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A critical evaluation of peripheral blood smear in the diagnosis of thalassemia syndrome

Sunarto
Child Health Department Gadjah Mada University/
Sardjito Hospital
Yogyakarta

ABSTRACT

Sunarto- *A critical evaluation of peripheral blood smear in the diagnosis of thalassemia syndrome*

The severe affected thalassemia syndrome is common in Southeast Asian countries. Considering that the feature of its peripheral blood smear is typical, this study was aimed at investigating the diagnostic value of the blood smear in thalassemia syndrome. Sixty five patients with severe anemia and splenomegaly who were admitted or came for follow up to Sardjito General Hospital in the period of 1996 and 1997 had been enrolled in this study. The peripheral blood smear was assessed, whether thalassemia or nonthalassemia, by two laboratory technicians, each of them read blindly each smear at two occasions with at least two weeks interval. The gold standard for diagnosis of thalassemia major was the evidence of increased HbA2 (>3,5% of total Hb) in both parents and for thalassemia-hemoglobin E disease was the increased HbA2 in one of the parents and the presence of HbE in the other. The measurements of HbA2 and HbE were carried out by quantification of HbA2 fraction following hemoglobin electrophoresis on cellulose acetate membrane (CAM) electrophoresis. The results of blood smear reading showed good intrarater agreement with kappa = 0.691 by the first rater and 0.634 by the second rater). The interrater agreement was high moderate to good (kappa = 0.567 - 0.728). The first reading by the first rater, the second reading by the first rater, the first reading by the second rater, and the second reading by the second rater showed sensitivities of 0.780, 0.780, 0.878, and 0.805 respectively; specificities of 0.708, 0.958, 0.542 and 0.792 respectively; positive predictive values of 0.820, 0.969, 0.766, and 0.868 respectively; and negative predictive values of 0.650, 0.719, 0.722, and 0.704 respectively. The peripheral blood smear has high sensitivity and specificity for diagnostic test of thalassemia syndrome.

Key words: thalassemia syndrome - blood smear - hemoglobin electrophoresis - sensitivity and specificity - predictive value

ABSTRAK

Sunarto - *Evaluasi kritis sediaan apus darah tepi sebagai uji diagnostik sindrom thalassemia*

Sindrom thalassemia berat sering terdapat di Asia Tenggara. Mengingat gambaran darah tepinya yang khas, penelitian ini dimaksudkan untuk mengetahui nilai diagnostik sediaan apus darah tepi pada sindrom thalassemia. Enam puluh lima pasien dengan anemia berat dan splenomegali yang dirawat inap atau dirawat jalan di Rumah Sakit Dr. Sardjito pada tahun 1996 dan 1997 dimasukkan dalam penelitian ini. Di samping pemeriksaan rutin, secara khusus sediaan apus darah tepi dibaca, dan disimpulkan thalassemia atau non-thalassemia oleh dua orang petugas laboratorium; masing-masing petugas membaca setiap sediaan secara buta dua kali dengan jarak waktu dua minggu atau lebih. Sebagai baku emas diagnosis thalassemia major adalah bukti pengemban bakat thalassemia (HbA2 > 3,5% dari total Hb) pada kedua orang tua dan untuk thalassemia-hemoglobin E adalah bukti pengemban bakat thalassemia pada salah satu orang tua dan pengemban bakat HbE pada yang lain. Pemeriksaan HbA2 dan HbE dilakukan dengan mengukur kadar fraksi HbA2 setelah elektroforesis Hb pada membran selulosa asetat (CAM). Hasil pembacaan sediaan apus darah menunjukkan kappa = 0,691 untuk pembaca pertama dan kappa = 0,634 untuk pembaca kedua. Kesepakatan *interrater* menunjukkan kappa

0,567-0,728. Pembacaan pertama oleh pembaca pertama, pembacaan kedua oleh pembaca pertama, pembacaan pertama oleh pembaca kedua, dan pembacaan kedua oleh pembaca kedua menunjukkan sensitivitas berturut-turut 0,780, 0,780, 0,878 dan 0,805 spesifisitas berturut-turut 0,708, 0,958, 0,543, dan 0,792; nilai ramal positif berturut-turut 0,820, 0,969, 0,766, dan 0,868; dan nilai ramal negatif berturut-turut 0,654, 0,719, 0,722, dan 0,704. Dapat disimpulkan bahwa sediaan apus darah mempunyai sensitivitas dan spesifisitas tinggi dalam diagnostik untuk sindrom thalassemia.

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INTRODUCTION

The most prevalent anemia in Indonesia, like in other developing countries, is iron deficiency anemia. In Southeast Asian countries, including Indonesia, severe anemia due to thalassemia syndrome (major thalassemia or ßthal/ßthal and thalassemia-hemoglobin E disease or ßthal/HbE both show similar clinical manifestation) is also commonly occurred[1,2,3]. Thalassemia cases, especially in region where malaria is prevalent, were easily misdiagnosed as malaria[4]. Other chronic hemolytic anemias may have clinical manifestations similar to thalassemia. Anemia with organomegaly is also commonly occurred in malignancies such as in leukemia, Hodgkin lymphoma and non-Hodgkin lymphoma. Recognizing thalassemic patients is important, because thalassemia brings specific problems individually as well as community and therefore needs specific management[2,5].

Both thalassemia and HbE disease are resulted from ß-globin gene defect and result in further mild to severe anemia. In SEA countries most of thalassemia major and half of thalassemia-HbE disease show severe anemia[6]. In daily practice thalassemia syndrome is diagnosed based on clinical signs (anemia, splenomegaly, often with hepatomegaly and lymphadenopathy, and other signs); increased HbF and or evidence of thalassemia trait or Hb E in the parents confirm the diagnosis. Thalassemia major is diagnosed if both parents are thalassemia carrier (show HbA2 > 3,5% of the total Hb), while thalassemia-hemoglobin E disease is diagnosed if one of the parents is thalassemia carrier and the other is HbE gene carrier[2]. Biochemical and molecular diagnosis are now possible, but it is done only for special purposes, e.g. population study including in Indonesia[7] and prenatal diagnosis, not for routine clinical practice.

Various blood diseases give specific pictures in the peripheral blood smear[8]. In thalassemia syndrome the blood smear reveals severe anisocytosis, hypochromia, polychromasia, poikilocytosis, bizarre cells, microspherocytes, target cells, and normoblast[9]. The blood picture is typical, eventhough some diseases show some slight similarities with thalassemia. Considering that in nearly all C Class Hospital and in all PUSKESMAS in Indonesia fascilities for HbF and HbA2 or HbE examination by either Hb electrophoresis, microcollumn chromatography or dichlorophenol indolphenol (DCIP) were not available, while the peripheral blood smear examination has been a routine procedure in all health centers, a question was raised if the blood smear might be of diagnostic value for thalassemia syndrome. This study was aimed at proving the diagnostic value of peripheral blood smear for thalassemia, comprising the sensitivity, specificity, the positive and negative predictive values, and the reliability on blood smear reading by laboratory technician.

MATERIALS AND METHODS

Patients with Hb less than 9 g/dL and showed splenomegaly, who were admitted or came for follow up in Dr. Sardjito General Hospital in the period of January 1996 to December 1997 were enrolled in this study. Evidence of thalassemia carrier (HbA2 > 3,5%) in both parents or thalassemia carrier in one parents and HbE gene carrier (Hb E band in Hb electrophoresis) in the other were used as gold standard.

The subjects underwent routine examinations, including routine laboratory investigations. Hemoglobin solution prepared from venous blood of the subjects were applied on cellulose acetate membrane (CAM) electrophoresis for HbF and

for HbE detection[10]. Blood samples of the parents were examined electrophoretically (CAM technique) for HbA2 level and HbE which were used as gold standard for the diagnosis of thalassemia syndrome. Peripheral blood smear of each subject, stained with May-Grunwald stain, was read by two laboratory technicians independently; each rater read each smear at two occasions with an interval of 2 weeks or more; both raters knew nothing about the patients, they knew only that the smears were obtained from anemic and splenomegalic patients. The raters were asked to interprete the morphology of the erythrocytes (anisocytosis, hypochromia, polychromatophilia, poikilocytosis, bizarre cells, microspherocytes, target cells, and increased normoblast) and to conclude whether the smear is of thalassemic or non-thalassemic patients. The blood smear readings were carried out in the laboratory of Child Health Department, whereas the Hb electrophoresis was done in the Biochemistry Department, Faculty of Medicine Gadjah Mada University.

Sensitivity, specificity, positive dan negative predictive values were analyzed with SPSS program. The inter- and intra-rater agreement were assessed by kappa statistical test. Kappa < 20 means poor, 21 - 40 fair, 41 - 60 moderate, 61 - 80 good, and 81 - 100 very good[11,12].

RESULTS

During the period of January 1, 1996 to December 31, 1997 a total number of 65 subjects with Hb < 9 g/dL and splenomegaly (these two clinical manifestations were found in nearly all thalassemic patients in Dr. Sardjito General Hospital previously) was enrolled in this study, consisted of thalassemic patients and non thalassemic patients. There were 29 males and 36 females with the ages of two to 12 years. Forty one patients were thalassemia syndrome based on Hb electrophoresis.

The interpretation of the blood smears by the first rater can be seen in TABLE 1.

TABLE 1. - Intraobserver variation in the blood smear readings

	Observer A			
	1st reading		Total	Po = 0.8461
	Thal (+)	Thal (-)		Pe = 0.501
				k = 0.691
2 nd reading				
Thal (+)	31	2	33	
Thal (-)	8	24	32	
Total	39	26	65	
	Observer B			
	1st reading		Total	Po = 0.8431
	Thal (+)	Thal (-)		Pe = 0.538
				k = 0.634
2 nd reading				
Thal (+)	37	1	38	
Thal (-)	10	17	27	
Total	47	18	65	

p_o = observed agreement
p_e = expected chance agreement
k = kappa

The agreement between the first and the second reading by the first rater showed k = 0.691. The agreement by the second rater showed k = 0.634

The interrater agreement between the first and the second reading by the first rater and the second rater is shown in TABLE 2. The agreement between the first reading by the first rater and the first reading by the second rater showed k = 0.728; between the first reading by the first rater and second reading by the second rater k = 0.650; between the second reading by the first rater and the first reading by second rater k = 0.567, and between the second reading by the first rater and the second reading by the second rater k = 0.721.

TABLE 3 shows the accuracy of blood smear reading for diagnostics of thalassemia syndrome. The first reading by the first rater, the second reading by the first rater, the first reading by the second rater, and the second reading by the second rater showed sensitivities of 0.780, 0.780, 0.878 and 0.805 respectively; specificities of 0.708, 0.958, 0.542, and 0.792 respectively; positive predictive values of 0.820, 0.969, 0.766, and 0.868 respectively; and negative predictive values of 0.654, 0.719, 0.722, and 0.704 respectively.

TABLE 2 - Interobserver variation in the blood smear readings

Observer B 1st reading	Observer A 1st reading: Thal (+)	Observer A 1st reading: Thal (-)	Total	
Thal (+)	39	8	47	Po = 0.877
Thal (-)	0	18	18	Pe = 0.543
Total	39	26	65	k = 0.728

Observer B 2nd reading	Observer A 1st reading: Thal (+)	Observer A 1st reading: Thal (-)	Total	
Thal (+)	33	5	38	Po = 0.831
Thal (-)	6	21	27	Pe = 0.517
Total	39	26	65	k = 0.650

Observer B 1st reading	Observer A 2nd reading: Thal (+)	Observer A 2nd reading: Thal (-)	Total	
Thal (+)	33	14	47	Po = 0.785
Thal (-)	0	18	18	Pe = 0.503
Total	33	32	65	k = 0.567

Observer B 2nd reading	Observer A 2nd reading: Thal (+)	Observer A 2nd reading: Thal (-)	Total	
Thal (+)	31	7	38	Po = 0.861
Thal (-)	2	25	27	Pe = 0.501
Total	33	32	65	k = 0.721

p_o = observed agreement
p_e = expected chance agreement
k = kappa

TABLE 3. - Accuracy of blood smear reading for thalassemia diagnostics

Observer A 1st reading	Hb electrophoresis: Thal (+)	Hb electrophoresis: Thal (-)	Total	
Thal (+)	31	7	38	Sensitivity = 0.780
Thal (-)	2	25	27	Specificity = 0.708
Total	33	32	65	PV_{pos} = 0.820
				PV_{neg} = 0.650

Observer A 2nd reading	Hb electrophoresis: Thal (+)	Hb electrophoresis: Thal (-)	Total	
Thal (+)	32	1	33	Sensitivity = 0.780
Thal (-)	9	23	32	Specificity = 0.950
Total	41	24	65	PV_{pos} = 0.969
				PV_{neg} = 0.719

Observer B 1st reading	Hb electrophoresis: Thal (+)	Hb electrophoresis: Thal (-)	Total	
Thal (+)	36	11	47	Sensitivity = 0.878
Thal (-)	5	13	18	Specificity = 0.542
Total	41	24	65	PV_{pos} = 0.766
				PV_{neg} = 0.722

Observer B 2nd reading	Hb electrophoresis: Thal (+)	Hb electrophoresis: Thal (-)	Total	
Thal (+)	33	5	38	Sensitivity = 0.805
Thal (-)	8	19	27	Specificity = 0.792
Total	41	24	65	PV_{pos} = 0.868
				PV_{neg} = 0.704

PV_{pos} = positive predictive value
PV_{neg} = negative predictive value

DISCUSSION

Thalassemia is a clinical syndrome, the etiology of which is the defect of the gene coding the synthesis of the globin polypeptide chain[13]. In Indonesia, one of the Southeast Asian countries, point mutation in the ß-gene resulting in ß-thalassemia and ßE or HbE are the most commonly found among ß-globin gene abnormalities. Homozygous thalassemia (ßthal/ßthal or major thalassemia) and thalassemia-hemoglobin E disease (ßThal/HbE) are frequently found, both present the manifestation of thalassemia syndrome, the severe form of which is severe anemia and other clinical manifestations. Among various types of thalassemia, however, there are mild forms which are not of clinical problems[14]. Thalassemia is now a worldwide problem. The birth of about 100,000 thalassemic babies every year throughout the world is of real clinical and community problems[15].

Wong estimated that thalassemia gene is found in about 3% and HbE gene in 4% of Indonesian population[3]. Iskandar Wahidiyat estimated there were more than 2000 major thalassemic babies born every year in Indonesia[5]. Somewhat equal prevalence is also very likely for thalassemia/HbE.[3] and about half of them have anemia and other clinical manifestations similar to major thalassemia[6]. Sunarto[16] reported that the number of thalassemic patients admitted to Dr. Sardjito General Hospital was too small compared to the estimated number for DIY province and south part of Central Java, the major service

area of Dr. Sardjito General Hospital. The most likely reason of the low admission is that many thalassemic cases might be unidentified by health center personnels due to the lack of diagnostic facilities. Considering that thalassemia is prevalent in Indonesia, therefore a sensitive and simple test for detecting thalassemia cases is highly desired.

In clinical practice the diagnosis of major thalassemia and thalassemia-HbE disease is based on physical manifestations and laboratory abnormalities, i.e. decreased Hb level, high HbF level or increased HbF together with the presence of HbE band in Hb electrophoresis. Hemoglobin electrophoresis of the parents will confirm the diagnosis of major thalassemia if both parents are thalassemia carrier (show level of Hb A2 > 3.5% of total Hb) or thalassemia-HbE disease if one parents has increased HbA2 and the other shows HbE band in Hb electrophoresis[2]. Biochemical analysis on globin synthesis and DNA analysis is very unlikely to be performed as routine procedure due its complexity and very high cost. Considering that many kinds of anemia are found in the community, and thalassemia was often misdiagnosed for other diseases[4], it is beneficial if peripheral blood smear may provide accurate diagnostic value for thalassemia syndrome. The use of simple tests is now advocated in areas with limited facilities, for instance the mid-arm and chest circumferences for estimating low birth weight infants[17], the assessment of skin, nail beds, and conjunctivae for the assessment of anemia[18], the chest indrawing and unable to drink as the key signs in acute respiratory infection cases for referral[19].

The intrarater reliabilities of rater I and rater II in blood smear readings were found good in this study as proved by the kappas, i.e. kappa = 0.691 and 0.634 respectively (TABLE 1). The interrater reliabilities in the readings showed kappas of 0.567 to 0.728 (TABLE 2). Kappa 0.41 - 0.60 is interpreted as moderate and kappa 61 - 80 is good agreement[12]. This evidence implies that reading the blood smear of thalassemic patients will give considerable high consistency either by a rater in different times or by different raters. Variation (variability in measurements on the same subject) in clinical observations and measurements may be due to : 1) variation owing to subject being measured, 2) variation owing to the examiner, and 3) variation owing to the instrument or method used[11]. Variation owing to subject can be great in finding data from anamnesis or physical examination[18]. Rater variability can be due to many factors, among others are the experience of the observer, fatigue, physical environment, and the absence of clear criteria. In interpreting a chest X-ray of hyaline membrane disease or of tuberculosis, variation owing to the material to be measured is absent. In reading thorax X-ray of the newborn suspected to suffer from hyaline membrane disease, Christiaty Surjono (1993) found intrarater kappa 0.63 by pediatricians and kappa 0.67 by radiologists, and interrater kappa 0.50 between pediatrician and the radiologist[20]. In this study the kappas found in blood smear reading is about the same as in the X-ray study. Suryono (1993) reported interrater agreement on Apgar score: weighted kappa value 0.82 was found between the measurement by resident in pediatrics and resident in obstetrics, weighted kappa 0.67 between midwife and resident in pediatrics, and weighted kappa 0.78 between midwife and resident in obstetrics[21]. Gjorup *et al.* (1986) found lower kappa value in detecting anemia on the basis of skin, nail and conjunctiva examination, i.e. 0.23 between the examiner I and II, 0.47 between the examiner I and III, and 0.23 between examiner II and III. The low kappas were especially found in mild cases[18]. The variation of any subject in the latter study from time to time was great, for instance due to emotional condition, temperature, activities, etc. Variation owing to examiner can be seen in Tjokrosonto study, that found high agreement between microscopist of Parasitological Department of Faculty of Medicine Gadjah Mada University (GMU) and microscopist of the Malaria Surveillance Program (PPM) with kappa 0.91 in the reading malaria parasite on the blood slides. Meanwhile, lower agreement (0.51) was found between microscopist of the Health Center (PUSKESMAS) and of GMU; and kappa 0.51 between microscopist of the Health Center and of PPM[22]. Reading erythrocyte morphology is likely very much easier than reading low density malaria on the blood slide. Microscopically, erythrocyte with its various abnormalities can be seen at a glance compared with malaria parasite

searching throughout almost the whole field of a blood slide.

In this study the observation was done on the erythrocyte morphology. A blood slide is a fixed material and general criteria to assess is also available. Experienced health center laboratory personnels will have no difficulty to identify anisocytosis, poikilocytosis, polychromatophilia, microspherocytes, fragmented cells, bizarre cells, normoblasts and other abnormalities of erythrocytes in thalassemia blood smear. This means that subjective variation would be likely minimal. The variation owing to instrument or method was also here nearly absent because both raters in this study used the same type microscopes and the same criteria. The difficulty might come up in deciding the conclusion, but the author believes that with training and experience there will no significant difficulty in reading thalassemia blood smear. In other words every experienced laboratory personnel can do it well.

The sensitivities in blood smear readings by rater I were 0.780 and 0.780; by the rater II were 0.878 and 0.805. The specificities were 0.708, 0.958, 0.542 and 0.792 respectively. High values can be expected if the rater is familiar with thalassemia blood picture. In fact there are some similarities between blood picture of thalassemia syndrome and that of other diseases[2,8]. Fortunately, such diseases are very rarely found compared to thalassemia. Furthermore, some of them have either clinical and/or specific laboratory characteristics, for instance high MCV and ring sideroblasts in sideroblastic anemia, while thalassemia syndrome shows hypochromia and microcytosis; red cell fragmentation syndrome (included thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, hemolytic uremic syndrome, drug induced hemolytic anemia, and also gram negative septicaemia) show fragmentocytes, polychromasia but no hypochromia, while thalassemia show prominent hypohromia; leucoerythroblastic change shows many metamyelocytes and myelocytes while no myelocytes in blood smear of thalassemia; myelodysplastic syndrome (type II) blood smear shows very much alike thalassemia major but anisocytosis in myelodysplastic syndrome consists of macrocytes and normocytes while thalassemia is microcytosis and hypochromia. Anyhow, if such diseases are prevalent, there might be more false positives resulting in decreased specificity.

Considering that sensitivity of blood smear as diagnostics of thalassemia can be as high as 0.878 (range 0.780 - 0.878) it seems that this procedure can be suggested as a valuable screening test. Even, in a high specificity (0.958) the sensitivity can be 0.780 (the first rater in the second reading). Nearly similar values of sensitivity and of specificity can be seen in the second reading by the second rater (0.805 and 0.792 respectively). The availability of a simple and reliable test with high sensitivity and specificity is beneficial for routine practice if diagnostic facility is limited. Achmad Surjono (1993) reported a very high sensitivity and specificity in using measurement of mid-arm of the newborn for estimating low birthweight, i.e. 0.818 and 0.957, respectively. The chest circumference showed sensitivity and specificity of 0.785 and 0.895, respectively. Therefore, these measurements can be used to estimate low birthweight if weighing instrument is not available in remote areas[17]. Likewise, peripheral blood smear examination is available in nearly all health centers in Indonesia, easy and practical, while gold standard for thalassemia diagnostics is rarely available.

CONCLUSION

The peripheral blood picture of thalassemic patient shows characteristic feature. The intrarater agreement was found to be considerably high (0.634 and 0.691). The interrater agreements in various reading show kappas of 0.567 to 728. The peripheral blood picture has high sensitivities (0.780 - 0.878) and the specificities were 0.542 - 0.958 as compared to the evidence of carrier in the parents as gold standard. The positive predictive values were 0.766 - 0.982 and the negative predictive values were 0.654 - 0.722. The epidemiological pattern of other hematologic diseases might decrease the values. The author is in opinion that the peripheral blood smear assessment can be used as the first line diagnostics for thalassemia syndrome.

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