

Standardization: What it Means and How it Affects Results (continued from page 3)

In the next issue of the READER (October 2005), we will discuss the various available antiphospholipid assays and recommendations for their use. As with other autoimmune diseases (i.e. SLE), one test may not be sufficient for an accurate serologic diagnosis.

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UPCOMING CONFERENCES

The XXth Congress of the International Society on Thrombosis and Haemostasis (ISTH) and the 51st Annual Meeting of the Scientific and Standardization Committee (SSC), will be held in Sydney, Australia from the 6 – 12 August 2005 at the Sydney Convention and Exhibition Centre, Darling Harbour. **Corgenix** will present two abstracts on oxidized low density lipoprotein / Beta 2 Glycoprotein I (oxLDL/B2GPI) complexes during the poster sessions.

Reminder: Dr. Luis Lopez will present, "Oxidized Low Density Lipoprotein and Beta 2 Glycoprotein I: Novel on Risk Factor for Autoimmune Mediated Athero-

READER PRODUCT FEATURE

Akers PIFA® Heparin/PF4 Rapid Assay For In Vitro Diagnostic Use

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UPCOMING CONFERENCES (cont.)

sclerosis at a Luncheon Symposium at 12:00 on August 21 during the annual meeting of the Association of Medical Laboratory Immunologists (AMLI) to be held in Denver August 21-24, 2005.

The University of New Mexico School of Medicine and the Office of Continuing Medical Education presents the Fifth Annual **Southwest Symposium on Thrombosis and Hemostasis** on October 7 and 8, 2005, at the Hilton Hotel in Albuquerque, NM. This year's symposium is intended for healthcare professionals who provide care to patients on anticoagulants such as warfarin, low molecular weight heparin, aspirin and other anti-thrombotic agents.

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Anti-Phospholipid Testing 101—Past, Present & Future Part II: Standardization: What it Means and How it Affects Results

The evolution of anticardiolipin (aCL) testing was discussed in the last issue of the READER (June 2005, Vol 15; No 3). More than 20 years have passed since the first ELISA method for aCL antibody detection was introduced to the clinical laboratory. Currently nearly 40 different commercial assays are available, in addition to many in-house methods still used by some laboratories. However, standardization continues to be a major issue when comparing results obtained by the different methods. While more is known about pathogenic mechanisms and clinical significance of antiphospholipid antibodies and more specific assays have been developed, significant performance differences still exist. Antiphospholipid assay standardization has been difficult to achieve due to differences in assay design (including reagent formulations and test procedures) and in the methods or materials used to calibrate the assays.

In 1986, E.N. Harris first proposed the use of standards for aCL antibody testing. He developed the first set of standards by mixing IgG and IgM positive serum samples (each from different patients) in various proportions with a normal serum; seven different levels or dilutions were prepared for each antibody isotype¹. The use of GPL (IgG anti-PhosphoLipid) and MPL unit systems for reporting IgG and IgM aCL levels was introduced; 1 GPL or MPL unit was defined as being equivalent to the binding of 1 ug/mL of affinity-purified antibody. Most aCL methods claim standardization to these preparations, which are commercially available through Louisville APL Diagnostics ("Harris' Standards"). One problem with these standards is that they are from a human (patient) source. When a particular lot is exhausted, a new lot of standards is prepared and values are assigned against the previous lot. Even if the same patient source is used, the affinity, avidity and specificity of the standards may vary from previous preparations. Multiple lots of these standards have been distributed since they were first introduced, and not all aCL methods (commercial or in-house) are standardized against the same preparation. Changing assay calibration to a new lot of standards can cause a noticeable shift in the recovery of patient samples². Some manufacturers currently use internal methods of calibration to maintain consistency across production kit

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These standards may react differently with other commercial kits due to several factors like the heterogeneity of antiphospholipid antibodies and the procedural differences.

lots, even though their assays were initially standardized against one of the Louisville preparations. Another challenge with these standards is how the values are assigned. Louisville APL Diagnostics also manufactures an ELISA kit for the detection of antiphospholipid antibodies.

This kit includes a mixture of phospholipids on the coated surface that does not include cardiolipin. The Louisville kit and an aCL kit from a different manufacturer are used to assign values to each new lot of standards. These standards may react differently with other commercial kits due to several factors like the heterogeneity of antiphospholipid antibodies and the procedural differences between aCL methods. If using the Louisville standards, each laboratory should consider both the recovery of the standards with their kit as well as the recovery of a healthy control population when establishing or confirming an appropriate cutoff value. The Louisville standards should be viewed

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more like reference preparations than actual standards. Finally, other antiphospholipid assays may show significant differences in recovery between methods because recognized standards were not available during the development of these tests, including aCL IgA, anti-phosphatidylserine, anti-beta 2 Glycoprotein I (aB2GPI), and anti-prothrombin assays.

The performance of various aCL antibody methods has been compared in a number of studies. A study by Reber et al. used the third preparation of the Louisville standards to compare the performance of nine commercial kits for the detection of aCL antibodies³. There were significant differences in the reactivity of the standards with the various kits. However, the performance of the standards did not predict how well the assays detected antibodies in patient populations. Furthermore, samples containing aB2GPI antibodies reacted similarly with all but one aCL kit, whereas the reactivity of samples without aB2GPI antibodies varied significantly between kits. This is important to consider when evaluating aCL methods. Further testing of discrepant samples for aB2GPI antibodies is recommended to aid in the interpretation of aCL results, especially when a patient's clinical history (i.e. of thrombotic events) is not available.

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Monoclonal antibodies have always offered a solution to lot-to-lot performance issues when human source standards failed. Recently, anti-human monoclonal antibodies to cardiolipin-bound B2GPI have been developed for both IgM (EY2C9) and IgG (HCAL) isotypes^{4,5}. The IgM monoclonal antibody was produced by Epstein-Barr virus transformation and somatic cell hybridization using lymphocyte cells from an APS patient. Production of an IgG monoclonal was more difficult; the variable region of a mouse IgG aB2GPI monoclonal antibody was fused (hybridized) to the constant region of human IgG immunoglobulin. Similar to the Louisville standards, these antibodies have been tested in various studies and their performance compared with different commercially available aCL and aB2GPI antibody kits. While the various kits gave positive results with these monoclonal antibodies, the difference in reactivity was still significant between methods^{6,7}.

These monoclonal antibodies have been proposed for use as standards for aCL and aB2GPI antibody assays, but technical issues need to be addressed and well understood before implementation:

1) These monoclonal antibodies are directed to a very specific region in domain I of the B2GPI molecule.

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There is still disagreement between researchers as to whether antibodies that bind to regions of B2GPI other than domain I are clinically relevant. Studies have demonstrated a stronger correlation with thrombosis by domain I aB2GPI antibodies compared to antibodies directed to other domains⁸. However, this does not rule out clinical relevance for all non-domain I aB2GPI antibodies. Also, not all aCL antibodies are B2GPI dependent. These antibodies may be caused by infectious or non-autoimmune diseases unrelated to

thrombosis, although some patients with non-B2GPI dependent aCL antibodies may have clinical events related to APS. In addition, monoclonal antibodies do not accommodate the heterogeneous nature of autoimmune antibodies. Most APS patients have multiple antibodies (polyclonal) that vary in specificity, avidity and affinity, as mentioned previously.

2) Studies are needed to demonstrate if lot-to-lot consistency during the production of these monoclonal antibodies can be achieved.

3) The monoclonal antibodies are currently available from two sources: a commercial company that also manufactures ELISA kits for both aCL and aB2GPI, and the Center for Disease Control (CDC, Bethesda, MD, USA). There is a difference in the form and concentration of the monoclonal antibody preparations they offer: one is liquid, the other lyophilized; the EY2C9 (IgM) preparations are similar in value (15.6 vs.

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20.0 µg/mL respectively), but the concentration of HCAL (IgG) standard differs significantly (18.8 vs. 1.1 µg/mL respectively). In fact, the concentration of the CDC IgG monoclonal is so low that it cannot be used to prepare a standard curve.

4) Lastly, with most current aCL or aB2GPI methods, values are reported in arbitrary "units" rather than in µg/mL. Converting current users into a new unit base would confuse both the laboratory and physicians. In order for these or any standard to be broadly accepted by laboratories and manufacturers, they need to be available from a non-commercial organization such as the CDC or World Health Organization (WHO), in one consistent form (either liquid or lyophilized) at a constant concentration. In addition, a monoclonal standard for IgA aCL or aB2GPI is still not available.

Different scientific groups interested in standardization issues have also made recommendations on aCL antibody assay design, testing procedures, and the interpretation of assay results. In 2000, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a guideline for the detection of aCL antibodies⁹. Several of the recommendations were found to be controversial including the mandatory use of a multi-point calibration curve, the use of bovine serum to block the cardiolipin-coated microtiter plate, and a minimum incubation time of 30-60 minutes. None of these suggestions were backed by scientific data. After much controversial debate, this effort was discontinued. One of the NCCLS issues, the use of bovine serum as a blocking agent on the coated microplate surface, comes from the aCL procedure originally described¹⁰. While bovine serum provides the cofactor B2GPI, it also introduces other proteins into the system that may cause non-relevant antibody reactivity. Many aCL ELISA assays (including the Corgenix kit) now use purified human B2GPI as part of the plate coating process.

The European Forum on antiphospholipid antibodies has made a consolidated effort to standardize aCL and aB2GPI antibody testing¹¹. They have suggested the following four minimal requirements: 1) all samples should be tested in duplicate; 2) each laboratory should determine its own cutoff using a population of at least 50 normal specimens; 3) laboratories should use 95, 98 or 99 percent confidence levels (percentiles) to calculate the assay cutoff; and 4) at least two reproducible external controls should be used, one below the assay cutoff and the other in the moderate positive range. Although these suggestions are good laboratory practices that many laboratories currently perform, they have done little to significantly improve detection discrepancies between different methods (kits).

Because of the heterogeneity of autoantibodies, laboratory tests for their detection are semi-quantitative at best. Even though most assays report results in unit values against an assigned cutoff, experts agree that interpretative ranges based on the level of reactivity (low, moderate or high) should be used to report positive results. Laboratories should either consult their manufacturer for guidelines or establish their own interpretive ranges based on their own patient populations. It has also been recommended that various comments be included when reporting patient results to assist the physician with interpretation¹². These include recommendations for additional testing with assays like lupus anti-coagulant or aB2GPI, the meaning of the various interpretive ranges and how they fit into the diagnostic criteria (Sapporo Criteria), and comments for IgM positive only results that may be associated with infectious disease, with the recommendation that aCL testing be repeated in 6-8 weeks to determine if the levels are transient.

Suggested Interpretive Ranges for the Corgenix READS anti-Cardiolipin Assays

	aCL IgG	aCL IgM	aCL IgA
Normal	< 23 GPL	< 11 MPL	< 22 APL
Low Positive (+)	23 - 35 GPL	11 - 20 MPL	22 - 35 APL
Moderate Positive (+)	36 - 50 GPL	21 - 30 MPL	36 - 45 APL
High Positive (+)	> 50 GPL	> 30 MPL	> 45 APL

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