Comparison of ANA Endpoint Dilution Using the PolyTiter™ Immunofluorescent Titration System and Conventional Serial Dilution

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The PolyTiter™ immunofluorescent titration system (PolyTiter system), an attachment for conventional fluorescent microscopes, facilitates immunofluorescent antinuclear antibody (IFA-ANA) testing by using calibrated light attenuation to determine the semiquantitative endpoint dilution or titer of ANA-positive specimens. The objective of this study was to compare the determination of ANA titer using the PolyTiter system to that of the conventional serial dilution method. A total of 172 consecutive ANA-IFA positive serum specimens, including the four most common pattern types, were evaluated in parallel by two readers to determine the dilution endpoint. The two meth-

ods produced equal dilution endpoints in 78 specimens (45%), within 1-dilution endpoint in 121 specimens (70%), and within 2-dilutions endpoint in 158 specimens (92%). The conventional method consistently produced a more conservative dilution endpoint than the PolyTiter system for three of the four patterns evaluated (i.e., centromere, nucleolar, and speckled vs. homogeneous). The PolyTiter system demonstrated satisfactory performance in comparison to the current standard for determining ANA dilution endpoints, while significantly reducing operator labor, time, and materials usage. *J. Clin. Lab. Anal.* 16:91–94, 2002. © 2002 Wiley-Liss, Inc.

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INTRODUCTION

Circulating antinuclear antibodies (ANA) are associated with many systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), mixed connective tissue disease, and rheumatoid arthritis. The detection of ANA in conjunction with clinical symptoms provides important diagnostic and prognostic information. Indirect immunofluorescence is the most commonly used method to screen for the detection of ANA (1,2). Patient serum is reacted with antigen localized in a tissue section on a glass slide. ANA in the serum binds to the antigens and, after removal of nonspecific antibodies by washing, is detected by the addition of fluorescence-labeled antihuman immunoglobulins. A positive reaction is seen as specific bright apple-green staining in a characteristic nuclear or cytoplasmic pattern when viewed under a fluorescent microscope, while negative specimens lack a specific pattern of fluorescence. Higher levels of ANA result in brighter staining than do lower levels. Currently, HEP-2 cell culture is the recommended substrate for immunofluorescent ANA screening. It is highly sensitive, such that negative results indicate a <0.14% chance of having SLE, although the specificity for SLE is low (20–30%) (3).

Screening for the presence of ANA is typically performed at

a 1:40 or 1:80 dilution. Specimens found to be positive are then serially diluted and retested, with the endpoint dilution reported as the highest dilution in which fluorescence is observed. While ANA titers vary depending on age and gender, with an increasing incidence in women and older persons, the level of antibody is generally higher in those patients with autoimmune disorders, and lower in apparently healthy individuals.

The PolyTiter™ immunofluorescent titration system (PolyTiter system) was developed by Polymedco, Inc. (Cortlandt Manor, NY) to facilitate ANA testing while utilizing the fluorescent microscope and ANA assay reagents in routine clinical laboratory use. The system is based on the principle that the intensity of the fluorescence on the ANA slide is related to the dilution endpoint of the specimen (4). The PolyTiter system parallels the dilution effect by utilizing filters to ad-

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just the illumination in the field of view. Installed between the lamp housing and the eyepiece, wide-spectrum filters quantitatively regulate the light source, allowing the determination of a "light attenuation endpoint" (LAE) from the initial screening dilution. By determining the LAE of four calibrator solutions, a standard curve can be constructed from which to read the titers of subsequent patient specimens.

In this study, the ANA dilution endpoints of 172 patient specimens were determined using the conventional serial dilution method and the PolyTiter system, and the results between the two methods were statistically compared.

MATERIALS AND METHODS

A total of 172 consecutive serum specimens found to be positive by ANA-IFA testing and with common immunofluorescent patterns were included in the study. An additional four diffuse grainy (anti-topoisomerase I antibody positive by extractable nuclear antigens (ENA) assay, two nuclear dots, one mitotic spindle apparatus (MSA), and four cytoplasmic positive sera (three antimitochondrial, one antiribosomal-like, and none anti-Jo-1) were excluded from the study, due to the low number of samples and limited statistical significance. Conventional twofold serial dilutions starting at 1:40 were performed to endpoint. The maximum serial dilution titer reported was 1:5120. Endpoint titers were then determined from a 1:40 dilution in these specimens using the PolyTiter system. Two experienced operators performed the testing in the Laboratory of Clinical Pathology of the Hospital of S. Donà di Piave (Venice, Italy). All specimens were evaluated using HEP-2 cells (INOVA Corp., San

Diego, CA) and a single fluorescent microscope (Olympus, BH-2). When discordant results were obtained in either method, the slides were re-read and a consensus was reached. Positive and negative quality control specimens were included in each test run on the first and last slides, and a control slide containing fluorescent beads at different fluorescence intensity, from 1+ to 4+ (FITC QC-slide; Immunoconcepts, Sacramento, CA), was used to check the efficiency of the microscope lamp.

The PolyTiter system includes hardware (the filter unit, microscope adapters, and control pad (Fig. 1)) and four prediluted ANA calibrators consisting of human serum with established ANA titers (1:40, 1:160, 1:640, and 1:2560). The calibrators are included in each test run and a light attenuation endpoint (LAE) is determined for each one. This is accomplished by using a control pad to adjust the filters in a step-wise fashion while viewing the field through the microscope objective in the usual manner. The light illuminating the slide is decreased by the user until no discernable fluorescent pattern is evident, then it is slowly increased to the point at which the cells are faintly visible again. This point is designated the LAE. The control pad monitors the position of the filters and converts it to a numeric readout, thus providing a quantitative PolyTiter value (1–100 U) corresponding to the LAE. The PolyTiter unit of each calibrator is plotted against the known dilution endpoint, creating a four-point standard curve (Fig. 2). Patient specimens are diluted 1:40 and assayed in the usual manner along with the calibrators, and their LAE determined. The PolyTiter units displayed on the control pad at the LAE for each patient specimen can then be read from the stan-

Fig. 1. PolyTiter system hardware.

Fig. 2. Example of a PolyTiter system standard curve.

dard curve. Dilution endpoints for the PolyTiter system are reported to the nearest conventional dilution endpoint value with a maximum titer of 1:2,560.

A one-sample test for binomial proportions was used to statistically compare the observed proportion of samples within ± 2 dilutions with respect to the proportion of those specimens having a PolyTiter system endpoint dilution higher than the serial dilution titer vs. lower than the serial dilution titer (5). Comparisons were made for the overall data as well as by pattern type, with serial dilution titers of 2,560 and 5,120 considered to be equivalent to a PolyTiter result of 2,560. The null hypothesis tested was that the proportion of “plus” 2-dilution samples would be equal to the proportion of “minus” 2-dilution samples, both of which theoretically should equal 50% vs. a two-sided alternative of not being equal to 50%. The exact binomial probabilities were calculated when np_0q_0 was less than 5. If np_0q_0 was greater than 5, then the normal theory method was used for determining the P -values for each comparison, i.e., overall and by pattern type. If the P -value was less than 0.05 then the null hypothesis (i.e., $H_0: P = 0.5$) was rejected and the alternative hypothesis ($H_1: P \neq 0.5$) was accepted, thereby indicating a statistically significant difference. A chi-square test for heterogeneity was performed to compare proportions across pattern types (5).

RESULTS

Seventy-eight of the 172 specimens (45%) were reported to have equal endpoint titers, with 121 dilution endpoints (70%) varying by ± 1 dilution, and 158 dilution endpoints (92%) varying by ± 2 dilutions. Tables 1 and 2 provide a summary of agreement by pattern type by frequency and by proportion. To compare proportions across pattern types for each of the three dilution endpoints, a chi-square test for heterogeneity was performed. For the equal titer endpoint, there ap-

TABLE 1. Summary of dilution endpoint agreement by pattern type (frequencies)

Pattern type	Sample size (n)	Equal titer endpoint	1-dilution endpoint agreement	2-dilutions endpoint agreement
Homogeneous	78	30	58	74
Speckled	50	30	38	47
Nucleolar	24	12	15	22
Centromere	20	6	10	15
All	172	78	121	158

Chi-square Test for Heterogeneity (equal titers): $\chi^2_{3df} = 7.95$, $P = 0.047$.

Chi-square Test for Heterogeneity (± 1 -dilution endpoint): $\chi^2_{3df} = 6.11$, $P = 0.107$.

Chi-square Test for Heterogeneity (± 2 -dilution endpoint): $\chi^2_{3df} = 9.09$, $P = 0.028$.

Chi-square Test for Heterogeneity (direction of differing titers): $\chi^2_{3df} = 19.22$, $P < 0.001$.

peared to be a borderline statistically significant difference in proportions between all pattern types ($\chi^2_{3df} = 7.95$, $P = 0.047$). For the ± 1 -dilution endpoint, no statistically significant difference in proportions between pattern types was observed ($\chi^2_{3df} = 6.11$, $P = 0.107$). For the ± 2 -dilution titer endpoint, the proportion for the centromere pattern type was found to be statistically less than that of the other three pattern types, i.e., 75% vs. 92–95%, respectively ($\chi^2_{3df} = 9.09$, $P = 0.028$).

When the titers differed, there was a statistically significant trend for the PolyTiter endpoint dilution to be lower than the serial dilution titer, but only for three of the four patterns evaluated (i.e., centromere, nucleolar, and speckled vs. homogeneous) using a chi-square test of heterogeneity ($\chi^2_{3df} = 19.22$, $P < 0.001$).

To statistically compare the observed proportion of samples within ± 2 dilutions with respect to the proportion of those specimens having a PolyTiter system endpoint dilution higher than the serial dilution titer vs. lower than the serial dilution titer, a one-sample test for binomial proportions was performed. These results are displayed in Table 3 and show a statistically significant difference overall and in all pattern types, except for the homogeneous pattern, indicating that

TABLE 2. Summary of dilution endpoint agreement by pattern type (proportions with 95% confidence interval)

Pattern type	Equal titer endpoint	1-dilution endpoint agreement	2-dilutions endpoint agreement
Homogeneous	38.46% (27.66–50.17%)	74.36% (63.21–83.58%)	94.87% (87.39–98.59%)
Speckled	60.00% (45.18–73.59%)	76.00% (61.83–86.94%)	94.00% (83.45–98.75%)
Nucleolar	50.00% (29.12–70.88%)	62.50% (40.59–81.20%)	91.67% (73.00–98.97%)
Centromere	30.00% (11.89–54.28%)	50.00% (27.20–72.80%)	75.00% (50.90–91.34%)
All	45.35% (37.76–53.10%)	70.35% (62.92–77.06%)	91.86% (86.72–95.48%)

TABLE 3. Summary of 2-dilution endpoint variance by pattern

Pattern type	Observed proportion of No. (+) to total no. within 2 dilutions	Expected proportion of No. (+) to total no. within 2 dilutions	Z-statistic	P-value for testing H (0): p ₀ = 50% vs. p ₀ ≠ 50%
Homogeneous	27/48 = 0.562500	24/48 = 0.50	0.8660	0.3865
Speckled	2/20 = 0.100000	10/20 = 0.50	-3.5777	< 0.001 ^b
Nucleolar	2/12 = 0.166667	6/12 = 0.50	N/A	0.0386 ^{a,b}
Centromere	2/14 = 0.142857	7/14 = 0.50	N/A	0.0129 ^{a,b}
Total	33/94 = 0.3511	47/94 = 0.50	-2.8880	0.0039 ^b

^aExact binomial probability.^bStatistically significant at $\alpha = 0.05$.

N/A, not applicable.

the PolyTiter system had consistently lower titers as compared to the serial dilution method.

DISCUSSION

To interpret an IFA-ANA test, an experienced operator uses a fluorescent microscope to analyze the intensity and pattern of fluorescent staining. Positive specimens exhibit more intense fluorescence than an established threshold, as well as a distinct staining pattern. The intensity of fluorescent staining is usually expressed as an endpoint titer. A very high titer is likely to be significant, but a low or absent titer cannot rule out the possibility of an autoimmune disorder. For example, high titers of homogeneous pattern are more strongly associated with SLE than with other diseases and correlate with SLE activity (6). Results from ANA testing may also be integral constituents of various ANA disease profiles, or suggest subsequent testing to be performed.

IFA-ANA is burdened by several disadvantages, including variations due to differences in microscopes, the influence of the quality of the optics used, subjective interpretation, high cost of reagents, labor-intensive procedures, requirements for highly trained personnel, little or no automation, and non- or semiquantitative results (2,7). Efforts to reduce these problems include procedural improvements, increased operator training, limitations to control operator fatigue, and automation for such things as slide preparation. ANA-EIA kits have recently been developed in an effort to increase objectivity and reproducibility, and make testing easier to automate. However, fluorescent-staining patterns cannot be determined in this method (8).

In this series, by using the PolyTiter system instead of sample titration, and including the use of the four calibrators in each test run, 75% fewer wells are required. However, we must consider that in a normal routine series, an average of 80% of the samples are negative and do not need further titration. Therefore, the total reduction of test wells would be approximately 42%. Accordingly, the same reduction applies

to the test cost per patient, which in this study translated into a cost reduction of \$1.92 to \$1.11 (U.S. dollars) per patient. While the savings in technologist time were not quantified in this study, it is clear that the amount of time saved is relative to the workload. Furthermore, it is estimated that the turnaround time would drop at least by 50%, since titration of positive samples is usually performed on subsequent days.

The PolyTiter system demonstrates agreement with the conventional serial dilution method in the determination of endpoint dilution. It requires less operator time, fewer reagents and materials, and results in higher throughput and faster turnaround time compared with the conventional method, while continuing to allow for pattern-staining identification. It may also enhance objectivity and reduce operator-to-operator variation; however, data from the present study did not permit this analysis. Further studies are needed to explore the possibility of additional benefits of the PolyTiter system.

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