Quantitation of commercial equine tetanus antitoxin by competitive enzyme-linked immunosorbent assay

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Abstract

In the USA, the potency of commercially prepared equine tetanus antitoxin is determined by the method outlined in the Code of Federal Regulations, Title 9, Part 113.451. In the current test, commercial equine tetanus antitoxin is tested by a toxin neutralization test in guinea pigs. The in vivo test measures antitoxin content through effectiveness of protection of guinea pigs injected with diluted mixtures of antitoxin and a standard toxin. A competitive enzyme-linked immunosorbent assay, designed as an in vitro alternative to the in vivo test, measures antitoxin content based on a competitive reaction between standard or unknown serum and murine monoclonal antibody specific for tetanus toxin. The monoclonal antibody used in the assay delayed death in mouse passive protection studies and reacted with the C fragment of tetanus toxin. No cross-reaction was observed when the antibody was tested with the toxins of Clostridium chauvoei, C. novyi, C. perfringens, or C. sordellii. The in vitro test will measure the antitoxin content of serum samples containing 100–1500 units of antitoxin. Tetanus antitoxin titers obtained by the competitive enzyme-linked immunosorbent assay compared favorably with the toxin neutralization test conducted in guinea pigs. The in vitro assay serves as a feasible alternative to the in vivo test because it can be completed in less time, is reproducible, and eliminates the use of test animals. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Competitive enzyme-linked immunosorbent assay; Equine tetanus antitoxin; Tetanus antitoxin

1. Introduction

Tetanus is a highly fatal, infectious disease of all species of domestic animals caused by the toxin of Clostridium tetani. The neurotoxin of C. tetani is exceedingly potent, but there is considerable variation in susceptibility between the animal species, the horse being the most susceptible. Horses suddenly placed at risk of infection from tetanus are commonly injected with approximately 1500 international units (IU) of tetanus antitoxin [1] for short-term passive immunity and an injection of tetanus toxoid for long-term active immunity.

The potency of equine tetanus antitoxin manufactured for veterinary use by licensees in the USA is tested according to the Code of Federal Regulations, Title 9 (9 CFR), Part 113, Section 451 [2]. As outlined in the regulation, a comparative toxin-antitoxin neutralization test is conducted using a standard antitoxin and a standard toxin. The ability of the antitoxin in the test sample to neutralize the standard toxin, as defined by prevention of tetanic para-
lysis or death of the guinea pigs injected with the toxin-antitoxin mixture, is compared to that of the standard antitoxin.

An indirect ELISA [3], developed by the Center for Veterinary Biologics-Laboratory and used to measure the antitoxin titer of serum from guinea pigs vaccinated with tetanus toxoid, was modified for use in measuring the antitoxin titer of serum from horses hyperimmunized with \textit{C. tetani} antigen. Although the ELISA accurately measures the antitoxin titers of guinea pig serum, it occasionally overestimates or underestimates the antitoxin titers of horse serum. This study describes the development of a competitive enzyme-linked immunosorbent assay (CELISA) that more accurately measures the antitoxin titer of serum from hyperimmunized horses and compares the CELISA results with those obtained by the toxin neutralization (TN) test in guinea pigs.

2. Materials and methods

2.1. Animals

Guinea pigs weighing 340–380 g were purchased from Central Iowa Cavies, Ames, IA, and Harlan Sprague Dawley, Indianapolis, IN.

BALB/c strain mice were obtained from the National Veterinary Services Laboratories (NVSL), Ames, IA.

2.2. Reagents

A crude preparation of tetanus toxin was used as a coating antigen in the CELISA. Briefly, \textit{C. tetani}, Harvard strain, was grown in a 14-l fermenter. The culture filtrate was concentrated using an ultrafilter with nominal molecular mass limits of 10,000 (Millipore Corporation, Bedford, MA). Crude tetanus toxin was inactivated with 0.4% formaldehyde (v/v) and mixed with aluminum potassium sulfate (2.0 mg Al ml$^{-1}$) to prepare the toxoid used for the initial immunization of BALB/c mice for monoclonal antibody production. Tetanus toxin was purified for subsequent injections in mice by preparative isoelectric focusing (Rotofor, Bio-Rad, Hercules, CA). Samples with isoelectric points between 5.5 and 5.7 were collected. Tetanus monoclonal antibody (mAb) 5C10 was produced by the method of Van Deusen and Whetstone [4]. Briefly, adult female BALB/c mice were injected subcutaneously with 0.3 ml of tetanus toxoid followed 4 weeks later with a 0.5-ml intravenous injection of purified toxin.

mAb 5C10 (isotype IgG1), which bound to the C fragment of tetanus toxin and provided passive protection to mice when challenged with tetanus toxin, was selected for use in the CELISA.

mAb 5C10 was conjugated with horseradish peroxidase (Sigma, St. Louis, MO) by the method of Ranger and Brown [5].

Hyperimmune horse serum was obtained from the Colorado Serum Company, Denver, CO, and diluted with normal horse serum (NVSL, Ames, IA) so the mixture contained 500 American antitoxin units (AU) per ml. The antitoxin titer of the mixture was confirmed by conducting TN tests in guinea pigs. The standardized serum was used to define a standard dose–response curve in the CELISA.

2.3. CELISA procedure

Flat-bottom 96-well microtitration plates (Nunc F96, Roskilde, Denmark) were coated with \textit{C. tetani} toxin diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6, and incubated 18–20 h at room temperature in a humidified chamber on an orbital shaker. The toxin was decanted, and blocking buffer (15 mM Na$_2$HPO$_4$, Na$_2$HPO$_4$, 345 mM NaCl, with 0.05% normal horse serum, pH 7.9) was added to each well. The plates were incubated 2.5 h at 35°C on an orbital shaker, then they were washed five times with PBS with 0.05% Tween 20, pH 7.2. Dilutions of the standardized hyperimmune horse serum (500 AU ml$^{-1}$) were made in 100 mM Na$_2$HPO$_4$, Na$_2$HPO$_4$, 345 mM NaCl, with 0.05% normal horse serum, pH 7.9) was added to each well. The plates were incubated 2.5 h at 35°C on an orbital shaker, then they were washed five times with PBS with 0.05% Tween 20, pH 7.2. Dilutions of the standardized hyperimmune horse serum (500 AU ml$^{-1}$) were made in 100 mM Na$_2$HPO$_4$, Na$_2$HPO$_4$, with 50 mM ammonium thiocyanate (NH$_4$SCN), pH 6.0. An initial dilution of 1:6000 was made to generate a 500 AU ml$^{-1}$ standard within the working sensitivity range of the CELISA. From this, incremental dilutions of 4:1, 3:2, 2:3, and 1:4 were made to generate standard samples corresponding to 400, 300, 200, and 100 AU ml$^{-1}$.

Normal horse serum was diluted 1:6000 to generate a 0 AU ml$^{-1}$ standard. Each of the standard serum preparations was added to the blocked plate (100 µl per well, six replicates per dilution). The anti-
The toxin to be tested was diluted in a similar manner, to generate five dilutions per serum sample, and was added (100 μl per well, six replicates per dilution) to the same plate as the standard serum preparation. The plates were incubated 18–20 h with shaking at room temperature in a humidified chamber, then washed as previously described. mAb 5C10 was diluted 1:14 000 in conjugate diluent (15 mM NaH₂PO₄, Na₂HPO₄, 345 mM NaCl, pH 7.2) and added (100 μl per well) to each well and incubated for 90 min at 35°C. After washing five times, 100 μl of tetrathenylbenzadine substrate was added to each well and incubated at room temperature for 10 min. The colorimetric reaction was stopped with 2.5 M sulfuric acid (100 μl per well). The absorbance (λ450 nm) of each well was measured (Molecular Devices, Sunnyvale, CA), and the replicate measurements were averaged. A best-fit linear regression line was calculated using the mean absorbance value for the standard serum dilutions. The mean absorbance values of the unknown serum samples were entered into the equation of the standard serum regression line to estimate the antitoxin titers of the unknown samples. Estimates of antitoxin titers derived from individual dilutions of the unknown sample were averaged to derive a mean estimate of antitoxin units per ml.

The following formula was used to determine the slope and correlation coefficient (r):

\[
\begin{align*}
    y &= \text{OD} \\
    x &= \text{antitoxin unit} \\
    n &= \text{antitoxin levels} \\
    \text{slope} &= \frac{\sum xy - (\sum x)(\sum y)}{\sum x^2 - (\sum x)^2} \\
    \text{y-intercept} &= \frac{\sum y}{n} - \text{slope} \frac{\sum x}{n} \\
    \text{correlation} (r) &= \frac{\sum xy - (\sum x)(\sum y)}{\sqrt{\sum y^2 - (\sum y)^2} \times \sqrt{\sum x^2 - (\sum x)^2}} \\
\end{align*}
\]

Antitoxin units = \frac{\text{unknown OD} - \text{y intercept}}{\text{slope}} \times \text{dilution factor}/6000

3. Results

The best reproducibility was obtained when the mean absorbance of the 0 AU ml⁻¹ standard was 1.5–1.8 and the mean absorbance of the 500 AU ml⁻¹ standard was 0.5–0.8 (Fig. 1). The correlation coefficient of the standard curve routinely was 0.99. Dilutions of the unknown serum samples with absorbance values of 0.5–1.10 were used to determine the average number of antitoxin units per ml; dilutions with absorbance readings outside this range were less accurate for estimating antitoxin titer. If the absorbance value of all five dilutions of an unknown sample was below 0.5, the test was considered invalid. The sample was retested at twofold greater dilutions, and estimations of titer from readings falling in the 0.5–1.10 range were then multiplied by 2. If the mean absorbance values of all five dilutions were above 1.10, the test was also considered invalid. In this case, the dilution factor of serum sample was reduced twofold, and the antitoxin readings calculated from dilutions with absorbance values of 0.5–1.10 were divided by 2.

3.1. CELISA vs. guinea pig TN test

Nine lots of equine tetanus antitoxin produced by five manufacturers and the standard C. tetani antitoxin were tested by the guinea pig TN test and

![Fig. 1. Typical titration curve calculated for the dilutions of tetanus antitoxin ranging from 0 to 500 AU ml⁻¹.](image)
CELISA (Table 1). The percent variation between the two tests ranged from −1% to +9%.

The effect of NH₄SCN on estimation of antitoxin titer was investigated. Four lots of equine tetanus antitoxin were diluted in buffers that did, or did not, contain NH₄SCN, and the antitoxin titers were quantitated by CELISA (Table 2). Higher antitoxin titers were obtained with three of the four lots when they were diluted in buffer without NH₄SCN.

4. Discussion

The CELISA quantitates the amount of tetanus antitoxin in horse serum in a simple, humane manner that compares favorably with results obtained by the guinea pig TN test. The CELISA varied −1% to 9% with the results of the TN test. In our laboratory, the guinea pig TN test routinely varies between 5% and 10%; the CELISA values obtained fall within this range.

While developing the CELISA, it was noted that antitoxin readings of given unknown serum samples could be affected by using different standard sera. Since all of the standard sera used were adjusted to 500 AU ml⁻¹ by the TN test, it was assumed the discrepancy was due to the difference in the affinity of the standard sera, which would be more apparent in an in vitro test. Ammonium thiocyanate has been used to measure the relative affinity of antibodies in other applications [6] by measuring the ability of antibodies to remain bound to specific antigen when treated with high concentrations of NH₄SCN. To reduce the variability introduced into the CELISA by low affinity antibodies in certain horse sera, we incorporated NH₄SCN into the serum diluent at a level sufficient to keep antibodies with very low affinity from binding to the toxin (Table 2) but not affecting the adsorption of toxin to the microtiter plate.

In conclusion, this study indicates that the CELISA would be a suitable replacement for the in vivo test. It is possible that the CELISA could be modified slightly to test serum samples containing lower levels of antitoxin.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Lot no</th>
<th>Serum diluted in buffer with NH₄SCN (AU ml⁻¹)</th>
<th>Serum diluted in buffer without NH₄SCN (AU ml⁻¹)</th>
<th>Guinea pig TN test (AU ml⁻¹)</th>
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*Midpoint between low and high antitoxin values was used for comparison purposes.
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References


