

INFLUENCE OF FRESHNESS OF EDTA BLOOD ON RELIABILITY OF THALASSEMIA SCREENING TESTS

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Abstract

Thalassemia screening tests include one tube (0.36% NaCl) osmotic fragility test (OFT), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH). Reliability of these parameters in stored EDTA blood is the important issue to be concerned. To address this issue, we analyzed the results of these thalassemia screening tests in 50 fresh and stored EDTA blood samples of collected in the Chiang Mai province of northern Thailand. These blood samples; 25 with MCV < 80 fl and 25 with MCV ≥ 80 fl, were stored at room temperature (RT) (approximately 30°C) and in cold (approximately 4°C). OFT, MCV, and MCH were tested in both groups of blood samples everyday for 7 days. It was found that falsely negative OFT results were seen after 1 day and 4 days of storage at RT and in cold, respectively. For MCV, acceptable values were obtained at 0 day and 2-3 days storage at RT and in cold, respectively. No change of MCH values was observed throughout 7-day storage at both RT and cold. It was concluded that when performing thalassemia screening tests in Thailand, fresh EDTA blood must be used for OFT test and MCV determination. However, although MCH seemed to be the most stable parameters, thalassemia screen cannot rely only on MCH value. Therefore, thalassemia screen using these three tests must be done within blood collection day if no cooling facility is available. This information would also be useful for countries in tropical region where ambient temperature is high.

Keywords: Mean corpuscular hemoglobin; Mean corpuscular volume; Red cell parameters; Thalassemia carriers; Thalassemia screen

Introduction

Thalassemia screening is the laboratory practice performed to search for carriers of severe thalassemia such as carriers of α -thalassemia 1, β -thalassemia and HbE. The conventional laboratory tests used for thalassemia screening consist of one-tube osmotic fragility test (OFT), mean corpuscular volume (MCV), mean

corpuscular hemoglobin (MCH), and HbE screening tests such as dichlorophenol indophenol precipitation (DCIP) test, HbE tube test and HbE screen (Karimi and Rasekhi, 2002; Winichagoon *et al.*, 2002; Wiwanitkit *et al.*, 2002; Sirichotiyakul *et al.*, 2005; Pranpanus *et al.*, 2009; Tatu and Kasinrerker, 2012). Blood samples having positive

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results for these tests are eligible for further confirmatory tests including hemoglobin studies and mutation analysis to obtain a specific diagnosis. Most importantly, MCV value of less than 80 fL was found to effectively screen in carriers of severe thalassemia, comprising α -thalassemia 1 and β -thalassemia carriers (Sirichotiyakul *et al.*, 2005). The blood samples of choice for thalassemia screening is EDTA blood which is venous blood stored in ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. EDTA prevents blood clotting by chelating calcium ion (Ca^{2+}), an essential cation for clot formation.

OFT is the test that evaluates the degree of hemolysis of blood samples which can clearly differentiate thalassemia carriers from normal or non-thalassemia individuals. MCV and MCH are the red cell indices that indicate the average size and hemoglobin content of an individual red blood cell. The mostly suitable EDTA blood samples for OFT, MCV, MCH determination are fresh blood samples. However, in some occasions, thalassemia screening tests cannot be performed immediately after blood collection, and stored blood samples are tested instead. Then, there are two main questions whether it is reliable to use OFT, MCV, MCH results of stored blood for thalassemia screening, and whether the storing condition affects red blood cells having different MCV equally?

Thailand is the country localized in tropical part of the world, the region having high temperature throughout the year. In this kind of climate, blood samples tend to degrade easily and blood test results for thalassemia screen will be unreliable. Therefore, we determined the impact of storing condition in Thailand on results of thalassemia screening tests.

Materials and Methods

Blood samples

This study was performed using 50 EDTA blood samples which were divided into 2 groups. The first group comprised 25 blood samples having MCV of < 80 fL, thus namely “*Microcytic Group*”. The second group consisted of 25 blood samples having MCV of ≥ 80 fL and named “*Normocytic group*”. Blood samples in each group were further divided into 2 groups to be stored at room temperature and the second at 4°C . The OFT, and automated RBC parameter determination were performed on blood collection day (Day 0) and every subsequent day for 7 days (Day1, Day 2, Day 3, Day 4, Day 5, Day 6, Day 7).

One-tube osmotic fragility test (OFT)

One-tube osmotic fragility test using 0.36% NaCl was performed for all 50 blood samples following the protocol described previously and routinely performed in our laboratory (Tatu and Sweatman, 2018). Briefly, 20 μL of EDTA blood was put in 2 mL of 0.36% NaCl solution. The suspension was mixed gently and incubated at room temperature for 5 minutes before visualizing the appearance of the RBC suspension by the naked eye. If the reddish RBC solution was clear and the black lines placed behind were visible, the OFT result was negative. However, if the RBC suspension was turbid and the black lines placed behind were invisible, the OFT result was positive. The appearance of RBC suspension in the middle of positive and negative was recorded as a suspicious result (Figure 1).

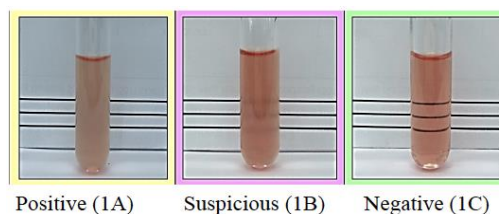


Figure 1. Results of one-tube osmotic fragility test (0.36% NaCl-OFT) showed “Positive (1A), Suspicious (1B), Negative (1C)”, respectively. In positive reaction (1A), the suspension is turbid so that the black lines placed behind the tube is invisible. The negative reaction (1C) has clear suspension and the black lines placed behind the tube can be visible. In contrast, it is difficult to judge as positive or negative for results in the suspicious reaction group (1B)

Automated blood cell count

RBC count (RBC), hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) were determined by an automated blood cell counter (Sysmex KX-21, Sysmex Corporation, Kobe, Japan). RBC count was performed by a DC detection method. Hb was measured by non-cyanide hemoglobin analysis method. Hct was determined by the RBC pulse height detection method. MCV in fL was calculated by the equation $(\text{Hct}/\text{RBC}) \times 10$. MCH in pg was calculated by the equation $(\text{Hb}/\text{RBC}) \times 10$ (Sysmex corporation, 1998).

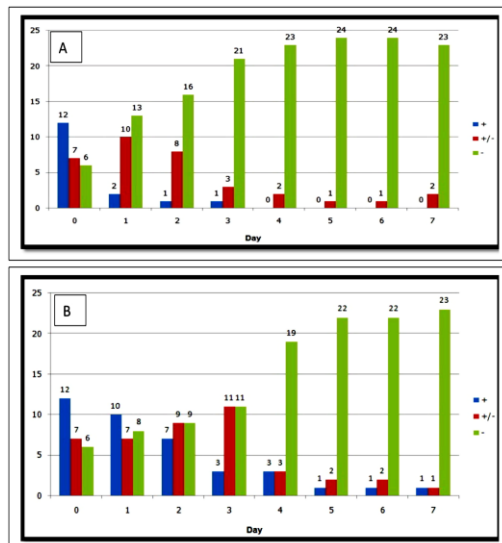


Figure 2. OFT results of EDTA blood of Microcytic group (n = 25) stored in RT (A) and 4°C (B). Blue bar indicates number of positive samples (+), red bar for suspicious samples (+/-) and green bar for negative samples (-). Numbers placed on top of each bar indicate amount of samples in those bars. Note that amount of positive samples decreased and that of negative samples increased by prolonged storage.

Statistical analysis

The z-score test at 0.05 level of significance was performed to determine differences of proportion of positive and suspicious OFT results obtained on subsequent days compared to those obtained on blood collection day or Day 0.

Results

One-tube osmotic fragility test

Stability of OFT results was evaluated for the *Microcytic Group*. For RT storage, the OFT test results changed overwhelmingly on Day 1 through Day 7. When considered only the OFT-positive result, its proportion of fresh blood samples (Day 0) was significantly different from that of Day-1 stored blood samples (p -value of z-score test = 0.002). When considered OFT-positive plus OFT suspicious results, the same finding was found (p -value of z-score test = 0.04) (Figure 2(a)).

For 4°C storage, the EDTA blood seemed to be more stable than the RT storage. Proportion of OFT- positive samples stored for 3 days was not different from that of fresh blood samples (Day 0) (z-score p -values were 0.57, 0.14, 0.05 for Day 1,

Table 1. Baseline values and acceptable range of RBC parameters according to the Clinical Laboratory Improvement Advisory (CLIA) guideline (2019)

RBC parameters		Baseline	Acceptable range (± 4)
RBC count (M/ μ L)	Normcytic group	5.11	4.90 - 5.31
	Microcytic group	4.78	4.59 - 4.97
Hb (g/dL)	Normcytic group	13.4	12.8 - 13.9
	Microcytic group	11.5	11.0 - 12.0
Hct (%)	Normcytic group	40.7	39.0 - 42.3
	Microcytic group	36.8	35.3 - 38.2
MCV (fL)	Normcytic group	85.3	81.9 - 88.7
	Microcytic group	72.5	69.6 - 75.4
MCH (pg)	Normcytic group	27.9	26.8 - 29.0
	Microcytic group	22.6	21.7 - 23.5

Note : Normcytic group; MCV ≥ 80 fL, microcytic group; MCV < 80 fL

Day 2, Day 3, respectively). This finding was the same when positive plus suspicious-OFT samples were considered together (z-score p -values were 0.52, 0.35, 0.13 for Day 1, Day 2, Day 3, respectively (Figure 2(b)). However, the proportions of positive and negative-OFT blood samples gradually declined as storing days increased.

RBC parameters

Analysis of effect of temperature and duration of storage on RBC parameters was performed for both the *Microcytic Group* and the *Normcytic Group*. Temperature and duration of storage affected stability of RBC count, Hb, Hct, MCV, but not MCH. RBC count and Hb of both groups of samples gradually increased, but the levels were still within $\pm 4\%$ acceptable limits set up by CLIA 2019 Guideline for Proficiency Testing (PT) Acceptable Limits (<https://www.westgard.com/2019-clia-changes.htm>) (Table 1). In contrast, Hct and MCV of both groups were increased sharply during storage for both temperature conditions. Therefore, according to CLIA 2019 Guideline for PT Acceptable Limits, Hct and MCV must be determined in Day 0 (blood collection day) if a cooling facility is not accessible. However, when stored at 4°C, Hct and MCV levels were still acceptable on Days 2-3 (Table 1, Figures 3). Interestingly, MCH was shown to be stable for all 7 days of storage at both RT and 4°C for both

Microcytic and Normocytic Groups. Its level was within $\pm 4\%$ CLIA 2019 Guideline for PT Acceptable Limits from Day 0 to Day 7 (Table 1, Figure 4).

Discussion

Accuracy of results of OFT, MCV, and MCH is important for effective and reliable thalassemia screening. Ideally, fresh EDTA blood should be used for this practice. However, blood test may be delayed due to lack of technology at the site and so blood must be stored and transported to the laboratory. Delayed blood tests of course could lead to erroneous test results due to changes of RBC quality in blood samples.

The 0.36%-NaCl based OFT is the classical test for thalassemia screening. It has been shown to

be simple and very effective to screen carriers of severe thalassemia; e.g. β -thalassemia, α -thalassemia 1 (Penman *et al.*, 2015; Allen *et al.*, 2019). However, it must be performed using only fresh blood as shown from the results of this study. Storing blood in EDTA should lead to depletion of adenosine triphosphate (ATP) which is necessary for effective work of the ATP-dependent Na^+/K^+ pump (Arif *et al.*, 2017). As a consequence, Na^+ plus water passed through the membrane into the RBC corpuscles, and hemolysis occurred. Keeping samples cold, in contrast, slowed down glycolysis and reduced the decline of ATP. Thus, the red blood cells became more osmotically resistant than those kept at RT. The observations of Antwi-Baffour, *et al.* in 2013 (Antwi-Baffour *et al.*, 2013) and Huang, *et al.* in 2015 (Huang *et al.*, 2015) showed the same findings. However, as shown in Figure 2 that 12 and 7 samples had positive and suspicious OFT results, respectively. The suspicious OFT results may be observed in carriers of HbE, α -thalassemia 1, α -thalassemia 2 and β -thalassemia (Chow *et al.*, 2005). Thus, the suspicious OFT should be judged as positive OFT. Therefore, the prevalence of positive OFT in this setting was 19 in 25 or 76%. The remaining 24% of samples with negative OFT should be the samples that had low ATP levels during storage before tested in this study. Hemolysis occurs easily in RBC having low ATP as mentioned before. Looking at Figure 2A, at Day 7 the number of negative samples was 23, while that in Day 6 was 24 and the number of suspicious samples increased from 1 to 2 from Day 6 to Day 7. This meant that one initially negative on Day 6 turned to be suspicious on Day 7. Some mistake might occur during the test in Day 7 such as the tube containing 0.36% NaCl was not properly mixed and percentage of NaCl might be slightly more than 0.36%. This might be the reason why the result of this sample was suspicious. However, most of the tubes containing 0.36% NaCl were properly mixed and the results were as expected.

MCV was the parameters calculated, in the Sysmex KX-21, from of RBC count and Hct. RBC count increased slowly, but Hct went up sharply. This should explain why the increase of MCV values was similar to that of Hct. In fact Hct in this Sysmex KX-21 was determined from the summation of pulse height of RBC electrical impedance. The height of pulse signals reflected volume of RBCs. RBCs should become swollen due to lack of ATP during prolonged storage and lead to increased Hct and, in turn, MCV. The reason of slow decrease in RBC count should be due to interference of debris of degraded white blood cells (WBCs) and platelet clumping in electrical impedance-based cell counting of an automated blood cell counter used in

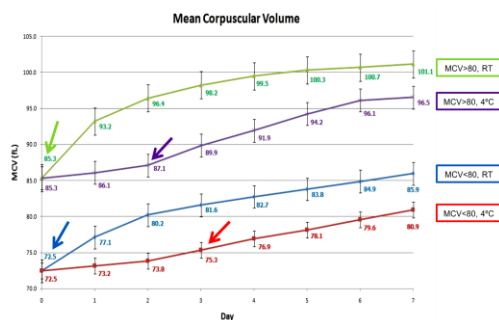


Figure 3. Changes of MCV during storage at RT and 4°C. Microcytic Group (n = 25) is indicated by $\text{MCV} < 80 \text{ fL}$, and Normocytic Group (n = 25) by $\text{MCV} \geq 80 \text{ fL}$. RT means room temperature storage. 4°C means 4°C storage. Numbers above and under lines indicate mean values.

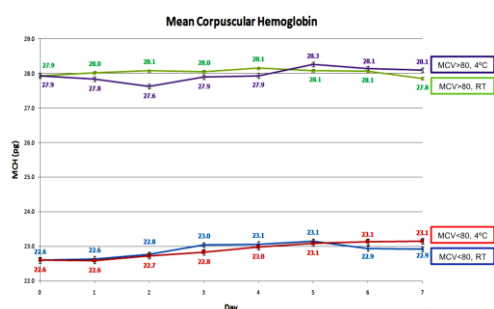


Figure 4. Changes of MCH during storage at RT and 4°C. Microcytic Group (n = 25) is indicated by $\text{MCV} < 80 \text{ fL}$, and Normocytic Group (n = 25) by $\text{MCV} \geq 80 \text{ fL}$. RT means room temperature storage. 4°C means 4°C storage. Numbers above and under lines indicate mean values.

this study. This phenomenon was evident by slow decrease of WBC count and platelet count observed in this study (data not shown). Storage in cold reduced the degree of ATP shortage, thus the MCV was more stable than with RT storage. Increased Hct and MCV during storage was also shown by Gulati GL in 2002 and Mane M in 2017 (Gulati *et al.*, 2002; Mane *et al.*, 2017).

MCH was found to be the most stable parameter at least for 7 days storage. This parameter was in fact calculated from RBC count and Hb concentration which had the same trend of change during storage at 4°C and the room temperature of Thailand. This finding was also shown by Gulati GL, *et al.* in 2002 by using a Coulter Gen S which counted cells using impedance principle and found that RBC count, Hb and MCH were stable for 7 days at the RT of USA (Antwi-Baffour *et al.*, 2013).

Conclusions

Storage of EDTA blood samples used for thalassemia screening must be carefully monitored. OFT and MCV tests must be performed as early as possible and not later than Day 2 after blood collection day (Day 0) even though cooling facility is accessible. MCH was the most stable parameter and EDTA blood samples could be kept at both 4°C and RT for at least 7 days. Both normocytic and microcytic RBCs were affected equally. This finding should be useful and applied in regions endemic with thalassemia and hemoglobinopathies. As MCH is a calculated parameter in all types of automated hematology analyzers, this finding should also be applicable for all platforms of automated hematology analyzers.

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