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Total combining power: Technique for the evaluation of the quality control process of clostridiosis vaccines☆

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ABSTRACT

An efficient technique for evaluation of the quality control of vaccines against clostridiosis is described in this study. This technique is capable of quantifying the toxoid of the bacterium *Clostridium perfringens* Type D, which is commonly found within these vaccines. The described method is performed in vivo to quantify the toxoid, replacing the current predominant approaches that use the titration of toxins before the inactivation process. This method is based on the partial neutralization of a determined dose of antitoxin by testing different doses of the toxoid. In order to guarantee its reliability, it is essential for the technique to be validated. Thus, the technique was tested using the following validation parameters: specificity and selectivity, detection limit, linear correlation, precision and robustness, in agreement with the requirements of regulatory agencies and international committees from around the world. The method was found to be specific, selective, robust, precise, and linear inside a specific concentration range. Therefore, it could be applied to the quality control of clostridiosis vaccines with satisfactory results.

1. Introduction

The bacterium *Clostridium perfringens* Type D is an anaerobic, heterofermentative, Gram-positive toxin producer, and it is an important agent that causes enteric diseases in animals (Lobato and Assis, 2000). *Clostridium perfringens* Type D strains are responsible for enterotoxemia, which mainly affects sheep, but also occurs in goats and cattle, due to changes in intestinal microbiota after the appearance of symptoms. Infected animals may suffer sudden death with heavy economic losses (el Idrissi and Ward, 1992; Songer, 1996; Uzal and Songer, 2008; Sobrinho et al., 2010; Bokori-Brown et al., 2013). Epsilon toxin (Etx) is a pore-forming toxin and is produced by *Clostridium perfringens* strains belonging to toxin types B and D (Bokori-Brown et al., 2011). Alpha and epsilon toxins are manufactured by these microorganisms after inactivation and are present in the composition of veterinary vaccines against clostridiosis (Lobato and Assis, 2000).

Owing to the economic impact caused by enterotoxemia, it is important to develop methods and techniques that successfully prevent such diseases. Quantitative kits allow for the detection and quantification of these toxins or the testing of the potency of the clostridial vaccine (Sobrinho et al., 2011, 2014). Some tests, including the toxin binding inhibition (ToBI) test and enzyme linked immunosorbent assay (ELISA), aim to evaluate the quality of veterinary vaccines in order to replace or minimize the use of laboratory animals (Sobrinho et al., 2010). Additionally, there are similar alternatives that have been developed with the same purpose (Sobrinho et al., 2009).

Variable classification is essential in order to analyze the results obtained in this study. Variables can be subdivided into two categories: continuous variables and discrete variables. Continuous variables are those that are measured and can therefore take any value within an

Abbreviations: International antitoxin unit (I.U.), The quantity of epsilon antitoxin that reacts with Lo and L+ doses of standard toxin according to their definitions; Lo dose, The largest quantity of toxin that can be mixed with one-tenth unit of standard antitoxin and not cause sickness or death when injected into mice; L+ dose, The smallest quantity of toxin that can be mixed with one-tenth unit of standard antitoxin and cause death in at least 80% of injected mice; Standard epsilon antitoxin, The epsilon antitoxin preparation, which has been standardized per antitoxin unit on the basis of the International *Clostridium perfringens* Epsilon Antitoxin Standard; Standard epsilon toxin, The epsilon toxin preparation standardized according to the procedure of the Code of Federal Regulations; Epsilon toxoid, Formaldehyde-inactivated *Clostridium perfringens* Type D epsilon toxin.

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interval, whereas discrete variables are those that are counted and therefore have a finite possibility of values (Sampaio, 1998).

However, it is necessary to have a validated method capable of evaluating the quality of inactivated antigen. This technique makes it possible to evaluate antigen production. The literature has little information about toxoid in vivo quality control, but makes reference to the technique known as total combining power [TCP].

The TCP test involves partial neutralization of a fixed dose of antitoxin with a series of variant doses of the toxoid being tested. The antitoxin which does not react with the toxoid is then mixed with a fixed dose equivalent to half of the dose of antitoxin used, and all series of mixings are injected in mice that are observed for two days (Walker et al., 1979).

The objective of this study is to develop and validate a technique for the quality control of epsilon toxoid in order to allow improved production of a toxoid vaccine (Brandi et al., 2014). Additionally, we sought to determine the concentration of the toxoid after inactivation in order to formulate the vaccine appropriately.

2. Material and methods

This work is in accordance with the ethical principles of animal experimentation, adopted by Brazilian College of Animal Experimentation Colégio Brasileiro de Experimentação Animal (COBEA) and was approved by the Commission of Ethics in Animal Experimentation - Comissão de Ética em Experimentação Animal (CETEA) of the Universidade de São Paulo - USP - Institute of Biomedical Sciences, registered with number 117, page 36 of 2nd book.

2.1. Quantification of Clostridium perfringens Type D epsilon toxoid

The quantification of the epsilon toxoid, which is a formaldehyde inactivated *Clostridium perfringens* Type D epsilon toxin (United States Department of Agriculture, 2006), was made through the antigen affinity to the standard epsilon antitoxin. The measurement was realized by the TCP technique, through indirect titration using two mice per dilution. The quantification of epsilon toxoid was started by serial dilution with factor 4, according to the Table 1. To conduct this technique, in addition to epsilon toxoid, we also used the epsilon toxin diluted for L0 dose that is the largest quantity of toxin which can be mixed with one-tenth unit of standard antitoxin and not cause sickness or death in injected mice (United States Department of Agriculture, 2006), and the L + dose being the smallest quantity of toxin which can be mixed with one-tenth unit of standard antitoxin and cause death in at least 80 % of injected mice (United States Department of Agriculture, 2006). The epsilon toxin preparation was standardized according to the procedure of the Code of Federal Regulations (United States Department of Agriculture, 2006).

Additionally, we use the Standard Epsilon Antitoxin expressed in I.U. mL^{-1}, acquired NCBI. The epsilon antitoxin preparation, which has been standardized as to antitoxin unitage on the basis of the International *Clostridium perfringens* Epsilon Antitoxin Standard (United States Department of Agriculture, 2006).

Table 1
Determination of TCP by epsilon toxoid serial dilution, using a factor of 4, until the dilution of 1:4096 was reached, using peptone saline, at 4 to 8 °C.

Toxoid dilution	1:1	1:4	1:16	1:64	1:256	1:1024	1:4096
Saline (mL)		1.5	1.5	1.5	1.5	1.5	1.5
Toxoid (mL)	2	0.5 (1:1)	0.5 (1:4)	0.5 (1:16)	0.5 (1:64)	0.5 (1:256)	0.5 (1:1024)
Total volume (mL)	2	2	2	2	2	2	2

Afterwards, 0.25 mL of specific standard antitoxin containing 2 I.U. mL^{-1} was added to a tube containing 0.25 mL of each dilution.

Table 2 describes the procedure for determination of TCP. First, diluted toxoid, shown in the second line, was mixed with the standard antitoxin 2 I.U. mL^{-1}, according to the third line of the table. The reaction was carried out over 1 h at 25 °C. Then, 0.25 mL of standard toxin and peptone saline was added to the tube, according to the fifth and sixth lines of the table, respectively. The mixture was then incubated at 25 °C for 30 min.

At the end of the reaction, 0.2 mL of the mixture was inoculated in two mice intravenously. The animals were observed for a period of seven days. At the end of the observation period, the TCP result was calculated based on the dilution at which all mice had died.

Table 2
Determination of TCP through the reaction of the epsilon toxoid with the standard antitoxin (2 I.U. mL^{-1}) and standard toxin (10 L + 10^{-1} mL^{-1})[a], diluted according to Table 1.

Toxoid dilution	1:1	1:4	1:16	1:64	1:256	1:1024	1:4096
Diluted toxoid (mL)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Standard antitoxin 2 I.U. mL^{-1} (mL)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Reaction 1 h at 25 °C							
Standard toxin 10 L + 10^{-1} mL^{-1}(mL)[a]	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Saline (mL)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Total volume (mL)	1	1	1	1	1	1	1

[a] 10 L + 10^{-1} mL^{-1} is the smallest concentration of toxin that induced death in at least 80% of inoculated mice when mixed with 0.1 I.U. mL^{-1} of standard antitoxin.

2.2. Validation of TCP methodology

The procedure for validation of analytical methods has been defined in different work groups of various International Committees and is described in the literature. The International Conference of Harmonization, ICH (ICH Harmonized Tripartite Guideline, 1996), and representatives from industry and regulatory agencies from the USA, Europe and Japan have defined parameters, requirements and, to some extent, methodologies used for the validation of analytical approaches in order to make their use reliable. The actual parameters have been determined by ICH and other regulatory agencies (Huber, 2001), as well as by ANVISA, National Agency of Sanitary Surveillance of Brazil (Brazil 2003). These parameters are: specificity and selectivity, linear correlation, limit of detection, precision and robustness.

The TCP methodology of epsilon toxoid quantification, for process control in manufacturing vaccines against *Clostridium perfringens* Type D, was validated using these parameters. The analyses were performed three times and the results presented are the mean values of each repetition.

For the TCP technique, a standard antibody with concentration of 2 I.U. mL^{-1} was utilized due to the use of a standardized toxin with 10 L + 10^{-1} mL^{-1}, in order to ensure that the presence of a dead animal always indicated the presence of toxoid. Preliminary tests were carried out to confirm the concentration of standard antibodies (2 I.U. mL^{-1} – data not shown).

For the validation steps in this study, Swiss mice weighing 17 to 22 g were used and were intravenously inoculated with 0.2 mL of the test solution.

2.2.1. Specificity and selectivity

Both specificity and selectivity of the TCP technique were evaluated using two groups of animals, the test group and control group. For the test group, animals were inoculated with serial dilutions of solutions containing toxoid, antitoxin and toxin. Two types of controls were used for the control group. For Control 1, solutions containing toxoid, antitoxin and bovine albumin 2% (replacing toxin) were inoculated. Control 2 was the same as Control 1, except that bovine albumin 2% was used, replacing the toxoid. Both controls 1 and 2 were inoculated in serial dilutions. The animals were observed over 4 days. Specificity and selectivity were assessed by comparing the results in the test group with the control group. In the control groups it was expected

that all animals would remain alive, while in the test group the expectation was that animal death would occur for at least one of the dilutions.

2.2.2. Detection limit and linear correlation

The detection limit was defined as the major dilution at which death occurred in animals. The linear correlation was measured using dilution factors of a concentrated reference sample. Five serial dilutions of the reference sample were made (pure, 1:4, 1:16, 1:64 and 1:256). From this pure sample and the other four dilutions, a new series of dilutions (factors 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) was performed, and 0.2 mL of the dilutions was inoculated in each group of two animals with a solution containing toxoid, antitoxin and toxin. The animals were observed for a period of seven days.

2.2.3. Precision

The precision of the TCP method was assessed on the same day (intra-batch precision) and on different days (inter-batch precision), as follows: Intra-batch precision: Animals were inoculated with a solution containing toxin in serial dilutions. This test was repeated three times on the same day. Inter-batch precision: Animals were inoculated with solution containing toxins in serial dilutions. This test was conducted over six consecutive days, using the same sample.

2.2.4. Robustness

The robustness of the TCP technique was evaluated in relation to pH, temperature and time. The following values for these parameters were used: pH: 6.7, 7.2, and 7.7. Temperature: 25 °C and 37 °C. Time: reaction between toxin and toxoid: 40, 60 and 80 min; reaction between antitoxin, toxoid and toxin: 10, 30 and 50 min. All tests utilized two mice per group, and 0.2 mL of the dilutions containing toxoid, toxin and antitoxin was inoculated into animals in each group. The animals were observed over seven days.

2.3. Statistical analysis

Descriptive statistics are reported for the calculated toxoid concentrations along with validation of TCP methodology. Each validation test of specificity and selectivity, linearity, precision, and robustness was performed using three independent experiments and evaluated by linear regression.

3. Results and discussion

3.1. Quantification of Clostridium perfringens Type D epsilon toxoid

A vaccine with a minimum specific amount of epsilon toxoid, that is, having a minimum specific TCP, was obtained from a single type of *C. perfringens*. This vaccine may be able to protect the vaccinated female and its progeny against the challenges caused by *C. perfringens*, independent on any additional lethal toxins produced by such a bacteria, that is naturally present in the environment. This is because of the fact that a vaccine with a good quality should result in a response with a minimum specific epsilon antitoxin in the vaccinated female, and also can protect its progeny (Lobato et al., 2006; Borrman et al., 2001).

Serum neutralization in mice is the standard technique used in the diagnosis of enterotoxemia and allows detection of the epsilon toxin, both in the intestinal contents of affected animals (Lobato et al., 2006) and in supernatants of cultures of *C. perfringens* Type D. Serum neutralization is used in industry and research centers (Brazil, Brazilian Ministry of Agriculture, 1997). However, this technique has disadvantages for use in the process and quality control of vaccines, as once it detects the presence of toxin it is unable to quantify and qualify the inactivated toxin (toxoid) at the end of the clostridial vaccine production process. In this context, the technical method of TCP appears to be a viable alternative for detecting toxoid after the inactivation process occurs.

As used in this study, the term "total combining power" is abbreviated to "TCP" and is defined and described by Batty [Toxin- 5 Antitoxin Assay, Methods in Microbiology, Chapter 8, Volume 5A (1971), ed. JR Norris e DW Ribbons]. In this assay, a specific volume of supernatant containing epsilon toxoid is brought into contact with a known quantity of antitoxins. After a suitable incubation period to allow the antitoxin and epsilon toxoid to bind, for example, 1 h at room temperature, a known amount of epsilon toxin is added. An appropriate time period is then given to allow the remaining free antitoxin to bind with the epsilon toxin, for example, 1 h at room temperature. The amount of free epsilon toxin is then determined for the death of mice. The toxoid can then be quantified by calculating the number of deaths observed in animals. A greater amount of epsilon toxoid present in the test solution indicates a larger amount of free toxin, which can cause death in animals.

Table 3 presents an interpretation of our results. Using the TCP technique, it is possible to identify the toxoid dose capable of neutralizing 0.1 I.U. of homologous antitoxin. In this example, the value of toxoid is 256 TCP in 0.1 mL of the mixture of toxoid plus the antitoxin, because at a 1:256 dilution all mice died and at the following dilution all mice survived.

Table 3
Results from the quantification of epsilon toxin of *Clostridium perfringens* Type D using the TCP technique.

Dilutions	Result[a]
1:4	++
1:16	++
1:64	++
1:256	++
1:1024	oo
1:4096	oo

[a] Interpretation of the result: 256 TCP in 0.1 mL^{-1} or 2560 TCP mL^{-1}. ++, Dead. oo, Alive.

In the standardization of the titration of epsilon toxoid we observed the death of mice given the most concentrated dilutions and the survival of mice given the less concentrated dilutions. Therefore, these events allow the estimation of the epsilon toxoid *C. perfringens* Type D concentration and demonstrate the biological phenomenon observed in the test. Therefore, the TCP technique allows the epsilon toxoid holder of *C. perfringens* Type D in an accurate, sensitive, practical, and prompt manner. Thus, the standardized test shown is a good option for screening culture supernatants of *C. perfringens* Type D used in the industrial production and or experimentation of toxoids. Compared to in vitro methods, the use of this model for in vivo cytotoxicity allows evaluation and study of the biological activity of the epsilon toxoid, which is not possible with other techniques such as ELISA and AGID (Borrmann et al., 2001).

3.2. Validation of TCP methodology

The same results were obtained using the TCP method regardless of whether other specific dilutions were used. The data obtained in this study were characterized as discrete variables because they could only assume certain values within a range. Treating our data as discrete variables for the evaluation of linear correlation, specificity, selectivity, robustness and precision was simpler, and the results remained unchanged in different conditions of analysis. We obtained a linear correlation result of almost 100%.

3.2.1. Specificity and selectivity

The specificity and selectivity of the TCP method for quantifying the epsilon toxin of *Clostridium perfringens* Type D are shown in Table 4. This

table indicates that when the toxin and the toxoid were replaced by albumin solutions at 2% in control groups 1 and 2, respectively, no animal deaths occurred. Nevertheless, in the test group, there were deaths until a dilution of 1024, which represents 10,240 TCP mL^{-1}. For quantitative analysis of the toxin or toxoid, specificity could be determined through the comparison of results from the toxin and toxoid samples and from nontoxic samples. Samples containing albumin were used to demonstrate that other inert substances, which are not of interest, do not affect the results of the test (Brazil ANVISA National Agency of Sanitary Surveillance, 2003). Based on the results obtained in Table 4, it could be verified that the reagents used in the sample matrix did not alter the quantification of toxoid. Other components, such as organic acids and ethanol present within the sample, are theoretically able to affect the sensitivity of the detector which measures the substances and to directly affect the response. In this case, these components were not able to influence the results. The replacement of toxin by albumin, which was used as a control, is a strategy for the analysis of component detection (Brazil Inmetro National Metrology Institute, 2003).

Table 4
Specificity and selectivity tests for TCP mL^{-1} obtained after inoculation of the dilution of epsilon toxin in the control groups and test groups of animals.

Group	TCP mL^{-1}
Control 1 (toxin replaced by albumin)	[a]
Control 2 (toxoid replaced by albumin)	[a]
Test	10,240

[a] Absence of animal death.

3.2.2. Linear correlation of the epsilon toxoid measurement technique by TCP

Fig. 1 indicates the result of the linear correlation of the mean of the epsilon toxoid from *Clostridium perfringens* Type D samples, measured by TCP mL^{-1}, acquired through toxoid dilution, with a dilution factor of 4, evaluating the pure sample until a 1:256 dilution. Linear regression revealed a correlation coefficient of 0.9999, affirming that the TCP technique for measurement of epsilon toxoid shows linearity until a dilution of 1:256. Practically, this result allows the quantification of samples with high concentrations of toxoid by serial dilutions of the sample. The number of points as well as dilution factors of the linear correlation analysis curve was determined as a function of the dilution table used for the toxoid quantification by TCP.

For any quantitative method used in the pharmaceutical field, there is a concentration range of the substance or property values at which that method can be applied. At the lowest limit of the concentration range, the limiting factors are the values of detection and quantification limits. At the highest limit, the limiting factors depend on the response system of the technique utilized for quantification. Within the working range there could be a linear response range, and inside of this linear range the signal response will have a linear relation with the substance or property value. The extent of this range is established during assessment of the method. The linear range of an assay method is the interval between, the lowest and highest levels of concentration of a substance for which precision, accuracy and linearity have been confirmed under specific assay conditions. The linear range is defined as the concentration range at which the sensitivity of the utilized method could be considered constant and normally expressed in the same units for all results (Brazil Inmetro National Metrology Institute, 2003). The results obtained in this work show that the technique used for toxoid quantification gives results directly proportional to the toxoid concentration used within a specific interval (Brazil – ANVISA - National Agency of Sanitary Surveillance, 2003), thus proving the linear correlation of the related technique.

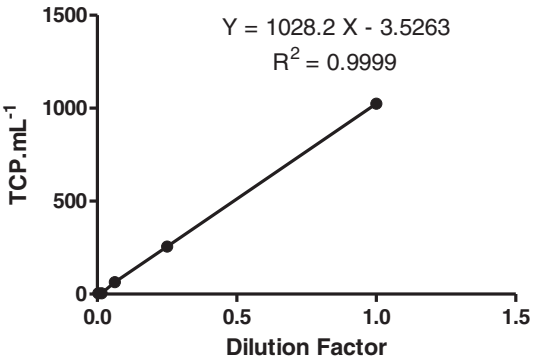

Fig. 1. Correlation between the measurements of *Clostridium perfringens* Type D epsilon toxin activity by TCP mL^{-1} as a function of the sample dilution factor.

3.2.3. Precision

Table 5 shows the precision analysis results of the TCP technique for the samples of *Clostridium perfringens* Type D epsilon toxoid, measured by TCP mL^{-1}, analyzed over a period of six days (inter-batch) and at different time points on the same day (intra-batch). We noticed that in both the inter-batch analysis and the intra-batch analysis, there was no variation in the toxoid title measured by TCP mL^{-1}. Based on the results in Table 5, it is possible to conclude that the toxoid could be quantified in distinct days, until six days, without loss of precision during this period.

Precision is a general term used to analyze the dispersion between independent assays, and is repeated from a single sample, similar samples or patterns, under defined conditions. It is normally determined under specific circumstances of measurement, and the two most common ways to express it are through repeatability and reproducibility, usually expressed using the standard deviation (Brazil Inmetro National Metrology Institute, 2003). In this work, we verified that the toxoid quantification technique gave results with great precision over a series of measurements of multiple sampling from a single sample. There was agreement using this technique between results within a short period of time with the same analyst and same instrumentation. Agreement was also verified between results acquired on different days. These results are in agreement with what ANVISA (Brazil – ANVISA - National Agency of Sanitary Surveillance, 2003) demands for a particular technique to be considered precise.

Table 5
Measurement of TCP of the epsilon toxoid on different days and on the same day.

Inter-batch		Intra-batch	
Days	TCP mL^{-1}	Hours	TCP mL^{-1}
1	10,240	0	10,240
2	10,240	4	10,240
3	10,240	8	10,240
4	10,240	–	–
5	10,240	–	–
6	10,240	–	–

3.2.4. Robustness

Table 6 indicates the results of the robustness analysis of the TCP technique for the samples of *Clostridium perfringens* Type D epsilon toxoid, measured by TCP mL^{-1}, which was analyzed as a function of temperature, toxoid and antitoxin reaction time, standard toxin, toxoid and antitoxin reaction time, and reaction pH. We found that none of the conditions studied caused variation of the toxoid measured by TCP mL^{-1}. Based on the results shown in Table 6, it can be concluded that the sample could be titrated either at 34, 37 or 40 °C; or titrated at pH values ranging from 6.6 to 7.7; or titrated with toxin and toxoid reaction times of 40, 60 or 80 min; or titrated with antitoxin, toxin and toxoid reaction times of 10, 30 or 50 min, all without a variation in the results.

Robustness measures the capacity of the technique to resist small and deliberate external variations, indicating reliability during its normal use. When developing methodology the robustness of variation should be considered because if the method is susceptible to variation across the displayed conditions, these conditions ought to be controlled and precautions should be implemented within the procedure (Brazil – ANVISA - National Agency of Sanitary Surveillance, 2003). Based on the results shown, it can be affirmed that the toxoid quantification technique is robust, because it was practically insensitive to small variations that may occur when this technique is being executed (Brazil - Inmetro National Metrology Institute, 2003).

Table 6
Measurement of TCP of epsilon toxoid at different temperatures, reaction times and reaction pH.

Temperature	TCP mL^{-1}	Reaction time[a]	TCP mL^{-1}	Reaction time[b]	TCP mL^{-1}	Reaction pH	TCP mL^{-1}
34	10,240	40	10,240	10	10,240	6.7	10,240
37	10,240	60	10,240	30	10,240	7.0	10,240
40	10,240	80	10,240	50	10,240	7.2	10,240
–	–	–	–	–	–	7.4	10,240
–	–	–	–	–	–	7.7	10,240

[a] Toxoid and antitoxin reaction times.
[b] Toxin and toxoid and antitoxin reaction times.

4. Conclusion

This study demonstrates that the detection of epsilon toxoid by TCP technique is a viable option to improve the animal bioassays that are normally used in epsilon toxin titration tests.

Despite the fact that it is not possible to consider that this technique is validated because it works with discrete variables, the results obtained in this work show that the TCP technique has specificity and selectivity to the epsilon toxin, precision on the same day and on different days, it has linear correlation across a dilution range, and it is robust in relation to common interference with analytical measurements, such as pH, temperature and reaction times.

Thus, it can be concluded that the TCP technique is applicable to process control in vaccine manufacturing.

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