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Performance Characteristics of the PolyTiter Immunofluorescent Titration System for Determination of Antinuclear Antibody Endpoint Dilution

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Conventional screening for circulating antinuclear antibodies (ANA) is generally performed by immunofluorescent (IF) microscopy with a 1:40 dilution of serum. Intensity of IF staining is then semiquantitated by using twofold serial dilutions, where the highest dilution in which staining intensity equals the endpoint control is expressed as an endpoint titer. The PolyTiter Immunofluorescent Titration system (Polymedco, Inc.) facilitates ANA-IF assay (IFA) testing by relating the intensity of IF staining to reference calibrators (defined in PolyTiter units), providing an endpoint titer directly from a 1:40 dilution. This study was conducted to assess the performance characteristics of the PolyTiter system. Two technologists each evaluated 10 replicates of three specimens and two controls on five sequential days. Endpoint dilution agreement (defined as ±2 dilutions) with the reference was 100% for all controls and for all specimens by one technologist. The second reader reported agreement of 98, 88, and 100% for the low, medium, and high specimens, respectively. Analysis of PolyTiter unit values yielded between-reader, between-run, and within-run precision coefficients of variation of less than 10%. The variance component in the lot-to-lot analysis was zero, indicating all of the variation was due to run-to-run differences. Overall endpoint dilution agreement between PolyTiter and serial dilution in the evaluation of 125 specimens at three sites was 90, 93, and 86%. Pattern identification with the PolyTiter was similar to that with serial dilution. The PolyTiter system demonstrates acceptable performance for routine ANA-IFA testing in the clinical laboratory.

The detection of circulating antinuclear antibodies (ANA) in human serum is an important tool in the investigation of several autoimmune diseases, the most frequent of which is systemic lupus erythematosus. The most widely used and accepted technique is the ANA immunofluorescent assay (ANA-IFA), which detects a wide range of autoantibodies to nuclear and cytoplasmic antigens. An initial screening for ANA, while not definitive, is useful in detecting systemic rheumatoid disorders. When positive, this assay may provide two key pieces of diagnostic information on which follow-up testing may be based: the intensity of the staining and an identifiable immunofluorescent pattern (2, 3, 4, 5, 6, 8, 9). Conventional ANA-IFA screening is generally performed using a 1:40 dilution of serum. The intensity of immunofluorescent staining is then semiquantitated using twofold serial dilutions. The highest dilution at which the intensity of the staining is equal to the endpoint control is usually expressed as an endpoint titer.

The PolyTiter Immunofluorescent Titration system (PolyTiter system; Polymedco, Inc.) was developed to facilitate ANA-IFA testing by providing an endpoint titer directly from a 1:40 dilution. Based on the principle that the intensity of the fluorescence on the ANA slide is related to the dilution endpoint of the specimen, the PolyTiter allows the technologist to regulate the light source in a linear manner so that the intensity of specimen staining can be related to the intensity of established calibrators. By controlling the illumination of an IFA slide in a manner parallel to specimen serial dilution, the system supports establishment of the titer from a single dilution. Unlike automated systems, in which all available light is measured, the technologist decides which features on the slide are to be evaluated by looking at the selected object (cytoplasm, nucleoli, etc). The ability of the user to change focus or objectives is unaffected, and the function of the microscope is unchanged regardless of whether the system is turned on or off. Therefore, the system does not require a dedicated fluorescent microscope. As with conventional ANA testing, the PolyTiter system is technician dependent, allowing the detection and characterization of specimen patterns.

This study was conducted to assess the performance characteristics of the PolyTiter system.

MATERIALS AND METHODS

PolyTiter system. The PolyTiter system consists of hardware (filter unit and control pad), calibrator solutions, and software. The filter unit, under the control of a stepper motor, is attached to the microscope using an adapter between the lamp housing and the body of the microscope or between the stage and eyepiece. The control pad attaches to the filter unit and allows the operator to increase or decrease the amount of light illuminating the slide in a stepwise manner while quantifying the corresponding level of illumination in units of 0 to 100 (Fig. 1).

The PolyTiter ANA calibrators are dilutions of positive sera characterized against known reference material. Prediluted calibrators are provided with endpoint titer values of 40, 160, 640, and 2,560 and are assayed along with 1:40 dilutions of kit controls and patient specimens using the HEp-2 assays routinely used for ANA-IFA. The technologist then determines the PolyTiter unit value for each calibrator, control, and patient specimen. The operator selects a representative field under the microscope and, while visually focusing on the fluorescent staining of the cells, adjusts the light via buttons on the control pad to the point where the pattern of fluorescence is barely visible. This degree of light attenuation is indicated by a corresponding PolyTiter unit value.

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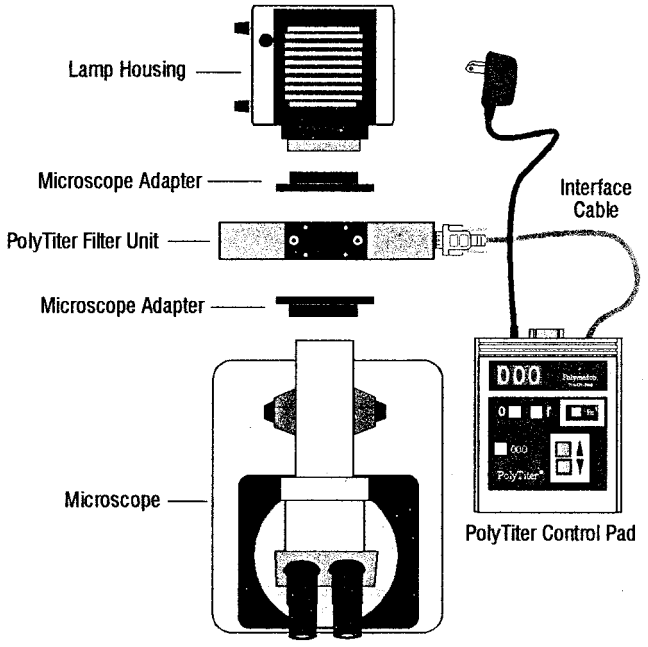

FIG. 1. PolyTiter system hardware.

A calibrator curve is plotted from the assigned calibrator concentrations (*y* axis) as a function of their corresponding PolyTiter values (*x* axis). The curve is either drawn point to point by the software or plotted by hand. The PolyTiter value for a given patient sample or kit control is located on the plot, and the corresponding titer is read from the graph. Specimens with titers greater than 2,560 may be reported as >2,560 or may be further diluted prior to reassaying. In the case of specimens with mixed patterns, a PolyTiter value for each individual pattern may be determined and the results reported.

The PolyTiter software application was created as an adjunct to the PolyTiter system to automate the generation of the calibration curve, determining control and specimen endpoint dilutions, and providing the results in a printable report format. PolyTiter values may be accepted into the computer software immediately from the control pad or recorded by the operator for input and data reduction after all the sample wells have been evaluated. The PolyTiter system is fully operational without the use of the software.

Reagents and equipment. DiaSorin Anafluor indirect fluorescent antibody tests were used for all ANA assays. Kallestad Laboratories Quantafluor fluorescent tests were included in the analysis comparing two manufacturer's HEp-2 slides. ANA kits were used according to the appropriate manufacturer's instructions. Study IF microscopes included an Olympus BH-Z, a Leitz Diaplan, and an Olympus BX 40F-3.

Analyses. Precision testing was conducted using a specimen set composed of three randomly ordered blind-coded specimens representing low (1:80), medium (1:320), and high (1:1,280) ANA titers. Testing was performed in one run per day by each of two technologists who used the PolyTiter to evaluate 10 replicates of the three specimens on five sequential days, a total of 50 replicates per specimen per reader. Positive and negative controls were included on each of the five slides each day, for a total of 25 replicates per reader. One reader performed all testing with one lot of ANA calibrators; the other reader included calibrators from two additional lots each day and read the result of testing for each replicate from three separate calibrator curves.

The average of three PolyTiter values per well was used to determine the titer from which overall agreement (defined as ±2 dilutions) was made against the reference result. Percent agreement was calculated for each set of replicate specimen and control values. In addition, a nested-components-of-variance approach was used to evaluate reader-to-reader, between-run (day), and within-run (day) precision using the average PolyTiter unit value and the first PolyTiter value only (single read). To evaluate lot-to-lot precision, the pooled estimate of the between-day variation across lots for percent agreement, defined as $(SD^2_{Lot\ 1} + SD^2_{Lot\ 2} + SD^2_{Lot\ 3})/3$ was calculated, and the lot-to-lot between-run (day) precision was determined for each sample using a coefficient of variation (CV) calculation, defined as [(pooled estimate of between-run variation)$^{1/2}$/(mean % agreement across lots)] × 100 or [(individual lot between-run variation)$^{1/2}$/(mean % agreement within an individual lot)] × 100.

A comparison between the PolyTiter and conventional serial dilution in the determination of endpoint titer was conducted at three laboratories, located in California, New Jersey, and Minnesota, using identical 125-member serum specimen sets. Specimens were identified by a commercial source as 90 specimens positive for ANA and 35 negative specimens. Positive specimens included single and multiple patterns, with some rare or unusual patterns as well as some cytoplasmic staining (Table 1). The specimen sets for each site were further divided, with each subset randomly ordered, blind-coded, and designated to be tested by the PolyTiter or by conventional serial dilution.

Positive specimens tested in the conventional serial dilution method were diluted 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1,280 at all sites, with titers reported as the reciprocal of the highest dilution at which a specific pattern of fluorescent staining was observed. At two sites, in the event of significant fluorescence in the 1:1,280 dilution, specimens were reported as >1,280. One site included a 1:2,560 dilution in the serial dilution testing and therefore included reports of 2,560 and >2,560; agreement was defined as ≥1,280 for these specimens. All specimens and controls tested with the PolyTiter system were diluted 1:40. Three PolyTiter readings were performed in each well using the five-step-mode function; thus, PolyTiter readings could range from 0 to 100 in units of 5. Endpoint titer reports of negative, 40, 80, 160, 320, 640, 1,280, 2,560, and >2,560 were possible with the PolyTiter system. Endpoint agreement between the two methods was defined as ±2 dilutions.

To evaluate the method comparison, the exact binomial probabilities derived from a one-sample binomial test and corresponding 95% confidence intervals (CI) were computed for the positive and negative samples by site. The null hypothesis tested for each of these comparisons (i.e., positive and negative titer samples) was H_0: $p = p_0$ versus H_1: $p < p_0$, where p is the observed acceptance proportion and p_0 is the expected acceptance proportion (i.e., 95% for positive titer samples and 99% for negative titer samples). Statistical significance was established as $P < 0.05$.

The 90 specimens identified as positive by the commercial source were also tested at one of the sites with a second manufacturer's ANA kit, and the results of each IFA were used to determine the observed proportions for both assays. A one-sample test for binomial proportions using a one-sided alternative was used for the statistical comparisons. The null hypothesis tested for each of the statistical comparisons was H_0: $p = p_0$ versus H_1: $p < p_0$, where p is the observed

TABLE 1. Specimen set *(n = 125)* identified by the commercial source for comparison of PolyTiter and serial dilution for determination of endpoint titer

Parameter	No. of specimens
Specimen type	
Negative	35
Positive	90
Staining pattern of positive specimens	
Speckled	28
Homogeneous	25
Nucleolar	7
Rim	3
Centromere	3
Golgi body	2
Mitochondrial (AMA)	1
Mixed	21
Staining pattern of mixed specimens	
Speckled & nucleolar	7
Homogeneous and nucleolar	2
Homogeneous and speckled	1
Homogeneous and spindle	1
Speckled and cytoskeletal	2
Speckled and mitochondrial	3
Centromere and mitochondrial	1
Centromere and cytoskeletal	1
Nucleolar, centromere, and mitochondrial	1
Nucleolar, homogeneous, and speckled	1
Homogeneous, rim, speckled, and cytoskeletal	1

TABLE 2. Precision testing of PolyTiter unit values

Specimen type and run	Avg of three PolyTiter reads/well					Single PolyTiter read/well				
	Reader 1 (avg)	Reader 2 (avg)	Overall (avg)	Source of variation	CV (%)	Reader 1 (avg)	Reader 2 (avg)	Overall (avg)	Source of variation	CV (%)
Low			40.84					40.41		
Run 1	36.17	43.27		Between-reader	5.48	35.50	41.60		Between-reader	5.70
Run 2	39.83	43.53		Between-run	3.98	39.50	43.40		Between-run	3.59
Run 3	39.83	42.47		Within-run	3.80	40.50	42.20		Within-run	6.24
Run 4	41.50	40.20				40.00	40.40			
Run 5	38.50	43.07				38.00	43.00			
Avg	39.17	42.51		Total	7.76	38.70	42.12		Total	9.18
Medium			55.77					55.62		
Run 1	49.00	58.00		Between-reader	6.33	49.00	58.60		Between-reader	6.88
Run 2	56.50	56.73		Between-run	4.75	56.00	56.60		Between-run	4.30
Run 3	55.83	58.60		Within-run	3.31	55.50	59.00		Within-run	4.46
Run 4	54.83	60.40				54.00	60.00			
Run 5	49.50	58.33				49.50	58.00			
Avg	53.13	58.41		Total	8.58	52.80	58.44		Total	9.26
High			64.41					64.14		
Run 1	66.33	65.87		Between-reader	5.14	66.50	65.20		Between-reader	5.39
Run 2	61.17	66.93		Between-run	2.69	60.50	66.80		Between-run	3.21
Run 3	61.33	67.07		Within-run	1.99	62.00	67.00		Within-run	3.28
Run 4	60.33	66.87				59.00	67.20			
Run 5	60.83	67.33				60.00	67.20			
Avg	62.00	66.81		Total	6.13	61.60	66.68		Total	7.08

acceptance proportion and p_0 is the expected acceptance proportion (i.e., 95% − 10% = 85% for positive titer samples).

RESULTS

The endpoint dilution agreement determined for the 25 replicates of both high and low controls was 100% for both readers, and all 50 replicates of the three precision specimens agreed for reader 2. For reader 1, the replicate titers for the low, medium, and high specimens agreed for 98, 88, and 100% of the known ANA values, respectively, with a between-run CV of 4.6, 20.3, and 0%, respectively.

The results of the precision evaluation using PolyTiter units are provided in Table 2, where the nested-components-of-variance approach yielded a CV of less than 10% for all sources of variation. As expected, the CVs were slightly higher (1 to 2.5%) for a single PolyTiter reading versus the average; however, results remained within acceptable limits, with neither method consistently higher or lower than the other. Furthermore, the variance component in the lot-to-lot analysis was equal to zero for all samples, indicating that all of the variation exhibited was due to run-to-run differences.

TABLE 3. Endpoint titer agreement between the PolyTiter and serial dilution methods for 125 samples

Agreement	% of samples		
	Site 1	Site 2	Site 3
±1 dilution	74	78	67
±2 dilutions	90	93	86
±3 dilutions	95	98	94

Overall endpoint titer agreement within two dilutions between the PolyTiter and conventional serial dilution in the 125 serum specimens was 90, 93, and 86% for the three sites (Table 3). Comparisons of the titer agreement between the two methods by specimen type (positive or negative) as defined by the commercial source produced P values of >0.05, indicating that the two methods yielded equivalent results (Table 4). Using an individual site's identification of specimens with multiple patterns, the proportions across sites for pattern one and pattern two were statistically equivalent (Table 5).

In the evaluation of endpoint titer agreement between the two manufacturer's kits, the observed proportion with the Anafluor kit was 90.0% (81 of 90, 95% CI = 81.9 to 95.3%), and with the Kallestad Quantiafluor kit it was 86.7% (78 of 90, 95% CI = 77.9 to 92.9%). The one-sample binomial test yielded a P of >0.05, indicating that the PolyTiter assay provides equivalent results in the commercially manufactured

TABLE 4. Titer agreement between conventional serial dilution and the PolyTiter system for 125 samples

Specimens	Site	% of samples (no./total)	95% CI (%)	P^a
Positive (n = 90)	1	86.67 (78/90)	77.87–92.92	0.3963
	2	90.00 (81/90)	81.86–95.32	0.1153
	3	81.11 (73/90)	71.49–88.59	0.1857
Negative (n = 35)	1	100 (35/35)	90.00–100	0.7034
	2	100 (35/35)	90.00–100	0.7034
	3	97.14 (34/35)	85.08–99.93	0.2966

[a] Exact binomial P values for testing H_0: P = 85% versus P < 85% for positive specimens and P = 99% versus P <99% for negative specimens.

TABLE 5. Pattern agreement between the PolyTiter system and serial dilution for multiple patterns as identified by site

Site	Pattern 1		Pattern 2	
	No. of samples/ total (%)	95% CI (%)	No. of samples/ total (%)	95% CI (%)
1	11/11 (100)	71.51–100	9/11 (81.8)	48.22–97.72
2	11/13 (84.6)	54.55–98.08	11/13 (84.6)	54.55–98.08
3	13/14 (92.9)	66.13–99.82	13/14 (92.9)	66.13–99.82

ANA-IFA assays evaluated. The PolyTiter values from the runs testing these 90 specimens in both kits were plotted by hand and the resulting titers were compared to the software-derived values. There were no discrepancies between the two calculation methods.

DISCUSSION

ANA-IFA testing, while problematic, is currently a commonly performed screening test for systemic rheumatic disease. An estimated 6,000 laboratories around the world perform ANA testing, with more than 90% using HEp-2 cell substrates (7). Difficulties with ANA-IFA include subjective interpretation, dependence on technologist experience, substrate fading or fluorescent photo bleaching, lack of standardized procedures, and technologist fatigue (1). However, reagent cost is generally more reasonable than other methods, and physicians are familiar with the reporting format of an endpoint titer and fluorescent staining pattern.

The PolyTiter system eliminates many of the drawbacks of conventional ANA-IFA while preserving its familiar report format. Regulation of the light source to give barely visible fluorescence allows a more objective determination of endpoint titer. Training of new technologists to determine endpoint titers consistently and accurately is more easily accomplished for the PolyTiter system compared to the traditional serial dilution approach. While the identification of staining patterns and the determination of endpoint titers of mixed pattern staining are not affected, the reader must still be properly trained in pattern recognition.

The PolyTiter system demonstrates acceptable precision and compares positively to conventional serial dilution in the determination of endpoint dilutions while using the same equipment and reagents currently used in the clinical laboratory. The ability of the PolyTiter system to supply an endpoint titer from a single serum dilution offers major advantages over conventional ANA titration methods. In addition to the obvious and significant reductions in reagent costs, those laboratories that perform a single dilution screen followed by titration of positives should see improvements in turnaround time using the PolyTiter system. Furthermore, use of the PolyTiter system should result in reduced technologist labor and fatigue.

Additional studies are needed to analyze specific cost reductions and to determine if the PolyTiter system may also offer increased intra- and/or interlaboratory reproducibility.

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