MODIFIED TOXIN-BINDING INHIBITION (TOBI) TEST FOR QUANTIFICATION OF EPSILON ANTITOXIN IN SERUM FROM IMMUNIZED SHEEP

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ABSTRACT - This study evaluated the use of the ToBI test as a substitute for the *in vivo* technique of the mouse serum neutralization test to manage quality control of vaccines against clostridiosis. Serum samples with known concentrations of anti-epsilon antibodies were obtained from 3 sheep immunized with vaccine against *C. perfringens* type D epsilon toxoid. The main results obtained indicate correlation coefficients above 97%, corroborating literature data. The inter and intraplate coefficients of variation were low (less than 1.3%). In conclusion, the *in vitro* ToBI test is suitable to evaluate the potency of clostridial vaccines. However, the ToBI test must be first validated before it can replace the *in vivo* serum neutralization methodology used in mice.

Keywords: Clostridiosis, *in vitro* assay, potency testing.

INTRODUCTION

Enterotoxaemia is the main syndrome caused by *Clostridium perfringens* type D. Soon after the first symptoms appear, it causes sudden death in sheep, goats and cattle subjected to changes in intestinal microbiota caused by drastic feeding changes such as weaning stress (El Idrissi & Ward, 1992). In young animals this disease usually progresses to acute or superacute degrees, a situation that makes treatment more difficult. However, immunization through vaccination can prevent this process. Current licensed vaccines are usually composed by several antigens adsorbed in aluminum hydroxide.

The *in vivo* neutralizing test is considered the standard procedure for determining anti-epsilon antibodies. Although the *in vivo* test is highly sensitive, it is costly, time consuming, and requires many test individuals along with specialized technicians. However, in many occasions, the diagnostic of enterotoxemia is done for a group of animals and lesions observed at post mortem are crucial to diagnose enterotoxemia. Therefore, *in vitro* tests have been used to control the quality of vaccines against tetanus toxoid (USDA , 2006). Besides correlating well with the *in vivo* tests, the *in vitro* procedure reduces the number of animals used in experimentation.

The ToBI test is an *in vitro* technique consisting of the inhibition of toxin binding to antitoxins adsorbed onto a microplate pre-incubated with antibodies obtained from immunized animals (Bonetti, 2002). The ToBI test was first developed to determine the antitoxin titers against tetanus and diphtheria toxins in human sera (Hendriksen et al., 1988). Hendriksen *et al.* (1989) used ToBI to test diphtheria toxoid potency. Later, Hendriksen *et al*. (1991) used this test to estimate tetanus toxoid potency in vaccines.

In spite of the many reports about the utilization of the ToBI test technique to titrate antitoxins induced by vaccinal antigens, it is necessary, in each process, the previous standardization of the technique prior to its application, in order to make it possible to enlarge its utilization in the quantification of different antigens.

The aim of the present study was to evaluate and standardize the use of the ToBI test for quality control of vaccines against with vaccine against *C. perfringens* type D epsilon toxoid. To achieve this,

the ToBI test was compared to the *in vivo* serum neutralization test performed in mice.

MATERIAL AND METHODS

Antibody and Toxin Production

Serum samples with known concentrations of anti-epsilon antibodies were obtained from 3 immunized sheep (Table 1).

The antitoxin used was the 2nd international standard for *Clostridium perfringens* type D epsilon antitoxin (2CpEpsilonAt), obtained from the National Institute for Biological Standards and Control – NIBSC (Potters Bar, United Kingdom). The *Clostridium perfringens* type D epsilon toxin was produced in culture medium as suggested by Brandi (2002), at 37ºC, centrifuged at 200 rpm for 15 h, and standardized using the L+/10 test (USDA, 2006). The toxins were clarified by crossflow filtration, concentrated by ultrafiltration and subjected to inactivation using 37% formaldehyde. After detoxification the suspensions were called toxoids. Both the toxins and the toxoids were produced and provided by the Vaccine Technology Laboratory of Vallée S. A, Montes Claros, Brazil.

Reagents for in vitro tests

To standardize the reagents used in the *in vitro* tests, dilutions of antigen, antibody and antitoxin were evaluated. We selected dilutions that provided absorbance near 1 within a 0 to 2 range, at 492 nm wavelength. The antigen used was the standard toxin with *in vivo* titer of 2560 L+/10/mL, prepared pure and at 1:4, 1:16, 1:64, 1:256 and 1:2560 dilutions. The antibody consisted of serum from rabbits immunized by anti-epsilon vaccine at 5 IU/mL (this titer was obtained through *in vivo* serumneutralization). This was prepared pure and at 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions. The standard NIBSC antitoxin (8 UI/mL) was used pure and at 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions.

Microtiter plates

The culture plate was coated with 100 µL standard NIBSC antitoxin diluted in carbonate and bicarbonate buffer (0.05M and pH 9.6). The plate was incubated for 12 h at 4 °C, washed, blocked with 200 µL of 3% albumin solution and incubated again for 60 min at 37 °C. Subsequently, 100 µL of the standard toxin was added to each well, each row received a different toxin dilution and the plate was incubated for 60 min at 37°C. After being washed, each pair of columns of the plate received 100 µL of the different antibody dilutions (from rabbit serum), and the plate was incubated for 60 min at 37 °C. A volume of 100 µL of rabbit anti-epsilon antibody conjugated diluted in buffer solution was added to the plate. The plate was incubated again for 60 min at 37 °C and washed 6 times with 100 µL washing solution. Finally, 100 µL of enzymatic substrate (OPD-ortophenyl-diamine) was added (10 mg of OPD dissolved in 25 mL citrate buffer 0.15M and pH 5 added to 12.5 µL of hydrogen peroxide 1:1150 from a pure sample). After incubation for 60 min at 37°C, 20 µL of 1M sulfuric acid was added, for 10 minutes.

The last row of the plate served as control for the combined reagents (antitoxin, standard toxin and substrate) and underwent all the processes described, except for the addition of rabbit antibody (Uzal et al., 1997).

Standard curve from rabbit serum

To produce the standard curve, the standard NIBSC epsilon antitoxin was diluted to 5.1; 2.55; 1.275; 0.213; 0.053 and 0.027 IU/mL concentrations. A volume of 50 µL of each antitoxin dilution was added to 50 µL of rabbit serum (50% of the previously standardized dilution of rabbit serum at 1:4) so that both could compete for the epsilon toxin in a dilution plate. Each dilution+serum antitoxin was introduced into a row of the plate after 100 µL of toxin was added to the dilution plate (at a previously determined dilution) and incubated for 1 h.

Two controls were performed in each plate used:
0% inhibition control, in which the standard antitoxin was not introduced into the next to last row in the rabbit serum plate;
100% inhibition control, in which the dilutions of standard antitoxin were introduced only in the last row of the plate, without rabbit serum. This was the site of the least dilution of standard epsilon antitoxin.

The potential of standard NIBSC epsilon antitoxin to inhibit antibody binding to the toxin was expressed in percentage values and used to build a standard curve by correlating inhibition percentage to standard serum (1:4 dilution) potency (IU/mL) (Uzal et al., 1997). The assay was carried out in 4 plates, being 8 times in a same plate.

The inhibition percentage used relative numbers (Uzal et al., 1997) and was calculated as

$$\text{Inhibition \%} = \frac{t \times 100}{z}$$

where:
x = 0% inhibition
y = 100% inhibition
t = x – absorbance of test serum
z = x – y

Antibody titration of sheep serum

The protocol used to determine serum antibody content in immunized sheep with vaccine against *C. perfringens* type D epsilon toxoid, using the modified ToBI test was similar to that used to produce the standard curve, except for the substitution of epsilon, a standard serum, by the test serum of immunized sheep (Uzal et al., 1997). Five different sheep serum samples were tested, total 15 serum samples. To assess test variability, 12 trials were carried out on each serum sample using 4 different plates of a same lot.

RESULTS

In reagent standardization, epsilon antitoxin at 8 IU/mL was efficient in sensitizing the plates after dilution at 1:4 ration. Sheep antirabbit immunoglobulin was conjugated to peroxidase at 1:20.000 dilution, as per manufacturer's recommendations. Thus, both a 1:4 dilution of rabbit immunoglobulin and standard toxin were selected to be used in the modified ToBI test.

The standard curve represented in Figure 1 shows the correlation between standard epsilon toxin and inhibition percentage in the ToBI test plate. The correlation coefficient of the standard curve is 95.67%.

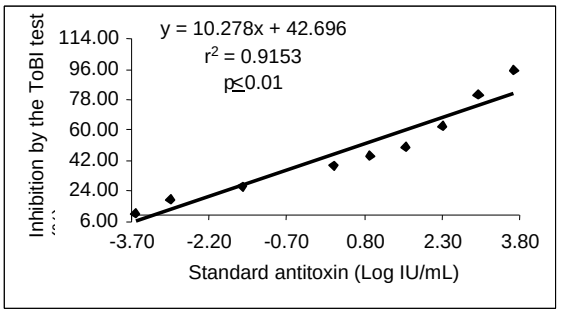

Figure 1. Standard curve indicating correlation between inhibition rate (%) obtained with the modified ToBI test and the logarithm values (Log) of the standard antitoxin.

The inhibition percentage of the modified ToBI test and the respective logarithmic values of epsilon antitoxin in sheep serum are shown in Tab. 1.

The coefficient of variation was lower than that reported in the literature. It ranged from 1.201% to 3.532% intraplate and from 3.779% to 9.717% interpolate for serum of immunized sheep, a nonsignificant difference (F test, alpha >0.01).

DISCUSSION

The results from the standard curve are similar to those of literature reports. The complete inhibition of toxin binding increases absorbance, as shown by the negative control. The complete inhibition was characterized by a decrease in absorbance levels (Hendriksen et al., 1988). The correlation coefficient of the standard is satisfactory for future use of this curve in the titration of sheep serum immunized with clostridial immunogens.

The values of epsilon antitoxin in sheep serum (log IU/mL) were lower than those obtained *in vivo* because a non-purified standard serum was used to bind the toxin. In addition, this serum was not used for *in vivo* test standardization. However, the high inhibition found for the *in vivo* test is in accordance with the percentage values of the standard curve used for serum titration. Therefore, a correlation coefficient of the standard curve close to 100% would better match the antitoxin titers found by the *in vivo* test. This is confirmed by the standard purified antitoxin titer, which is below the expected value of 2 IU/mL, despite its providing 40.04% to 42.23% inhibition.

Fayez et al. (2005) obtained similar results with the ToBI test and mouse serum neutralization. According to this study, antitoxin values (IU) measured by toxin neutralization and by the ToBI test were 10 and 11 for beta toxin, 5 and 5 for alpha toxin, 5 and 5 for epsilon toxin and 20 and 21 for tetanus antitoxin, respectively.

As shown in Table 1, the results obtained are consistent. Binding inhibition increased linearly with epsilon antitoxin titers in serum samples of immunized sheep. Sera with the highest epsilon antitoxin titers had the maximum inhibition rate in all the plates analyzed, whereas an inverse effect was obtained for the lowest titers of epsilon antitoxin.

Corroborating results from other studies, the correlation coefficient between results on the ToBI test and mouse serum neutralization was higher than

Table 1. Inhibition rate (%) of the modified ToBI test, with the respective values of epsilon antitoxin (Log IU/mL) and absorbance values obtained in plates 1 to 4 and the standard deviation (SD) obtained from each point.

Serum sample	Mean A_{492}nm	% Inhibition	Log IU/mL	IU/mL	Log IU/mL	IU/mL	*in vivo* *In vitro*	SD (δ)
	In vitro – Plate 1				*In vivo*			
S1	1.092	37.617	-0.494	0.610	0.693	2	3.279	0.038
S2	1.034	40.896	-0.175	0.839	0.693	2	2.388	0.048
S3	0.892	48.992	0.613	1.846	1.609	5	2.708	0.018
S4	0.887	49.291	0.642	1.900	1.609	5	2.631	0.029
S5	0.651	62.774	1.954	7.057	2.302	10	1.417	0.017
Standard	1.049	40.040	-0.258	0.772	0.693	2	2.590	0.077
Mean	0.934	46.602	0.380	2.171	1.267	4.333	2.502	0.038
	In vitro – Plate 2				*In vivo*			
S1	1.095	37.422	-0.513	0.599	0.693	2	3.339	0.070
S2	1.051	39.935	-0.269	0.764	0.693	2	2.618	0.055
S3	0.877	49.882	0.699	2.012	1.609	5	2.485	0.012
S4	0.868	50.400	0.750	2.117	1.609	5	2.362	0.015
S5	0.623	64.383	2.110	8.248	2.302	10	1.212	0.093
Standard	1.019	41.753	-0.092	0.912	0.693	2	2.590	0.076
Mean	0.922	47.296	0.448	2.442	1.267	4.333	2.368	0.054
	In vitro – Plate 3				*In vivo*			
S1	1.109	36.637	-0.590	0.554	0.693	2	3.610	0.048
S2	1.043	40.368	-0.227	0.797	0.693	2	2.509	0.047
S3	0.870	50.291	0.739	2.094	1.609	5	2.388	0.014
S4	0.867	50.434	0.753	2.123	1.609	5	2.355	0.016
S5	0.623	64.383	2.110	8.248	2.302	10	1.212	0.093
Standard	1.011	42.229	-0.045	0.956	0.693	2	2.590	0.030
Mean	0.921	47.390	0.457	2.462	1.267	4.333	2.361	0.041
	In vitro – Plate 4				*In vivo*			
S1	1.019	41.753	-0.492	0.611	0.693	2	3.273	0.039
S2	1.052	39.849	-0.277	0.758	0.693	2	2.638	0.054
S3	0.868	50.358	0.745	2.107	1.609	5	2.373	0.016
S4	0.867	50.434	0.753	2.123	1.609	5	2.355	0.016
S5	0.623	64.383	2.110	8.248	2.302	10	1.212	0.093
Standard	1.021	41.667	-0.100	0.905	0.693	2	2.590	0.031
Mean	0.908	48.074	0.457	2.459	1.267	4.333	2.343	0.042

97%, confirming that these techniques are equally efficient in detecting neutralizing antibody titers, in contrast to ELISA, which is effective in detecting total antibodies (Hendriksen et al., 1991; Fayez et al., 2005; Weddell & Worthington, 1984). This is essential data that can be used in tests of vaccine potency. The correlation coefficient found is similar to that of 99.4% reported by Fayez et al. (2005), who used serum from sheep immunized against epsilon toxin. A good correlation (96%) was also found when other type of antigens were used, such as the diphteria toxin (Weddell & Worthington, 1984).

The coefficient of variation was lower than that reported in the literature. Fayez et al. (2005) tested 10 samples of different sera at 5 different times and found an inter plate coefficient of variation between 1% and 4%, and interplate coefficient of variation between 0 and 36%.

The benefits of replacing the serum neutralization technique in mice by *in vitro* methodologies include the significantly lower number of animals used in experimentation (Weddell & Worthington, 1984), less animal suffering and a decrease in the costs incurred in the quality assessment of epsilon toxoid and anti-clostridiosis vaccines (Wood, 1991). Moreover, the results were obtained in a few hours, whereas this process requires several days for serum neutralization in mice.

In conclusion, the *in vitro* ToBI test is suitable for evaluating clostridial vaccines because its results have proven to be highly correlated with the traditional *in vivo* metodologies currently employed. Therefore, the ToBI test can be used by vaccine producers and regulatory agencies as an alternative to the serum neutralization test to determine vaccine potency. However, this technique must be previously validated.

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