LAL Update
ASSOCIATES OF CAPE COD, INCORPORATED

November 2006 • Volume 23, No.2

Letter From the Editor

Dear LAL User:

In this issue of the LAL Update we present articles written by two of our Technical Service representatives. Mark Fields addresses testing of blood samples (whole blood, serum, or plasma). His article expands on a topic that was included in the article on interferences with the LAL test in the October 2005 issue of the LAL Update (Volume 22, no. 3). Kathleen Brosnahan writes about calculations performed by the software used in photometric LAL tests and discusses the importance of knowing what is being calculated, how it is being calculated and the appropriateness of the calculations.

If you wish to discuss the issues raised in these articles, or any other LAL / endotoxin related issues, or to talk about our Northstar BioProducts™ line of products for glycobiology and glycoprotein characterization, please call our Technical Services department at 800-848-3248, or one of our main numbers, 888-395-2221 / 508-540-3444.

With best wishes,

Michael E. Dawson, Ph.D.

Testing Blood Samples for Endotoxin

By: Mark Fields

Introduction

Blood samples (whether whole, serum, plasma, or fractions) can be among the most difficult samples to analyze by the LAL test. This article describes some of these difficulties and offers tips on how to prepare and test such samples.

Difficulties

Blood samples contain a number of substances that can interfere with the LAL test.[1] For example, certain proteins in blood have the ability to neutralize endotoxin,[2,3] which can be troublesome when attempting to quantify the endotoxin concentration of a sample. Furthermore, blood contains serine proteases that are also known to interfere with the LAL assay. Some of these proteases degrade the proteins of the LAL enzyme cascade, resulting in inhibition; others activate the cascade, resulting in false positives.

Although it is usually necessary to treat the sample in some way, as will be discussed, it is worth testing the sample before employing any inactivation treatment. Samples can vary widely in the degree of interference and, depending on the dilution at which the sample is being tested, treatment may not be necessary. As with many samples in which there is interference with the LAL test, sometimes the best solution for testing blood or serum is to dilute the samples until the interference is overcome. However, it is quite likely that it will be necessary to treat the sample prior to testing to neutralize interference. There are several treatments that denature or inactivate the interference-causing proteins.[4, 5]

LAL Technical Report

The first procedure to try is heat inactivation, which is both effective and relatively easy to perform. This protocol for heat inactivation is described in detail in a previous LAL Update,[6] and we have used it successfully in our laboratory for testing plasma and sera. In some cases, however, the heat inactivation protocol can affect the apparent concentration of endotoxin because certain proteins bind endotoxin to a greater degree when heated. One approach is to test a sample that has been heat inactivated and compare it with an untreated sample to see if this protocol is useful.

Other inactivation protocols involve chemical treatments, such as the addition of acid or chloroform,[5] but these are recommended only when heat inactivation is not effective. A treatment that we have found to be successful in reducing interference in a wide variety of whole-blood samples includes the use of nitric acid, detergent, and sodium hydroxide.[7] Whichever treatment is used, it is important that positive product controls be included for every sample tested.

In addition to proteins, normal blood contains low levels of (1,3)-ß-D-glucan (BG), a component of fungal cell walls that activates the LAL enzyme cascade. Fungal infections can increase BG levels, and even low levels of BG can introduce a testing artifact when BG-sensitive LAL reagents are used to measure endotoxin.[7] Therefore, when testing blood or serum samples for endotoxin, an endotoxin-specific test is strongly recommended. ACC's Glucashield™ product is a reconstitution buffer that can be used in conjunction with all of our multitest LAL reagents (Pyrotell® 5mL and 2mL, Pyrotell®-T, Pyrochrome®, and Chromo-LAL). When reconstituted with Glucashield™, the LAL enzyme cascade is rendered insensitive to BG, producing a more accurate measurement of endotoxin in the sample.

Blood chemistry is highly variable; it is influenced by the physiological state of the patient or donor, which is itself influenced by many factors, including age, diet, genetic make-up, health, fatigue, and lifestyle. Consequently, although a treatment may be effective for many or most samples, it is quite likely that there will be some samples for which the treatment will not overcome interference.

As was noted in a recent LAL Update on interference,[6] treatments other than dilution should be validated by adding a known amount of endotoxin to a known volume of sample, performing the treatment, and demonstrating recovery of the added endotoxin within pre-specified limits (usually 50%-200%).

Recommendations

Of the LAL methods (gel clot, chromogenic, and turbidimetric), gel clot is widely considered to be the most robust and least prone to interference, making it a logical choice for such difficult samples as blood and serum. The chromogenic method is also a good choice for testing these samples, provided that potential optical interference is taken into account.[8] Because the chromogenic method is read at 405 nm, certain plasma or serum samples that absorb at this wavelength may interfere with the technique. Although sufficient dilution will eliminate this problem, another possible solution is to use ACC's Pyrochrome® kit with Diazo reagents. The color produced in this variant of the test is magenta, which absorbs in the 540-550 nm range, thus avoiding the interference at 405 nm.[9] Note, however, that this is an endpoint test and so has a narrower range of detection than kinetic methods.

When using one of the photometric methods (i.e., the chromogenic and turdidimetric techniques) to quantify endotoxin in whole-blood samples, it is generally necessary to centrifuge the sample to avoid the optical interference caused by blood cells. The photometric methods are also likely to provide valid results from several of the dilutions tested (that is, the positive product control "spike" is recovered within the specified limits, and the sample result falls within the standard curve). However, it is not unusual to find that although spike recovery is consistent from dilution to dilution, the measured endotoxin concentration (after correction for dilution) changes with dilution of the unspiked sample. Usually the concentration increases with dilution. Thus, although spike recovery indicates that there is no interference, the endotoxin measured in the sample clearly indicates that interference is still present. The added endotoxin spike is behaving differently from the endotoxin in the sample. The interference can sometimes be removed by diluting the sample until the endotoxin concentrations are consistent in both the spike and the unspiked sample. At other times dilution is not effective and another treatment must be sought to overcome the interference. In the absence of an effective alternative treatment, the only appropriate course is to report the result as greater than or equal to the highest endotoxin concentration detected.

Summary

In conclusion, blood (or blood-derived) samples can present a challenge to the LAL test. Some method development work is often necessary to find an appropriate test technique. A number of possible strategies are available for overcoming interference, but the simplest approach that can be validated is always the best. Thus the choices, in order, should be dilution, heat inactivation, and chemical treatments. The Technical Service staff at Associates of Cape Cod are always ready to advise you on a testing strategy, and the Contract Test Service has a wide range of experience testing different types of blood-derived samples from a range of different species.

References

1. Ketchum, P. A. and T. J. Novitsky. 1999. Assay of endotoxin by Limulus Amebocyte Lysate. In "Septic Shock: Methods and Protocols," Methods in Molecular Medicine, Vol. 36. Pp. 3-12.
2. Warren, H. S., T. J. Novitsky, P. Martin, P. F. Roslansky and G. R. Siber. 1987. Endotoxin neutralizing capacity of sera from different patient populations assessed by the Limulus lysate test. In "Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test." Pp. 341-348.
3. Warren, H. S., T. J. Novitsky, P. A. Ketchum, P. F. Roslansky, S. Kania and G.R. Siber. 1985. Neutralization of bacterial lipopolysaccharides by human plasma. J. Clin. Microbiol. 22(4): 590-595.
4. Roth, R. I., F. C. Levin, and J. Levin. 1990. Optimization of detection of bacterial endotoxin in plasma with the Limulus test. J. Lab. Clin. Med. 116: 153-161.
5. Novitsky, T. J. 1994. Limulus Amebocyte Lysate (LAL) detection of endotoxin in human blood. J. Endotoxin Res. 1: 253-263.
6. Dawson, M. E. 2005. Interference with the LAL test and how to address it. LAL Update 22(3): 1-5.
7. Tamura, H, S. Tanaka, T. Obayashi, M. Yoshida and T. Kawai. 1991. A new sensitive method for determining endotoxin in whole blood. Clin. Chim. Acta 200: 35-42.
8. Roslansky, P. F. 1990. Serum testing using chromogenic and gel-clot methods. LAL Update 8(4): 1-3.
9. Novitsky, T. J. 1994. Diazo coupling option with Pyrochrome® Chromogenic LAL. LAL Update 16(2): 1-3.

Note: Since writing this article, Mark has changed careers. We thank him for his good work with Associates of Cape Cod and wish him well.

Understanding Correlation Coefficients and Coefficients of Variation in Photometric LAL Testing.

Or: Do you know what your software was doing last night?

By: Kathleen Brosnahan

Introduction

Software is supplied with the various instruments for performing photometric LAL tests, as well as with instruments for other assays. These software packages process the data and perform statistical functions. Although the statistical functions may be similar, the algorithms used may be different or applied differently and these differences can affect the results reported.

The term statistics refers to "the analysis and interpretation of data with a view toward objective evaluation of the reliability of the conclusions based on the data."[1] In the case of automated laboratory systems such as those used for photometric LAL tests, it is important to know how the software is processing data and determining results. It is particularly important when comparing systems, because they do not all calculate the same statistical parameters in the same way. One should recognize that a correlation coefficient closer to unity or a smaller coefficient of variation (CV) may be a result of the way data are processed rather than an indication of better linearity or greater precision in a given system.

In this article, we aim to clarify some of the most common statistical parameters used in LAL testing and explain the different calculations performed. It is essential that users understand the implications of the values that their software reports and how they may affect results.

The **correlation coefficient, *r***, indicates the strength and direction of a linear relationship between two random variables-in this case endotoxin concentration and onset time, or OD. The FDA Guideline states that "the absolute value of the correlation coefficient, |r|, must be greater than or equal to 0.980 for the range of endotoxin concentrations."[2]

The equation used to calculate *r* is below:[1]

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{n}}{\sqrt{(\sum X^2 - \frac{(\sum X)^2}{n})(\sum Y^2 - \frac{(\sum Y)^2}{n})}}$$

Calculation of the correlation coefficient examines the relationship between the mean value and the actual *Y* values in a regression analysis. Some software packages use each individual replicate of the onset time to calculate the correlation coefficient. Other software takes the mean of the replicates and performs a regression analysis on the mean. The latter method minimizes the deviations of the *Y* value (which in this case is the log of the onset time) from the line of best fit and results in a higher *r* value than if the individual replicates were used. Consequently, from the same raw data, the software will report different correlation coefficients, depending on whether individual replicates or means of replicates were used in the calculation.

Consideration of each replicate for a given concentration separately results in a more conservative assessment of linearity. This is the approach utilized in all software produced by Associates of Cape Cod Inc. (ACC). We contend that the correlation coefficient should include a measure of the variability of the replicates. The 1991 FDA Guidance on the testing of human and veterinary drugs by kinetic LAL techniques specifically states "DO NOT AVERAGE THE REACTION TIMES OF REPLICATES OF EACH STANDARD BEFORE PREFORMING REGRESSION-CORRELATION ANALYSIS" (FDA's capitals).[3]

The **coefficient of variation (CV)** is an estimate of the variability within a population about the mean. It is the ***standard deviation*** expressed as a percentage of the mean. The standard deviation is the most common measure of statistical dispersion - that is, of how tightly various data points are clustered around the mean in a set of data. If the data points are all close to the mean, then the standard deviation, and hence the CV, is low (closer to zero). If many data points are very different from the mean, then the standard deviation is high (further from zero). If all the data values are equal, then the standard deviation is zero.

When a parameter is measured for every individual in a population, the standard deviation can be calculated for that population. The result is an actual measure of the dispersion (variability) in the population. In this case, the following equation,[1] is used:

$$\text{Standard Deviation}_{pop} = \sqrt{\frac{\sum X_i^2 - \frac{(\sum X_i)^2}{N}}{N}}$$

Where $\boldsymbol{X_i}$ is a value in the data set, ***N*** is the total number of values in the population.

Alternatively, a number of samples (n) can be taken from that population and an **estimate** of the standard deviation of a population determined by the following equation.[1]

$$\text{Standard Deviation}_{sample} = \sqrt{\frac{\sum X_i^2 - \frac{(\sum X_i)^2}{n}}{n-1}}$$

In this case the divisor is "*n*-1", where the "-1" has the effect of adding a correction to compensate for additional variability that might not have been captured in the sample.

This is the more appropriate model to use for an LAL test in which a limited number of "samples" (replicates) are assayed, and it is the equation utilized by our software. This equation gives an increased value for the standard deviation - leading to a more conservative CV. The effect of the "-1" is greatest for a small number of replicates, which is usually the case for LAL tests. Note that calculation of the CV for two or three replicates is of little statistical value. However, the CV does provide a measure of the variability of the replicates in an individual test.

Other software may make use of the first equation, resulting in smaller values for the CV. This result does not mean that the method is any more precise, just that

LAL Technical Report

the data are processed differently.

There is also a difference in the values used to calculate the CV in different software. The CV of the endotoxin concentration should be calculated for the mean of the endotoxin concentration, not the onset time. Because the LAL assay determines endotoxin concentration, the CV should be a measure of the variability of that parameter. The onset time is not the result being reported. This would be an academic consideration if there were a linear, arithmetic relationship between onset time and endotoxin concentration. In endpoint assays, in which endotoxin concentration is directly proportional to OD, there is a linear relationship, and the CV of the OD is the same as that for the endotoxin concentration. In kinetic tests, on the other hand, in which a log transformation is performed on the onset time to construct the standard line and to determine endotoxin concentrations, the relationship is not linear. The CV of the onset time is consistently less than that of the endotoxin concentration and gives a misleading indication of the magnitude of the replicate value distribution. This issue was discussed in more detail and demonstrated in a previous issue of the LAL Update.[4]

To conclude, it is important to understand how calculations are being performed by the software and to be satisfied that the correct equations are being applied in the right way. It is also important that specifications be appropriately set. If a specification that was set for a CV based on the standard deviation for a population is applied to software that calculates CVs for samples, this could result in unnecessary failures to meet the specification. It is important to understand what the software is doing so that data is properly interpreted and so that limits for test acceptability or validity are correctly set.

References

1. Zar, J. H. "Biostatistical Analysis," Fourth Edition. Prentice Hall, Upper Saddle River, NJ.

2. US Food and Drug Administration. 1987. Guideline on Validation of the Limulus Amebocyte Lysate test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices.

3. US Food and Drug Administration. 1991. Interim Guidance for Human and Veterinary Drug Products and Biologicals: KINETIC LAL TECHNIQUES.

4. Richardson, K. W., and T. J. Novitsky. 2002. Simple statistics for the LAL user-standard deviation, repeatability, reproducibility, and a clarification of the coefficient of variation. LAL Update 20 (4).

LAL News and Events

NOVEMBER 2006
Society for Glycobiology Annual Conference
November 15-18, 2006
Sheraton Universal Hotel
Los Angeles, CA
Booth: 7

DECEMBER 2006
American Society for Hematology (ASH) Annual Meeting
December 9-11, 2006
Orange County Convention Center
Orlando, FL

MARCH - 2007
LAL Testing: Drugs, Medical Devices and Biopharmaceuticals
Presented by the Center for Professional Advancement.
March 12 - 14, 2007. Amsterdam, Netherlands

A three day course directed by Dr. Dawson, presented with Karen McCullough, Roche Molecular Systems

For details and registration see www.cfpa.com or call: 732-238-1600 in the US; 31-20-638-28-06 in the Netherlands.

APRIL 2007
Hyaluronan 2007
April 22-27, 2007
Charleston, SC

MAY 2007
ARVO
May 6-10, 2007
Fort Lauderdale, FL

PEGS Summit
May 14-15, 2007
Boston, MA

JUNE 2007
ASCO
June 2-4, 2007
Chicago, IL

For more information or to register for a workshop, visit our website at www.acciusa.com or call (888) 395–2221

Customer Service: custservice@acciusa.com **Technical Service:** techservice@acciusa.com **www.acciusa.com**

LAL Update®
November 2006 • Volume 23, No.2

ASSOCIATES OF CAPE COD INCORPORATED

Corporate Headquarters
Associates of Cape Cod, Inc.
124 Bernard E. Saint Jean Drive,
East Falmouth, MA 02536
T (508) 540–3444
F (508) 540–8680

UK Office
Associates of Cape Cod Int'l Inc.
Deacon Park, Moorgate Road,
Knowsley, Liverpool L33 7RX
United Kingdom
T (44) 151-547-7444
F (44) 151-547-7400

German Office
Pyroquant Diagnostik GmbH
Opelstrasse 14
D-64546 Mörfelden-Walldorf,
Germany
T (49) 61 05-96 10 0
F (49) 61 05-96 10 15