ORIGINAL ARTICLE

High Performance Liquid Chromatography (HPLC) Screening among Filipinos with Suspected Thalassemia

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ABSTRACT

Introduction. Thalassemias and hemoglobinopathies are autosomal-recessive red blood cell disorders affecting hemoglobin (Hb) quantity and/or quality. Clinical manifestations vary from clinically asymptomatic to transfusion dependent individuals. These disorders are global in scope and is prevalent in Southeast Asia hence screening in the Philippines is very crucial for its prevention and control.

Objective. Our retrospective study aimed to determine the frequency of thalassemias and hemoglobinopathies in patients referred to the Molecular Genetics Unit, Institute of Human Genetics, National Institutes of Health, University of the Philippines, Manila for High Performance Liquid Chromatography (HPLC) screening.

Methods. Blood samples from patients (n=622) sent by hematologists from different parts of the country from October 2008 to February 2015 were analyzed. Extracted whole blood samples from the subjects were anticoagulated with ethylenediaminetetraacetic acid (EDTA) and were analyzed using BIORAD VARIANT™ HPLC Testing System and VARIANT™ Beta Thalassemia Short (BTS) Program kit for the detection of abnormalities in hemoglobin. Interpretation of results were based on the submitted mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) values, and Hb typing via HPLC of the patients.

Results. Approximately twenty-nine percent (29.10%, n=181) of subjects were presumptively identified with thalassemias and hemoglobinopathies by HPLC. Beta-thalassemia trait, Hb E trait, and beta-thalassemia/Hb E disease were detected in 65 (10.45 %), 14 (2.25 %), and 3 (0.48 %) subjects, respectively. While suspected alpha-thalassemia, presumably Hb H disease, was found in 99 (15.92 %) patients. Interestingly, seventy-two percent (72.11%, n=318) of the patients with normal Hb typing via HPLC have low MCV and MCH values.

Conclusion. Results of this study provide the spectrum and frequency of thalassemias and hemoglobinopathies in patients referred to our laboratory for HPLC analysis.

Key Words: thalassemia, hemoglobinopathies, high performance liquid chromatography, Filipino, red cell indices

INTRODUCTION

Thalassemias and hemoglobinopathies rank among the most common blood disorders in the world.[1] The World Health Organization (WHO, 1989) reported that 7% of the world's population is a carrier of the disorders.[2] Cases of thalassemias and hemoglobinopathies, and some of its interactions, are reported to be a major health problem.[3,4] Moreover, these disorders are both prevalent and widely distributed across Africa, the Mediterranean region, the Middle East, the Indian subcontinent, China, and Southeast

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Asia, therefore screening programs in these areas are necessary for the prevention and control of the disorders.[2,5]

Thalassemias are groups of autosomal recessive inherited blood disorders characterized by quantitative decreased or absence of production of globin chains of the hemoglobin (Hb) molecule.[6] Patients with aberrations in the beta globin chain are beta thalassemics while those with abnormalities in the alpha globin chain are alpha thalassemics.[6] Beta thalassemia is further classified as trait, intermedia and major whereas alpha thalassemia is categorized as carrier, trait, Hb H disease and Hb Barts hydrops fetalis depending on the severity of the disease.[6,7] Clinical manifestations of thalassemia disorders vary from clinically mild anemia with mild persistent microcytosis to severe life-threatening anemia that causes death even in utero.[4] In contrast, hemoglobinopathies such as Hb C, Hb S, and Hb E, result in abnormalities in the globin chains affecting the structure of hemoglobin molecules within red blood cells[4,5] which also cause significant clinical manifestations in some cases.[4]

The presumptive diagnosis of thalassemias and hemoglobinopathies is important in the prevention and control of the disorder and is done through the assessment of erythrocyte morphology and indices particularly the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), detection of Hb variants and quantification of Hb levels, detection of Hb H inclusion bodies[2] and genetic analysis. Specifically, the BHES protocol is used in the presumptive diagnosis of thalassemias where (B) stands for the assessment of blood film and blood count; (H) for Hb subtyping by high performance liquid chromatography (HPLC); (E) for analysis of blood sample via electrophoresis, and (S) for stability analysis of samples via the H-inclusion test and/or sickle cell solubility test. Ultimately, definitive diagnosis is done via genetic analysis.[8]

The Bio-Rad VARIANT™ HPLC (Bio-Rad Laboratories, California, USA) Testing System, an automated cation exchange HPLC instrument used together with The VARIANT™ Beta Thalassemia Short (BTS) Program (Bio-Rad Laboratories) is a sensitive and precise method that is capable of accurately quantifying Hb A_2 and Hb F concentrations within the validated reportable range of 1.0% to 13.0% and 1.0% to 40%, respectively. It is capable of detecting hemoglobinopathies such as Hb E, Hb D, Hb S, and Hb C.[4,9,10] The program was also reported to be useful in the qualitative diagnosis of common alpha-thalassemias such as Hb H, EA Barts, and EF Barts diseases.[2] Furthermore, hemoglobin fraction analysis by cation exchange HPLC has the advantage of quantifying Hb F and Hb A_2 along with hemoglobin variant in a single highly reproducible system.[11]

Cases of thalassemia and hemoglobinopathies were found to be particularly prevalent in tropical and subtropical countries.[12] In Southeast Asia alone, the approximate number of affected births is 0.66 per 1,000.[13] In the Philippines, there is lack of available data on the frequency of thalassemias and other hemoglobinopathies. Our study aimed to determine the frequency of these disorders in Filipino patients referred to our laboratory for HPLC analysis. The results gathered in this study will provide initial data on frequencies and spectrum of thalassemia and hemoglobinopathy cases screened in our laboratory. The result of this study will also guide and aid future studies, particularly in the application of HPLC in presumptive diagnosis of thalassemias and other hemoglobinopathies in the Philippines.

METHODS

Six hundred twenty-two (622) patients with available MCV and MCH, values referred by hematologists from different parts of the country from October 2008 to February 2015, two years old and above, regardless of gender and ethnic origin, were included in this retrospective study. The subjects included were patients referred by hematologists as part of the work up for anemia and for confirmation of clinically suspected thalassemia or hemoglobinopathy. The study was approved for implementation by the Research Ethics Board of the University of the Philippines, Manila (UPMREB 2017-229-01).

Five (5) ml of whole blood in ethylenediaminetetraacetic acid (EDTA) tube was collected from the subjects and sent to the Molecular Genetics Laboratory, Institute of Human Genetics (IHG), National Institutes of Health (NIH), University of the Philippines, Manila (UPM) for HPLC analysis. The samples collected were prepared using the BTS Program kit according to the manufacturer's instruction and were loaded on the VARIANT™ HPLC Testing System. The testing system used the 3.0 x 0.46 cm cation exchange cartridge and a dual wavelength filter photometer for separation and detection of the different hemoglobin variants.[2] Results were reported as chromatograms with time vs absorbance parameters. Hemoglobin types were determined based on Hb A_2, A, F, S, C, H and Barts levels (Figure 1).

Interpretation of patients' results were either normal hemoglobin type or non-clinically significant thalassemia, normal hemoglobin type with low MCV and MCH values, beta-thalassemia trait, Hb E trait, beta-thalassemia/Hb E disease and suspected Hb H disease, and were based on the submitted MCV and MCH values and Hb types seen in the HPLC chromatograms of the subjects (Table 1).

RESULTS

Demographic Profile

A total of 622 patients referred from October 2008 to February 2015 were included in this retrospective analysis. The patients screened included 346 (55.63%) females and 265 (42.60%) males. Majority of the patients were from the ages 2 to 5 years old (n=137, 22.03%) followed by patients with ages 6 to 10 years old (n=134, 21.54%) and 31 to 40

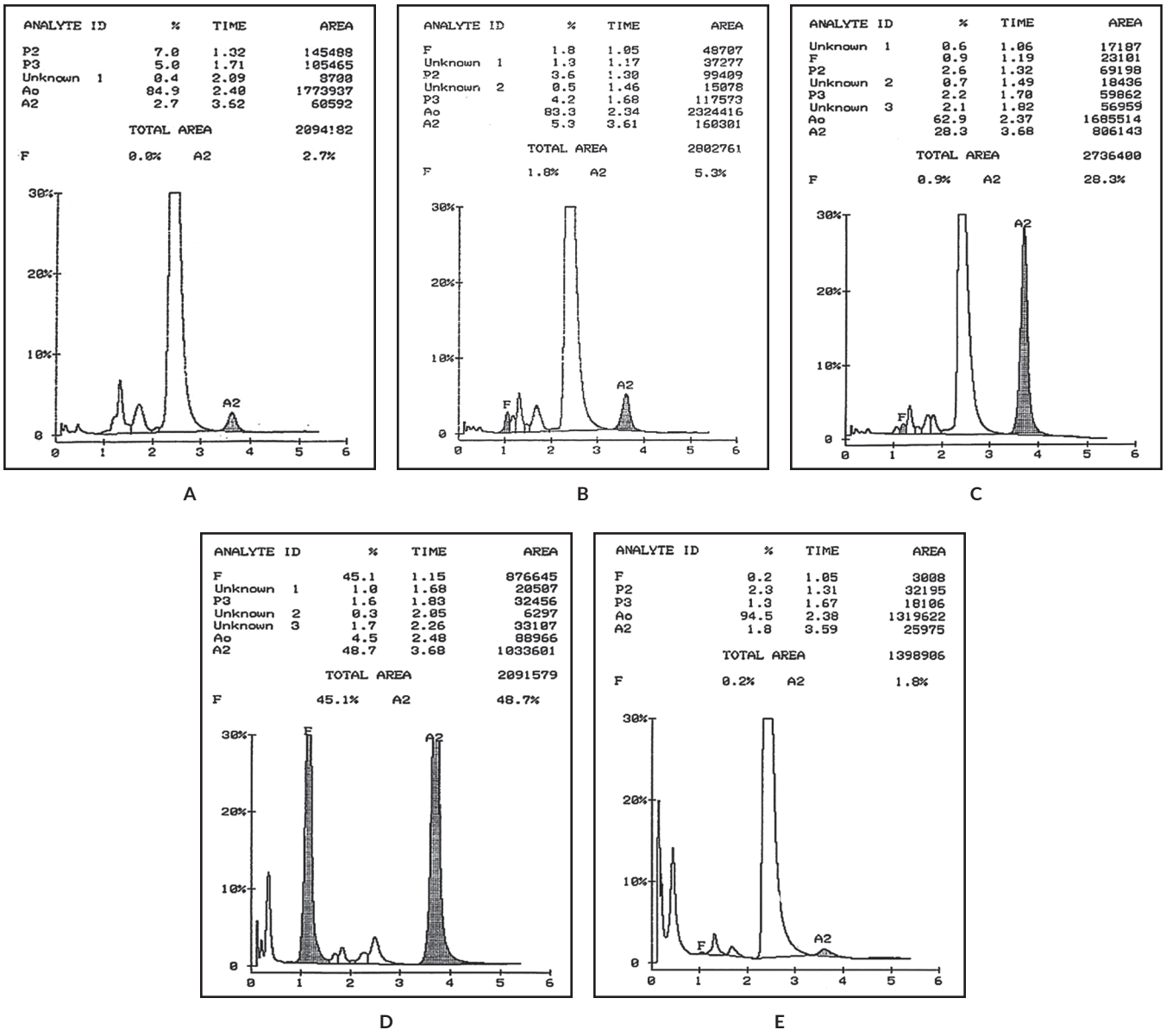

Figure 1. Hemoglobin typing via HPLC analysis. HPLC chromatograms of patients with hemoglobin type **(A)** A_2A, Hb A_2 ≤ 3.5% **(B)** A_2A, Hb A2 3.6 – 8 % **(C)** EA, Hb A_2 ≥ 25 % **(D)** EFA and **(E)** A_2A Barts H or A_2A H. X and Y axes represent the retention time in minutes and percentage hemoglobin. Eluted peaks are Hb Barts and H, F, A, A_2 with retention times at less than 1 minute, 1.00 – 1.30, 2.20 – 2.30, 3.68 – 3.98 minutes, respectively.

Table 1. Criteria used for presumptive screening of thalassemia

MCV (fL)	MCH (pg)	Hb Type	Interpretation
≥ 80	≥ 27	A_2A, Hb A_2 ≤ 3.5%	Normal hemoglobin type or non-clinically significant thalassemia
< 80	< 27	A_2A, Hb A_2 ≤ 3.5%	Normal hemoglobin type with low levels of MCV (< 80 fL) and MCH (< 27 pg)
< 80	< 27	A_2A, Hb A_2 3.6–8 %	Beta thalassemia trait with or without alpha thalassemia
< 80	< 27	A_2F	Beta thalassemia major with or without alpha thalassemia
< 80 or ≥ 80	< 27 ≥ 27	EA, Hb A_2 ≥ 25 %	Hb E trait
< 80	< 27	EE, Hb E ≥ 80%, Hb F ≤ 5 %	Hb E disease with or without alpha thalassemia
< 80	< 27	EFA	Beta thalassemia/Hb E disease with or without alpha thalassemia
< 80	< 27	A_2A Barts H or A_2A H	Suspected Hb H disease

years old (n=90, 14.47%) (Table 2). Most patients were from Luzon (n=361, 58.04%), particularly Region II, the National Capital Region (NCR), Region IV-A and Region III (Table 3). Approximately five (5.31%, n=33) and two (1.61%, n=10) percent of the patients were referrals from Mindanao and the Visayas, respectively (Table 3).

Frequency

One hundred eighty-one (n=181, 29.10%) subjects referred to our laboratory presented with abnormal hemoglobin fractions on HPLC and were presumptively screened with thalassemias or hemoglobinopathies. Sixty-five (n=65, 10.45%) subjects were with the beta-thalassemia trait, fourteen (n=14, 2.25%) with Hb E trait, and three (n=3, 0.48%) with Beta thalassemia/Hb E disease. Ninety-nine (n=99, 15.92%) of the patients seen were suspected with Hb H disease. Interestingly, approximately seventy-two percent (72.11%, n=318) of patients with normal hemoglobin type (n=441) have low levels of MCV and MCH as seen in Table 4.

DISCUSSION

In the Philippines, this study is the first to report on the frequency data of thalassemias and hemoglobinopathies determined through the analysis of MCV and MCH values and Hb type of patients. This study determined that most of the patients referred to our laboratory for HPLC analysis were suspected Hb H disease (15.92%), beta thalassemia trait (10.45%), Hb E trait (2.25%) and beta thalassemia/Hb E disease (0.48%) cases. Moreover, it is important to note that majority (72.11%, n=318) of patients sent for testing with normal Hb type have low levels of MCV and MCH.

The frequency of suspected Hb H disease (15.92%) reported in our study is higher than the reported gene frequency (5%) of alpha thalassemia in the Philippines by Fucharoen et al.[14] However, the number of Hb H cases reported in this study might not be reflective of the true frequency of the disorder because though the HPLC method is considered a sensitive and accurate method for detecting normal and abnormal levels of hemoglobin, Hb H and Hb Barts levels are only qualitatively analyzed using the HPLC method and the BTS Program.[2] The evaluation via H-inclusion test, peripheral blood film and confirmation via the molecular analysis are highly advised for these patients. On the other hand, the frequency of beta thalassemia trait (10.45%, n=65) reported in this study is comparable with the carrier frequency of beta thalassemia in Southeast Asia (0-11%) reported by Weatherall et al. in 2001.[6] No beta thalassemia major was detected in the patients tested yet there were ten (n=10, 15.38%) beta thalassemia trait patients with increased Hb F values ranging from 5.10 to 11.10%. The elevation of Hb F of these patients could be due to co-inheritance of delta thalassemia or hereditary persistence of fetal hemoglobin (HPFH, OMIM #141749).

Table 2. Age distribution of patients

Age group	No. of patients (n = 622)	Percentage (%)
2 to 5 years old	137	22.03
6 to 10 years old	134	21.54
11 to 20 years old	89	14.31
21 to 30 years old	75	12.06
31 to 40 years old	90	14.47
41 to 50 years old	47	7.56
51 to 60 years old	24	3.86
61 and above	26	4.18

Table 3. Distribution of patients based on region of origin

Region	No. of patients (n = 622)	Percentage (%)
Luzon		
NCR	86	13.83
CAR	5	0.80
Region I	16	2.57
Region II	129	20.74
Region III	53	8.52
Region IV-A	57	9.16
Region IV-B	6	0.96
Region V	9	1.45
	361	***58.04***
Visayas		
Region VI	4	0.64
Region VII	5	0.80
Region VIII	1	0.16
	10	***1.61***
Mindanao		
Region IX	1	0.16
Region X	0	0
Region XI	19	3.05
Region XII	10	1.61
Region XIII	1	0.16
ARMM	2	0.32
	33	***5.31***
Data not available	218	35.05

Table 4. Age distribution of patients

Case	No. of patients (n = 622)	Percentage (%)
Normal hemoglobin type or non-clinically significant thalassemia	**123**	**19.77**
Normal hemoglobin type with low MCV and MCH values	**318**	**51.13**
Beta thalassemia trait with or without alpha thalassemia	**65**	**10.45**
Beta thalassemia major with or without alpha thalassemia	**0**	**0.00**
Hb E trait	**14**	**2.25**
Hb E disease with or without alpha thalassemia	**0**	**0.00**
Beta thalassemia/Hb E disease with or without alpha thalassemia	**3**	**0.48**
Suspected Hb H disease	**99**	**15.92**

The presentation of Hb E trait (n=14, 2.25%) and the absence of Hb S and Hb C cases in the patients analyzed is consistent with literature.[6] Hb E is the most common Hb variant reported in Southeast Asia with a carrier rate of more than 60%. The detection of Hb E in this study was via detection of Hb A_2 at 25.00% to 35.00% levels,[2] since Hb E co-elutes with Hb A_2, and this is comparable with the values detected in this study (25.00% to 31.4%). Carrier frequency of Hb C in the region was reported to be 0% while Hb S was reported to be common in the Sub-Saharan Africa, the Indian subcontinent, and the Middle East only.[6] Despite Hb S not being reported, the inclusion of sickle cell solubility tests in routine testing or screening for thalassemias and hemoglobinopathies is still recommended. The WHO recounted that inherited hemoglobin disorders including the sickle cell disorders are now common worldwide due to an increase in migration.[13]

The detection of beta thalassemia/Hb E disease in 0.48% (n=3) of the subjects in this study is important to note. Although Hb E disease is benign, it is considered clinically important when co-inherited with beta-thalassemia because it results to a clinical condition similar to beta thalassemia intermedia and major.[6,15,16] In 2011, Olivieri et al. even indicated that globally, about fifty percent of the most severe form of beta thalassemia that requires transfusion from birth is due to its co-inheritance with Hb E disease.[17]

Finally, three hundred eighteen patients (n=318, 72.11%) with normal Hb type (n=441) were observed to have low MCV and MCH values. These patients are advised to have further analysis via electrophoresis on cellulose acetate to rule out beta thalassemia, H-inclusion test to rule out alpha-thalassemia and serum ferritin to rule-out anemia due to iron deficiency.[18] In Canada, carrier screening is done in women with low MCV (microcytosis) and low MCH (hypochromia) values in the presence of normal hemoglobin type. These group of women were advised to have an H-inclusion test and were simultaneously tested for serum ferritin to rule out iron deficiency anemia. This is important to consider as couples who are carriers of thalassemias and hemoglobinopathies has a 25% chance of conceiving newborns with clinically significant disease such as Hb H, Hb Barts hydrops fetalis and beta thalassemia major.[5] Moreover, about one percent (1.13%, n=5) of these samples also have elevated Hb F, ranging from 6.3 to 34.8%, despite normal Hb A_2. Two patients from this group have Hb F values of 22.00% and 34.80% and marginal Hb A_2 levels of 3.30% and 3.40%, respectively. Delta-thalassemia causes elevation of Hb F and decrease of Hb A_2 levels of beta thalassemia trait,[19] suggesting that these two patients could be delta-beta thalassemia cases. This should be emphasized, as co-inheritance of delta thalassemia may have obscured the detection of the beta thalassemia trait, thus, carrier screening for beta thalassemia is also advised for these patients.

CONCLUSION

A significant proportion of thalassemias and hemoglobinopathies were noted in Filipinos referred to our laboratory for HPLC screening. Results of this study provide spectrum and initial data on the frequency of these diverse groups of diseases in Filipino patients tested in our laboratory. This study also showed that Hb typing via HPLC with evaluation of MCV and MCH values is an effective tool in screening thalassemias and hemoglobinopathies. However, for comprehensive work-up of suspected patients, adherence to the BHES protocol and confirmatory testing via genetic analysis is highly recommended. The authors also suggest for the confirmation of the thalassemias and hemoglobinopathies identified as well as its possible co-occurrence with other thalassemias and hemoglobinopathies as it may affect patient's course of disease and prognosis.

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Statement of Authorship

All authors have approved the final version submitted.

Author Disclosure

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