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Original Article

Clot waveform analysis to differentiate mild, moderate, and severe hemophilia A

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Abstract

Background Clot waveform analysis can be used to evaluate clot formation profiles. This waveform can be obtained from activated partial thromboplastin time (APTT) assays without additional reagents and shows different patterns in hemophilia patients with coagulation factor VIII (F VIII) deficiency or abnormality.

Objective To determine the clot wave pattern and its process in clot formation phases (pre-coagulation, coagulation, and post-coagulation) in normal and hemophilia A subjects, analyze for possible correlations between clot wave parameters and F VIII activity, and obtain the pattern of coagulation curves in hemophilia subjects as a step to assess clot waveform analysis as a possible screening tool for hemophilia.

Methods In this cross-sectional study, we performed clot wave analysis in 145 adult and pediatric subjects with hemophilia to obtain the clot wave pattern in this condition. Clot wave analysis was also done in 160 subjects with normal hemostasis to obtain reference clot wave parameters.

Results In this study, the starting point of coagulation phase in normal subjects was between 30-40 seconds, with a shorter pre-coagulation phase and steeper slope. Hemophilia patients had a longer pre-coagulation phase and flatter slope, especially in severe hemophilia A patients, who had longer and more variable coagulation starting points ($P<0.001$). The absolute values of maximum coagulation velocity (Min1), maximum coagulation acceleration (Min2), and maximum coagulation deceleration (Max2) of hemophilia A patients were also lower than those of normal hemostasis patients, with lower absolute value seen in severe than in mild-moderate hemophilia A patients. A moderate correlation was found between Min1, Min2, and Max2 with F VIII activity ($P<0.001$).

Conclusion Clot wave analysis may be considered as a method for screening hemophilia patients to distinguish mild-moderate and severe hemophilia A patients in health facilities that lack the ability to perform F VIII assays. **[Paediatr Indones. 2024;64:325-31; DOI: 10.14238/pi64.4.2024.325-31].**

Keywords: *APTT; clot wave analysis; CWA; coagulation; hemophilia*

Hemophilia is a congenital blood clotting disorder associated with the X chromosome. There are two types of hemophilia, namely, hemophilia A due to factor VIII (F VIII) deficiency and hemophilia B due to F IX deficiency.[1] Based on F VIII activity, hemophilia A can be further classified as mild (F VIII 5-40 IU/dL or 5%-40%), moderate (F VIII 1-5 IU/dL or 1%-5%), and severe (F VIII <1 IU/dL or <1%).[2] From January to July 2019, Cipto Mangunkusumo National Hospital, Jakarta, Indonesia had 90 hemophilia A and 32 hemophilia B patients registered in the Pediatric Outpatient Clinic, of which 72.2% had severe hemophilia A.[3] The identification of various types of bleeding disorders, including hemophilia, is quite difficult and requires sophisticated laboratory facilities. For these reasons, most people with bleeding disorders throughout the world, including in Indonesia, have difficulty getting a proper diagnosis.[4]

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The evaluation of F VIII activity is the most important test in the management of hemostasis in patients with hemophilia A. Clot wave analysis (CWA) has considerable potential as a basis for thoroughly assessing hemostasis in hemophilia patients. CWA can evaluate clot formation qualitatively in wave patterns, and quantitatively when expressed as absolute values or in units of seconds. The process of clot formation is divided into three phases: pre-coagulation, coagulation and post-coagulation. Pre-coagulation is defined as the first segment from the moment of signal start until the start of coagulation. After the coagulation has started, light transmission decreases (or absorbance increases) with fibrin formation, producing a slope in the clot wave. At the end of coagulation, the transmission of light or absorbance tends to stabilize, characterized by the return of the slope to a more linear segment. Clot wave analysis parameters consist of clotting time (CT), maximum coagulation velocity (Min1), maximum coagulation acceleration (Min2), and slowing or maximum coagulation deceleration (Max2).[5,6] Clot wave patterns in hemophilia A show variations in coagulation abnormalities compared to normal plasma. In normal plasma, the pre-coagulation phase is short and steep, while deficiencies in F XII, X, IX, VIII, V and/or II lengthen the coagulation phase, with varying slopes.[5] Clot wave analysis may be derived from the APTT assay without increasing examination costs, thereby assisting clinicians in diagnosing hemophilia through simpler measures, especially in healthcare facilities with limited hemostasis testing capabilities.[6] Clot wave patterns and values derived from APTT can aid clinicians in screening for coagulation factor deficiencies and monitoring therapy results.[6,7]

The CWA is practical for diagnostic purposes and may also be useful for monitoring hemostasis in cases of replacement therapy for massive bleeding or surgery in hemophilia patients. Furthermore, it is also valuable for assessing very low F VIII and F IX activity, as well as monitoring emicizumab therapy.[7,8] Nevertheless, little research has been done on clot wave patterns and CWA parameters in hemophilia patients, especially in Indonesia, due to unfamiliarity with the assays. The patterns formed can be used as a reference to interpret CWA results. We aimed to determine the clot wave patterns and CWA parameters in patients with normal hemostasis and in mild, moderate, and severe hemophilia A patients, as well as evaluate their association with F VIII activity.

Methods

This cross-sectional study was conducted from August to December 2019 and consisted of two stages: a reference values study on normal subjects and a second stage to characterize CWA parameters in hemophilia A subjects. For the reference values study, we enrolled 160 subjects (60 adult males, 60 adult females, and 40 children <18 years of age) who had PT and APTT values within the normal reference range, were admitted for medical check-up or routine preoperative evaluation, and were not diagnosed with anemia, liver disorders, or diabetes mellitus. The exclusion criteria for adult and pediatric patients with normal hemostasis were incomplete medical records, hemoglobin <10 g/dL, leukopenia or leukocytosis, thrombocytopenia or thrombocytosis, or elevated aspartate aminotransferase (AST), alanine aminotransaminase (ALT), or blood glucose levels. Some hemophilia subjects have a history of positive antibody (inhibitor) to F VIII. This antibody will also inhibit the clotting process due to the antibody binding.

For the second stage of the study, a total of 139 hemophilia A subjects were enrolled, consisting of 15 mild, 28 moderate, and 96 severe hemophilia A patients. Patients were excluded if their APTT examination results were unavailable.

F VIII activity was examined at the Central Laboratory of Cipto Mangunkusumo National Referral Hospital, Jakarta. Patient laboratory data were collected from the laboratory or hospital information system. F VIII activity assays were performed in patients who did not have such assay results. We used *Pathromtin® SL*, Owren's veronal buffer (OVB), Owren's buffer saline (OBS), and plasma deficient F VIII (*Siemens Healthcare Diagnostics*, Marburg, Germany) as reagents for the APTT assay. A *CS-5100* automatic coagulometer (*Sysmex*, Kobe, Japan) is used to examine the F VIII activity (performed using the one stage clotting assay method) and to obtain the CWA data. This assay measured APTT in the F VIII deficiency plasma mixture. F VIII activity was calculated using a standardized curve made

with several dilutions of human plasma from normal controls.

Clot wave analysis takes advantage of changes in light transmission that occur during clot formation. These changes in light transmission or absorbance for CWA were determined during APTT measurement. The instrument recorded coagulation time based on the percentage of coagulation immediately after the reagent was added, but before coagulation occurred, thus setting the degree of transmitted light intensity to 0%. After the coagulation was completely formed, the degree of intensity was considered to be 100%. Coagulation time was defined as the time needed to change the intensity of the transmission light by 50%.[5]

We collected Min1, Min2, and Max2 data of both the normal and hemophilia A subjects. As these values were abnormally distributed, they were presented as median and range of 2.5-97.5 percentile. Spearman's correlation test was used to analyze how Min1, Min2, and Max2, respectively, correlated with F VIII activity. Results of the clot formation were calculated as area under the curve and in seconds. We used the Mann-Whitney test to assess differences in maximum velocity, maximum acceleration, and maximum deceleration between the normal hemostasis and hemophilia A groups. The clot wave patterns of the two groups were analyzed using *Statistical Product and Service Solutions (SPSS) ver. 20* (*IBM*, Armonk, New York) and *Microsoft Excel* (*Microsoft*, Redmond, Washington) software, and clot wave patterns were presented in a scatterplot. A P value of <0.05 was considered to be statistically significant. The study protocol was approved by the Medical Research Ethics Committee of the Faculty of Medicine, University of Indonesia and the Ethical and Research Division of Cipto Mangunkusumo Hospital.

Results

We enrolled 160 subjects with normal coagulation for the reference values study, consisting of 51.9% males and 48.1% females, 75% of whom were adults . The median age of our subjects was 32 years. Median PT and APTT values were 10.6 seconds and 34.1 seconds, respectively. Subjects' characteristics are shown in **Table 1**. The median age of hemophilia patients in this study was 8 years; 86.3% of the patients were children and 94.2% were male.

The clot wave patterns of the normal hemostasis group are shown in **Figure 1**. The starting point of the coagulation phase in normal subjects was 30-40 seconds, with a shorter pre-coagulation phase and steeper slope compared to the hemophilia subjects.

Table 1. Characteristics of subjects

Characteristics	Adult	Children	Total
Normal hemostasis group	**(n=120)**	**(n=40)**	**(N=160)**
Median age (range), years	41 (18-70)	6 (1-17)	32 (2-69)
Sex, n (%)			
Male	60 (50)	23 (57.5)	83 (51.9)
Female	60 (50)	17 (42.5)	77 (48.1)
Median PT (range), seconds	10.45 (9.8-11.59)	10.9 (10-11.8)	10.6 (9.8-11.8)
Median APTT (range), seconds	33.80 (31-40.77)	35.1 (31-44.3)	34.1 (31-41.5)
Hemophilia A group	**(n=19)**	**(n=120)**	**(N=139)**
Median age (range), years	38 (18-88)	7 (0.8-16)	8 (0.8-65.5)
Sex, n (%)			
Male	15 (78.9)	116 (96.7)	131 (94.2)
Female	4 (21.1)	4 (3.3)	8 (5.8)
Hemophilia A, n (%)	19 (95)	120 (96)	139 (95.9)
Mild	5 (25)	10 (8)	15 (10.4)
Moderate	3 (15)	25 (20)	28 (19.3)
Severe	11 (55)	85 (68)	96 (66.2)
Median APTT (range), seconds	116.1 (44.4-190)	136.4 (46.8-155.5)	134.5 (45.2-190)

Ranges presented are 2.5th to 97.75th percentiles

The clot wave patterns of the hemophilia A subgroups [15 mild (**Figure 2a**), 28 moderate (**Figure 2b**), and 96 severe hemophilia A patients (**Figure 2c**), as well as 6 severe hemophilia subjects with F VIII inhibitor (**Figure 2d**)] showed longer starting points of 40, 50, and 80 seconds, respectively, compared to the normal subjects. The inhibitor subgroup had an even slower starting point than that of the hemophilia A group.

Of our hemophilia A group, 69% were categorized as severe. This data was based on hospital data of hemophilia A patients in January-July 2019.[3]

Both in adults and children, the Min1, Min2, and Max2 absolute values were much lower and more prolonged in hemophilia A patients than in the normal hemostasis group, as shown in **Tables 2** and **3**, respectively. The overall values of Min1, Min2, and Max2 parameters in mild, moderate and severe hemophilia can be seen in **Table 4**.

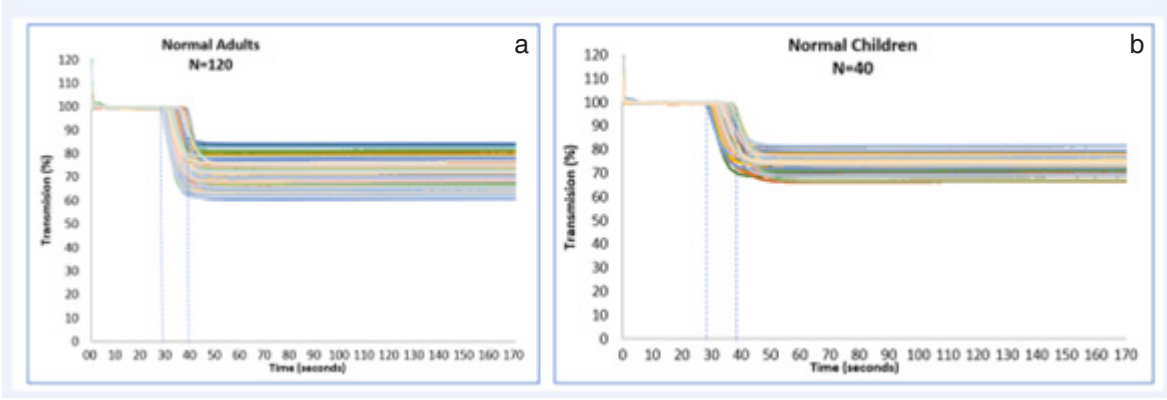
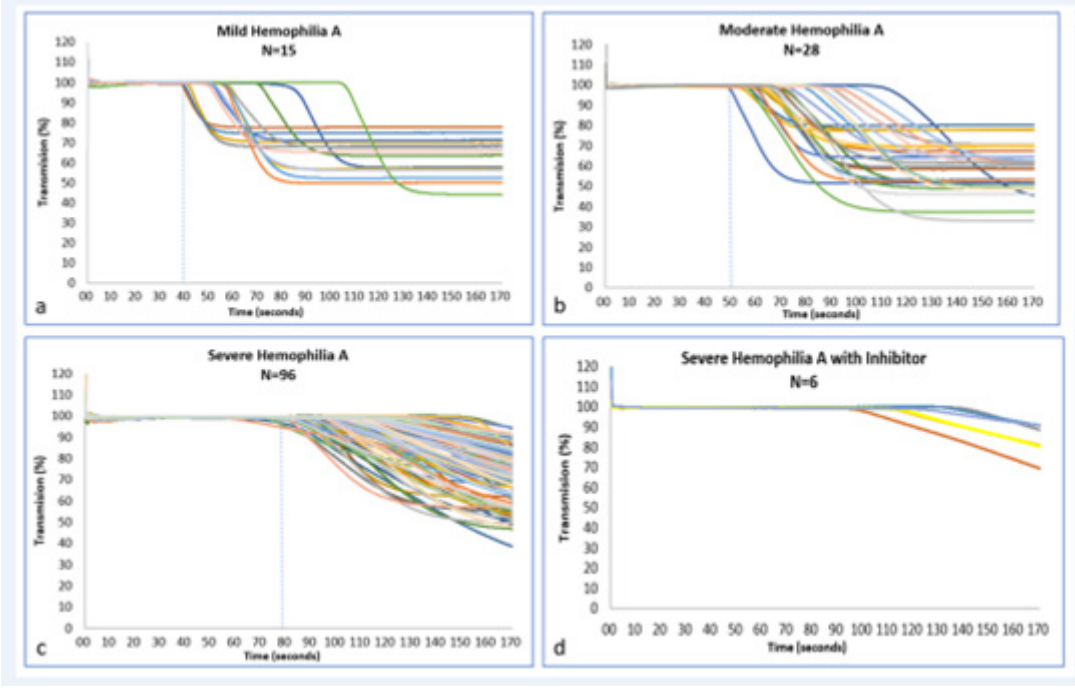


Figure 1. Clot wave patterns of (a) normal adults and (b) normal children.
The starting point of the coagulation slope in normal adult and children ranged from 30 to 40 seconds.



Figures 2. Clot wave patterns in hemophilia A patients: (a) mild hemophilia A; (b) moderate hemophilia A; (c) severe hemophilia A; (d) severe hemophilia A with F VIII inhibitor.

Table 2 . Min1, Min2, and Max2 values in hemophilia A *vs.* normal adults

Parameter	Hemophilia A adults (n=19)	Normal adults (n=120)	P value
Min1 (absolute)	0.959 (0.35-2.88)	3.289 (2.03-4.52)	<0.001
Min1 (seconds)	102.1 (43.7-165.9)	33.7 (31-40.58)	<0.001
Min2 (absolute)	0.097 (0.03-0.41)	0.549 (0.33-0.77)	<0.001
Min2 (seconds)	86.3 (39.4-157.2)	30 (27.3-36.58)	<0.001
Max2 (absolute)	0.018 (0.0-0.23)	0.434 (0.28-0.65)	<0.001
Max2 (seconds)	124.6 (0.0-164.1)	37.35 (34.6-44.8)	<0.001

Mann-Whitney test, data presented in median (range)

Table 3. Analysis of Min1, Min2, and Max2 in normal and hemophilia A children

Parameters	Hemophilia A children (n=120)	Normal children (n=40)	P value
Min1 (absolute)	0.573 (0.23-24.5)	2.789 (1.94-3.54)	<0.001
Min1 (seconds)	123 (45.83-166)	34.95 (31-43.5)	<0.001
Min2 (absolute)	0.048 (0.02-0.34)	0.464 (0.32-0.60)	<0.001
Min2 (seconds)	97.05 (39.15-145.2)	31.2 (27.41-39.05)	<0.001
Max2 (absolute)	0.012 (0.0-0.22)	0.361 (0.25-0.48)	<0.001
Max2 (seconds)	140.6 (0.0-164.2)	38.6 (34.62-48.4)	<0.001

Mann-Whitney test, data presented in median (range)

Table 4. Min1, Min2 and Max2 values based on severity of hemophilia A

Parameter	Mild hemophilia A		Moderate hemophilia A		Severe hemophilia A		P value
	Adult (n=5)	Children (n=10)	Adult (n=3)	Children (n=25)	Adult (n=11)	Children (n=85)	
Min1 (absolute)	2.645 (2.2-2.88)	1.992 (1.63-2.95)	1.881 (1.11-2.01)	1.275 (0.86-2.46)	0.741 (0.35-1.06)	0.459 (0.19-1.17)	<0.001
Min1 (seconds)	61.9 (43.7-112.3)	58.75 (42.3-92.4)	65.1 (59.7-72.3)	81.1 (55.7-134.2)	114.7 (96.9-165.9)	132.4 (86.94-166.42)	<0.001
Min2 (absolute)	0.337 (0.33-0.41)	0.261 (0.23-0.44)	0.249 (0.15-0.29)	0.147 (0.07-9.34)	0.06 (0.03-0.115)	0.037 (0.01-0.14)	<0.001
Min2 (seconds)	56.4 (39.4-105.8)	51.5 (38.1-86.3)	60 (54.3-60)	68.8 (49-112.3)	91.4 (81-157.2)	107.5 (73.23-148.79)	
Max2 (absolute)	0.194 (0.18-0.23)	0.147 (0.09-0.32)	0.107 (0.07-0.15)	0.059 (0.03-0.18)	0.009 (0-0.03)	0.004 (0-0.04)	<0.001
Max2 (seconds)	72 (48.4-124.6)	68.95 (46.8-101.2)	75.4 (72.5-82.2)	94.7 (65.6-155.6)	135.7 (0-164.1)	147.6 (0-164.2)	<0.001

Data presented in median (range)

Discussion

Hemophilia is a congenital coagulation disorder associated with the X chromosome, hence, it is more commonly found in males.[1] In our study, the median age of hemophilia patients was 8 years; 86.3% of patients were children and 94.2% were male. According to the *World Federation of Hemophilia Report* on the *Annual Global Survey 2017*, 57% of hemophilia A patients were children aged <18 years, and 88%-90% of them were male.[9]

The F VIII activity assay for the diagnosis, management, and monitoring of therapy for hemophilia patients is not readily available in most healthcare facilities in Indonesia. Clot wave analysis using an optical coagulometer, such as in our study,

is expected to help overcome these limitations. This APTT-derived clot wave does not require the addition of special F VIII or F IX deficient reagents. The automatic optical coagulometer is equipped with software that converts raw data into CWA parameters. A qualitative assessment of clot wave patterns can be used to interpret CWA quantitative parameters.[5]

A study reported that clot wave patterns in normal patients had a short pre-coagulation phase and steeper slope than in factor-deficient patients.[6] Clot wave patterns in normal-hemostasis adults and children in our study were similar to the study.[6] The starting point for the formation of coagulation slope in both normal adults and children was 30-40 seconds. However, there was a slight variation in the height of the slope in this group. These variations can be caused by the disparity in the speed of clot formation, hence, the time needed to achieve a fully formed clot for each subject is different and is influenced by the number of coagulation factors in each sample.[6,7]

Patients with hemophilia A had a longer pre-coagulation phase and a flatter slope compared to the clot wave pattern of the normal group. The coagulation starting points for slope formation in mild, moderate, and severe hemophilia A were ± 40 seconds, ± 50 seconds and ± 80 seconds, respectively. Slope patterns in severe hemophilia A patients varied as well (**Figure 2a, 2b**, and **2c**).

We identified a subgroup of mild hemophilia A patients with prolonged pre-coagulation phase and longer slopes, resembling clot wave patterns of severe hemophilia A. This phenomenon may have been due to the presence of inhibitors and was found in cases of acquired hemophilia A (**Figure 2d**). Acquired hemophilia A may also cause more severe clinical manifestations and prolongation of APTT that could not be corrected by the addition of normal plasma in a mixing study.[11]

There were significant differences between the Min1, Min2 and Max2 values in normal adults and children compared to adult and pediatric hemophilia patients (**Tables 2** and **3**). The absolute Min1, Min2 and Max2 values in hemophilia A children were lower than those in hemophilia A adults (**Table 4**). These findings were consistent with those in a previous study that confirmed that the APTT of adults and children was, indeed, significantly different. Adults have lower APTT and higher fibrinogen levels than children.[12] In our study, the Min1, Min2 and Max2 parameters were significantly different in hemophilia A patients compared to normal subjects. A study found that Max2 value can distinguish hemophilia patients from healthy patients, as well as between moderate and severe hemophilia A patients.[13]

Min1, Min2 and Max2 values also differed between mild, moderate, and severe hemophilia A (**Table 4**). There were lower Min1, Min2 and Max2 absolute values and more prolonged Min1, Min2 and Max2 in seconds in patients with severe hemophilia A compared to those with mild and moderate hemophilia A. Increased factor activity was followed by an increase in the absolute value and decrease of the Min1, Min2 and Max2 in seconds. A previous study found that the speed of clot formation decreased in patients with severe hemophilia A. Consequently, the clotting time was also prolonged, causing a significant decrease in the absolute value of Min1 and Min2.[14] The parameter values obtained in our study may differ from others, which may have been caused by several factors such as differences in the coagulometer and reagents used, the study population, and the sample size.[6,7]

Analysis of Min1, Min2 and Max2 absolute values and F VIII activity in hemophilia A patients showed a moderately positive correlation that was directly proportional to F VIII activity, which meant an increase in F VIII activity would be followed by an increase in the Min1, Min2 and Max2 absolute values. On the other hand, the correlation between the Min1, Min2 and Max2 values in seconds and F VIII activity in hemophilia A patients was moderately negative, i.e., inversely proportional to F VIII activity, which meant that an increase in F VIII activity would be followed by a decrease in the Min1, Min2 and Max2 values in seconds.

As reported by Shima *et al.*,[15] there was a good correlation (r=0.720) between F VIII <1 IU/dL and the Min2 parameter and there was a difference in slope between normal and severe hemophilia A patients. A study also showed a good correlation (r=0.760) between F VIII activity and peak acceleration.[7] Our findings resembled those of the aforementioned studies, but there were differences in research subjects, reagents, coagulometers, and the range of measurement of F VIII activity of the coagulometer used in this study, namely 1-150%.

With differences observed in clot wave patterns and the CWA parameters of normal hemostasis and hemophilia A patients, we suggest that CWA can be a valuable means for assessing coagulation function in hemophilia patients. Clot wave analysis can be considered as a method for screening hemophilia patients to distinguish mild-moderate hemophilia A from severe hemophilia A in health facilities that have limited F VIII assay availability, but have an automatic coagulometer equipped with a clot wave analysis. This clot wave analysis can also be considered to provide clinicians with more in-depth understanding of patients with severe hemophilia A with F VIII less than 1% activity.

Conflict of interest

None declared.

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