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**Title page**

**Title:** The prevalence of anemia, thalassemia and iron deficiency in Phnom Penh, Cambodia

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**The prevalence of anemia, thalassemia and iron deficiency in a group of Phnom Penh Cambodian students**

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**Abstract**

**Background**: Anemia is a serious public health problem in developing countries. A number of factors might contribute to the condition including iron deficiency and thalassemia. In Cambodia, prevalence data on anemia, iron deficiency and thalassemia is not available.

**Objectives:** To determine the prevalence of anemia, thalassemia and iron deficiency in Phnom Penh, Cambodia and to demonstrate the health burden of anemia in relation to thalassemia and iron deficiency among anemic subjects.

**Methods:** A cross-sectional analytic study assessed anemia, thalassemia, as well as iron deficiency among Phnom Penh Cambodian students.Anemia was defined by WHO criteria adjusted for age and sex.Blood samples of 400 students enrolled the blood donation at faculty of Pharmacy, University of Health Sciences, Phnom Penh, Cambodia will be recruited consecutively during August to October 2013. All subjects will be initially screened for thalassemia at the faculty in Phnom Penh using either OF/DCIP or MCV/DCIP (MCV < 80 fl). Blood and plasma samples of both positive and negative-screening subjects will be collected and sent to the Center for Research and Development in Medical Diagnostic Laboratory (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Thailand, for investigation of thalassemia and hemoglobinopathies. The project was approved both by the National Ethic Committees on Health Research (NECHR), Phnom Penh, Cambodia and by the Ethical Committee of Khon Kaen University, Khon Kean, Thailand. Informed consent was obtained from all participants.

**Results:** XXX out of the 400 students (?%; 95% CI = …to…) were anemic. The overall prevalence for thalassemia alone, iron deficiency alone, and both thalassemia and iron deficiency was % (95% CI = …to…), % (95% CI = …to…), and % (95% CI = …to…), respectively. To demonstrate the extent of anemia in relation to thalassemia and iron deficiency among anemic subjects, the proportions of thalassemia and iron deficiency in the anemic group were compared. It is found that the proportion of iron deficiency was two times lower than the proportion of thalassemia.

**Conclusion:** A high prevalence of anemia, thalassemia and iron deficiency indicates a need for appropriate measures to prevent and control anemia, iron deficiency as well as thalassemia in the country. Comparison of proportions of thalassemia and iron deficiency among anemic indicates that thalassemia is the major contributing factor for anemia among Cambodian students.

**Key words:** Prevalence, Screening, Thalassemia, Iron deficiency, Anemia, Phnom Penh, Cambodia

**Introduction**

Anemia is a global public health problem affecting both developing and developed countries with major consequences for human health as well as social and economic development. It occurs at all stages of the life. One in four people in the world is affected by anemia and three most vulnerable high risk groups are preschool-age children, pregnant women, and non-pregnant women of childbearing age [[[1]](#endnote-2)]. Hemoglobinopathies have been widely reported in many populations of Southeast Asia, with the prevalence of α-thalassemia ranging from 5-15%, β-thalassemia from 1-20%, and Hb E from 4-60%, according to the region and country sampled [[[2]](#endnote-3)]. Anemia is most health problem in many countries, in particular, developing country [[[3]](#endnote-4)].

To date, very few studies have been conducted in Cambodia and prevalence rates for hemoglobinopathies have only been accessed in a small number of subjects, living either in rural communities or in refugee camps outside the country [[[4]](#endnote-5), [[5]](#endnote-6), [[6]](#endnote-7)]. It is generally assumed that the major contributing factor for anemia is iron deficiency [[[7]](#endnote-8)]. However, recent study on anemia among reproductive age women reported a half of anemic group are still anemic after iron-folic acid supplementation [[[8]](#endnote-9)].

It is therefore in question whether thalassemia and hemoglobinopathies are responsible for the existing anemia. Symptomatic thalassemia major and intermedia requires life long-term medical care and support [[[9]](#endnote-10)]. Therefore, the present study was undertaken to assess the current prevalence of anemia, thalassemia (including hemoglobinopathies), and iron deficiency in a group of Phnom Penh Cambodian students and to demonstrate the health burden of anemia in relation to thalassemia and iron deficiency among anemic subjects in this region.

**Materials and Methods**

**Subjects and samples**

A cross-sectional analytic study assessed anemia, thalassemia, as well as iron deficiency among Phnom Penh Cambodian students. This study analyzed data collected of blood samples of 400 students enrolled the blood donation at faculty of Pharmacy, University of Health Sciences, Phnom Penh, Cambodia will be recruited consecutively during August to October 2012. Five milliliters of blood sample were collected from venous blood anti-coagulated with ethylenedi-aminotetraacetic acid (EDTA) from all subjects. After collection, blood samples were put on ice or stored at 40C and transferred to the laboratory of the faculty of pharmacy, Phnom Penh, Cambodia on the same day of sampling to determine the hematological parameters by using automated blood cell counter and to be initially screened for thalassemia using either OF/DCIP or MCV/DCIP (MCV < 80 fl). Plasma samples will be separated and kept at -20 oC for determination of ferritin. The remaining blood and plasma samples of both positive and negative-screening subjects will be collected and sent to the Center for Research and Development in Medical Diagnostic Laboratory (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Thailand, for further investigation of thalassemia and hemoglobinopathies such as Hb and DNA analysis.

All participants were assumed to be healthy based on physical appearance and self-report. Individuals with chronic illness, recent surgery receiving or giving blood, or taking any supplements that contain irons will be excluded. No subject had a known prior diagnosis of thalassemia or a hemoglobinopathy. This research study protocol was approved both by the National Ethic Committees on Health Research (NECHR), Phnom Penh, Cambodia and by the Ethical Committee of Khon Kaen University, Khon Kean, Thailand. The participants were informed of the nature and purpose of the study and the consent form were read to each subject. Consent was given by signature or by thumb print. Written informed consent was obtained from the participants as an indication of their willingness to participate, and each participant received a written report of the results of the screening tests.

Before taking their blood, a short questionnaire about their health was administered by a student researcher to obtain ancillary information such as age, sex, residence, height, weight, and medical history. The items noted the presenting illness, diagnosis, and whether the student previously had received a blood transfusion or not. Weight and height are also measured and recorded.

**Laboratory investigations**

**Screening test**

Initial screening techniques provide a qualitative result “yes/no”. Positive samples need further confirmatory test while negative samples can be eliminated from further complicated and expensive testing.

Primary screening for thalassemia and hemoglobinopathies usually involves an accurate blood count using an expensive electronic blood cell counter. It is called the complete blood count (CBC). The key components of the CBC include: Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), and red cell distribution width (RDW). Among these parameters MCV < 80 fl and MCH < 27 pg are the most important ones that can indicate the existence of α- or β- thalassemia trait [[[10]](#endnote-11)].

A cheaper alternative method was tested by using a modified osmotic fragility (OF) test which was specific for α0- or β-thalassemia and the modified dichlorophenolindophenol (DCIP) test which was specific for Hb E. Briefly, in the OF test, a sample of 20 µL of whole blood was mixed with 2 mL of 0.34% buffered saline solution in the test tube and left at room temperature for 15 minutes before being interpreted. For the DCIP precipitation test, 20 µL of whole blood was added to 2 mL of a modified DCIP reagent, and the mixture was incubated at 37oC for 15 minutes before the addition of 20 µL of stopping reagent supplied by the manufacturer to eliminate and decolorize the excess DCIP dye. Both tests were interpreted by visualization as negative or positive. Negative samples are characterized by a clear solution and positive samples by a cloudy appearance.

**Hemoglobin Typing**

Hb types and levels of all Hb components were determined using by an automated capillary electrophoresis (CE). The automatic capillary electrophoresis system can separate and quantitate Hbs A, F, E, A2, Constant Spring (CS), H, and Bart’s. With this technique, charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation occurs according to the electrolyte pH and electroosmotic flow, from cathode to anode. Briefly, plasma was discarded, and packed red cells were hemolyzed automatically with hemolyzing solution injected by aspiration at the anodic end of the capillary. A high-voltage protein separation is performed in silica capillaries. Direct detection of the Hb fractions is made at 415 nm at their cathodic end. The resulting electrophoregrams are evaluated visually for pattern abnormalities [[[11]](#endnote-12)].

**DNA Analysis**

Genomic DNA was prepared from peripheral blood WBCs by using a standard method. All common α-thalassemia mutations, including α0-thalassemia (SEA type), α+-thalassemia (3.7- & 4.2 kilobase deletions), Hb Constant Spring (Hb CS) and Hb Paksé (Hb Ps) were identified by polymerase chain reaction (PCR) and related methods [[[12]](#endnote-13),[[13]](#endnote-14),[[14]](#endnote-15),[[15]](#endnote-16)].

EA

A2A

EE; E>80

Hb Analysis

Normal or non-clinically significant thalassemia

MCV/DCIP or OF/DCIP

Screening (Students)

Negative screening

Positive screening

Hb E > 35% 🡺 HE with/without β-thal disease

Hb E=25-35% 🡺 Hb E trait

Hb E<25% 🡺 Hb E trait (not R/O αo-thal )

Homonzygous Hb E (not R/O αo-thal)

Hb A2A~4-10%

Hb A2A<4%

βo-thal trait

(not R/O αo-thal)

αo-thal trait?

DNA Anylysis

Other types: A­2AH, EF, EFA, etc

FIGURE 1: A flow diagram demonstrating Thalassemia Screening Strategy

**Statistical analysis**

Data processing was done using the Minitab statistical software program (Minitab Inc., State College, PA, USA). Demographics and baseline characteristics of students enrolled in this study were characterized by descriptive statistics. Mean and standard deviation or median and interquartile range were used to describe hematological parameters and ferritin level. Prevalence of anemia and iron deficiency as well as thalassemia and hemoglobinopathies was demonstrated using inferential statistics, the proportion and 95% confidential interval. Student t-test or Mann-Whitney U-test was applied for testing the difference between the two different groups. The area under the curve (AUC), sensitivity and specificity were calculated to determine the validity of various formulas. Statistical analysis was performed by using the MedCalc software (version 11.3.1.0, Medcalc software, Mariakerke, Belgium). To test the proportion difference, Z-test will be applied. Logistic regression will be employed to identify the potential risk factors for anemia. A P-value of < 0.05 was considered as statistically significant difference.

**Results**

A total of 1500 students studying at Faculty of Pharmacy, Phnom Penh, Cambodia were the population of this study. From the 430 students who were selected, responded to the survey, and agreed to participated as members of this study. 30 were excluded for this paper due to being excluded; hence, 400 students were included in the analysis (FIG. 2).

Total number of registered students at Faculty of Pharmacy, Phnom Penh, Cambodia

(N = 1500)

Sampled and responded the questionnaires

 (n = 450)

20 could not be contacted due to wrong phone number

Contactable (n= 430)

Returned questionnaires and enrolled

(n= 430)

30 excluded due to being met the exclusion criteria

Study participants

(n=400)

FIGURE 2: Consort diagram of description how the study sample look like or how they were selected

From August to October 2012, 400 students were screened for this study. Four hundred and fifty students were enrolled and 50 failed screening. One of the failed screening students met the exclusion criterion of hemoglobin <7.5g/dl. The baseline clinical characteristics are shown in table 1.

TABLE 1: Baseline characteristics

|  |  |
| --- | --- |
| Baseline data | Total (N=400) |
| Gender Male Female | 50350 |
| Age (years), | 16-25  |
| Family history of thalassemia | 10 |
| Weight | 35-80 kg |
| Height | 135-180cm |

Hematological parameters were determined using an automated hematologic analyzer (Sysmex SF-3000, Japan). Serum ferritin (SF) was determined based on the chemiluminescent immunoassay using the Access Immunoassay Systems Serrum ferritin (Beckman Coulter, USA.)[[16]](#endnote-17). Individual with SF < 20 ng/ml was considered as having iron deficiency[[17]](#endnote-18). All samples were screened for the common α-thalassemia genes, α0-thalassemia (SEA&THAI deletion), α+-thalassemia (3.7 and 4.2-kb deletion), Hb CS and Hb Paksé using the polymerase chain reaction (PCR)-based methods [[[18]](#endnote-19),[[19]](#endnote-20),[[20]](#endnote-21),[[21]](#endnote-22)]. Beta-thalassemia and other hemoglobinopathies were determined using automated capillary zone electrophoresis (Capillarys, Sebia, France). An individual with MCV <80 fl and Hb A2 > 4.0% was diagnosed as a thalassemia carrier[[22]](#endnote-23). In case abnormal Hb profiles, an allele-specific PCR developed in the laboratory of the investigators was used for searching for known Hb variants [[[23]](#endnote-24),[[24]](#endnote-25)]. Anemia was defined by WHO criteria adjusted for age and sex as hemoglobin < 12 g/dl for women and < 13 g/dl for men[[25]](#endnote-26). IDA was defined as anemia with serum ferritin (SF) is lower than 20 ng/ml accompanied by hemoglobin (Hb) level < 12.0 g/dl for women, and < 13.0 g/dl for men [[[26]](#endnote-27),[[27]](#endnote-28)]. All automated measurements were done in full agreement with manufacturers instructions. Commercial quality-control samples were used to monitor the assay performance.

XXX out of the 400 students (?%; 95% CI = …to…) were anemic. The overall prevalence for thalassemia alone, iron deficiency alone, and both thalassemia and iron deficiency was % (95% CI = …to…), % (95% CI = …to…), and % (95% CI = …to…), respectively. Of 400 participants who responded for thalassemia screening, the majority was females (80%). The hemoglobin (Hb) levels of all the students were normally distributed with the mean Hb of 14g/dl in males; and 13g/dl in females. Forty students (10%) had low Hb level of whom only one was a male student (cut off Hb < 12 g/dl for women and < 13 g/dl for men). To demonstrate the extent of anemia in relation to thalassemia and iron deficiency among anemic subjects, the proportions of thalassemia and iron deficiency in the anemic group were compared. It is found that the proportion of iron deficiency was two times lower than the proportion of thalassemia.

TABLE 2: Prevalence of anemia, iron deficiency, iron deficiency anemia, iron deficiency anemia and thalassemia trait, thalassemia trait, thalassemia disease among 400 Phnom Penh Cambodian students

|  |  |  |  |
| --- | --- | --- | --- |
| Condition | Number | Percentage | 95% CI |
| Anemiaa | XXX | XXX | XXX |
| Iron deficiencyb | XXX | XXX | XXX |
| Iron deficiency anemia | XXX | XXX | XXX |
| Iron deficiency anemia and Thalassemia trait | XXX | XXX | XXX |
| Thalassemia trait | XXX | XXX | XXX |
| Thalassemia disease | XXX | XXX | XXX |
| Normalc | XXX | XXX | XXX |

a: based on WHO criteria adjusted for age and sex, Hb < 12 g/dl for women and < 13 g/dl for men

b: based on SF < 20 ng/ml

c: Individual who had no anemia, iron deficiency, and thalassemia

TABLE3: Results of the initial screening using combined OF/DCIP tests, hematological analysis, and globin genotypes for 400 students in Phnom Penh, Cambodia

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| OF/DCIP | Hb (g/dl) | Hct (%) | MCV (fl) | MCH (pg) | MCHC (g/dl) | RDW (%) | Hb type | Hb A2/E (%) | Hb F (%) | α-genotype | β-genotype |
| (-/-) (120) | XX | XX | XX | XX | XX | XX | XX | XX | XX | XX | E / α |
| (+/-) (250) | XX | XX | XX | XX | XX | XX | XX | XX | XX | XX | E trait |
| (-/+) (50) | XX | XX | XX | XX | XX | XX | XX | XX | XX | XX | β trait |
| (+/+) (80) | XX | XX | XX | XX | XX | XX | XX | XX | XX | XX | E / β |

**\*\*\* *Because of confidentiality of this project, I would not be able to put the real results***

**Discussion**

It is usually assumed that the most important cause for anemia is iron deficiency [[[28]](#endnote-29),[[29]](#endnote-30)]. Other nutritional deficiencies of importance for being anemic include vitamin A, vitamin B12, and folate; however, these micronutrient deficiencies are relatively low in university student in Cambodia [ref]. The other significant factors considered to be causes of anemia in the study area might be thalassemia and hemoglobinopathies.

It might be that iron deficiency nowadays is less common in the study area so that only a few students were iron deficiency; therefore, they are not representative of the general school students population in Phnom Penh, Cambodia.

As this purpose of study was not to determine the prevalence of thalassemia in general community, the high occurrence of thalassemia in our subjects could be due to significant participation from students who had a family history of thalassemia.

Hemoglobinopathies were found to be very common in the Phnom Penh region of Cambodia with XXX% of students affected. Overall, 20 different phenotypes were identified; however, severe thalassemic lesions were uncommon and only a few cases of each of Hb H disease and Hb E/β-thal were detected. This contrasts with neighboring Thailand where Hb E/β-thal and Hb H disease are major health problem[[30]](#endnote-31). However, some of them Hb E/β-thal may survive without any treatment depending on thegenetic background of β-thalassemia alleles and other

genetic modifiers [[[31]](#endnote-32)].

It should be notified that, though Hb, MCV, and MCH are significantly reduced, heterozygous states of thalassemia may or may not cause anemia. In cases with anemia, it is clear that anemia is mild, which explains why children are apparently healthy. It is well established that the severity of clinical symptoms of thalassemia depends on the severity of imbalanced globin chain synthesis. While the excess α-chain precipitates in the red-cell precursors and leads to ineffective erythropoiesis, the excess β-chains are capable of forming the homotetramer of hemoglobin H (β4). In heterozygous states, globin chain imbalance is mild so that the excess globin is almost completely destroyed by the red-cell proteolytic enzyme, hence, there are no clinical symptoms [[[32]](#endnote-33)].

**Conclusions**

The study findings indicate that anemia in a group of Phnom Penh Cambodian students is not associated with iron deficiency. Instead, thalassemia and hemoglobinopathies are the major contributing factors. The data will be useful for appropriate care for students in the region as well as enabling individuals to receive information about their personal health, future health and potential health of their offspring.In addition, a high prevalence of anemia, thalassemia and iron deficiency indicates a need for appropriate measures to prevent and control anemia, iron deficiency as well as thalassemia in the country. Comparison of proportions of thalassemia and iron deficiency among anemic indicates that thalassemia is the major contributing factor for anemia among Cambodian students. Thalassemia is prevalent in Cambodia; this study supports the opinion that population screening for common types of thalassemia is recommended.

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