (H positive) (golf-ball shaped) are demonstrated. Haemoglobin H disease (H inclusion bodies 30% and above) has high incidence in the South-East Asia and occurs sporadically in the Middle East [9]. H inclusion bodies less than 10% are α-thalassaemia trait, H inclusion positive 25-30% is α-thalassaemia intermedia and H inclusion bodies more than 30% is haemoglobin H disease [6]. In 1956, the first report of haemoglobin H disease in Myanmar was made by Aung Than Batu and colleagues. They indicated a high prevalence of Hb H disease [5].

In this study, we found that H inclusion positivity was seen in 8.77% and H inclusion was negative in 91.23%. Among H inclusion positive cases, 21 cases (6.82%) showed H inclusion less than 10%, three cases (0.97%) showed H inclusion 25-30%

and three cases (0.97%) showed H inclusion more than 30%. Hb H and Hb Bart's were associated with a severe form of α-thalassaemia, Hb Bart's is γ_4 and Hb H is β_4. The cases with Hb Bart's may die either in utero or soon after birth [10]. Taking into consideration of hemoglobin Bart's hydrops and Hb H in the sever form of a thalassaemia, probability of risk couples must be definitely increased. If a mother is a carrier for α^0-thalassaemia her pregnancy is at risk for the Bart's hydrops fetalis syndrome, whereas the worst possible outcome of a pregnancy involving a woman homozygous for α^+-thalassaemia is the much milder condition, Hb H disease.

Weatherall (1998) has been suggested that over the next decade, it will be essential to make the thalassaemia problem more visible to governments and international health agencies that are involved in health care in the developing countries. This will require detail population surveys to determine the gene frequencies of the important forms of thalassaemia, together with a better understanding of their natural history and of the factors that modify their clinical phenotypes [11].

Haemoglobin H disease (β_4) indicates that the α-thalassaemia gene would be present in these patients who form Hb H inclusions in red cells on exposure to brilliant cresyl blue staining test. In normal adult blood, haemoglobin A consists of more than 90% of total haemoglobin and haemoglobin F is less than 1% and the remaining portions consist of haemoglobin A_2 2.5-4%.

In the present study, haemoglobin F was present alone or combined with other bands in newborn cord blood. It is normal finding for newborn babies. In normal newborn cord blood, haemoglobin F accounts for 70-90% of total haemoglobin [3].

Aung Than Batu *et al.* reported that approximately 10% of Burmese cord blood had Hb Bart's indicating a high prevalence of alpha thalassaemia trait and possibility of Hb H being frequent [5]. Therefore, according to this study, on cord blood electrophoresis, α-thalassaemia prevalence is comparable to those previously reported.

Health education and genetic counseling are the important initial steps of effective prevention program of thalassaemia which should be developed in Myanmar in the near future. Standards for appropriate Hb testing by various electrophoresis techniques must be set up, Hb A_2 estimation should be encouraged as a routine screening test. These options should be developed before considering prenatal diagnosis. We suggest that intensive community education is of prime importance which should precede the institution of genetic screening programmes. At least family planning and birth spacing practice after health education and genetic counseling may hopefully reduce the rate of birth of an infant with thalassaemia/ haemoglobinopathy.

This study highlights the fact that screening and genetic counseling, can support prevention and control programs for thalassaemia. Systemic prevention and control program should be planned by the cooperation of both the public and private sectors.

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REFERENCES

1. DJ Weatherall. The Thalassaemias. *British Medical Journal* 1997; 314: 1675-1677.
2. De Gruchy. Disorders of haemoglobin structure and synthesis. In: *Clinical Haematology in Medical Practice*. Firkin F *et al.* (eds). Blackwell Scientific Publication Oxford London Edinburgh 1989:137-171.
3. Weatherall DJ & Clegg JB. The thalassaemia syndromes. In: *Historical Perspectives*, 3rd ed. Blackwell Scientific Publications, Oxford UK. 1982: 147.

4. Aung Than Batu, Hla Pe, Nyunt Mg & Khin Kyi Nyunt. The thalassaemia in Burma, Union of Burma. *Journal of life Sciences* 1968; 241-247.
5. Aung Than Batu, Hla Pe & Khin Kyi Nyunt. Haemoglobinopathies in Burma II Haemoglobin H disease. *Tropical and Geographical Medicine*. 1971; 19-25.
6. Brozovic M & Henthorn J. Investigation of abnormal haemoglobin and thalassaemia. In: *Practical Haematology:* Dacie JV.& Lewis SM. (eds) 8th ed. Churchill Livingstone, Edinburg. 1995; 249-286.
7. Ne Win, Thein Myint Thu, Phyu Phyu Aung *et al*. Antenatal screening of beta thalassaemia and haemoglobin E from the perspectives of detection of couples at risk for a newborn with severe thalassaemia. *Journal of Myanmar Military Medicine* 2003; 009: 25-28.
8. Wong HB. Prevention of thalassaemia in South East Asia. *Annals of Academic Medicine Singapore* 1985; 14 (4): 654-65.
9. Minnich, V, , Nakorn SN, Tuchinda S, Wasi P& Moore CV. Inclusion body anaemia in Thailand: Haemoglobin H disease. Proceedings of the 6th International Society of Haematologis, Boston, Grune and Stratton, New York 1958.
10. Hunt JA & Lehmann H. Abnormal human haemoglobins, haemoglobin Bart's: A foetal haemoglobin without α chains. *Nature* 1959: 872.
11. Weatherall DJ. Thalassaemia in the next millennium. Key note address. *Ann-N Acad-Sci.* 1998; 30: 8501-8509.