Sensitivity, Specificity and Predictive Value of Modified Assays for Activated Protein C Resistance in Children

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Summary

Very little data is available assessing the clinical utility of coagulation-based APC resistance assays compared to DNA-based analysis for the factor V Leiden mutation in children. Therefore, the clinical utility of four aPTT-based assays for APC resistance was evaluated in 169 children, ages 3 months through 16 years. The prevalence of the Arg506 to Gln mutation was 7/169 (4.1%). Using cutoff points derived from the normal PCR-screened population (n = 162), two assays for APC resistance (APC-SR and n-APC-SR) gave poor concordance with the PCR assay (sensitivity 29% and 57%, respectively). Two modified assays (FDAPC-SR and n-FDAPC-SR), in which patient plasma was prediluted 1:5 in factor V deficient plasma, gave excellent concordance (sensitivity 100%). The predictive value of a positive test was 0.25, 0.44, 1.00 and 0.88 for the APC-SR, n-APC-SR, FDAPC-SR and n-FDAPC-SR, respectively. The FDAPC-SR and n-FDAPC-SR tests gave excellent discrimination using cutoff values derived from the total population (n = 169) without regard to previous PCR screening results.

Introduction

Resistance to activated protein C (APC) is caused by an amino acid substitution at the cleavage site of factor V (Arg506 to Gln, factor V Leiden), and is the most frequent inherited cause of thrombosis in adults (1-3). APC resistance has been recently recognized as a cause of thrombosis in infants and children, with manifestations including venous and arterial thrombosis, purpura fulminans, stroke, and osteonecrosis (4-9). The most specific method (“gold standard”) for identification of the factor V Leiden mutation in children. Therefore, the clinical utility of coagulation-based assays for APC resistance may not be as reliable in this population. In fact, a modified APC resistance assay using dilutions of patients' plasma in factor V deficient plasma has been recently recommended for newborn testing (18). There has been no study, however, assessing the utility of coagulation-based APC resistance assays compared to DNA-based analysis for the factor V Leiden mutation in children of various ages. Therefore, the purpose of this study was to evaluate the diagnostic sensitivity and accuracy of the plasma APC resistance assay in predicting the Arg506 to Gln, factor V Leiden mutation in children.

Materials and Methods

Subjects

Otherwise healthy children ages three months through 16 years (inclusive) who were admitted for elective same-day surgery at Children’s Hospital Medical Center were eligible for study. Children were clinically well with no serious underlying illnesses nor history of bleeding or thrombotic problems in the family. Informed consent was obtained from the parents of all children and the study was approved by the Review Board on Investigations Involving Human Beings of the Children’s Hospital Medical Center. A total of 180 children had blood obtained for the study, of which 169 had adequate samples obtained for all testing procedures. Race was specified in 148 of the 169 children, there being 126 (85%) white and 22 (15%) black children. At least 10 children at each year of age were selected. Blood was collected after routine venipuncture usually prior to anesthesia induction.

Blood Sampling and Plasma Preparation

Blood was collected in evacuated glass tubes containing 0.105 M sodium citrate (9 parts blood to 1 part anticoagulant) and centrifuged at 2000 g for 20 min to obtain platelet-poor plasma, which was frozen in aliquots and stored at -70° C until it was analyzed. For the normal plasma pool, an equal volume of plasma from each of forty adult controls (20 males, 20 females previously screened and found to be normal for the factor V Leiden mutation) was pooled, then separated into aliquots, snap frozen, and stored at -70° C until use.

Assays

The assays for anticoagulant response to APC were performed using two methods. The first was a commercial method using the Coatest for APC Resistance (Chromogenix AB, Mölndal, Sweden). Plasma was incubated with an
equal volume of the aPTT reagent for 5 min at 37 °C. Coagulation was triggered by the addition of CaCl₂ in the absence and presence of human APC and the time for clot formation was recorded. Clotting times were measured on a BBL Precision Coagulation Timer, Lancer Coagulyzer II (Sherwood Medical Industries, St. Louis, Missouri.) The results were expressed as follows: (1) APC-sensitivity ratio (APC-SR) expressed as the ratio of the clotting time with APC to the clotting time without APC; (2) normalized APC-sensitivity ratio (n-APC-SR) expressed as the ratio of the APC-SR of the patient sample to the APC-SR of the normal plasma pool.

The second method for assessing sensitivity to APC utilized a predilution of one volume sample plasma with four volumes of APC deficient plasma (V-DEF Plasma, Chromogenix AB, Mölndal, Sweden.) The testing and calculation of the factor V-deficient APC-SR (FDAPC-SR) on the factor V-deficient sample plasma mixture was then performed, as described in the first method. A normalized FDAPC-SR (n-FDAPC-SR) on the diluted patient sample was calculated as in the first method utilizing the normal plasma pool. The inter-assay coefficients of variation (CV%) calculated on the basis of the results of a commercial lyophilized normal plasma (Chromogenix AB, Mölndal, Sweden) were 7.6%, 4.1%, 6.4% and 6.4% for the APC-SR, n-APC-SR, FDAPC-SR and n-FDAPC-SR, respectively.

Genomic DNA and Factor Leiden Determination

Genomic DNA was prepared from peripheral-blood leucocytes (19). A 224 basepair fragment of the factor V gene was then amplified using the following primers:

5' Amplimer: ATG ATG CCC AGT GCT TAA CAA GAC CAT ACT
3' Amplimer: CTT GAA GGA AAT GCC CCA TTA

The 5' amplimer was designed from reported cDNA of exon 10 (20). The 3' amplimer was the cDNA to a 21 base pair fragment found in intron 10, as used by Koeleman et al. (21). The amplified fragment was then digested with Mnl-I restriction enzyme and the products loaded on a 2% Agarose gel. The following fragments were obtained:


**Statistical Methods**

To determine the sensitivity, specificity and predictive value of the tests for APC resistance, the cutoff for the lower limit of normal was defined in two ways. In one method of analysis this cutoff was set at \( \leq 2 \) SD from the mean utilizing the data from the 162 children with normal “wild-type” factor V. In a second method, the data was analyzed using a cutoff point which was determined as \( \leq 5 \)th percentile for the entire \( n = 169 \) data set. This analysis was performed to determine the utility of the APC resistance assays utilizing data from a large cohort of normals without the need to determine PCR analysis for each member of the cohort.

**Results**

Concordance between the Factor V Leiden Mutation and Clotting Assays for APC Resistance

One hundred sixty-nine children were analyzed by the DNA PCR-based test for the factor V Leiden mutation as well as by the four coagulation-based tests, the APC-SR, n-APC-SR, FDAPC-SR and n-FDAPC-SR. Seven children (6 white, 1 black) of a total of 169 children were heterozygous for the mutant factor V Leiden gene, with a prevalence for heterozygosity of 4.1%. There were no homozygotes detected. The 162 children with a normal factor V genotype had a mean APC-SR of 3.02 (SD 0.45), mean n-APC-SR of 1.01 (SD 0.16), mean FDAPC-SR of 2.35 (SD 0.22), and mean n-FDAPC-SR of 1.03 (SD 0.05). The distribution of the APC resistance ratios for the four tests in the normal (1691 GG) versus heterozygous (1691 AG) children is displayed in Fig 1.

Sensitivity of the Test

Using a cutoff value \( \leq 2 \) SD from the mean in the 162 children with normal factor V genotype, the sensitivity of the four tests for APC resistance is shown in Table 1. Similar results were obtained using a cutoff for APC resistance ratio \( \leq 5 \)th percentile derived from the full data set \( n = 169 \). In this case, an abnormal APC ratio was present in 3/7 and 4/7 heterozygous children for the APC-SR and n-APC-SR tests, respectively, while all 7 heterozygotes had resistance to APC by the FDAPC-SR and n-FDAPC-SR tests.

Specificity of the Test

Using a cutoff value \( \leq 2 \) SD from the mean in the 162 children with normal factor V genotype, the specificity of the tests (proportion of those with normal (1691 GG) factor V who have a normal ratio) is shown in Table 1. Using a cutoff value \( \leq 5 \)th percentile derived from the total cohort \( n = 169 \), the specificity was 96%, 97%, 99% and 98% for the four tests, respectively.
Table 1  Sensitivity and specificity of the coagulation-based tests for APC resistance in 169 children. The PCR test for the factor V Leiden mutation determined 162 children to be “wild-type” normal (1691 GG) and 7 children to be heterozygous (1691 AG). Cutoff for the APC ratio was ≤ 2 SD from the mean for n = 162 (normal) children

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cutoff for APC ratio (≤ 2 SD)</th>
<th>Factor V Leiden Heterozygous (%)</th>
<th>APC ratio ≤ 2 SD and Factor V Leiden Heterozygous (%)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC-SR</td>
<td>2.13</td>
<td>7</td>
<td>2</td>
<td>2/7 (29%)</td>
</tr>
<tr>
<td>n-APC-SR</td>
<td>0.70</td>
<td>7</td>
<td>4</td>
<td>4/7 (57%)</td>
</tr>
<tr>
<td>FDAPC-SR</td>
<td>1.91</td>
<td>7</td>
<td>7</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>n-FDAPC-SR</td>
<td>0.92</td>
<td>7</td>
<td>7</td>
<td>7/7 (100%)</td>
</tr>
</tbody>
</table>

Table 2  Predictive value of a positive and negative test for APC resistance in 169 children. The PCR test for the factor V Leiden mutation determined 162 children to “wild-type” normal (1691 GG) and 7 children to be heterozygous (1691 AG). Cutoff for the APC ratio was ≤ 2 SD from the mean for n = 162 (normal)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cutoff for normal APC ratio (≤ 2 SD)</th>
<th>Factor V Leiden (wild-type) (%)</th>
<th>Normal APC ratio and Factor V Leiden wild-type</th>
<th>Predictive value of positive test</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC-SR</td>
<td>2.13</td>
<td>162</td>
<td>156</td>
<td>0.25</td>
</tr>
<tr>
<td>n-APC-SR</td>
<td>0.70</td>
<td>162</td>
<td>157</td>
<td>0.44</td>
</tr>
<tr>
<td>FDAPC-SR</td>
<td>1.91</td>
<td>162</td>
<td>162</td>
<td>1.00</td>
</tr>
<tr>
<td>n-FDAPC-SR</td>
<td>0.92</td>
<td>162</td>
<td>161</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Predictive Value of a Positive Test

Using the cutoff value ≤ 2 SD in the 162 children with normal factor V genotype, the predictive value of a positive test is shown in Table 2. Using the cutoff value ≤ 5th percentile for the full data set (n = 169), the predictive value of a positive test was 0.33 for APC-SR, 0.44 for n-APC-SR, 0.78 for FDAPC-SR, and 0.70 for n-FDAPC-SR.

Predictive Value of a Negative Test

Using the cutoff value ≤ 2 SD in the 162 children with normal factor V, the predictive value of a negative test is shown in Table 2. The predictive value of a negative test was similar using the cutoff point ≤ 5th percentile for the full data set of 169 children: APC-SR 0.98, n-APC-SR 0.98, FDAPC-SR 1.00, n-FDAPC-SR 1.00.

Discussion

Screening tests in epidemiologic studies should be cost-effective instruments able to identify the highest possible proportion of target subjects, i.e. those with the mutant factor V Leiden who are positive by the test being studied (coagulation test). Tests for population screening should be able to include all true positives (sensitivity) while having the lowest possible false positive rate (specificity). The original APC resistance test and its modification using the “normalized ratio” showed poor sensitivity (29% and 57%, respectively) in this study of normal children. In contrast, excellent diagnostic sensitivity and specificity was obtained for the FDAPC-SR and n-FDAPC-SR assays. In this study all subjects were tested for the presence of the factor V Leiden mutation. If the genotypic analysis of a group of children is unknown, however, excellent discrimination can still be obtained using the ≤ 5% cutoff value, as shown by our analysis of the entire cohort of 169 children.

In conclusion, as shown in the current study, there are serious limitations of the original APC resistance test (10, 11), and its modification using the normalized ratio (12). In fact, these tests appear to have little clinical usefulness as a screening test for the factor V Leiden mutation in children. The modified APC resistance tests (14-16), which utilize the dilution of patient plasma with factor V deficient plasma, on the other hand, show excellent predictive value, probably making additional genotyping unnecessary, except as confirmation for those children with an abnormal n-FDAPCR.

References


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