A Spectrophotometric Method to Precisely Determine Endpoint Titers in Complement Fixation Assays

Ryan J. Welch,^{†*} Stephen D. Merrigan,[†] and Julio C. Delgado

ARUP Institute for Clinical and Experimental Pathology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah

Since the early 20th century, complement fixation (CF) testing has been used to quantify the humoral response to various pathogens. The qualification of a positive result is based on a subjective determination of 30% lysis of sheep red blood cells, which can lead to variability in the analysis. A spectrophotometric reading of

a standard with a known 30% lysis was used to standardize the currently used CF method and tested with controls and patient sera for various fungal assays. By utilizing this method a precise and non-subjective determination of endpoint titers was achieved. *J. Clin. Lab. Anal.* 26:190–193, 2012. © 2012 Wiley Periodicals, Inc.

Key words: Complement Fixation; Titer; Spectrophotometry; Mycoses

INTRODUCTION

In 1899 Jules Bordet discovered that hemolysis was induced by complement that led to the development of the complement fixation (CF) test in 1901 (1). In 1965 protocols were formally established by the Centers for Disease Control (CDC) to standardize many aspects of CF testing and published as Laboratory Branch Complement Fixation (LBCF) (2). Since then CF testing has been used to aid in the diagnosis and to monitor treatment for many diseases, including the mycoses histoplasmosis, coccidioidomycosis, and aspergillosis (3).

Complement fixation testing is based on the ability of antibodies, primarily of the IgM and IgG (IgG1 and IgG3) class, to activate the classical complement pathway (4, 5). Antibodies present in the patient sera will bind to the antigens in solution that have been selected based on the disease of interest. Sheep red blood cells (SRBC), anti-SRBC antibody, and complement are then added. In the absence of antigen-specific antibody, the anti-SRBC antibody will bind to C1 and initiate the classical complement pathway; this leads to downstream synthesis of the membrane attack complex (MAC) from multiple C9 molecules and SRBC lysis. When patient antibodies bound to antigen in solution are at a concentration significant enough to minimize anti-SRBC complement activation, minimal SRBC lysis occurs. Samples are titrated and an endpoint is established by observing the titer at which 30% lysis is reached. The percent lysis is determined by a trained

laboratory technologist; however, this leads to subjective differences in establishing when 30% lysis is reached (6).

To standardize endpoint titer determination in complement fixation assays, we developed a spectrophotometric method that objectively determines when 30% lysis has been reached. To accomplish this, a standard was created with a known percent of lysed cells. This standard was then analyzed on a spectrophotometer at a wavelength known to be absorbed by hemoglobin, a marker for red blood cell lysis. Utilizing this methodology, we hypothesize that 30% hemolysis can be accurately determined on known positive control material and patient samples for histoplasmosis, coccidioidomycosis, and aspergillosis. Correlation was done between laboratory technologist readings and the spectrophotometric method. We show that by implementing this method differences in qualitative and quantitative results between personnel can be eliminated, and the precision of the assay greatly improves.

[†]Both authors contributed equally to this work.

*Correspondence to: Ryan J. Welch, ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108. E-mail: ryan.welch@aruplab.com

Received 3 August 2011; Accepted 24 February 2012

DOI 10.1002/jcla.21509

Published online in Wiley Online Library (wileyonlinelibrary.com).

MATERIALS AND METHODS

Sera Collection

Two sets of serum samples were collected from samples that had been sent to ARUP's Microbial Immunology Laboratory for fungal CF testing: 11 samples sent for *Coccidioides* CF testing were used to assess laboratory technologist endpoint analysis precision and 60 samples sent for *Coccidioides*, *Aspergillus*, and *Histoplasma* mycelia CF testing were used to assess the utility of the spectrophotometric method. All patient samples included in this study were de-identified according to a University of Utah Institutional Review Board approved protocol (#7,275) to meet the Health Information Portability and Accountability Act patient confidentiality guidelines. Specimens were stored at 2–8°C while all evaluations were performed.

Laboratory Technologist Precision

Endpoint titers were determined on the 11 CF samples tested for *Coccidioides* antibodies by four different laboratory technologists and precision was assessed.

Hemoglobin Color Standard

A hemoglobin color standard was prepared freshly with each run as described by the CDC (2). In brief, a hemoglobin solution was prepared with 1.0 mL of well-mixed 2.8% SRBC (Hema Resource and Supply, Inc., Aurora, OR; these were internally prepared and standardized) and 7.0 mL deionized (DI) water that was shaken until the cells lysed and the solution was clear. The final volume was then raised to 10.0 mL by adding 2.0 mL of internally prepared CF saline containing DI water with NaCl, CaCl₂, and MgCl₂, with a final pH between 7.3 and 7.4. A 0.28% cell suspension was also prepared by adding 1.0 mL of well-mixed 2.8% SRBC suspension to 9.0 mL of cold CF saline. The hemoglobin color standards were then prepared by adding the hemoglobin solution to 0.28% SRBC as shown in Table 1.

Complement Fixation Procedure

The CF procedure is described by the CDC (2). In brief, optimal concentrations of hemolysin (Lonza Walkersville, Inc., Walkersville, MD), CF antigen, and complement were predetermined by previous titrations. Patient samples and patient pools were diluted 1:4 by adding 0.1 mL sera to 0.3 mL CF saline. Diluted samples were then heat inactivated in a 56°C water bath for 30 min. Patient samples were further serially diluted two fold from 1:8 to 1:1,028 by adding 25 µL to wells, of a clear round bottom 96 microtiter plate, already containing 25 µL of CF saline. To each well, 25 µL of working dilution of antigen was

TABLE 1. Preparation of Color Standards

Hemolysis (%)	Hemoglobin (mL)	0.28% SRBC (mL)	OD ₄₀₅ ^a
0	0.0	1.0	0.194 ± 0.051
10	0.1	0.9	0.378 ± 0.034
20	0.2	0.8	0.506 ± 0.017
30	0.3	0.7	0.656 ± 0.011
40	0.4	0.6	0.816 ± 0.042
50	0.5	0.5	0.957 ± 0.113
60	0.6	0.4	1.063 ± 0.150
70	0.7	0.3	1.153 ± 0.028

^a Average optical density at 405 nm plus or minus the standard deviation.

added. Next 50 µL of working complement was added to each well. Plates were then sealed and incubated at 4–8°C overnight (12–18 hr). After overnight incubation, 50 µL of sensitized SRBC (equal volumes of working hemolysin and 2.8% SRBCs) were added to each well and incubated in 37°C water bath for 30 to 60 min.

Sample results for each well were determined by degree of lysis. Lysis greater than 30% was considered to be negative for assay antibodies. Lysis less than 30% was considered positive for assay antibodies. As modified from Kaufman, 1992 and internally determined, a well with lysis of less than 30% at a titer of at least 1:32, 1:8, and 1:8 was required for *Coccidioides*, *Aspergillus*, and *Histoplasma* mycelia, respectively, to be considered positive (3).

Spectrophotometric Methodology

From samples, analyzed by CF as described previously, 100 µL of supernatant (avoiding the fixed complement precipitate) from each well was pipetted into a clear round bottom 96-well microtiter plate. On the same plate, 100 µL of color standards ranging from 0 to 70% lysis into eight wells in increments of 10% lysis. The plate was then analyzed by a spectrophotometer (Tecan Rainbow F39200, Tecan Group Ltd., Männedorf, Switzerland). The absorption spectrum of the 30% lysis standard was determined to have a maximum OD at a wavelength of 414 nm (Fig. 1). However, a wavelength of 405 nm was chosen to read all samples as this is a standard wavelength available on common laboratory spectrophotometers and showed a significant OD above background. The hemolysis standards ranging from 0 to 70% lysis were determined to have significantly different ODs from that of the 30% lysis standard (Table 1). The distinct separation of ODs created a value that could be used as a cutoff point for the controls and patient sera. The optical density at 405 nm (OD₄₀₅) of the samples was compared to the OD₄₀₅ of the color standard, and the OD₄₀₅ of the 30% lysis was considered to be the cutoff.

Fig. 1. Absorption spectrum of the 30% lysis standard.

RESULTS AND DISCUSSION

Laboratory Technologist Precision

Each sample endpoint titers ranged from $<1:2$ to $1:64$. To evaluate the results, a single titer difference was given a value of one. For example, a titer difference between $1:2$ and $1:8$ would be assigned a value of 2. The 11 samples collected for the precision study had standard deviations (SDs) ranging from 0.00 to 1.53 titers, with the average SD being 0.89 titers. This equates into an average coefficient of variation (CV) of 60.0% between laboratory technologists. This high variation justifies exploring a method that standardizes the interpretation method. When these 11 samples were interpreted using the spectrophotometric method, no variation in titer endpoint determination was observed between laboratory technologists.

Analysis of Fungal Positive controls and Patient Sera

Each fungal positive control displayed a clear OD difference between the endpoint titer and the first negative titer (Fig. 2). A definitive OD above the 30% lysis standard was used to determine the endpoint titer for all patient sera. Of the 60 patient serum samples collected, 29 were positive and 31 were negative for CF antibodies against one of the fungal specific antigens by the non-spectrophotometric method (see Materials and Methods). The endpoint titers as determined by spectrophotometric analysis were then compared with the endpoint titers determined by laboratory technologist readings. Of the patient serum samples with negative antibody titers, all 31 samples were verified to have negative titers by spectrophotometric analysis and did not vary from the original negative titer value. Of the 29 samples with positive CF antibody titers, 19 samples had titer endpoints that matched, and nine samples only varied endpoints by plus

Fig. 2. Complement fixation positive controls compared to the 30% lysis standard. The solid line (—) indicates the 30% lysis standard; the dotted line with crosses (•••×•••) indicates the *Aspergillus* positive control; the dashed line with triangles (—▲—) indicates the *Histoplasma* positive control; the broken dashed line with squares (—■—) indicates the *Coccidioides* positive control.

Fig. 3. Bar graph illustrating the difference between the endpoint interpretation of laboratory technologist and the endpoint called by the spectrophotometric method. A total of 29 positive samples were analyzed with the number of samples in each difference category shown above the respective bar.

or minus one titer. One sample that had a negative titer of by spectrophotometric analysis had an endpoint difference of more than four titers from what was determined by a laboratory technologist (Fig. 3); however, this patient tested negative by immunodiffusion for the specific fungal antibody being tested for by CF, likely indicating an incorrect determination by laboratory technologist.

Accurate titer endpoint determination is important in CF testing. An inherent flaw with CF testing is the subjective nature in which the test is read that can lead to poor inter-technologist precision. If titer endpoint is inaccurately assessed there is the potential for false positive or false negative results. Moreover, differences in titer values over the course of monitoring may have an impact on patient care, either through the incorrect assumption that an individual being monitored is improv-

ing or by not responding to therapy (7). The goal of this study, therefore, was to provide an objective method that increases the precision of CF testing. By producing a standard curve that can be read spectrophotometrically and by comparing it to samples with unknown lysis percentages, a fully objective method was developed. While this method has been shown to work for three fungal assays, it could be applied to any complement fixation assay where an endpoint is defined by a lysis level.

It is important to note that the accuracy of this methodology was not assessed as that was not the goal of the study. The goal of utilizing the spectrophotometric method was to provide a standard reference by which 30% lysis could be accurately determined and reproduced. Although the number of samples was small, it is clear that there are differences in how laboratory technologists interpret individual samples and that the spectrophotometric method eliminates this subjectivity. We, therefore, conclude that the method described here provides an objective means to obtain a titer endpoint, allowing for a reduction in errors caused by human endpoint assessment.

ACKNOWLEDGMENTS

This study was supported by the ARUP Institute for Clinical and Experimental Pathology®.

REFERENCES

1. Bialynicki-Birula R. The 100th anniversary of Wassermann-Neisser-Bruck reaction. *Clin Dermatol* 2008;26(1):79–88.
2. United States. Public Health Service. Standardized diagnostic complement fixation method and adaptation to micro test. Washington: For sale by the Superintendent of Documents, U.S. Govt. Print. Off. 1965; vi, 34 p.
3. Kaufman L. Laboratory methods for the diagnosis and confirmation of systemic mycoses. *Clin Infect Dis* 1992;14 Suppl 1:S23–29.
4. Bruggemann M, Williams GT, Bindon CI, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med* 1987;166(5):1351–1361.
5. Dangel JL, Wensel TG, Morrison SL, Stryer L, Herzenberg LA, Oi VT. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO J* 1988;7(7):1989–1994.
6. CDC. Standardized diagnostic complement fixation method and adaptation to micro test. *Public Health Rep* 1965;80(7):652.
7. Rubin RH, Young LS. Clinical approach to infection in the compromised host. New York: Kluwer Academic/Plenum. 2002; xxv, 719 p.