Original Article

A Modified Mixing Test with a Proposed Cutoff Value to Screen for Clotting Factor Inhibitors

Benjaporn Akkawat and Ponlapat Rojnuckarin

Department of Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital

Abstract

Background: *Mixing studies are helpful for clotting factor inhibitor screening in cases with activated partial thromboplastin time (APTT) prolongation. However, the standard consensus procedure is lacking.* ***Objective:*** *The aim is to develop a sensitive inhibitor screening assay in bleeding patients. The test is preferably insensitive to lupus anticoagulants (LA) in this setting.* ***Methods:*** *Plasma samples investigated for APTT prolongation (N = 90) were tested for factor VIII or IX inhibitors and LA. Positive mixing was defined as an ability of one part of patient plasma to prolong APTT of one part of pooled normal plasma (PNP) after 2-hour incubation. Two controls, which were also incubated for 2 hours, were 1) PNP mixed with test plasma immediately before the APTT test and 2) PNP mixed with buffer. Time-dependent inhibitors showed longer clotting time than those of 2 controls, while time-independent inhibitors had similar clotting time to the first control but longer than the second control. Three lots of APTT reagents were investigated.* ***Results:*** *There were 18 (20%) clotting factor inhibitor cases. The mean titer was 1.7 ranging from 0.67 to 4.0 Bethesda units. Fifteen samples of LA were regarded as negative. The most appropriate cutoff was the APTT ratio difference of 0.10 or higher. The mixing study using any lots of APTT reagents showed 100% sensitivity for clotting factor inhibitors. The sensitivity for LA was lower. The majority of factor inhibitors were time-dependent, while the LA inhibition was mostly immediate. One lot of the reagent displayed 93.1% specificity, but the other 2 lots showed higher false positive rates.* ***Conclusion:*** *We described a sensitive screening test for clotting factor inhibitors. However, optimal lots of APTT reagents need to be determined to obtain excellent specificity.*

Keywords : ● *Mixing test* ● *Clotting factor inhibitor* ● *Lupus anticoagulants* ● *Survival*

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Introduction

Mixing studies are useful screening tests for inhibitors in patients with prolonged coagulation times. Inhibitors can be a specific clotting factor antibodies or lupus anticoagulants (antiphospholipid antibodies). Because positive test results may suggest bleeding or thrombotic risks, respectively, clinical implications are strikingly different. There has been a consensus guideline for the mixing tests to diagnose lupus anticoagulants,[1] but the standard mixing procedures for coagulation factor inhibitor screen are lacking. Therefore, we aimed to investigate the roles of mixing studies to screen for clotting factor autoantibody in non-hemophiliacs or alloantibody in hemophilia patients who acutely bleed or require invasive procedures. The test is preferably

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Requests for reprints should be addressed to Ponlapat Rojnuckarin, Department of Medicine, King Chulalongkorn Memorial Hospital, Rama IV Rd., Patumwan Dist., Bangkok 10330, Thailand

e-mail: rojnuckarinp@gmail.com

insensitive to lupus anticoagulant as the detections are not helpful in these clinical settings.

Although some reference laboratories use factor inhibitor assays directly without a prior step of mixing test. Bethesda inhibitor assay is more expensive because it needs factor-deficient plasma and several dilutions of test plasma may be required. Higher cost will be incurred, if the Nijmegen modification is used.[2] In addition, this special assay may not be available in general laboratories. If a mixing test is sensitive for low-titer inhibitors, the inhibitor assay can be avoided in case of a correctable mixing study. Additionally, factor replacement can be immediately given to the patients who need urgent clotting factor concentrates for active bleeding or invasive procedures. The inhibitor assay is required only to confirm an uncorrectable mixing test.

Some factors can interfere with the mixing test results.[3] For examples, labile clotting factors in plasma continuously degrade during incubation possibly causing inability to detect a clotting time correction. On the other hand, residual factor activity in test plasma when mixed with normal plasma may yield a shorter clotting time compared with a mixture with buffer, which contains no clotting activity. Appropriate control tubes are required to limit these false negative and false positive results, respectively.

In this study, the sensitivity and specificity of a screening mixing study were investigated. In addition, 3 lots of activated partial thromboplastin time (APTT) reagents were tested for their performances.

Materials and Methods

Materials and equipment

Owren Veronal buffer (OVB) and 3 lots (538403, 538407 and 538414) of activated partial thromboplastin time (APTT) reagents (Actin FS™) were obtained from Siemens (Dade Behring), Germany. APTT, lupus anticoagulant (LA), factor VIII and IX inhibitor assays were performed using CA1500 coagulation analyzer (Sysmex, Japan). LA was detected by Silica Clotting Time™ (SCT™, Instrumentation Laboratory Company, USA) also using CA1500.

Plasma samples

Plasma samples of 90 patients with prolonged APTT were included. The Ethical Committee of the Faculty of Medicine, Chulalongkorn University did not require informed consent for the study of clinical samples with no additional specimen collection, as long as the report was in aggregate. Thrombin time (TT) must have been normal to exclude heparin effects. Blood specimens were collected in 0.109 M sodium citrate at the ratio of 9:1. After centrifugation, all plasma was snap-frozen in liquid nitrogen and kept at -80°C until test. Pooled normal plasma from 30 healthy donors was similarly prepared and stored. The pooled normal plasma was also being used as a reagent in our service laboratory. These normal subjects had signed consent forms to use their plasma as a laboratory reagent.

Mixing study

The screening method was modified from Kasper technique[4] using a commercial APTT reagent. Patient plasma and an equal volume of pooled normal plasma were incubated both together (T) and separately (S) for 2 hours at 37°C. The 1:1 mixture of pooled normal plasma and the Owren Veronal buffer (OVB) as the control (C) was also incubated at the same temperature for the same duration. At 2 hours, the separately-incubated plasma (S) was mixed for immediate APTT testing. This separately-incubated tube (S) was used to control for the variable residual factors in test plasma and some degradation of the labile factors during incubation. Both might interfere with the assay results when inhibitor titers were low.[3]

The APTT ratios of all mixtures (T, S and C) were calculated by dividing the clotting time of each mixture by the clotting time of the pooled normal plasma from the same lot of APTT reagent on the same day.

The coefficients of variation (%CV) of APTT varied between the reagent lots. However, the recommended %CV of APTT should not exceed 4%.[5] As the tests

and controls contained the same plasmas with a similar incubation time, differences below 0.05 might be due to variations in the test. The appropriate cut point remained to be determined. In cases of time-dependent inhibitors, the APTT ratio of T (together-incubated tube) should be longer than those of S (separately-incubated and immediately-mixed tube). For samples with time-independent inhibitors, both T and S should yield longer APTT ratios compared with C (pooled normal plasma and buffer). Both time-dependent and time-independent inhibitors were considered positive.

Confirmatory tests for inhibitors

As the gold standard, patient plasmas were tested for both lupus anticoagulant (LA) and Bethesda inhibitor assay for clotting factors. LA was determined by SCT™ according to the manufacturer instruction. LA results were interpreted using the cutoff value validated in our laboratory.[1] Factor VIII, IX and XI inhibitors were determined using one-stage assay.[6] The inhibitor titers of 0.6 Bethesda units or over were considered positive.

Results

There were a total of 90 specimens with APTT prolongation. All samples were tested for clotting factor inhibitors using the Bethesda assay, as well as a lupus anticoagulant test. Eighteen cases (20%) were positive for clotting factor inhibitors, 15 factor VIII and 3 factor IX inhibitors. Four factor VIII inhibitors were from non-hemophiliacs. The higher titer inhibitors were diluted to be below 5.0 Bethesda units (BU) in order to investigate the sensitivity of the mixing test for low-titer inhibitors. The mean (± SD) inhibitor titer was 1.70 ± 1.07 BU ranging from 0.67 to 4.0 BU. One factor VIII inhibitor patient also had positive lupus anticoagulants.

There were 72 samples that were negative for clotting factor inhibitors. Fifteen of them were positive for lupus anticoagulants (16.7%). The known cases of factor deficiencies without inhibitors were hemophilia A (27), von Willebrand disease (12), hemophilia B (3), factor XII deficiency (3), disseminated intravascular coagulation (3), factor XI deficiency (2), liver disease (1), warfarin (1) and erythrocytosis causing excessive citrate anticoagulation (1). Information could not be obtained in 4 cases.

All plasma specimens underwent 3 mixing tests using 3 lots of APTT reagents (538403, 538407 and 538414) in order to screen for clotting factor inhibitors. Lupus anticoagulant positivity alone was considered negative.

Different APTT ratio cutoff points were explored in 3 lots using the receiver operating characteristic (ROC) analysis varying the cutoff from 0.01 to 0.15 as shown in Figure 1. The lot 538414 [Figure 1(C)] showed the best performance. The most appropriate cutoff points with the best specificity while maintaining 100% sensitivity was 0.10. Higher cutoffs could improve specificity but decrease sensitivity.

Every case of clotting factor inhibitor had positive screening mixing tests by all 3 lots of APTT reagent yielding the sensitivity of 100%. On the other hand, the test had lower sensitivity for lupus anticoagulants, especially the reagent lot number 538414 (Table 1). The 3 lots of APTT reagents displayed differences in specificity. The lot 538414 showed a specificity of 93.1%, while lot number 538403 and 538407 had higher false positive rates for clotting factor inhibitors (Table 1). After excluding LA-positive cases, the APTT reagents lot 538403 and 538407 gave the specificity of 54.4% and 47.4%, respectively.

To sub-classify the inhibitors, time-dependent inhibition was defined as the APTT ratio of the mixing followed by 2-hour incubation (T) subtracted with the APTT ratio of 2-hour separate incubation followed by immediate mixing (S), over 0.10. The majority of factor inhibitor cases showed time-dependent inhibition (Table 2). On the other hand, lower proportions of LA-positive samples and other false positive samples showed time dependency. These differences in time dependency between types of inhibitors were more pronounced in the APTT reagent lot number 538403 and 538407.

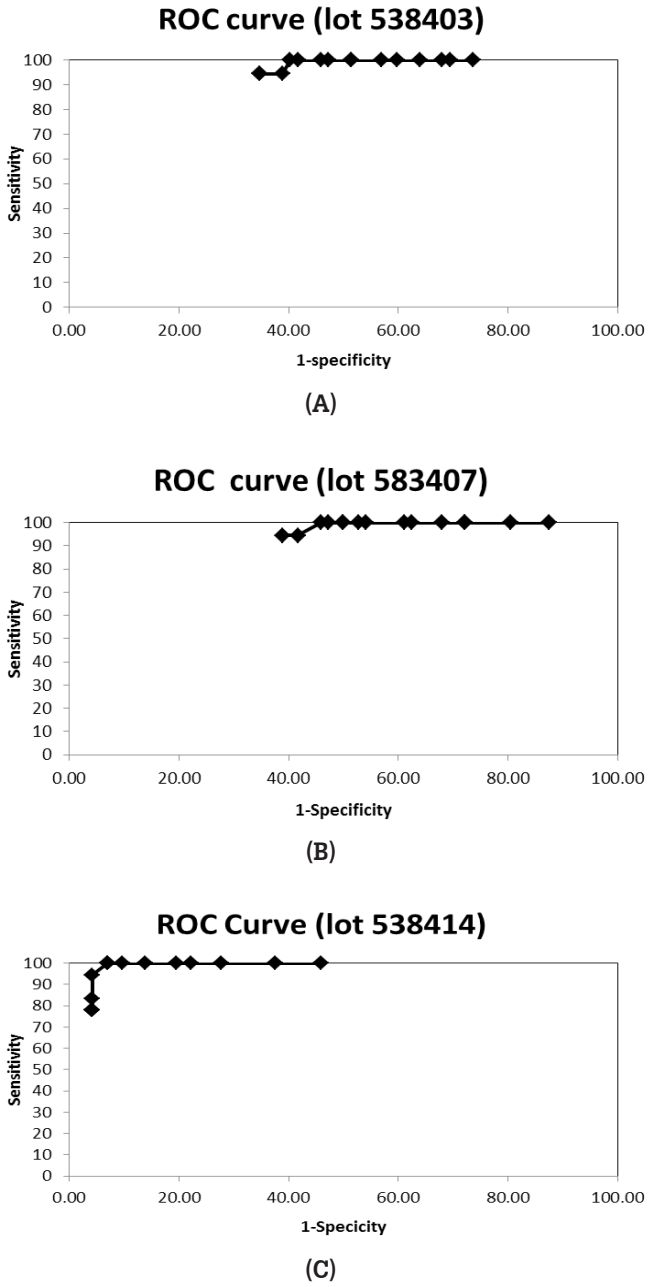

Figure 1 The Receiver Operating Characteristic (ROC) analyses of the APTT reagents lot 538403 (A), 538407 (B) and 538414 (C) varying the cutoff points between 0.01 and 0.15

Table 1 Sensitivity and specificity of the mixing test[1]

APTT Reagent Lot	Factor inhibitor		Lupus anticoagulants		Factor inhibitor or Lupus anticoagulants	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
538403	100	54.2	50.0	41.9	75.8	54.4
538407	100	50.0	43.8	36.5	72.7	47.4
538414	100	93.1	18.8	27.0	60.6	94.7
Positive cases	18		16		33[2]	

[1]*Both time-dependent and time-independent inhibitors were considered positive. The cutoff APTT ratio difference was 0.10.*

[2]*One patient was positive for both factor VIII inhibitor and lupus anticoagulant.*

Table 2 Time-dependency of inhibitors as detected by the mixing study

APTT reagent (Lot number)	Time-dependent factor Inhibitors	Time-dependent lupus anticoagulants	Time dependency in false positive mixing test
538403	77.8% (14/18)	12.5% (1/8)	6.1% (2/33)
538407	55.6% (10/18)	14.3% (1/7)	5.6% (2/36)
538414	77.8% (14/18)	66.7% (2/3)	40.0% (2/5)

Time dependency was defined as the difference between the APTT ratios of 2-hour incubated mixing and immediate mixing over 0.10

Discussion

In this study, we have developed a modified screening assay for factor VIII and IX inhibitors in patients who have APTT prolongation. The test is intended to be used in cases with bleeding or requiring invasive procedures. It was highly sensitive (100% sensitivity) even to low-titer inhibitors (under 1.0 BU). Therefore, the negative results can obviate cumbersome factor inhibitor assays. However, the specificity of the test depended on the lots of APTT reagent. The lot-to-lot variations in mixing test performances have never been described. The reason for this difference remains to be determined. One of the explanations is a variation of the APTT reagents in sensitivities for lupus anticoagulants (Table 1).

In order to obtain a good accuracy, the reagents need to be evaluated for their performances. In this paper, the lot number 538414 showed very good specificity and, thus, it was chosen for further mixing study in our laboratory. The majority of lupus anticoagulants did not cause positive mixing tests using this APTT reagent lot (Table 1). This is beneficial because detection of antiphospholipid antibody is not useful in cases when hemorrhage is the main concern. After excluding LA cases, the specificity of APTT reagent lot 538403 and 538407 were still only approximately 50%. Therefore, their low accuracy was not solely due to the LA-sensitivity. These 2 lots may be used in a screening test, but fewer factor inhibitor assays can be avoided.

Instead of measurement of the correction of clotting time of test plasma, we determined an ability of test plasma to prolong a clotting time of pooled normal plasma. An immediate mixture of test and normal plasma and a mixture of buffer and normal plasma were used as controls. All tests were incubated at 37°C for 120 minutes to control for clotting factor degradation. In order to obtain a highly sensitive screening test, ROC analyses were performed to determine the specific cutoffs while the sensitivity remained 100%. The most appropriate point was 0.10.

Consistent with previous reports,[3,7] we found that the majority of clotting factor inhibitors was time-dependent, i.e. APTTs were more prolonged after 2-hour incubation compared with immediate mixing. On the other hand, lupus anticoagulants usually work promptly after mixing. Furthermore, false positive mixing tests did not show time-dependency. This was more pronounced using APTT reagents with lower specificity (Table 2). Therefore, the comparison of APTT between immediate and pre-incubated mixtures is helpful in differential diagnosis of inhibitors. Nevertheless, this distinction was not absolute.[3,7] As we needed a highly sensitive screening assay, the time-independent inhibitors were also regarded as positive in this study.

There are some variations in mixing procedures, e.g. the ratio between patient and normal plasma and the duration of incubation. The 4:1 ratio may be more sensitive to a low-titer inhibitor. However, one study showed that this ratio did not work well in diagnosing lupus anticoagulants[8] and it required more patient plasma possibly causing insufficient specimens for further tests.[9] The 1: 1 ratio of patient to normal plasma was chosen in this study showing excellent sensitivity. Regarding the incubation duration, sufficient time is needed for the time-dependent clotting factor inhibitors, but too

long incubation may result in excessive degradation of clotting factors without inhibitor. The 2-hour duration is generally used in clotting factor assays as it has been shown to be the most optimum.[10]

A mixing study has been one of the most difficult tests to interpret mainly because of the poor definition of 'correctable mixing'. Formulae, such as 'percentage change'[11] and 'Rosner index',[12] have been devised. However, cutoff points need to be determined in individual laboratory. The best cutoff value in our study was 0.10 showing that it is safe to exclude the presence of clotting factor inhibitor.

In mixing studies, corrections are usually obvious in cases of severe single coagulation factor deficiency and may be less clear in mild multiple factor deficiency or antiphospholipid.[13] Consequently, some authors suggested omission of the mixing step in LA detections.[14,15] However, our study suggested that the mixing test is useful at least in the settings of bleeding.

In conclusion, a sensitive screening test for clotting factor inhibitor in the intrinsic pathway has been developed. However, appropriate brand and lot of APTT reagent need to be pre-selected preferably by the reagent manufacturers for this use.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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การปรับปรุงการตรวจ Mixing Test และ เสนอจุดตัดในการตรวจกรองหาสารต้านปัจจัยการแข็งตัวของเลือด

เบญจพร อัคควัฒน์ และ พลภัทร โรจน์นครินทร์

สาขาโลหิตวิทยา ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และ โรงพยาบาลจุฬาลงกรณ์ สภากาชาดไทย

บทคัดย่อ

ที่มา *การทำ mixing study มีประโยชน์ในการตรวจกรองหาสารต้านปัจจัยการแข็งตัวของเลือดในกรณีที่ activated partial thromboplastin time (APTT) ยาวผิดปกติ แต่ยังขาดมาตรฐานในขั้นตอนการทำและการแปลผลที่ชัดเจน* ***วัตถุประสงค์*** *เพื่อพัฒนาการทำ mixing study ให้มีความไว (sensitivity) ต่อการตรวจคัดกรองหาสารต้านปัจจัยการแข็งตัวของเลือดในผู้ป่วยภาวะเลือดออกง่าย แต่ทั้งนี้ต้องไม่ไวต่อ lupus anticoagulants (LA)* ***วัสดุและวิธีการ*** *ใช้พลาสมาที่มี APTT ยาวผิดปกติและ Thrombin time (TT) ปกติ 90 ราย นำมาตรวจหา LA ด้วยหลักการ APTT ตรวจหาสารต้านต่อ factor VIII หรือ IX แล้วทำ mixing study ด้วยการผสมพลาสมาผู้ป่วย 1 ปริมาตรกับ pooled normal plasma (PNP) 1 ปริมาตร นำไปอุ่น 37 °ซ. 2 ชั่วโมง พร้อมๆ กันก็ทำหลอดควบคุม 2 หลอด โดย (1) อุ่นพลาสมาผู้ป่วยและ PNP แยกกันคนละหลอดเมื่อครบเวลาจึงผสมกัน และ (2) ผสม PNP 1 ปริมาตรและ buffer 1 ปริมาตรอุ่นไปพร้อมๆ กัน เปรียบเทียบค่า APTT ratio ของทั้ง 3 หลอดจากน้ำยา APTT 3 lot หากหลอดที่อุ่นด้วยกันยาวกว่าอุ่นแยกกันบ่งชี้ว่ามีสารต้านปัจจัยการแข็งตัวของเลือดชนิดขึ้นกับเวลา (time dependent) หากเท่ากันแต่ยาวกว่าหลอดควบคุมที่ 2 ก็เป็นชนิดไม่ขึ้นกับเวลา (immediate)* ***ผลการศึกษา*** *พบสารต้านปัจจัยการแข็งตัวของเลือด 18 ราย (ร้อยละ 20) มีระดับตั้งแต่ 0.67 ถึง 4.0 หน่วย Bethesda (เฉลี่ย 1.7) ค่าจุดตัดที่เหมาะสมที่สุด คือ APTT ratio ยาวกว่า 0.10 ผล mixing study เพื่อตรวจคัดกรองสารต้านปัจจัยการแข็งตัวของเลือด แสดงถึงความไวร้อยละ 100 ทั้ง 3 lot แต่ความไวต่อ LA จะต่ำกว่านี้ สารต้านปัจจัยการแข็งตัวของเลือดที่ตรวจพบส่วนใหญ่เป็นชนิดขึ้นกับเวลา ขณะที่ LA จะเป็นชนิดไม่ขึ้นกับเวลา น้ำยา APTT lot หนึ่งมีความจำเพาะ (specificity) ถึงร้อยละ 93.1 แต่ในอีก 2 lot กลับพบผลบวกปลอม (false positive) มากกว่า* ***สรุป*** *ผู้วิจัยได้เสนอการตรวจคัดกรองที่มีมาตรฐานและมีความไวสูงในการตรวจหาสารต้านปัจจัยการแข็งตัวของเลือด แต่ทั้งนี้การเลือก lot น้ำยาที่เหมาะสมก็จะช่วยให้การตรวจมีความจำเพาะสูงด้วย*

คำสำคัญ : ● *Mixing test* ● *สารต้านปัจจัยการแข็งตัวของเลือด* ● *Lupus anticoagulants*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2557;24:137-43.