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Evaluation of Activated Partial Thromboplastin Time Mixing Studies Using Several Methods

Chaonan Liu, MD; Liqin Ling, MD; Xunbei Huang, MD; Jian Mi, MD; Juan Liao, MD; Jin Jia, MD; Xia Wang, MD; Jing Zhou, MD

**Context.**—The prolonged activated partial thromboplastin time (APTT), a vital screening test for coagulation, can be due to deficiencies in coagulation factors and the existence of factor inhibitors or antiphospholipid antibodies. APTT mixing studies are being optimized to help find the cause.

**Objective.**—To optimize APTT mixing studies, we evaluated existing standards and explored when and how to combine 1:1 and 4:1 mixing.

**Design.**—Patients with a prolonged APTT but otherwise normal prothrombin time and thrombin time were enrolled in our hospital from January 1, 2018, to December 31, 2019. All samples were subjected to 1:1 mixing studies, while 134 were subjected to 4:1.

**Results.**—A total of 251 samples were involved, including 116 with factor deficiencies, 75 with FVIII inhibitors, and 60 with antiphospholipid antibodies. A Rosner index less than 11% or an extended incubation time of more than 3 seconds was better than other existing standards in differentiating factor deficiencies from inhibitors and in differentiating time-dependent inhibitors from time-independent inhibitors, but the approach presented here improves upon those. For the best diagnostic accuracy, samples with a Rosner index between 5.0% and 9.1% need a 4:1 mixing study, while others need 1:1. A combination of Rosner index and percent-extended incubation time-P seemed to offer objective and effective criteria for interpreting the results.

**Conclusions.**—APTT mixing studies had overall good sensitivity and specificity in differentiating factor deficiencies from inhibitors, or time-dependent from time-independent inhibitors. The combination of 1:1 and 4:1 mixing studies can improve the diagnostic ability compared with 1:1 alone.


Activated partial thromboplastin time (APTT) is a vital screening test for any disease related to abnormal hemostasis or thrombosis. A prolonged APTT usually results from deficiency of coagulation factors, the existence of coagulation factor inhibitors, or the presence of antiphospholipid antibodies (APLs). Clinical treatments can be extremely different because of these different causes. However, not every hospital has a laboratory that can run all the tests for coagulation factors, factor inhibitors, and APLs. This can cause a delayed or even a wrong diagnosis and treatment, which could be life-threatening in severe cases.

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Fortunately, APTT mixing studies have been carried out to explore the cause of a prolonged APTT. Briefly, patient plasma is mixed with a normal plasma pool (NPP) (usually at a 1:1 ratio) to see if the prolonged APTT is corrected. Correction of the APTT by addition of NPP means the coagulation factors in the patient’s plasma are either low or absent. In contrast, failure of the APTT to correct by addition of NPP indicates the presence of factor inhibitors or APLs in the patient’s plasma. Moreover, prolongation of the APTT after incubation of the mixed plasma at 37°C for 1 to 2 hours indicates the presence of an inhibitor of coagulation Factor VIII (FVIII) rather than other factor inhibitors or APLs. This is because FVIII inhibitors are usually time dependent while the others are usually time independent.

Nevertheless, APTT mixing studies are not widely used because there is no general consensus about how to define “a corrected APTT result” and how much longer “a longer APTT” is when used to differentiate time-dependent and time-independent inhibitors. Second, some studies have demonstrated that a patient plasma:NPP ratio of 4:1 might be better than a patient plasma:NPP ratio of 1:1 in detecting inhibitors because it gives a better sensitivity in screening out factor inhibitors or APLs, but it is still not clear when and how a 4:1 mixing study should be applied. To solve the problems mentioned above, our study evaluated each existing standard and explored when and how to combine the 1:1 and 4:1 mixing studies.
MATERIALS AND METHODS

Samples

Patients, who had a prolonged APTT, enrolled in our hospital from January 1, 2018, to December 31, 2019. The inclusion criteria were as follows: (1) patients with a prolonged APTT, and (2) patients with normal prothrombin time and thrombin time. The exclusion criteria were (1) abnormal liver function, (2) anticoagulant medication (including APLs undergoing treatment), and (3) with any other abnormal coagulation tests (such as prothrombin time and/or thrombin time). Samples were collected into vacutainer tubes (BD Vacutainer; Becton Dickinson and Company) with 3.2% sodium citrate. Citrated whole blood was centrifuged at 1500 g for 15 minutes to obtain platelet-poor plasma with a platelet count of less than $10 \times 10^9/\mu L$. All tests of each sample were completed within 1 month. NPP was from at least 20 healthy individuals. This study was approved by the Ethics Committee of Sichuan University (Chengdu, China).

Reagents and Analyzers

A SYSMEX CS-5100 coagulation analyzer (Sysmex) was used to test APTT, dilute Russell viper venom time, and coagulation factors. The following reagents were used: (1) for APTT, APTT reagent (Siemens Healthcare Diagnostics, Marburg, Germany); (2) for factor-deficient plasma, factors VIII, IX, XI, and XII (Siemens Healthcare Diagnostics); and (3) for dilute Russell viper venom time, Lupus Anticoagulant Screen and Confirm Kit (Siemens Healthcare Diagnostics).

A YHLO iFlash 3000-A chemiluminescence analyzer (Shenzhen YHLO Biotech Co., Ltd.) was used to test the anti-cardiolipin antibody (ACA; YHLO) and anti-β2-glycoprotein I antibody (anti-β2GP1) (Shenzhen YHLO Biotech Co., Ltd.). Both kits were used for detecting total antibodies, including IgM, IgG, and IgA.

Outcomes

All samples were screened for coagulation factors, factor inhibitors, and APLs (LA, ACA, and anti-β2GP1). Patients with FVIII, FIX, FXI, or FXII less than 40% were considered to have coagulation-factor deficiency. Patients with a FVIII inhibitor greater than 0.6 Bethesda units/mL by Bethesda assay were diagnosed with a FVIII inhibitor. Patients with a positive APL were identified as having APL (when the lupus anticoagulant test was above 1.2, when ACA was more than 24 U/mL, or when anti-β2GP1 was more than 24 U/mL).

Interpreting APTT Mixing Studies and Results

Patient’s plasma was A, and NPP is B. A 1:1 mixing study of A and B is C, while a 4:1 mixing study of A and B is D. Then incubated at 37°C for 2 hours before being tested again (A’, B’, C’, D’). There are 2 more special mixing samples tested for activated partial thromboplastin time (APTT): a 1:1 mixing study of A’ and B’ is E, and a 4:1 mixing study of A’ and B’ was F. We compared the results between “mixing and then incubation samples” (C’ and D’) and “incubation and then mixing samples” (E and F), which should address the incubation problem mentioned above. In total, 10 APTT results were obtained for 1 patient (A, B, C, D, A’, B’, C’, D’, E, and F) (Figure 1).

Factor deficiency was identified as follows if:

1. The result of C was within the normal reference interval (24.8–33.8 seconds); or
2. The extended time, the 1:1 mixing study or the 4:1 mixing study was calculated as (C – B) seconds or (D – B) seconds, and (C – B) was less than 5 seconds; or
3. The Rosner index, the 1:1 mixing study or the 4:1 mixing study was calculated as either (C’ – B’)/A × 100% or (D’ – B’)/A × 100%, and the value of (C’ – B’)/A × 100% was more than 11% (or 15%); or
4. The percent-extended time, the 1:1 mixing study or the 4:1 mixing study was calculated as either (C’ – B’)/B × 100% or (D’ – B’)/B × 100%, and the value of (C’ – B’)/B × 100% was more than 15%; or
5. The percent correction of the 1:1 mixing study, calculated as (A’ – C’)/(A’ – B’) × 100%, was more than 70% (while a value less than 58% suggested an inhibitor); or
6. The percent correction of the 4:1 mixing study, calculated as (A’ – D’)/(A’ – B’) × 100%, was more than 50%; or
7. The incubated percent correction of in the 4:1 mixing study, calculated as (A’ – D’)/(A’ – B’) × 100%, was more than 10%.

Inhibitors were considered when the patient was excluded from having factor deficiency, and when the extended incubation time was more than 3, 7, 14, 15 or 10 seconds, the inhibitors were defined as time-dependent inhibitors.

For the extended incubation time, the 1:1 or 4:1 mixing study was calculated as (C’ – E) seconds or (D’ – F) seconds. For the percent-extended incubation time-P, the 1:1 or 4:1 mixing study was calculated as either (C’ – E)/A × 100% or (D’ – F)/B × 100%. For the percent of the extended incubation time-N, the 1:1 or 4:1 mixing study was calculated as either (C’ – E)/B’ × 100% or (D’ – F)/B’ × 100%. For the percent of the extended incubation time, the 1:1 or 4:1 mixing study was calculated as either (C’ – E)/E × 100% or (D’ – F)/F × 100%.
Abbreviations: NPV, negative predictive value; NRI, normal reference interval; PPV, positive predictive value.

Considering the area under the receiver operating characteristic (ROC) curve (AUC) is a global measure to evaluate the diagnostic abilities of a test, and Youden Index, which is the point nearest to the top-left most corner of the ROC curve, represents the optimal compromise between sensitivity and specificity.18 ROC curves and abilities of a test, and Youden Index, which is the point nearest to (ROC) curve (AUC) is a global measure to evaluate the diagnostic negative.20–22

Table 1. The Diagnostic Ability for Each Existing Standard

<table>
<thead>
<tr>
<th>Evaluation Indices</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1 mixing study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentate factors deficiency from inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosner index &lt;11% for factor deficiency</td>
<td>100</td>
<td>47.4</td>
<td>62.0</td>
<td>100</td>
<td>71.7</td>
</tr>
<tr>
<td>Rosner index &lt;15% for factor deficiency</td>
<td>100</td>
<td>25.2</td>
<td>53.5</td>
<td>100</td>
<td>59.8</td>
</tr>
<tr>
<td>Percent correction &gt;70% for factor deficiency</td>
<td>54.3</td>
<td>26.7</td>
<td>58.3</td>
<td>90.0</td>
<td>39.5</td>
</tr>
<tr>
<td>Extended time &lt;5 s for factor deficiency</td>
<td>96.6</td>
<td>40.0</td>
<td>58.0</td>
<td>93.1</td>
<td>66.2</td>
</tr>
<tr>
<td>Percent-extended time &lt;15% for factor deficiency</td>
<td>94.8</td>
<td>56.3</td>
<td>65.1</td>
<td>92.7</td>
<td>74.1</td>
</tr>
<tr>
<td>Within NRI for factor deficiency</td>
<td>95.7</td>
<td>34.1</td>
<td>55.5</td>
<td>90.2</td>
<td>62.6</td>
</tr>
<tr>
<td>Differentate time-dependent from time-independent inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extended incubation time &gt;3 s for time-dependent inhibitor</td>
<td>90.7</td>
<td>70.0</td>
<td>79.1</td>
<td>85.7</td>
<td>81.5</td>
</tr>
<tr>
<td>Extended incubation time &gt;7 s for time-dependent inhibitor</td>
<td>44.0</td>
<td>96.7</td>
<td>94.3</td>
<td>58.0</td>
<td>67.4</td>
</tr>
<tr>
<td>Extended incubation time &gt;10 s for time-dependent inhibitor</td>
<td>17.3</td>
<td>96.7</td>
<td>86.7</td>
<td>48.3</td>
<td>52.6</td>
</tr>
<tr>
<td>4:1 mixing study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentate factors deficiency from inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent correction &gt;50% for factor deficiency</td>
<td>16.7</td>
<td>97.8</td>
<td>93.3</td>
<td>39.1</td>
<td>45.4</td>
</tr>
<tr>
<td>Incubated percent correction &gt;10% for factor deficiency</td>
<td>82.1</td>
<td>71.7</td>
<td>84.1</td>
<td>68.8</td>
<td>78.5</td>
</tr>
</tbody>
</table>

Data Analysis

Considering the area under the receiver operating characteristic (ROC) curve (AUC) is a global measure to evaluate the diagnostic abilities of a test, and Youden Index, which is the point nearest to the top-left most corner of the ROC curve, represents the optimal compromise between sensitivity and specificity.18 ROC curves and Youden indices were used to identify the optimal cutoff value for distinguishing the “factor deficiency” from the “inhibitor” or the “time-independent inhibitor” from the “time-dependent inhibitor.” A larger AUC indicates better discrimination. Youden Index is the point that corresponds to the maximum value of (specificity + sensitivity – 1).19–22 The AUC, accuracy, sensitivity, specificity, negative predictive value, and positive predictive value were calculated to evaluate each standard. Accuracy is calculated as follows: sensitivity × prevalence + specificity × (1 – prevalence). The sensitivity is defined as the proportion of people with disease who will have a positive result. The specificity is the proportion of people without disease who will have a negative result.5 Positive predictive value assesses how well the standard predicts patients who actually have disease. The numerator gives the number of those who are actually positive, while the denominator gives the number of patients classified by the standard as being at positive. Negative predictive value assesses how well the standard predicts patients who do not have the disease. The numerator gives the number of those who are actually negative, while the denominator gives the number of patients classified by the standard as being at negative.20–22

All statistical analyses were performed using SPSS 19.0 (IBM). In all analyses, *P < .05* was considered significant.

RESULTS

A total of 251 samples were involved in our study. There were 116 patients with coagulation factor deficiency, including 97 patients with mild deficiency, 9 with moderate deficiency, and 10 with severe deficiency. There were 75 patients with FVIII inhibitors, including 66 with a low titer and 9 with a high titer. A total of 60 patients had APLs, including 50 patients who were only LA-positive, 4 who were only ACA-positive, and 6 who were LA-positive, ACA-positive, and anti--¿2GP1-positive. Detailed information on the samples is shown in the Supplemental Figure 1 and Supplemental Table (see Supplemental Digital Content).

All the samples were subjected to 1:1 APTT mixing studies, while 134 of them were subjected to 4:1 APTT mixing studies because the others were insufficient for 4:1 mixing studies. A total of 88 had factor deficiencies, 22 had FVIII inhibitors and 24 had APLs.

Identifying Coagulation Factor Deficiency

The 1:1 APTT mixing study.—The Rosner index, extended time, and percent-extended time of patients with factor deficiency were lower than those with an inhibitor. However, the percent correction and incubated percent correction were higher than those with an inhibitor.

We evaluated each existing standard as indicators of factor deficiency. A Rosner index less than 11% and percent-extended time less than 15% were better than the others. For a Rosner index less than 11%, the sensitivity and negative predictive value were up to 100%, accuracy, specificity, and positive predictive value were 71.7%, 47.4%, and 62.0%, respectively (Table 1). For the percent-extended time less than 15%, the sensitivity and negative predictive value were 94.8% and 92.7%, and the accuracy, specificity, and positive predictive value were 74.1%, 56.3%, and 65.1%, respectively (Table 1).

The cutoff value in our laboratory of each existing standard was calculated by ROC curve analysis (Table 2 and Supplemental Figure 2). The AUC values of the Rosner index, extended time, percent-extended time, and incubated percent correction were similar at 0.82, 0.85, 0.85, and 0.85, respectively. They are better than the percent correction with an AUC of 0.67. The best cutoffs of the Rosner index, extended time, percent-extended time, and incubated percent correction were 9.1%, 3.3 seconds, 11.5%, and 53.8%, respectively. The sensitivity and specificity of each standard for factor deficiency were 62.5% to 96.6% and 51.9% to 95.7%, respectively. It is obviously better to use the appropriate cutoff value by the laboratory itself than the established cutoff value.
The 4:1 APTT mixing study.—If inhibitors were at low levels, a 1:1 mixing study would give a false “corrected” value and thus an incorrect diagnosis of coagulation-factor deficiency. The 1:1 dilution by NPP will dilute factor inhibitors or APLs, which will not be enough to cause a prolonged APTT. Therefore, Chang et al\textsuperscript{10} conducted a 4:1 mixing study to improve the detection sensitivity of factor inhibitors or APLs, as only 1-part NPP was diluted by 20% with 4 parts of the patient’s plasma, which was confirmed in our data (Table 1). On the other hand, the 4:1 mixing study was not sensitive in detecting coagulation-factor deficiency, especially when the coagulation factor activity was lower than 25% (the sensitivity of factor deficiency detected by our laboratory was approximately 40%), because 1-part NPP is not enough to fully complement the APTT. Therefore, we recommend only a 4:1 mixing study in patients with an inhibitor and a factor activity above 25% and to calculate the cutoff value of the 4:1 mixing study by ROC curve analysis.

The sensitivity of almost all standards in detecting an inhibitor increased (Rosner index from 63.0%–95.7%, percent correction from 51.9%–87.4%, extended time from 77.8%–91.3%), and percent-extended time from 83.0%–96.4%), while the specificity hardly changed (Table 2).
Combining the 1:1 and 4:1 APTT mixing studies.—As mentioned above, the 1:1 mixing study is better than the 4:1 mixing study in detecting coagulation factor deficiency, while the 4:1 mixing study is better than the 1:1 mixing study in detecting factor inhibitors or APLs. Therefore, using a combination of the 1:1 and 4:1 is a better choice than using only 1 of the mixing studies alone.

Because the diagnostic accuracy of percent correction is poor in our laboratory, incubated percent correction needs the results after incubation, and the diagnostic ability is not significantly improved compared with others. We explored how to combine the 1:1 and 4:1 mixing studies in the Rosner index, extended time, and percent-extended time.

The specificity of diagnostic factor deficiency or inhibitor reached 90% in the 1:1 mixing study by ROC curve analysis and was selected as the boundary to decide which mixing ratio to use for a sample (Table 3). In addition, 9.1%, as the best cutoff value in the ROC curve of the 1:1 mixing study, was selected as the cutoff to diagnose the inhibitors.

The samples with a Rosner index of 5.0% to 9.1%, extended time of 2.3 to 3.6 seconds, or percent-extended time of 7.9% to 12.8% in a 1:1 mixing study were selected for a 4:1 mixing study. For this, the evaluation criteria of the 4:1 mixing study were used (Table 2). The sensitivity of diagnostic inhibitors by combining the 1:1 and 4:1 mixing studies was increased from 62.2% to 87.4%, 83.0% to 86.6%, and 73.3% to 86.4% for the Rosner index, percent-extended time and extended time, respectively, compared with those that used only the 1:1 mixing study (Table 4).

**Table 3. The 4:1 Mixing Studies Criteria of Rosner Index, Extended Time, and Percent-Extended Time**

<table>
<thead>
<tr>
<th>Samples Needed</th>
<th>Rosner index (%)</th>
<th>Cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Extended time, s</th>
<th>Cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Percent-extended time, %</th>
<th>Cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosner index, %</td>
<td>5.0</td>
<td>26.7</td>
<td>90.4</td>
<td></td>
<td>9.1</td>
<td>63.0</td>
<td>96.6</td>
<td></td>
<td>5.0–9.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extended time, s</td>
<td>2.3</td>
<td>34.5</td>
<td>90.4</td>
<td></td>
<td>3.6</td>
<td>71.1</td>
<td>90.5</td>
<td></td>
<td>2.3–3.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent-extended time, %</td>
<td>7.9</td>
<td>33.6</td>
<td>90.4</td>
<td></td>
<td>12.8</td>
<td>71.1</td>
<td>90.5</td>
<td></td>
<td>7.9–12.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4. The Diagnostic Ability of 1:1 Mixing Study Only and Combination of 1:1 and 4:1**

<table>
<thead>
<tr>
<th>Evaluation Indices</th>
<th>1:1 Mixing Only</th>
<th>Combination of 1:1 and 4:1 Mixing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity (%)</td>
<td>Specificity (%)</td>
</tr>
<tr>
<td>Differentiate factors deficiency from inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosner index</td>
<td>96.6</td>
<td>62.2</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>62.2</td>
<td>96.6</td>
</tr>
<tr>
<td>Percent-extended time</td>
<td>84.5</td>
<td>83.0</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>83.0</td>
<td>84.5</td>
</tr>
<tr>
<td>Extended time</td>
<td>87.1</td>
<td>73.3</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>73.3</td>
<td>87.1</td>
</tr>
</tbody>
</table>

| Differentiate time-dependent from time-independent inhibitors | | | | | | | | | | |
| Percent-extended incubation time-P | 95.8 | 100.0 | 100.0 | 95.7 | 97.8 | 95.8 | 100.0 | 100.0 | 95.7 | 97.8 |
| Time independent | 100.0 | 95.8 | 95.7 | 100.0 | 97.8 | 100.0 | 95.8 | 95.7 | 100.0 | 97.8 |
| Percent-extended incubation time-N | 95.8 | 100.0 | 100.0 | 95.7 | 97.8 | 95.8 | 100.0 | 100.0 | 95.7 | 97.8 |
| Time independent | 100.0 | 95.8 | 95.7 | 100.0 | 97.8 | 100.0 | 95.8 | 95.7 | 100.0 | 97.8 |
| Percent-extended incubation time | 90.0 | 84.0 | 81.8 | 91.3 | 86.7 | 87.8 | 84.4 | 81.1 | 90.0 | 85.9 |
| Time independent | 84.0 | 90.0 | 91.3 | 81.8 | 86.7 | 84.4 | 87.8 | 90.0 | 81.1 | 85.9 |
| Extended incubation time | 78.3 | 88.0 | 83.9 | 83.5 | 83.7 | 77.4 | 87.8 | 82.0 | 84.4 | 83.6 |
| Time independent | 88.0 | 78.3 | 83.5 | 83.9 | 83.7 | 87.8 | 77.4 | 84.4 | 82.0 | 83.6 |

**Abbreviations:** NPV, negative predictive value; PPV, positive predictive value.

*Extended incubation time, percent-extended incubation time-P, percent-extended incubation time-N, and percent-extended incubation time, that need to be tested using 4:1 mixing, were determined by extended time, Rosner index, and percent-extended time.
extended incubation time were evaluated in our laboratory. The diagnostic accuracy of 3 seconds was better than that of the other 2 times in our laboratory (Table 1).

The cutoff values of the four standards (extended incubation time, percent-extended incubation time-P, percent-extended incubation time-N, and percent-extended incubation time) in our laboratory were calculated by ROC analysis (Table 2 and Supplemental Figure 2). The AUC of percent-extended incubation time-P (0.96, 95% CI 0.88–1.00), percent-extended incubation time-N (0.96, 95% CI 0.88–1.00), and percent-extended incubation time (0.91, 95% CI 0.85–0.96) were better than those of extended incubation time (0.89, 95% CI 0.83–0.95). The best cutoffs of the percent-extended incubation time-P, percent-extended incubation time-N, percent-extended incubation time, and extended incubation time were 10.8%, 21.3%, 11.0%, and 3.4 seconds, respectively. The sensitivity and specificity of each standard for time-dependent inhibitors were 84.0% to 100.0% and 78.3% to 95.8%, respectively. These percentages are better than simply using them in the extended time.

The 4:1 mixing APTT studies with an incubation time.—The mixing ratio of the incubation was the same as that before incubation to ensure the continuity of the experiment. The diagnostic ability of 1:1 and 4:1 was similar in determining whether the inhibitor was time dependent (Table 2).

**Integration of the APTT Mixing Studies**

We evaluated the 1:1 mixing study alone and in combination with the 4:1 mixing study (Table 4). The sensitivity of the Rosner index, percent-extended time, and extended-time diagnostic inhibitors was improved by combining the 1:1 and 4:1 mixing studies, while there was little change in specificity (<3.3%). Considering the applicability and operability, we suggest using the Rosner index and percent-extended incubation time-P for the evaluation (Figure 2).

In conclusion, we suggest correcting the testing procedure as follows: Rosner index of samples are obtained by 1:1 mixing study first; according to the value of Rosner index, the samples with a Rosner index less than 5.0% are defined as factor deficiency; the samples with a Rosner index between 5.0% and 9.1% are further tested by 4:1 mixing study. The samples with the Rosner index of the 4:1 mixing study less than 15.9% are defined as factor deficiencies, otherwise the presence of inhibitors is indicated. To identify the nature of the inhibitor, the percentages of the 4:1 mixing study were calculated as follows: Rosner index, the extended incubation time was evaluated in our laboratory. The diagnostic accuracy of 3 seconds was better than that of the other 2 times in our laboratory (Table 1).

**Figure 2.** The testing procedure for determining the cause of long activated partial thromboplastin time (APTT) by mixing study. Rosner index, the 1:1 mixing study, or the 4:1 mixing study was calculated as either $(C - B) / A \times 100$ (%) or $(D - B) / A \times 100$%. For the incubated percent-extended time-P, the 1:1 or 4:1 mixing study was calculated as either $(C' - E) / A' \times 100$% or $(D' - F) / A' \times 100$%.

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**Evaluation of APTT Mixing Studies**—Liu et al.
whether the inhibitors are time dependent, the samples are further incubated and tested by 4:1 mixing study. Samples with percent-extended incubation time-P less than 13.5% are defined as time-independent inhibitors, otherwise the presence of time-dependent inhibitors is indicated. The samples with the Rosner index of 1:1 mixing study greater than 9.1% are further incubated and retested by 1:1. The samples with the percent-extended incubation time-P less than 10.8% are defined as time-independent inhibitors, otherwise the presence of a time-dependent inhibitors are indicated.

**DISCUSSION**

APTT mixing studies are useful in helping to find the specific cause of a prolonged APTT. Correction of the APTT by addition of NPP means the coagulation factors in the patient’s plasma are either low or absent; otherwise, it means there are inhibitors present. Moreover, prolongation of the APTT after incubation of the mixed plasma at 37°C for 1 to 2 hours indicates the presence of an inhibitor of coagulation Factor VIII (FVIII) rather than other factor inhibitors or APLs.9 APTT mixing studies do not need extra reagents and instruments, and only the APTT test itself can help to identify the cause of a prolonged APTT; this means that there are many local small hospitals without a large laboratory that can conduct APTT mixing studies to make primary diagnosis. However, there is no consensus on when and how to apply APTT mixing studies, which limits their application.7,14

Most of the cutoff values of diagnostic standards were recommended based on a 1:1 mixing study.7,10–12 This is because 1-part NPP mixed with 1-part patient plasma can cause the coagulation factor activity in the mix to be more than 50%, which is enough for a “corrected” result.7 However, if there are factor inhibitors or APLs, which can also block coagulation factors from both the patient’s plasma and NPP, the APTT result cannot be “corrected.”9

The best sensitivity for detecting inhibitors was 56.3% when evaluated using existing standards. A total of 53.3% (72 of 135) of inhibitors were incorrectly classified into the factor deficiency group by having a Rosner index less than 11%, including 88.5% (23 of 26) of inhibitors with a weakly positive LA, 16.7% (3 of 18) of inhibitors with a moderately positive LA, 4 ACL-positive inhibitors, and 54.7% (41 of 75) of inhibitors with low titer for FVIII.

The AUC is a global measure to evaluate the diagnostic ability of a test. A larger AUC indicates better discrimination. Youden Index, which is the point nearest to the top-left most corner of the ROC curve, represents the optimal compromise between sensitivity and specificity. So, it is a commonly used approach when selecting a cutoff point.18

We selected the optimal cutoff (Youden Index) suitable for our laboratory by ROC. The sensitivity for detecting an inhibitor was improved after using a local cutoff (Rosner index, extended time, and percent-extended time are 63.0%, 77.8%, and 83.0%, respectively). A total of 37.8% (51 of 135) of inhibitors were incorrectly classified into the factor deficiency group by a Rosner index that was lower than 9.1%, including 73.1% (19 of 26) weakly positive LA, 16.7% (3 of 18) moderately positive LA, 4 ACL-positive, and 33.3% (25 of 75) low-titer FVIII inhibitors. The sensitivity and specificity are improved by local cutoff values, but it is still low. It may be that the inhibition was diluted at a 1:1 dilution ratio, resulting in reduced sensitivity. A 4:1 mixing study was used to improve the detection sensitivity of factor inhibitors or APLs.10,25–27 The sensitivity for detecting inhibitors was increased from 62.2% to 83.0% to 86.4% to 87.4% by using a combined 1:1 and 4:1 mixing study. Only 10.4% (14 of 135) of inhibitors were incorrectly classified into the factor-deficiency group by the Rosner index, including 38.5% (10 of 26) weakly positive LA, 50% (2 of 4) ACL-positive, and 2.7% (2 of 75) low-titer FVIII inhibitors. The combination of the 1:1 and 4:1 mixing studies can improve the sensitivity of factor inhibitors or APLs without significantly reducing the sensitivity of factor deficiency (<3.1%).

Moreover, approximately 85% of APLs are time-independent anticoagulants23 because they interact very quickly with phospholipids, and thus can immediately cause a prolonged APTT; incubation with APLs for more time will not make the prolonged APTT longer.28 In contrast, FVIII inhibitors are time dependent and temperature dependent, and incubation for 1 to 2 hours at 37°C can lead to a better interaction between the FVIII inhibitor and FVIII, which can make the prolonged APTT longer.25,29 Based on this theory, we incubated our mixed samples at 37°C for 2 hours to make them fully reflective of this and then performed the APTT test again to differentiate time-independent and time-dependent inhibitors.

However, incubation itself might cause a longer APTT because unstable coagulation factors (FV and FVIII) might be devitalized. Therefore, we compared the results between “mixing and then incubation samples” (C’ and D’) and “incubation and then mixing samples” (E and F), which should address the incubation problem mentioned above. The difference between the 2 samples was the extended time, which was considered the influence of time-dependent inhibitors. The local cutoff used in our laboratory was 3.4 seconds, which is close to 3 seconds,13 as reported in the literature. It is inappropriate to generalize local extended incubation time, because of the differences in instruments, reagents, and testing conditions among laboratories. We use the 3 percentage forms to reduce the impact of some of the differences as follows: the numerator is the extended incubation time, the denominator is the patient’s plasma after incubation (A), the NPP after incubation (B), or the mixing plasma after incubation (E or F). The extended incubation time-P, whose denominator is the patient plasma after incubation, showed better diagnostic accuracy and better continuity with the Rosner index. Therefore, the Rosner index and extended incubation time-P should be used to evaluate the results of mixing studies.

More importantly, in this study, we focused only on patients who had a prolonged APTT without any other abnormal coagulation tests, such as prothrombin time and thrombin time, as these patient should be very different from other types of patients and might need a totally different standard.13 In addition, different laboratories have different reagents, instruments, and NPPs, which will lead to different ways of interpreting the results of APTT mixing studies.13 We should be more careful when using extended times and extended incubation times.

Our study showed that the APTT mixing study had an overall good sensitivity and specificity for detecting factor deficiency and inhibitors, even time-dependent and time-independent inhibitors. The combination of the 1:1 and 4:1 mixing studies can increase diagnostic accuracy compared with the 1:1 mixing study alone. A combination of the Rosner index and percent-extended incubation time-P seemed to offer objective and effective criteria for evaluating the results.
References