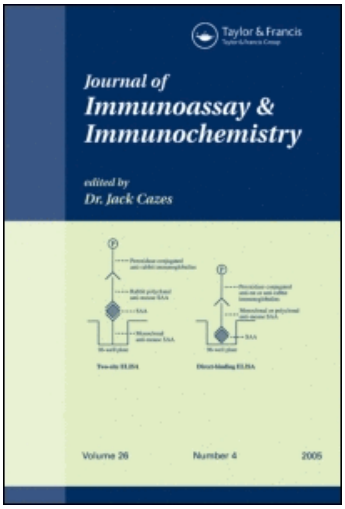

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APPLICATION OF A TIMING PROTOCOL TO THE REDUCTION OF INTER-PLATE VARIABILITY IN THE INDIRECT ENZYME IMMUNOASSAY FOR DETECTION OF ANTI-*BRUCELLA* ANTIBODY

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KEY WORDS: *Brucella*, enzyme immunoassay, assay variability, timing protocol.

ABSTRACT

A simple timing protocol was developed to monitor chromogen conversion in an enzyme immunoassay, performed in microtiter plates, for the detection of antibody to *Brucella abortus* in bovine serum. Application of this protocol decreased the inter-plate coefficient of variation from 28.6% to 6.8% when optical density (OD) values, subsequent to the reaction of a standard antibody reagent, were compared to a static development time. Substantial reductions in variation were also observed for low titered seropositive and for seronegative control reagents. The timing protocol was based on the mathematical relationship of the OD value at 4 minutes of development to a predetermined target OD value (1.0) for a standard antibody reagent. Application of this relationship to the calculation of a variable, final development time eliminated the need for extensive data manipulation and assay calibration.

INTRODUCTION

The end result of any enzyme immunoassay (EIA) technique is the enzymatic generation of a measurable product. In heterogeneous assays, such as the indirect EIA described in this communi-

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cation, all biological reactions occur at the surface of a solid matrix. The majority of these assays are performed in microtiter plates. As well, the majority culminate in the formation of a chromogenic product which is quantified photometrically by means of a through-the-well plate scanner. Several factors have been reported to affect assay performance as ultimately reflected in the formation of the final product. Differential adsorptive and desorptive characteristics of polystyrene microtiter plates may contribute to both inter-plate and intra-plate variability (1,2). Fluctuations in buffer pH and temperature may affect reaction kinetics and stability at one or more stages in the assay procedure (3,4,5,6). Errors in reagent preparation or instability of reagents upon storage may influence assay performance (3). Multiple washing procedures required to remove unreacted or weakly reacting materials may also be subject to variability in efficiency (7,8). Timing of reactions, especially the enzymatic reaction, is a critical factor in reproducibility (6,9,10). The efficiency of reagents employed to stop some enzymatic reactions may be questionable (9,11). Lastly, the stability of some substrates and enzymatic products may also affect assay performance (12,13). Given appropriate quality control, the effects of each of these factors may be minimal; however, the sum total of individual variabilities may result in significant assay variation. This report describes a protocol designed to compensate for inherent variability during the course of performing the assay.

MATERIALS AND METHODS

'Brucella' Antigen

Smooth lipopolysaccharide (S-LPS) was extracted from freeze-dried, heat-killed B. abortus cells (strain 413) by the method of Redfearn as previously described (14). For use in the EIA, freeze-dried S-LPS was reconstituted to 1.0 mg/ml in 0.06 M carbonate buffer, pH 9.6, chilled to 4°C and sonicated at a power setting of 6 watts by six bursts of one minute duration separated by one minute intervals of cooling. After centrifugation at 2000 x g for 10 minutes at room temperature (RT), the supernatant was stored in 1.0 ml amounts at -20°C until required.

Standards and Quality Controls

A pool of high titered bovine sera was selected as a standard antibody reagent. After repeated testing by the EIA protocol described below, it was determined that this reagent, at a dilution of 1:1,000, would yield an average optical density (OD) of 1.0 units given a uniform substrate development time of 10 minutes. A pool of medium titered bovine sera was selected as a seropositive quality control reagent and a pool of sera from a Brucella-free herd was selected as a seronegative quality control reagent. Quality control sera were tested at a dilution of 1:100. The standard, diluted 1:10 in 0.01 M phosphate buffered saline (PBS) and the quality control reagents, undiluted were stored in 1.0 ml amounts at -20°C. A third quality control reagent consisted of diluent buffer only (no serum).

Conjugated Antiglobulin

Rabbit anti-bovine IgG (H+L) conjugated with horseradish peroxidase (Cappel Laboratories, lot 13970) was used at an optimal working dilution of 1:2,000. The optimal working dilution was that dilution which, when reacted with 200 µl of bovine IgG (500 ng/ml) passively absorbed to a microtiter well, resulted in chromogen conversion equivalent to 1.0 OD units after 10 minutes of development.

Substrate/Chromogen

A substrate/chromogen solution of 4.0 mM H_2O_2 plus 1.0 mM ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)] in 0.05 M citrate - NaOH buffer, pH 5.0 was prepared fresh daily from separate stock solutions of 3% H_2O_2 (~880 mM) and 40 mM ABTS.

EIA Protocol

General purpose, flat bottom, 96 well, polystyrene microtiter plates (Linbro/Titertek, lot 76301036) were prewashed with distilled water before a working dilution of S-LPS antigen (1.0 µg/ml) in 0.06 M carbonate buffer, pH 9.6 was dispensed in 200 µl volumes to all wells. The plates were sealed and incubated overnight (~18 hours) at RT. In quantities sufficient for the day's assay, the standard and quality control sera were diluted in 0.01 M phosphate buffered saline, pH 7.2 plus

0.05% Tween 20 (PBS-T) and maintained at RT. Immediately prior to sample application, the antigen-coated plates were washed four times with PBS-T using a 96 well microtiter plate washer (Flow Laboratories). Each microtiter plate was divided horizontally and vertically into four by six well quadrants. Standard and quality control samples in 200 µl volumes were applied to single wells in each of the 4 quadrants. Samples in diagonally opposed quadrants were considered to be duplicates for the purposes of analysis. Forty bovine test sera, each at a dilution of 1:100 (data not presented), were assayed as diagonally opposed duplicates in the remaining wells of each plate. The plates were sealed and incubated for 3 hours at RT. The conjugated antiglobulin was diluted (1:2,000) in PBS-T and maintained at RT and after the plates were again washed four times with PBS-T, the conjugated antiglobulin was applied in 200 µl volumes to all wells. The plates were then sealed and incubated for one hour at RT. After each plate had again been washed, the substrate/chromogen solution, prepared and maintained at RT, was applied in 200 µl volumes to all wells and each plate was immediately transferred to a microtiter plate shaker. Chromogen conversion at RT was measured photometrically at 414 nm using a Multiskan plate reader (Flow Laboratories) which had been previously blanked on 200 µl of substrate/chromogen in a separate microtiter plate which had been prerinsed with PBS-T.

Timing Protocol

The enzymatic reaction was timed with a stop watch. Four minutes after the delivery of substrate/chromogen, the optical density (OD) values of all wells were recorded and the mean amount of chromogen conversion in the four wells containing the standard was determined. An approximate estimation of the development time required to achieve an OD of 1.0 was derived from the following relationship:

$$T_{1.0} = \frac{[OD_{1.0} \times T_4] + C}{OD_4} = \frac{4.7}{OD_4}$$

where $T_{1.0}$ = time to achieve an OD of 1.0 (y min.)

$OD_{1.0}$ = target OD (1.0 units)

T_4 = time of first reading (4 min.)

OD_4 = OD at 4 minutes (x units)

C = correction factor (0.7)

Because of the non-linearity of the kinetic reaction of horseradish peroxidase with its substrate/chromogen, a correction factor (empirically estimated) was required to achieve a close approximation of the final development time. At the estimated final development time, the OD values of all wells were again recorded.

Data Analysis

The Multiskan plate reader was indirectly interfaced with a time sharing minicomputer (Vax 11/780, Digital Equipment Corporation) as previously described (15). Two custom programs were

used to read and store the raw data output from the Multiskan and to calculate from the raw data file, the mean, standard deviation and % coefficient of variation for each of the sample duplicates. Statistical and curve fitting programs on the Agriculture Canada computer system were then applied to the duplicate values and final development times.

RESULTS

Timing Protocol

The relationship between the OD of the standard at 4 minutes and the final development time required to achieve an OD of 1.0 is shown in Figure 1. From least-squares curvefit analysis of 84 data pairs, the final development time was found to be represented by a hyperbolic curve as a function of the OD at 4 minutes and defined by the following equation:

$$Y = A + (B/X)$$

where Y = final development time (min.)

X = OD of standard at 4 minutes

A = 0.1394

B = 4.5939

The line defined by this curvilinear equation is superimposed upon the scatter plot shown in Figure 1.

The frequency distribution of final development times for 84 test plates is shown in Figure 2. Between 4 and 6 test plates were run on any given day. Final development times seldom varied

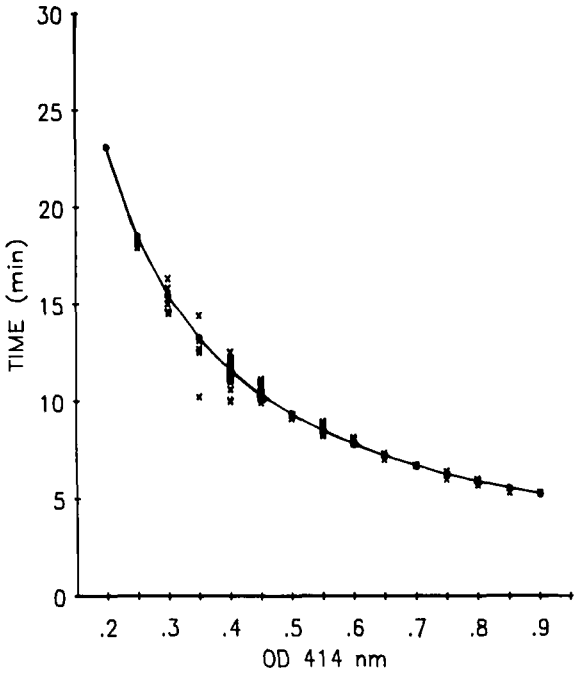

FIGURE 1. Final development time as a function of the optical density of the standard at a 4 minute development time for 84 test plates. Median value of optical density class interval shown on abscissa. Solid line through closed circles (●) defined by curvilinear regression.

by more than 1.5 minutes from the mean development time for the day. The mean development time for the 84 plates was 10.2 ± 2.7 minutes.

Inter-plate Variation

The inter-plate variation of the standard and of the 3 controls are compared in Table 1. The mean OD of the standard was 1.02 ± 0.07. The percent coefficient of variation of the stan-

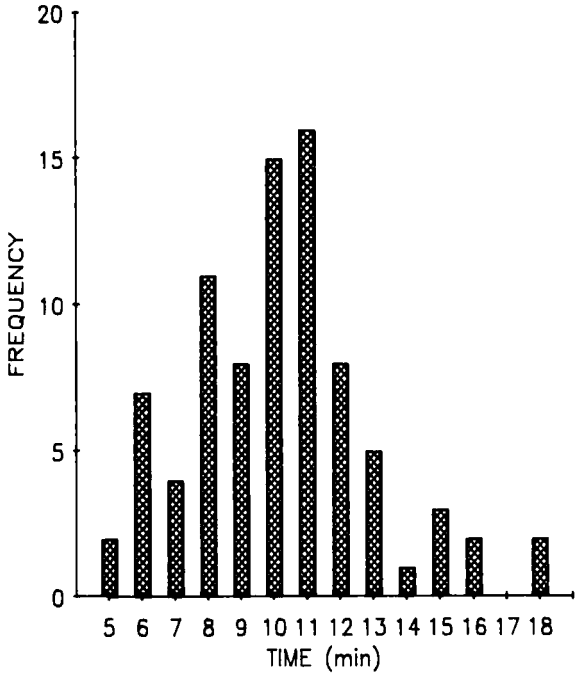

FIGURE 2. Frequency distribution of final development times for 84 test plates as defined by the timing protocol. Median value of development time class interval shown on abscissa.

dard was reduced from 28.6% at the 4 minute development to 6.8% by the timing protocol. Based on the ratio of the average OD of the standard at an average development time of 10.2 minutes to the average OD at 4 minutes (2.08), the OD values at the 4 minute reading were extrapolated to the expected values at a 10.2 minute development time. Actual values at a static final development time of 10 minutes were not recorded so as not to interfere with the plate shaking of the timing protocol. The expected mean OD of the standard at a static development time of 10.2 minutes was

TABLE 1.

Inter-plate Variation of Optical Density Values at a Static Development Time of 4 Minutes Followed by Application of the Timing Protocol.

Protocol[a]		STD[c]	PC	NC	BC
STATIC	N[b]	84	84	84	84
	$\bar{X}$	0.49	0.14	0.09	0.05
	SD	0.14	0.04	0.04	0.02
	%CV	28.6	28.6	44.4	40.0
TIMING	N	84	84	84	84
	$\bar{X}$	1.02	0.27	0.13	0.06
	SD	0.07	0.03	0.03	0.02
	%CV	6.8	11.1	23.1	33.3

a optical densities recorded at a static development time of 4 minutes followed by a final reading as determined by the timing protocol.

b N, number of plates; $\bar{X}$, mean optical density; SD, standard deviation; %CV, percent coefficient of variation.

c STD, standard; PC, seropositive control; NC, seronegative control; BC, buffer control.

also 1.02; however, the standard deviation was 0.30. The expected percent coefficient of variation of the standard was then 29.4%. The frequency distributions of observed OD values of the standard using the timing protocol and the expected OD values at a static development time of 10.2 minutes are compared in Figure 3. The OD values of the standard appeared to be far more normally distributed about the mean with the timing protocol than the expected OD values at a final, static development time.

The percent coefficient of variation of the seropositive control was reduced from 28.6% at the 4 minute development to 11.1% by the timing protocol of the standard. The variation of the seronegative control was reduced from 44.4% to 23.1% and of the buffer control from 40.0% to 33.3%. The same criteria used to extrapolate the OD values of the standard to a static development time of 10.2 minutes were not applicable to the controls. However, the timing protocol was effective in reducing the standard deviation from 0.04 to 0.03 OD units for both the seropositve and the seronegative controls. A reduction in the standard deviation was also observed for the standard (from 0.14 to 0.07 OD units). The standard deviation of the buffer control was not reduced but remained constant.

Intra-plate Variation

The mean intra-plate variation of the standard and of the 3 controls are compared in Table 2. The grand mean represents the

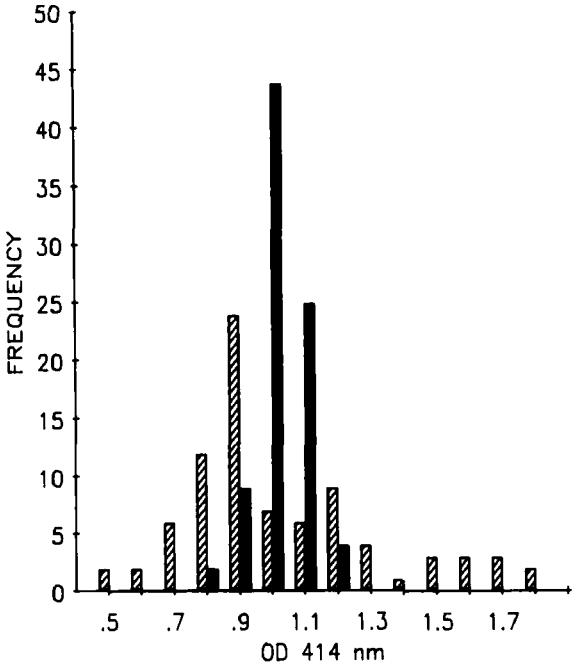

FIGURE 3. Frequency distribution of optical density values of the standard. Median value of optical density class interval shown on abscissa. Hashed bars represent expected values at a static development time of 10.2 minutes. Solid bars represent actual values derived from the timing protocol.

mean of 168 duplicates on 84 plates, as described for the sample placement configuration. Standard deviations and percent coefficients of variation were calculated for each duplicate at the final development time as described in the timing protocol. The mean percent coefficient of variation was less than 10% for the standard and the seropositive and seronegative controls and less than 20% for the buffer control.

Table 2.

Intra-plate Variation of Optical Density Values of Duplicate Samples After Application of the Timing Protocol.

	STD[e]	PC	NC	BC
No. of Duplicates[a]	168	168	168	168
Grand mean[b]	1.02	0.27	0.13	0.06
Mean Std. Deviation[c]	0.06	0.02	0.01	0.01
Mean % CV[d]	5.9	7.4	7.7	16.7

[a] 168 duplicates on 84 microtiter plates

[b] mean optical density of duplicates

[c] mean standard deviation of duplicates

[d] mean percent coefficient of variation of duplicates

[e] STD, standard; PC, seropositive control; NC, seronegative control; BC, buffer control.

DISCUSSION

Disease surveillance, eradication and import/export programs require the testing of extremely large numbers of serum samples. Although bovine brucellosis in Canada nears eradication, as many as 300,000 dairy cattle are serologically screened in this laboratory, annually. Another 50,000 cattle require additional confirmatory tests for certification of brucellosis-free status. As currently conducted, conventional agglutination and complement fixation tests require reading, interpretation and recording of

test results, manually. This is laborious and creates a significant paper burden. Primary binding assays, such as the indirect EIA are especially attractive because of their potential for automation. Photometric reading and electronic data output, processing and interpretation are key features of this automation. In order to maximize this potential, the quality of data must be examined with respect to assay performance and the necessity for data manipulation prior to interpretation. This study was undertaken to improve the quality of raw data and therefore minimize the requirement for data manipulation.

Inter-plate variability is an inherent problem of microtiter, heterogeneous EIA techniques (13,16). This problem is especially apparent in single serum dilution assays such as the indirect EIA for the detection anti-Brucella antibodies. Several approaches have been taken in order to standardize the expression of data with reference to antibody activity at a single dilution. Activity has been expressed as a positive/negative ratio (16) or a convalescent/acute-phase ratio (17). Optical density values have been normalized with respect to a positive reference serum (13) or transformed into a percentage of a positive reference serum (10). Reference sera have also been used to establish standard curves based on titrations (15,18) or on the kinetics of the enzymatic reaction (9,19). All of these methods require at least three levels of data manipulation if samples are to be tested in replicate.

The majority of indirect EIA techniques for the detection of antibody activity are based on the quantitation of an enzymatic product at a static point in time. We have found that inter-plate variability under these circumstances is unacceptably high. The timing protocol effectively reduces inter-plate variability to a more acceptable level without the need for extensive data manipulation or assay calibration. The flexible development time compensates for inherent factors such as those described in the introduction which are ultimately reflected in the formation of the final product. In addition, the timing protocol does not appear to compromise intra-plate variability even during prolonged development. The timing protocol also facilitates a simple quality control program in that the mean OD values of the seropositive, seronegative and buffer controls can be directly monitored for each plate.

Although OD values are not highly quantitative in the expression of antibody activity, they do provide valid criteria for the determination of seropositive or seronegative status. In addition, OD values are readily applicable to seroepidemiological studies. The timing protocol facilitates a direct comparison of mean OD values of duplicate test samples. Classification of serological status can be derived from the direct comparison of test sample OD values to frequency or percentile distributions of OD values from large reference populations.

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