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ZOOLOGY

A study on incidence of thalassemia in Port Said - Egypt using complete blood counts (CBC), a modified osmotic fragility test (OF-test) and a direct PCR on whole blood cell lysates

Akram M. Abouzied[1,#], Amany A. Ibrahim[2], Ahmed A. El-Lwah[3], Zohour I. Nabil[1]

1. Zoology Department, Faculty of Science, Suez Canal University, Ismailia, Egypt. 2. Medical Lab El-Lwah, Saad Zaghloul St, Port Said, Egypt. 3. Department of Clinical Pathology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.

Corresponding author: Akram M. Abouzied
Tel: 00201002848459 Fax: 0020643230416 email: abouakr@iit.edu

ABSTRACT

In order to study the incidence of thalassemia in Port Said - Egypt, we have used complete blood counts (CBC) and a combination of a modified onetube osmotic fragility test (OF-test) followed by a direct PCR analysis on whole blood cell lysates. One hundred and eighty adult Port Said subjects from different regions were screened. Fifty nine of the 180 subjects (32.8%) were positive for a modified OF-test. They all had significant CBC analysis as compared to the OF-test negative group. A successful identification of thalassemia using a 0.34% buffered saline OF-test prior to PCR-based genotyping on cell lysates was demonstrated. Among the 59 OF-test positive subjects, 8 resided unrelated cases were successfully observed to be thalassemic subjects. Our prospective study in 180 adult subjects confirms the expected incidence of α-thalassemia and β-thalassemia types in Port Said limited population which has not been reported before. The purpose to study the incidence of thalassemia in Port Said is making it applicable to a large-scale screening program in Egypt population, to give more insight into the origin and thalassemia mutations underlying these incidences, and to meet the short and long term goals of thalassemia prevention and management.

Keywords: Modified osmotic fragility test, thalassemia, thalassemia incidence, whole blood PCR

INTRODUCTION

Thalassemias are Hb genetic disorders in which there is unbalanced synthesis of different globin chains (Angatiniotis and Modell, 1998) and thus excessive destruction of red blood cells. In a normal scenario, the α/β-globin chains synthesis is ~ 1.0. When there is an imbalance in the synthesis of globin chains, it is defined as thalassemia.

Thalassemia is classified according to the globin chain for which the synthesis is impaired, e.g. α-thalassemia and β-thalassemia affecting the α- and β-globin chains respectively (Weatherall, 2001). The genes coding for α-like and β-like globin chains are located on chromosome 16 and 11 respectively. The globin genes share a common structure consisting of three exons and two introns (Fig. 1). The intron-exon junction is characterized by the presence of a conserved nucleotide sequence GU ("donor" splice sequence) at 5` end and AG ("acceptor" splice sequence) at 3` end (Feng *et al*., 1997; Orkin and Goff, 1981; Molchanova *et al.*, 1994).

Thalassemias are caused by a diverse array of mutations. The α-thalassemias are most commonly due to gene deletions while β-thalassemias are generally due to gene mutations at various sites of the β-cluster genome (Weatherall, 2001; Temtamy *et al*., 2009).

It is a life-threatening disease and each year hundreds of babies are born with α-thalassemia as well as severe forms of β-thalassemia (Angatiniotis and Modell, 1998; Weatherall, 2001; Renzo *et al*., 2010). It is a fairly common single gene disorder (Flint *et al*., 1993) and its current therapy consists of frequent blood transfusions combined with iron chelation treatment to combat anemia. The only cure, bone marrow transplantation, is limited by the scarcity of suitable

histocompatible donors (Lucarelli *et al*., 1999; Shawky and Kamal, 2012). Over the last century, because of mass population migration around the world, the prevalence of the thalassemia has become a global issue with particularly high frequencies in the Mediterranean basin (Weatherall and Clegg, 1981). In Egypt, β-thalassemia is considered the commonest chronic hemolytic anemia (85.1%) (Sabri, 1973; Rady *et al*., 1997; Shawky and Kamal, 2012). It is a significant public health problem in Egypt and the incidence varies from region to another. There is a high rate of consanguineous marriage in Egypt which helps to accumulate deleterious genes in families, reaching 35.3% with an average inbreeding coefficient of 0.019 which could be considered high (Shawky *et al*., 2011; Shawky and Kamal, 2012).

There are no published thalassemia-frequency data for Port Said, Egypt. Moreover, at the molecular basis the disease is not studied and the mutations not identified. The purpose of this study was to provide up-to-date information about the incidence of thalassemias and the nature of mutations that occurred in this Mediterranean basin city. This study reported successfully the incidence of thalassemias and analyzed mutations in Port Said governorate using complete blood counts (CBC), as well as a modified osmotic fragility test (OF-test) and a direct PCR on whole blood cell lysates.

Materials and methods

Complete Blood Counts (CBC):

One milliliter each of peripheral blood was put in standard EDTA-containing tube as anticoagulant and sent for complete blood count (CBC) in the Medical Lab El-Lwah, Saad Zaghloul St, Port Said, Egypt. One hundred fifty adolescent from both genders and fifty children aged 1-13 years (with a mean of 7 years) were sampled.

Our CBC tested the following parameters: hemoglobin (Hb) level, hematocrit (Hct), red blood cell (RBC) count, mean cell volume (MCV), mean cell Hb (MCH), mean cell Hb concentration (MCHC) using the automated Sysmex KX-21N hematology analyzer (Sysmex corporation, Japan).

Leishman's-stained blood smears from each tube were evaluated under a light microscope as usual.

Osmotic Fragility test (OF-test):

The osmotic fragility test (OF-test) was done as described by Panyasai *et al* (2002). The test was applied to the groups of subjects who were also investigated for complete blood counts (CBC). A sample of 20 µl whole blood was pipetted and mixed well with 2 ml of 0.34% buffered saline solution at pH 7.4 and allowed to stand at room temperature for 15 min before interpretation. The tests were evaluated by visualization as negative and positive. Negative samples were characterized by a clear red hemoglobin solution indicating complete hemolysis of the red cells in the solution whereas positive samples were identified by a cloudy or smoky appearance because of incomplete hemolysis of the red cells (figure 1). Suspicious samples with a very fine cloudiness considered as positive. To estimate the percentage of hemolysis, the test tube was centrifuged for 5 minutes at 500 x g and the hemoglobin in the supernatant solution was measured manually by absorbance (optical density) at 540 nm using UV-visible spectrophotometer. The results of the visualized interpretation and percentage of hemolysis in the solution were as follows: negative corresponded to 91 to 100% hemolysis and positive to 86 to 90 % hemolysis. Means and standard deviations of the hematologic analysis data and the osmotic fragility test (OF-test) of the healthy and anemic values were calculated and then compared by a paired *t*-test.

Fig. 1. Interpretation of the osmotic fragility test (OF-test). Clear (left: negative test) or turbid (right: positive test) solution, based on readable or unreadable black texts on the white background paper.

DNA Extraction:

DNA samples were prepared from whole peripheral blood of subjects using the Blood DNA Preparation Kit, Jena Bioscience, Egypt. 900 µl RBC Lysis Solution was added to 300 µl of whole blood in 1.5 ml microcentrifuge and vortexed and centrifuged at 15,000 rpm for 1 min. The supernatant was removed by suction up to about 20 µl; 1 ml of water was added, after centrifugation for 1 min as above, the supernatant was removed again up to about 20 µl and vortexed. 300 µl Cell Lysis Solution was added and vortexed. From a Jena Bioscience, Blood DNA Preparation Kit, 100 µl Protein Precipitation Solution was added to the suspension and vortexed vigorously for 30 seconds to mix well and centrifuged at 15,000 rpm for 1 min. The supernatant was transferred into a cleaned 1.5 ml microcentrifuge containing 300 µl isopropanol >99% and then vortexed and centrifuged again at 15,000 rpm for 1 min. The DNA white pellets were taken and kept at 4 °C until they were used as templates in PCR.

Polymerase Chain Reaction (PCR):

For PCR, all amplifications were made in final volumes of 50 µl with 40 µl of PCR TaqMan Master Mix (Applied Biosystems), contained 1 µl each of the 2 sets of amplification primers (figure 2) at a final concentration of 1 pmol and 4 µl of genomic DNA template (0.075µg/1µl). We optimized the PCR in a way that all primers can be used in one single amplification reaction. The PCR tubes were placed in a thermal Cycler (Perkin-Elmer). When the temperature in the block reached 94 °C and were kept at that temperature for 2.5 min for strand separation. After that, 35 cycles of amplification were performed. The cycle of denaturation at 94 °C for 0.5 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. When the last cycle was completed, the reactions were kept for another 5 min at 72 °C. The total amount of PCRs was analyzed by electrophoresis on 2% agarose gels containing 0.5 µg/ml of ethidium bromide; the DNA bands were detected by UV light and documented using a Biorad gel doc 1000 system. All products obtained were sized using a standard 100-bp molecular weight ladder.

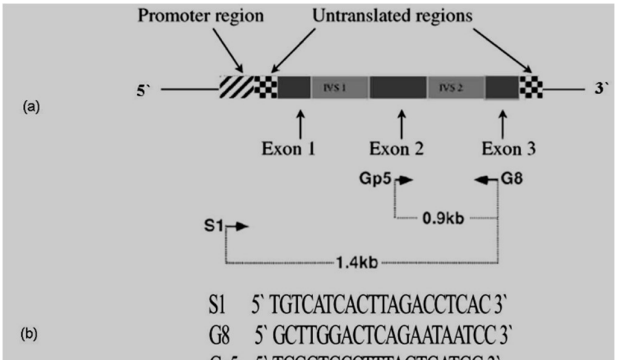

Fig. 2. (a) Schematic structure of human globin gene with three exons separated by two intervening sequences (IVS). The location and direction of amplification primers are shown. (b) Nucleotide sequences of each primer. S1 + G8 and Gp5 + G8 which span the 1.4 and 0.9 kb regions respectively.

Direct Sequencing of Amplified DNA:

Direct sequencing of PCR amplified genomic DNA samples was performed. Briefly, the amplified products obtained by a single amplification reaction were extracted once with ethanol precipitation after the phenolchloroform treatment. Direct sequencing of the PCR products was performed with the automatic sequencing assay using the Perkin-Elmer ABI 3700 DNA sequencer following the manufacturer's protocol for the sequencing reaction. One strand of DNA was sequenced. The sequencing results have been further grouped and analyzed.

Results

Complete Blood Counts (CBC):

The majority of the Hb variants do not infer any specific morphological features to the erythrocytes and the complete blood count (CBC) may well present normal. Although there is no simple phenotypic test available for the detection of thalassemia, the red cell indices are important indicators in the assessment of mild thalassemia characterized by normal or slightly subnormal Hb levels. Reduced mean corpuscular volume (MCV < 72 fL) of red cells and mean corpuscular hemoglobin (MCH <27 pg) are indicative of possible thalassemia (Clarke and Higgins, 2000; Traeger-Synodinos et al., 2002).

One hundred fifty adolescent of both genders from unrelated Port Said families were sampled along with fifty children aged 1-13 years. Tests performed for the detection of hemoglobin variants included Leishman's-stained blood smears from each subject. Results of the tests for hemoglobin variants are summarized in table 1. Among the One hundred fifty adults only fifty seven (38 %) in male and female were anemic subjects. Eighteen adults (13 males, 5 females) had significantly lower microcytic MCV (< 72 fL) of red cells and hypochromic MCH (<27 pg) as compared to those of the 132 adult subjects ($p < 0.001$) which indicative of possible thalassemia incidence in the genetics of a Port Said population and generally regarded as a case of further tests. None were transfusion-dependent but most had been transfused sporadically. Inclusion bodies and hypochromic red blood cells were readily detected by Leishman's-stained blood smears from each. The results of hemoglobin variants in children are shown in table 2. Gender in children does not detect any significance difference by t-test, and therefore these are not included in table 2. The parameters: hemoglobin (Hb) level, hematocrit (Hct), red blood cell (RBC) count and mean cell Hb concentration (MCHC) did not differ significantly between study groups.

Table 1. Results of hematologic values in normal adults compared with anemic adults in Port Said subjects.

Gender/Age (yr) Analysis data	normal ♂ >13	affected ♂ >13	normal ♀ >13	affected ♀ >13
	n = 75	*n* = 26	*n* = 75	*n* = 31
RBCs (X 10^{12}/*l*)	4.6 ± 0.5	3.8 ± 0.34	4.4 ± 0.26	4.01 ± 0.16
Hb (*g*/*dl*)	13.98 ± 0.17	9.5 ± 0.6 ¥	12.8 ± 0.2	8.8 ± 0.24 ¥
Hct (*%*)	43.03 ± 1.1	29.9 ± 2.7 ¥	39.2 ± 2.2	28.5 ± 0.9 ¥
MCV (*fL*)	85.3 ± 1.4	74.3 ± 4.2	84.7 ± 2.7	72.8 ± 1.9 ¥
MCH (*pg*)	30.6 ± 0.9	26.6 ± 1.6	29.03 ± 1.3	22.5 ± 0.9 ¥
MCHC (*g/dl*)	34.1 ± 0.7	31.7 ± 0.6	31.6 ± 1.3	31.4 ± 0.8

n number of individuals tested

numbers represent means ± SE values (n = 75)

¥ Significant according to unpaired *t*-test ($P \leq 0.01$)

Discussion

Preliminary observations on tissue polypeptide pattern indicated the differences between infected and control snails by both qualitative and quantitative techniques. The total protein contents of *B.alexandrina* generally decreased significantly as a result of infection. While, the total protein contents of *B.glabrata* increased due to infection compared with control group. Loker and Hertel (1987), revealed a significant increase in total protein in the plasma of *B.glabrata* infected for 4 and 8 days with *Echinostoma paraensei*. Previous studies on *B.glabrata* infected with *Schistosoma mansoni* indicated that after 11-14 days of infection, the plasma protein content of infected snails is significantly reduced (Lee and Cheng, 1972; Gress and Cheng, 1973; Michelson and Dubois, 1975; Stanislawski and Becker, 1979). So, those previous studies are tolerant with the present study of infected *B.alexandrina*. While, total protein of infected *B.glabrata* in the present study is coordinated with the results of Loker and Hertel (1987). *Schistosoma mansoni* therefore seems to evoke different response in different hosts as *B.alexandrina* or *B.glabrata*.

The electrophoretic pattern by SDS-PAGE revealed that, there were 13 different protein bands with molecular weights ranged from 15-300 KDa. Each group has a specific band, *B.alexandrina* has a specific band with 300 KDa, whereas, *B.glabrata* has a specific bands with molecular weights of 75 and 65 KDa. A band with molecular weight of 180 KDa in the present study considered to be hemoglobin similar to that described previously by Loker and Hertel (1987). In contrary, Granath et al. (1987) described this band with molecular weight of 160 KDa. This band with 180 KDa diffused completely in 14 days postexposure to parasite in both *Biomphalaria* species, this may be due to consumption of hemoglobin by parasite after prolonged exposure to S.mansoni. Uchikawa and Loker (1992), designed a group of molecules G1M (200 KDa) and G2M (80-120 KDa) as a response to infection with Echinostoma paraensei. But in the present study there were only two bands (275 and 150 KDa) appeared in B.glabrata after prolonged exposure (14 days) to parasite. A band with molecular weight of 65 KDa appeared in both control and 7days PE. B.glabrata and disappeared in 14 days PE.

Adema et al. (1997 a & b) demonstrated that 65 KDa band appeared in infected B.glabrata, and this band was strongly bound by anti-fibrinogenes antibodies, comprised of at least two members of the fibrinogene related protein (FREPs) and responsible for the internal defence of the snail. Also FREPs have been identified by Stout et al. (2008). These apparent bands indicate the presence of immune polypeptides that bind to protein of S.mansoni miracidia and sporocyst and ensured by the study of previous authors who demonstrated that FREPs, G1M, and G2M may agglutinate different types of erthrocytes and other particulate materials or bind protein of echinostome miracidia and sporocyst both in vivo and in vitro (Hertel et al., 1994 and Locker et al., 1994). The 35 and 15 KDa protein bands of the present study appeared in B.alexandrina and B.glabrata only 7 days PE respectively.

According to similarity matrix in the present study, there is an obvious high similarity index (S=0.8) between the two species of Biomphalaria, despite of their morphological and molecular differences. Also higher values of similarity indices (S= 0.8 & 0.75) obtained between all the groups of B.glabrata compared to control. However, moderate values of similarity (S= 0.5 & o.6) obtained between B.alexandrina groups compared to control. El- Dafrawy et al. (2006) demonstrated the highest similarity index 0.667, in 2 weeks and 5 weeks post exposure to S.mansoni miracidia groups of B. alexandrina and the lowest one was in 3-days post exposure (0.5) compared to control group.

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الملخص العربي

"ألاختلافات النوعيه بين قوقع بيومفلاريا الكسندرينا و بيومفلاريا جلابراتا في وجود و غياب البلهارسيا المعويه باستخدام تحليل البروتين."

تعتبر البلهارسيا من أكثر الامراض الشائعه في مصر منذ قديم الاذل. و قد تم التعرف علي نوعين من القواقع هما بيومفلاريا الكسندرينا و بيومفلاريا جلابراتا و التي تعتبر من انجح العوائل الوسيطه في مصر. في هذة الدراسه تم قياس كلا من البروتين الكلي بأستخدام طريقة برادفورد, و قياس الحمل الكهربي لفصل البروتينات بأستخدام صوديوم دودوسيل سلفات بولي أكريلاميد جل في أنسجة المجموعات الغير مصابه و المصابه بالبلهارسيا المعويه لكل من قوقع بيومفلاريا الكسنرينا و جلابراتا. و قد أظهرت النتائج ان العدوي و ايام الاصابه يمكن ان تؤثر علي التحليل الكمي و النوعي للبروتين. حيث وجد انخفاض معنوى ملحوظ في نسبة البروتين الكلي في قوقع بيومفلاريا الكسندرينا نتيجة الاصابه بالبلهارسيا المعويه, علي عكس ما حدث في قوقع بيومفلاريا جلا براتا حيث ظهر زياده في نسبة البروتين الكلي تحت تأثير العدوي. أوضح قياس الجهد الكهربي للبروتين ان هناك اختلافات بين النوعين من القواقع سواء المصابه او الغير مصابه بالبلهارسيا المعويه. حيث وجد عدد 13 شريط من البروتينات لها اوزان تتراوح بين 15-300 كيلو دالتون, مع وجود أشرطه مميزه لكل مجموعه. و قد اوضحت مؤشرات التشابه ان اعلي نسبة تشابه0.8 هي بين مجموعات بيومفلاريا الكسندرينا و بيومفلاريا جلابراتا الغير مصابه.

تعتبر هذه الدراسه هي محاوله لتعريف الانواع المتشابهه من الحيوانات باستخدام تقنيات حديثه و بسيطه بدلا من الطرق القديمه التي تعتمد علي الشكل الظاهري فقط. و ايضا اكتشفت الدراسه تنوع في اشرطة البروتين نتيجة الاصابه بالبلهارسيا المعويه.