Cut-off Levels Based on Deviation from Standard Negative Control is Better than Moderate Level Based on Fixed Cut-off for ACA Assessed Using ELISA for The Diagnosis of Antiphospholipid Syndrome

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ABSTRACT

Aim: to assess the consistency of the standard negative control of IgG and IgM ACA levels within runs and batches of tests, and levels of ACA agreement between those established according to deviation from standard negative control and those established based on a fixed level cut off.

Methods: serum samples of 148 patients who presented an INR <0.9 or prothrombin activity of >130% or aPTT below 0.8 times control or thrombosis with aPTT below 1.2 times control were tested in a 22-time running test to determine IgG and IgM ACA levels using Quanta Lite™ ACA IgG (HRP) and Quanta Lite™ ACA IgM (HRP) commercial reagents.

Results: coefficients of variant within runs and batches of standard negative control IgG and IgM ACA levels were 19.30% and 29.17% respectively. Using kappa statistics to determine degree of agreement between cut-off levels by deviation from standard negative control and fixed cut-off level of ACA identified using ELISA, the disagreement in IgM and IgG were k 0.30, and 95% CI of k 0.27 to 0.34 (z =1.033, p =0.3015), and k 0.63, and 95% CI of k 0.53 to 0.73 (z =1.411, p =0.1584) for cut-off levels based on deviations from standard negative control and fixed cut-off levels respectively. Cut-off levels based on deviation from standard negative control was more sensitive, with a 92% predictive true positive value, compared to a 69% predictive true positive value by fixed cut-off levels of IgM ACA detected using ELISA, and nearly equivalent to IgG ACA, with 84.4% and 87.1% predictive true positive values respectively.

Conclusion: cut-off points based on fixed levels of ACA detected using ELISA cannot be applied, because both IgG and IgM ACA levels of standard negative control were inconsistent among runs and batches. Cut-off points based on the deviation of 3 standard negative control levels for IgG ACA and based on deviations of 2.5 times from standard negative control levels for IgM ACA were better than cut off by fixed levels of ACA in producing true positive results.

Key words: anti-cardiolipin antibodies, ELISA, commercial reagent

INTRODUCTION

Activated protein C resistance (APC resistance) and persistent anti-cardiolipin antibody (ACA) and/or lupus anticoagulant (LA) are strongly associated with an increased risk of recurrences of thromboses.1 APC resistance defined as an insufficient prolongation clotting time of plasma, was first measured with a modified activated partial thromboplastin time (aPTT) test on the addition of exogenous APC.1 Anti-phospholipid antibody syndrome (APS) is defined by the presence of an antiphospholipid antibody (ACA and/or LA) and recurrent clinical events such as arterial or venous thromboses, or fetal loss.1,2 The thrombogenic activity of antiphospholipid antibodies may be led by endothelial perturbation and inactivate function of β2-GPI, Annexin V, APC, Protein S, and Thrombomodulin, as shown in Table 1.1,3-6

Antiphospholipid antibodies are defined as immunoglobulins that react with biological membranes whose main component is phospholipids.1 Types of antiphospholipid antibody develop when different antigens bind to exposed phospholipids epitopes. The prothrombin-dependent antiphospholipid antibody is generated by the binding of exposed phospholipid epitopes
and lupus inhibitor (LA). The β2-Glycoprotein I (β2-GPI)-dependent antiphospholipid antibody is generated by the binding of phospholipid epitopes and β2-GPI, while the annexin V-dependent antiphospholipid antibody is generated by the binding of exposed phospholipid epitopes and Annexin V. The many types of the antiphospholipid antibodies were found to be redirected by diphosphatidylglycerol (Cardiolipin) and can be detected using ELISA or radioimmunoassay (RIA).3

The results of semi-quantitative detection of ACA using ELISA should be expressed in GPL units for IgG of ACA and in MPL units for IgM of ACA. Based on an evaluation of 224 normal samples and 65 SLE patients, suggested fixed cut-off levels of 15 GPL and 12.5 MPL units were established. Harris et al. suggest an alternative semi-quantitative method of expressing results where 10 to 20 GPL or MPL units are considered low positive, 20 to 80 GPL or MPL units moderate positive, and above 80 GPL or MPL units high positive. Many authors report ACA levels detected using ELISA as continuous data to compare different clinical conditions. On the other hand, many other authors report various cut-off levels according to deviation from standard negative control of ACA detected using ELISA associated with several diseases, as shown on table 2.2

Therefore, we initiated a study to assess the consistency of the standard negative control of IgG and IgM ACA levels within runs and batches of tests, and levels of ACA agreement between those established according to deviation from standard negative control and those established based on a fixed level cut-off.

METHODS

Serum Sample

Serum samples from 148 patients presenting with an INR of <0.9 or prothrombin activity of >130% or

Table 1. Phospholipid-binding Protein Antigens as ACA Target

<table>
<thead>
<tr>
<th>Cells</th>
<th>Phospholipids</th>
<th>Cofactors (Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothel</td>
<td>Anionic PL:</td>
<td>Lupus inhibitor (LA)</td>
</tr>
<tr>
<td>Trophoblast</td>
<td>− Phosphatic acid</td>
<td>β2-Glycoprotein I</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>− Phosphatidylserin</td>
<td>Annexin V7</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>− Phosphatidylinositol</td>
<td>Protein C (PC)</td>
</tr>
<tr>
<td></td>
<td>− Phosphatidylglycerol</td>
<td>Protein S</td>
</tr>
<tr>
<td></td>
<td>− Diphosphatidylglycerol</td>
<td>Prothrombin</td>
</tr>
<tr>
<td></td>
<td>Neutral PL:</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td></td>
<td>− Phosphatidylcholine</td>
<td>Factor Xic</td>
</tr>
<tr>
<td></td>
<td>− Phosphatidylethanol</td>
<td>GM-CSF &amp; IL-3</td>
</tr>
<tr>
<td></td>
<td>amin</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Frequency of Positive Result Of ACA By ELISA On Different Subject by Various Cutoff Level by Deviation from Standard Negative Control2

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Subject</th>
<th>No. of patients</th>
<th>Cut-off level*</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manoussakis, et al</td>
<td>Healthy elderly</td>
<td>64</td>
<td>2</td>
<td>52</td>
</tr>
<tr>
<td>Meyer, et al</td>
<td>Syphilis</td>
<td>48</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>Malia, et al</td>
<td>Systemic sclerosis</td>
<td>28</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Frampton, et al</td>
<td>Guillain-Barre syndrome</td>
<td>92</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>Vaarala, et al</td>
<td>Acute infection</td>
<td>149</td>
<td>2.5</td>
<td>32</td>
</tr>
<tr>
<td>Manoussakis, et al</td>
<td>Healthy Blood donors</td>
<td>267</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Derkshen, et al</td>
<td>SLE</td>
<td>275</td>
<td>3</td>
<td>44</td>
</tr>
<tr>
<td>Ford, et al</td>
<td>Osteoarthritis</td>
<td>63</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Shergy, et al</td>
<td>Juvenile RA</td>
<td>12</td>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td>Keane, et al</td>
<td>Rheumatoid arthritis</td>
<td>99</td>
<td>2</td>
<td>49</td>
</tr>
<tr>
<td>Ford, et al</td>
<td>Rheumatoid arthritis</td>
<td>57</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Loizou, et al</td>
<td>Rheumatoid arthritis</td>
<td>47</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

* IgG and/or IgM in times to standard negative control level
aPTT below 0.8 times control or thrombosis with aPTT below 1.2 times control were tested in a 22-time running test to determine IgG and IgM ACA levels.

1. Materials
   Commercially available reagents by Quanta Lite™ ACA IgM/G (HRP) in every batch contained:
   • Cardiolipin-coated polystyrene microwell strips, 12 (1x8 wells) with holder
   • ACA HRP IgM/G Calibrator, containing human antibodies to cardiolipin
   • ACA Negative control, buffer containing no human auto-antibodies
   • ACA HRP IgM/G Control, containing human antibodies to cardiolipin
   • ACA Sample Diluents
   • ACA PBS, rinse buffer
   • HRP IgM/G Conjugate, containing anti-human antibodies to cardiolipin
   • TMB Chromogene
   • HRP stop solution

2. Methods
   • Separate serum from clot and keep in 2-8°C until the running of test and store at ≤ minus 70°C for future studies.
   • Bring all reagents and samples to room temperatures 20-25°C and mix well.
   • Make Buffer PBS ACA by adding 25 parts of ACA PBS Concentrate to 975 parts of Aquadest. Buffer PBS ACA should be made up fresh daily, and any unused portion should be thrown out at the end of the day.
   • Prepare 5 points of standard fit curve solution. For the first point standard solution (S1), remove 100 μL of ACA HRP IgM/G Calibrator directly from vial into a well of Polystyrene cardiolipin coated microwell strips. Prepare the remaining 4 points S2 through S5 of 5 point standard fit curve solutions by diluting the ACA HRP IgM/G Calibrator with the ACA negative control in external tube of Polystyrene cardiolipin coated microwell strips as shown in the table 3.
   • Move 100 μL each of S2 through S5 standard fit curve solutions into Polystyrene cardiolipin coated micro-well strips as shown in the table 4.
   • Add 1 μL of ACA negative control into Polystyrene cardiolipin coated micro-well strips containing 100 μL of ACA Sample Diluents as shown in the table 4.
   • Add 1 μL of Sera sample into Polystyrene cardiolipin coated micro-well strips containing 100 μL of ACA Sample Diluents as shown in the table 4.
   • After the last serum has been inserted to the well, start the timer to count the incubating time then close the micro-well plate and incubate for 30 minutes at room temperature (20-25°C).
   • Wash three times, and to each well add 210 μL Buffer PBS ACA, then mix it using an aspirator. Make sure that each well is really empty and devoid of waste at the end of each washing, and leave the blot in an absorbent material.
   • Add 100 μL HRP Conjugate into each well. HRP Conjugate will always be expelled from the bottle only as much as it is needed for that moment using aseptic standard principle and correct laboratory technique. Never reinsert the expelled conjugate in order to avoid contamination by microbial and chemical contaminant.
   • Repeat the three times washing as indicated at point 10.
   • Add 100 μL TMB Chromogene to each well to stain the blot and incubate in a dark room for 30 minutes at room temperature (20-25°C).
   • Add 100 μL HRP Stop liquid to stop staining reactions in each well. HRP stops conduction as well as conducting on HRP Conjugate.
   • Close the well plate carefully by finger for the well mix.
   • Read the absorbance (Optical Density) of each well using chromogenic photometer on 450 nm wave length immediately. The reading has to be done within 1 hour after stopping the staining reaction. If a biochromatic photometer reader is utilized, it is operable when the wave length is 620 nm.

3. Absorbance Reader
   Spectrophotometer reader by 450 nm Optical density.
   $\text{ACA level} = \frac{\text{Absorbance level}}{\text{Slope of calibrator fit curve}}$

4. Quality Control
   • ACA HRP IgM/G Calibrator, ACA HRP IgM/G Control, and ACA Negative control reagents should be run with every batch of sample to ensure that all reagents and procedures are performing properly.
   • The absorbance level of the first point standard solution (S1) ACA HRP IgM/G Calibrator must be greater than 1.0.
• The absorbance level of the first point standard solution (S1) ACA HRP IgM/G Calibrator must be greater than ACA HRP IgM/G Control, and the absorbance level of ACA HRP IgM/G Control must be greater than ACA standard negative control.

• The absorbance level of ACA standard negative control cannot be greater than 0.2.

**Statistical Analysis**

Dispersion of central tendency is analyzed with a coefficient of variant (COV) to assess the stability of the standard negative control of IgG and IgM ACA levels in every running of test by several batches of reagents.

\[
COV = \frac{s}{\bar{x}} \times 100\%
\]

Kappa statistics were used to determine inter-observer agreement for multinomial data to assess level of ACA agreement between that based on the deviation of the standard negative control and by fixed level.

The predictive true positive value based on standard negative control refers to positive results based on deviation of the standard negative control according to positive result based on fixed cut-off divided by a positive result based on deviation from the standard negative control.

The predictive true positive value by fixed cutoff is positive results by fixed cut-off according to positive result based on deviation of the standard negative control divided by positive result by fixed cut-off.

**RESULTS**

From 148 serum samples run 22 times in the ACA test, the median level of standard negative control IgG ACA has 4.93 GPL units, and the median level of standard negative control IgM ACA has 7.66 MPL units. The range and mean level of standard negative control IgG and IgM ACA is shown in table 5 and figure I, while the coefficients of variant of IgG and IgM ACA standard negative control levels are 19.30% and 29.17% respectively.
DISCUSSION

Kappa statistics was used to determine the degree of agreement between cut-off levels by deviation from standard negative control and fixed cut-off levels of ACA detected using ELISA, resulting in a disagreement in both IgM and IgG as shown in table 6 and table 7 respectively.

Cut-off levels based on deviation from standard negative controls were more sensitive, with a 69/75 (92%) predictive true positive value than 69/100 (69%) predictive true positive value by fixed cut-off levels of IgM ACA detected using ELISA, and IgG ACA produces nearly equal predictive true positive values of 27/32 (84.4%) based on deviation from standard negative control and 27/31 (87.1%) based on a fixed cut-off level. The predictive true negative value of IgM ACA based on deviation to standard negative control was 112/116 (96.55%) and 112/117 (95.73%) for IgG ACA based on a fixed cut-off. The overall positive levels of IgG ACA antiphospholipid antibodies based on deviation from standard negative controls on 148 serum samples was 21.62% and 20.94% based on fixed cutoff, where normal levels of IgG ACA were <3 times of IgG standard negative control levels, positive levels of IgG ACA were ≥ 3 times of IgG standard negative control level; and normal level of IgG ACA based on fixed cut-off was <15 GPL, and positive level of IgG ACA based on fixed cut-off was ≥ 15 GPL, as shown in table 8.

Referring to the diagnostic criteria established by the International Consensus of Antiphospholipid Syndrome in October 1998, antiphospholipid syndrome is diagnosed based on fixed cut-off of IgG and/or IgM ACA titers demonstrating at least moderate levels by more than 20 GPL/MPL. This study demonstrated positive predictive value for IgG by fixed cutoff of 18/148 (12.16%) and
32/148 (21.62%) based on deviation from standard negative control, and positive predictive value for IgM by fixed cutoff of 39/148 (26.35%) and 75/148 (50.68%) based on deviation from standard negative control. This finding suggests that cut-off low level based on deviation from standard negative control is more sensitive and accurate than moderate levels by fixed cut-off.

Thrombophilic state is defined as a predisposing condition to both first and recurrent episodes of thrombosis. Thrombogenic activity of antiphospholipid antibodies may be triggered and aggravated by the inhibition of protein C activation mediated by the thrombin-thrombomodulin complex on endothelial cell surfaces. Although sometimes APC resistance can be found in patients with antiphospholipid antibodies, the simultaneous presence of both abnormalities in one patient may lead to severe thrombotic tendency.

The overall prevalence of thrombosis in 245 of persistent ACA positive patients, as shown in table 9, is 53.88%. The Dallas/Fort Worth metroplex clinical center reports 8.2% patients with PC defects and 63.1% patients with antiphospholipid antibodies defects were found among 160 patients with recurrent miscarriage syndrome without anatomical and/or hormonal defects. Ismail reported 13.3% in 30 acute coronary syndrome patients with protein C activity levels below 70%. PC deficiency is usually classified into two types; Type I (quantitative) deficiency is characterized by reduced antigenic levels of protein C in three assays, while Type II (qualitative) deficiency is
characterized by normal antigenic levels of protein C. In both types, there is reduced functional activity to activate degraded factor Vc and VIIIc, and associated thrombophilic state.3 Antigenic levels of PC can be detected by chromogenic assay or immunoenzymatic assay (ELISA) for PC.10

On the other hand, APC resistance and ACA positive or both can be found in thrombophilic patients with or without APC deficiency. Clinical studies and in vitro experiments indicated that some types of antiphospholipid antibodies are capable of inducing factor V independent APC resistance.1 The overall prevalence of APC resistant and ACA positive on 326 thrombophilic patients as shown in table 10 has 51.43% and 23.62% respectively.1  The difference in the prevalence of ACA positivity among thrombophilic patients may be caused by different sampling frames or attainable population and ethnic groups, where APC resistance in the Netherlands and the USA is most frequently found in thrombophilic patients by 20% to 52%.13

Functional PC activity to activate degrading factor Vc and VIIIc can be detected by diluting the Russel Viper Venom Time (dRVVT) test with additional exogenous APC commercial reagent, where shortened dRVVT results in reduced functional activity of APC to activate degraded factor Vc and VIIIc.16

### CONCLUSION

Cut-off based on fixed levels of ACA by ELISA cannot be applied, because both IgG and IgM ACA levels of standard negative control are unstable within runs and batches, where coefficients of variant within runs and batches for both IgG and IgM ACA level of standard negative control are more than 7.45%.

Cut-off based on deviation from 3 times standard negative control levels for IgG ACA and by deviation from 2.5 times standard negative control level for IgM ACA is better than cut-off by fixed level of ACA in producing true positive results.

### ACKNOWLEDGEMENT

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### REFERENCES


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**Table 10. Clinical Studies on Antiphospholipid Antibodies and Activated Protein C Resistance in Thrombophilic Patients**

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>APC deficient</th>
<th>APC resistant</th>
<th>ACA Positive</th>
<th>Both*</th>
</tr>
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<tbody>
<tr>
<td>Walker et al, 1994</td>
<td>17/82 (20.73%)</td>
<td>13/17 (76.47%)</td>
<td>15/82 (18.29%)</td>
<td>13/82 (15.85%)</td>
</tr>
<tr>
<td>Bokarewa et al, 1995</td>
<td>30/78 (38.46%)</td>
<td>17/30 (56.67%)</td>
<td>24/78 (30.77%)</td>
<td>17/78 (21.79%)</td>
</tr>
<tr>
<td>Bokarewa et al, 1996</td>
<td>58/166 (34.94%)</td>
<td>24/58 (41.38%)</td>
<td>38/166 (22.89%)</td>
<td>24/166 (14.46%)</td>
</tr>
<tr>
<td>Over all</td>
<td>105/326 (32.21%)</td>
<td>54/105 (51.43%)</td>
<td>77/326 (23.62%)</td>
<td>54/326 (16.56%)</td>
</tr>
</tbody>
</table>

* Both APC resistant and ACA positive
deficient in acute coronary syndrome. In press.