Original Article

Evaluation of Kaolin Clotting Time for the Diagnosis of Lupus Anticoagulants by Using Different Calculation Methods

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Abstract

Background: *Lupus anticoagulants (LA) are a group of antibodies that prolongs phospholipid-dependent coagulation assays. They are associated with a wide variety of clinical settings such as thrombosis and recurrent spontaneous abortion.* ***Objective:*** *To evaluate the results of kaolin clotting time (KCT) obtained from different calculation methods and cut-off values to find the optimal way of identifying of LA.* ***Methods:*** *Plasma samples of 38 LA positive patients and of 50 LA negative patients were analyzed.* ***Results:*** *For the screening test, the calculations that showed 100% in both sensitivity and specificity were KCT, ratios of KCT of test sample to mean KCT of normal pooled plasma (KCT/mNP), or to mean KCT of normal subjects (KCT/mNor). For the confirmatory test, the sensitivity of any calculation methods demonstrated wide discrepancies (23.7-89.5%), while the specificity was high (90-98%). There were variations of the results when using mean+2SD as cut-off but not with the percentiles. The difference between normal saline control and platelet neutralization procedure (NSS-PNP) was the best method for the confirmatory test.* ***Conclusion:*** *The appropriate calculation method of the result should be selected by careful consideration concerning the sensitivity and specificity of the test including cut-off value.*

Keywords : ● *Kaolin clotting time* ● *Lupus anticoagulants*

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Introduction

Lupus anticoagulants (LA) are clinically important because they are associated with venous and arterial thrombosis,[1-4] pulmonary embolism,[2,4] recurrent spontaneous abortion,[3,5,6] pregnancy complications,[1,3,5-7] neurological diseases (ischemic stroke,[4,8,9] migraine,[8] diplopia,[8] memory loss,[8] ataxia,[8] dementia,[9] epilepsy[9], etc), thrombocytopenia,[5,10] and a wide variety of clinical situations such as myocardial infarction,[4] acute coronary syndrome,[11] renal involvement[12,13] and infectious diseases.[13,14] They are a heterogenous group of antibodies belonging to antiphospholipid antibody family and they react against various phospholipid-protein complexes or lipid-protein products.[15-17] Some antigenic targets of these antibodies are β_2 - glycoprotein I,[16,18] prothrombin,[17] annexin V,[19] protein C and protein S.[20] LA are identified by their ability to prolong one or more phospholipid-dependent coagulation assays.[15] Due to their heterogeneities, some LA react better in some test systems than in others. Thus, more than one test system should be used for the identification of LA.[21,22] The laboratory evaluation of LA antibodies shows the correlation between coagulation profiles and distinct type of phospholipid-dependent inhibitors. The diluted Russell viper venom time (dRVVT) is mostly abnormal in anti-β_2-glycoprotein I positive patients, whereas kaolin clotting time (KCT) is more sensitive in anti-prothrombin positive patients. No difference is

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observed in activated partial thromboplastin time (APTT) in this group of patients.[23] Among the coagulation tests, various methods for calculating the results are used to identify the presence of LA.[24,25] The difference of the methods may influence the sensitivity of the tests. Therefore, we evaluated KCT which has been shown to be a highly sensitive test for detecting LA[26,27] by comparing the results obtained from different calculation methods and cut-off values to find the optimal ways for the diagnosis of LA.

Materials and Methods

Test samples

We studied plasma samples of 38 LA positive patients (25 females, 13 males; age ranged from 9-65 years) and 50 LA negative patients (36 females, 14 males; age ranged from 11-82 years). LA positive plasma samples are the samples that are positive by the diluted Russell viper venom time (dRVVT) and/or activated partial thromboplastin time (APTT) tests according to the International Society on Haemostasis and Thrombosis (ISTH) 2009 guideline.[28] None of the plasmas contained heparin or warfarin. The dRVVT (LAC screen and LAC confirm) and APTT reagents are from Instrumentation Laboratory, Milano, Italy. The platelet neutralization procedure (PNP) was used as APTT confirmatory test.[29] The plasma samples with negative dRVVT and APTT tests were also obtained from 40 healthy subjects to serve as reference controls. The research project was approved by the Ethics Committee of Faculty of Medicine Ramathibodi Hospital, Mahidol University.

Blood collection

Nine parts of blood samples from patients and healthy subjects were collected into plastic tubes containing one part of 0.109 mM/L sodium citrate (9:1 ratio). The samples were centrifuged twice at 2,000 g for 20 minutes, 4°C. The plasmas were analyzed immediately or kept frozen at -80°C until assay. Normal pooled (NP) plasma from 30 healthy subjects was prepared in the same manner.

Reagents

Kaolin (Sigma, St. Louis. MO) was suspended in 0.9% NSS at a concentration of 20 mg/mL. Frozen platelet suspension (phospholipid; PL, source) was prepared as previously described by Triplett DA, et al.[29]

Screening test

KCT was performed manually according to Exner T, et al.[30] Briefly by pre-incubating the mixture of 0.2 mL plasma and 0.1 mL kaolin suspension for 3 min at 37°C after which 0.2 mL 0.025 M calcium chloride was added. The duration between addition of calcium chloride and clot formation was recorded.

Confirmatory test

Platelet neutralization procedure (PNP)[29] was performed by adding 0.1 mL frozen platelet suspension to the mixture of 0.2 mL plasma and 0.1 mL kaolin suspension. Then the mixture was incubated for 3 min at 37°C after which 0.2 mL 0.025 M calcium chloride was added. Finally, the clotting time was recorded. The control was performed by replacing frozen platelet suspension with normal saline solution (NSS).

Data analysis

Screening and confirmatory of KCT tests were performed on plasmas of each 40 normal subjects. Cut-off values were calculated as mean+2SD, 97.5 percentile and 99.0 percentile distribution. Abnormal KCT values of test samples were defined by various expressions of the test values.

Screening test

1. *Values against the upper reference limit*

Mean, standard deviation, 97.5 and 99.0 percentile were calculated from KCT of 40 normal subjects. If KCT of the test sample exceeded the upper limit of reference range, LA positive would be considered.

2. *Ratio of KCT of test sample to KCT of NP (KCT/NP)*[31]

The NP plasma collected from 30 healthy donors was tested in the same run of KCT screening test. The KCT/NP ratio of each sample was positive when the calculated ratio was above the upper range of reference population.

3. Ratio of KCT of test sample to mean KCT of NP (KCT/mNP)

Mean KCT of NP was calculated. The KCT of test sample was divided by mean KCT of NP and the upper range of the reference population was used as the cut-off value.

4. Ratio of KCT of test sample to mean KCT of normal subjects (KCT/mNOR)

The ratio between KCT of test sample and mean KCT of 40 normal subjects was determined. LA are considered positive when the ratio exceeds the upper range of normal subjects.

Confirmatory test

Data was assessed in various calculation methods of the clotting times from confirmatory assays.

1. The difference between KCT of NSS and PNP (NSS-PNP)

PNP method was described by Triplett DA, et al.[29] The shortening of clotting time (after adding of PL) of each sample compared to saline mixture control was determined. We calculated the difference between KCT of NSS mixture and KCT of PNP of each test sample. The result exceeded cut-off value of reference population was considered to be LA positive.

2. The difference between KCT of test sample and PNP (KCT- PNP)

The difference of clotting times with low and high PL for each sample was recommended by Staclot LA (Diagnostica Stago, Asnieres, France).[32] We employed the difference between KCT in screening test and confirmatory test (KCT-PNP) to assess our data. The value exceeded upper limit of reference population was indicative for LA.

3. The ratio of KCT of NSS to KCT of PNP (NSS/PNP)

The shortening of the clotting time compared to saline mixture control was expressed in the KCT ratio of NSS and PNP. The plasma sample was considered positive of LA when the ratio was above cut-off value.

4. The ratio of KCT of test sample to KCT of PNP (KCT/PNP)

The calculated ratio of clotting time with low and high PL concentration (test/confirm ratio) was recommended by the manufacturer (DVV confirm, American Diagnostica, CT, USA). We calculated KCT ratio from screening test with no added PL and confirmatory test with high concentration of PL (KCT/PNP). Cut-off value was the upper range of the reference population ratio.

5. The normalization ratio (Screen Ratio/Confirm Ratio)

A commercial kit from Instrumentation Laboratory, Milano, Italy recommends the determination of Normalized LAC ratio. The ratio of patient's clotting time to mean clotting time of reference population in screening test (Screen ratio) and in confirmatory test (Confirm ratio) were calculated (normalized LAC ratio = Screen ratio/ Confirm ratio). If the normalized LAC ratio ≥ 1.2, positive of LA would be considered. We calculated screen ratio as KCT/mean KCT of reference population and confirm ratio as KCT of PNP/mean KCT of PNP of reference population. The normalization ratio was determined as Screen Ratio/Confirm Ratio. Cut-off value of the normalization ratio was also 1.2.

6. Index of circulating anticoagulant (ICA)[33]

The index was calculated according to the formula: [(KCT of 1:1 NP + test plasma) - KCT of NP)/KCT of test plasma] x 100.

Value above 15% was considered to be positive for LA.

7. The lupus ratio (LR)[34]

The ratio of screen/confirm of patient divided by screen/confirm of NP was calculated (KCT/PNP of patient)/(KCT/PNP of NP). Result above upper limit of reference range was interpreted as LA positive.

8. Percentage correction of ratio

A commercial kit (Lupus anticoagulant test kit, Unicorn diagnostic, London, UK) recommends the calculation of clotting time ratio of test/ NP for diluted phospholipid reagent (DPL ratio) and for the confirm

reagent (Con ratio). Percentage correction of ratio was calculated in the formula of [(DPL ratio - Con ratio)/ DPL ratio] x 100. We used the ratio of KCT/NP as DPL ratio and PNP/mean PNP of NP as Con ratio. A ratio exceeded cut-off value of normal subjects was considered to be LA positive.

Results

The cut-off values of each calculation method derived from 40 healthy subjects were shown in Table 1.

Screening test

The KCT, KCT/NP and KCT/mNOR demonstrated 100% in both sensitivity and specificity with all cut-off values. The KCT/NP expressed low sensitivity (78.9-84.2%). However, its specificity was 100% with all cut-off values (Table 2).

Confirmatory test

The ways of data analysis in confirmatory tests revealed some sensitivity variations. The NSS-PNP and the KCT-PNP had high sensitivity (84.2-89.5%), while other five calculation methods (the NSS/PNP, KCT/PNP, normalized ratio, LR and the % correction ratio) had low sensitivity (23.7-65.8%, Table 2). However, all methods demonstrated high specificity (92-98%). Similar sensitivity and specificity were observed in each calculation method with both 97.5 and 99.0 percentile cut-off values. On the contrary, the use of mean+2SD cut-off gave rise to the discrepancy of the results which most of them were lower than that of the percentiles (Table 2). A poor sensitivity was obtained from the ICA (50%) but this method gave a good specificity (94%).

Results of KCT tests on factor VIII deficient plasmas

For screening tests, plasma samples containing factor VIII level < 10% revealed abnormal results in all samples of all calculating methods. On the contrary, samples with FVIII level > 10% had low positive results (Table 3). For confirmatory tests, most of calculation methods expressed low positivity rates in samples with FVIII level < 10%, only two of them (NSS-PNP and KCT-PNP) showed high abnormal results. The positivity rates also decreased in samples with FVIII level > 10%. The variation of values on samples containing factor VIII level > 50% might be due to the small number of samples (Table 3). The results of ICA were negative in all samples containing factor VIII level < 10% and factor VIII level 11-50%. No data were available for the samples with FVIII level > 50%.

Discussion

Since the recognition of the antiphospholipid syndrome, many clinical laboratories are faced with an increase in requests for LA determinations. Laboratory confirmation of the diagnosis is significant, particularly in a patient with a history of thrombosis. Moreover persistently positive LA indicates a high risk for recurrence of thrombosis.[35,36] Failure to detect LA results in an inappropriate treatment of anticoagulant. In contrast, a false-positive result may lead to unnecessary treatment with anticoagulant with its associated risk of bleeding. The diagnosis of LA remains variable with respect to sensitivity and specificity of laboratory assays. This is partly due to difference in reagents, procedures, instruments, cut-off values and result interpretations as pointed out by numerous studies.[25,37-42] The most common LA antibodies are anti-prothrombin and anti β_2 - glycoprotein I which are associated with different coagulation test systems. They are characterized by the sensitivity of the KCT and dRVVT to the presence of anti-prothrombin and anti β_2 - glycoprotein I, respectively.

The ISTH 2009 guideline proposed the use of two assays to measure LA: the dilute Russell viper venom time and a sensitive partial thromboplastin time (PTT-LA).[28] The guideline does not support the use of KCT, citing difficulties in performing the KCT in some laboratories. The problems of KCT are the turbidity of kaolin suspension and the need of a manual technique, make it unsuitable for large-scale testing. However, KCT might be useful for small-scale performing since the method is simple and uses low cost resources. Moreover, it is a sensitive measure of LA. The depletion

Table 1 Cut-off values of KCT tests in each calculation method as determined from 40 healthy subjects

Cut-off values	Screening tests				Confirmatory tests						
	KCT (sec)	KCT/NP	KCT/mNP	KCT/mNOR	NSS-PNP (sec)	KCT-PNP (sec)	NSS/PNP	KCT/PNP	Nor ratio	LR ratio	Per cor ratio
Mean + 2SD	92.3	1.513	1.403	1.293	52.5	39.9	39.9	2.123	1.267	1.388	39.135
97.5 percentile	92.7	1.425	1.409	1.298	47.9	37.5	37.5	2.266	1.274	1.401	28.616
99.0 percentile	92.7	1.425	1.425	1.299	48	37.5	37.5	2.27	1.274	1.403	28.706

KCT, Kaolin Clotting Time; NP, Normal plasma; mNP, mean Normal plasma; mNor, mean Normal subject; NSS, Normal saline solution;

PNP, Platelet neutralization procedure; Nor ratio, Normalized ratio; LR ratio, Lupus ratio; Per cor ratio, Percentage correction of ratio

Table 2 Sensitivity and specificity of KCT test of each different calculation methods with different cut-off values

	Screening tests				Confirmatory tests						
	KCT	KCT/NP	KCT/mNP	KCT/mNOR	NSS-PNP	KCT-PNP	NSS/PNP	KCT/PNP	Nor ratio	LR ratio	Per cor ratio
Sensitivity (%)											
Mean + 2SD	100	78.9	100	100	84.2	84.2	65.8	63.2	60.5	47.4	23.7
97.5 percentile	100	84.2	100	100	89.5	84.2	52.6	63.2	60.5	44.7	44.7
99.0 percentile	100	84.2	100	100	89.5	84.2	52.6	63.2	60.5	44.7	44.7
Specificity (%)											
Mean + 2SD	100	100	100	100	96	90	94	92	92	98	98
97.5 percentile	100	100	100	100	92	90	96	94	94	98	98
99.0 percentile	100	100	100	100	92	90	96	94	94	98	98

KCT, Kaolin Clotting Time; NP, Normal plasma; mNP, mean Normal plasma; mNor, mean Normal subject; NSS, Normal saline solution;

PNP, Platelet neutralization procedure; Nor ratio, Normalized ratio; LR ratio, Lupus ratio; Per cor ratio, Percentage correction of ratio

Table 3 Frequencies of abnormal KCT tests on samples with the various levels of factor VIII in different calculation methods with various cut-off values

	Screening tests				Confirmatory tests						
	KCT	**KCT/NP**	**KCT/mNP**	**KCT/mNOR**	**NSS-PNP**	**KCT-PNP**	**NSS/PNP**	**KCT/PNP**	**Nor ratio**	**LR ratio**	**Per cor ratio**
Factor VIII 0-10%											
Mean + 2SD	1/11	1/11	1/11	1/11	10/11	8/11	3/11	3/11	3/11	3/11	0/11
97.5 percentile	1/11	1/11	1/11	1/11	10/11	9/11	0/11	2/11	3/11	3/11	3/11
99.0 percentile	1/11	1/11	1/11	1/11	10/11	9/11	0/11	2/11	3/11	3/11	3/11
Factor VIII 11-15%											
Mean + 2SD	4/12	3/12	4/12	4/12	1/7	1/7	0/7	0/7	0/7	1/7	0/7
97.5 percentile	4/12	3/12	4/12	4/12	2/7	1/7	0/7	0/7	0/7	1/7	0/7
99.0 percentile	4/12	3/12	4/12	4/12	2/7	1/7	0/7	0/7	0/7	1/7	0/7
Factor VIII >50%											
Mean + 2SD	3/13	2/13	3/13	3/13	2/3	2/3	1/3	1/3	1/3	2/3	0/3
97.5 percentile	3/13	2/13	3/13	3/13	3/3	2/3	0/3	1/3	1/3	2/3	1/3
99.0 percentile	3/13	3/13	3/13	3/13	3/3	2/3	0/3	1/3	1/3	2/3	1/3

KCT, Kaolin Clotting Time; NP, Normal plasma; mNP, mean Normal plasma; mNor, mean Normal subject; NSS, Normal saline solution;

PNP, Platelet neutralization procedure; Nor ratio, Normalized ratio; LR ratio, Lupus ratio; Per cor ratio, Percentage correction of ratio

of phospholipid in KCT makes it more sensitive to LA and increases the ability to detect low titer of the inhibitors. Lo et al reported that KCT was the only test that demonstrated LA in all dilutions (1:1, 1:3, 1:6, and 1:9) of patient plasmas.[43] This finding suggested that KCT was the most sensitive test for LA detection. The KCT had an effectiveness to detect LA similarly to dRVVT[44,45] and the combination of them showed a higher positivity rate in plasma samples than the single test.[27] There were some variations in the diagnosis of LA between KCT and APTT due to the difference of reagents and test system used.[44,45] In the addition, the results obtained from KCT revealed the significant correlation with silica clotting time (SCT).[46]

In this study, we evaluated the calculation methods of KCT tests to find the suitable way to indicate the presence of LA in plasma samples. Our finding demonstrated that for screening tests all calculating methods and cut-off values (except KCT/NP) had 100% sensitivity and specificity. However, there were some discrepancies among the results for confirmatory tests. The NSS-PNP and KCT-PNP showed high sensitivity (84.2-89.5%) and specificity (90-96%). The calculation of ratios between clotting times (NSS/PNP and KCT/PNP) revealed low sensitivity (52.6-65.8%) although the specificity was favorable (92-96%). When normal plasma was introduced into the test in order to detect the weak positivity, the sensitivity of the normalized ratio, the LR and the % correction ratio were low (23.7-60.5%). On the other hand, their specificities were very high (92- 98%). If the mixture of test and normal plasma was used to exclude deficiency of coagulation factors, the sensitivity of ICA was low (50%) but the specificity was high (94%). The influence of cut-off value to the result was demonstrated by decreased positivity rate of the clotting time when using mean+2SD as cut-off value compared to 97.5 or 99.0 percentile.

The frequencies of abnormal results on plasma with various levels of factor VIII were also varied. All calculation methods of screening tests and two of confirmatory tests (NSS-PNP and KCT-PNP) showed high abnormal results in samples with the factor VIII level < 10%. Other methods of confirmatory tests presented a few positive results on test plasmas. The samples with factor FVIII level > 10% gave rather low positivity rates with all calculation methods. Since the ICA was performed on the mixture 1:1 of patient plasma and NP, it showed the correction of prolonged clotting times on factor deficient plasmas. The cut-off values seemed to have no effect to the results.

The discrepancies of the results were also reported by several studies. The problem of interpreting results was caused by various equations and calculations recommended by some studies or manufacturers. Gardiner[25] reported that if using mean+2SD as cut-off, the percentage correction of ratio of dRVVT test gave sensitivity slightly lower than using the British Committee for Standards Haematology (BCSH)'s recommended cut-off value for Unicorn Diagnostic, Manchester UK, and American Diagnostica, whereas the specificity was higher. The percentage correction of clotting time expressed very poor sensitivity despite good specificity for these kits. Even the test/confirm ratio gave higher sensitivity for the Unicorn and the American Diagnostic kits but it was much lower for the Manchester kit. However, the ratio gave high specificity for all test kits. This study suggested that the ways in which the screen and confirmation data analyzed had a greater impact on the interpretation of the results than the choice of test kit.

Another study[47] also found that the difference in calculating data and cut-off gave rise to the difference in sensitivity. The report showed that the sensitivity of dAPTT was very low with 97.5 percentile cut-off. If a sensitivity of (near) 100% should have been achieved, more than 50% of the tests on the fifth international survey of lupus anticoagulants (ISLA-5) cut-off would have been false-positive. When the results of test/NP ratio were compared to the upper limit of the hospital, the sensitivity was high. If using an upper reference limit of 1.1 as recommended by commercial assays, it would be very good. The sensitivities of the confirmed methods which obtained from the difference between

clotting times of test samples with low and high PL content (test-confirm) or ratio between the clotting times (test/confirm ratio) were rather low. The percentage correction of the ratio and the percentage correction of clotting time gave lower sensitivity compared to the calculation of an LR. The study concluded that the variation in sensitivity and specificity between various LA tests may be due to not only to differences in reagents, phospholipid concentrations, and instrumentation, but also to the way of the data are analyzed.

In conclusion, the methods of choice should be KCT or KCT/mNP or KCT/mNOR and NSS-PNP for screening and confirmatory tests, respectively, with either 97.5 or 99.0 percentile cut-off. The test panel should consist of screening, mixing, and confirmatory steps. This study confirmed that calculation method and cut-off value played an important role for the variation of sensitivity and specificity of LA testing. Therefore, the selection of the appropriate method should be made by careful consideration and could not be generalized to all test systems.

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การประเมินการตรวจ Kaolin Clotting Time สำหรับการวินิจฉัย Lupus Anticoagulants โดยใช้วิธีคำนวณที่แตกต่างกัน

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บทคัดย่อ

ที่มา *Lupus anticoagulants (LA) เป็นกลุ่มของ antibodies ที่ทำให้การทดสอบการแข็งตัวของเลือดที่อาศัย phospholipid ยาวผิดปกติ LA มีความสัมพันธ์กับอาการทางคลินิกหลากหลาย เช่น การเกิดลิ่มเลือดอุดตันและการแท้งลูกซ้ำซ้อนที่เกิดขึ้นเอง* ***วัตถุประสงค์*** *ในการศึกษาครั้งนี้ได้ประเมินผลของ kaolin clotting time (KCT) ที่ได้จากวิธีคำนวณและค่า cut-off ต่างๆ หลายแบบเพื่อจะหาแนวทางที่เหมาะสมในการตรวจวินิจฉัย LA* ***วิธีการ*** *ทำการทดลองในพลาสมาที่เป็น LA positive 38 รายและเป็น LA negative 50 ราย* ***ผลการศึกษา*** *สำหรับ screening test พบว่าวิธีคำนวณที่ให้ทั้งความไวและความจำเพาะร้อยละ 100 คือ KCT และอัตราส่วนของ KCT ของสิ่งส่งตรวจกับ mean KCT ของ normal pooled plasma หรือกับ mean KCT ของคนปกติ ส่วน confirmatory test ความไวของวิธีคำนวณต่างๆ มีความแตกต่างกันมาก (23.7-89.5%) ในขณะที่มีความจำเพาะสูง (90-98%) มีความแปรปรวนของผลการทดลองเมื่อใช้ mean+2SD เป็น cut-off แต่ไม่มีกับ percentile การหาความแตกต่างระหว่าง normal saline ที่เป็นตัวควบคุมกับ platelet neutralization procedure (NSS-PNP) เป็นวิธีคำนวณที่ดีที่สุดสำหรับ confirmatory test* ***สรุป*** *การเลือกวิธีคำนวณผลการทดลองที่เหมาะสม ควรจะพิจารณาด้วยความระมัดระวังโดยควรคำนึงถึงความไวและความจำเพาะของ test รวมทั้งค่า cut-off ที่จะใช้ด้วย*

Keywords : ● *Kaolin clotting time* ● *Lupus anticoagulants*

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