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Correlation between ToBI test and *in vivo* titration for diphtheria and tetanus

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ABSTRACT

Alternatives to animal testing for quality control of biologicals have been a goal since 1959. Instituto Butantan has been developing such methods for quality control of biologicals for human use (vaccines and hyperimmune equine sera) for the last 13 years. In this paper we compare the modified ToBI test and the *in vivo* seroneutralization test to assess immunogenicity of diphtheria and tetanus vaccines and hyperimmune sera. Data from the last 10 years were statistically analyzed to compare the results for *in vivo* and *in vitro* titrations (diphtheria, $n = 525$ and tetanus, $n = 455$). The agreement between the tests depended on the serum titer range. For both diphtheria and tetanus components, the correlation and concordance coefficient was higher as the serum titer increased. Overall, the *in vitro*/*in vivo* titer ratio did not vary systematically over the range of measurements. These results indicate that although the *in vitro* ToBI test is not completely able to replace the *in vivo* serum titration, it is a useful tool to guide the tests during the production process, which can reduce the number of animals used for lot release.

1. Introduction

The Brazilian Pharmacopoeia requires either the NIH or the WHO method for diphtheria and tetanus vaccine immunogenicity assays, leaving it up to the producers to use their discretion in choosing which method to use, according to economic and practical criteria [1]. While adoption of the NIH method (also known as the L + /10/50 test) expends fewer animals than the WHO method, the advantages of introducing an alternative method such as the ToBI test to assess vaccine immunogenicity are unquestionable. Rapidity, low cost and reproducibility are only a few of many advantages that can be enumerated.

Development of alternative methods for quality control of biologicals has been mandatory during the last years and many efforts have been dedicated worldwide to test development [2] and regulatory aspects [3].

Based on the publication by Hendriksen et al. in 1989 [4], the utility of the ToBI test was validated at Instituto Butantan [5]. Since then, we have standardized and used a modified ToBI test to determine immunogenicity of diphtheria and tetanus vaccines from Instituto Butantan as well as equine sera potency, in order to reduce guinea pig and mouse use. In this paper, we compare the results of serum titration of diphtheria and tetanus vaccines and equine sera from the last 10 years, by *in vitro* modified ToBI and *in vivo* test (L + /10/50).

These data were obtained from the parallel tests performed for lot release and applied the 3Rs concept (Replacement, Reduction and Refinement) for Instituto Butantan's quality control. This current work analyzed vaccines (toxoids, and combined vaccines for adult (dT) and infant use (DT and DTP)) and horse hyperimmune sera. Statistical tools (Lin's correlation and concordance coefficient [6] and Bland–Altman ratio plots [7]) were used to establish the agreement level between the titration methods over three ranges of sera titers (less than 2 IU/mL, from 2 IU/mL to 25 IU/mL and more than 25 IU/mL).

2. Materials and methods

2.1. Animals

Immunization step: guinea pigs (*C. porcellus*, English strain, albino) of both sexes weighing 450–550 g were used. Diphtheria

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component sera titrations (toxin neutralization test) were carried out in guinea pigs (*C. porcellus*, English strain, albino) of both sexes weighing 250–350 g, and the tetanus component was tested in mice (*Mus musculus*, swiss webster strain) weighing 18–20 g. All animals were obtained from Instituto Butantan Animal Facilities, Sao Paulo, Brazil.

Animal tests were performed in accordance to institutional guidelines for animal welfare [8].

2.2. Reagents

Chemicals were obtained from Sigma–Aldrich and Merck, unless otherwise stated. When needed, vaccines and sera were diluted in Phosphate Buffered Saline (PBS) containing 117 mM NaCl, 3.2 mM KH_2PO_4 and 10.4 mM Na_2HPO_4, pH 7.4. Toxoids and equine sera used for the ToBI test were provided by Instituto Butantan's Production Division. Reference antitoxins developed in horses were provided by National Authority (INCQS). Bio-Manguinhos/Fundação Oswaldo Cruz (Rio de Janeiro, Brazil) kindly provided the horseradish peroxidase-labeled equine anti-sera that were diluted in PBS containing Tween 20® 0.05% at 1:7000 (anti-diphtheria conjugate) and 1:5000 (anti-tetanus conjugate) prior to use. All washes in the ToBI test were performed twice with saline solution (0.9%) containing Tween 20® (0.05%).

2.3. Tested products

Diphtheria and tetanus vaccines alone and combined as well as hyperimmune horse sera were used in this study (Table 1). Vaccines were adsorbed onto 0.7 mg_{Al3+}/mL of aluminum hydroxide and hyperimmune sera against diphtheria and tetanus consisted of pepsin fragmented horse $F(ab')_2$ purified by salting-out with ammonium sulfate. All products were prepared and supplied by Instituto Butantan's Production Division.

2.4. Animal immunization

This test was carried out according to the Brazilian Pharmacopoeia [1].

Six guinea pigs (weighing 450 g–550 g) received 0.75 mL of the testing vaccine subcutaneously. After the immunization period (4 weeks for the diphtheria component and 6 weeks for the tetanus component), each animal was bled by cardiac puncture to separate the sera which was pooled for each vaccine lot and kept at −20 °C or less until *in vivo* (L + /10/50) or *in vitro* titration.

Table 1
Vaccines and sera samples used in this study. Since the experiments were independent and happened over the course of 10 years, the sample number for each component varied.

Sample	Sample number for each component assay	
	Diphtheria	Tetanus
Diphtheria toxoid (AnD)	195	–
Tetanus toxoid (AnT)	–	88
Diphtheria and tetanus combined vaccine (adult or infant use) (DT)	117	132
Diphtheria, tetanus and whole cell pertussis combined vaccine (DTP)	169	174
Diphtheria, tetanus, whole cell pertussis and Hepatitis B combined vaccine (DTPHB)	19	38
Diphtheria, tetanus, whole cell pertussis, Hepatitis B and *Haemophilus influenza* b combined vaccine (DTPHBHib)	11	13
Hyperimmune serum	14	10
Total	525	455

2.5. In vivo serum titration

This method was performed according to the Brazilian Pharmacopoeia [1]. Briefly, four solutions (serial dilution factor 1.2) of the serum pool were mixed with one L + /10/50 dose of diphtheria or tetanus toxin (L + /10/50 is the toxin dose that in the presence of 0.1 IU of antitoxin causes morbidity or death in 50% of the animals in 96 h). After incubation at 37 °C for 45 min, each mixture was subcutaneously injected in six guinea pigs (0.75 mL/guinea pig) or ten mice (0.1 mL/mouse) per dilution. The same procedure was done with national standard antitoxin for which concentrations were adjusted in order to have the minimum required titer between the highest and the lowest doses. Typical symptoms or animal death were recorded within 96 h. Effective dose 50 (ED_{50}) values of tested samples and reference antitoxin were determined by Probit analysis by using WHO software for vaccine control for MS-DOS® or Combistats© software version 4.0 for MS-Windows (EDQM). The potency was determined by: (A/B)xC, where: A was the ED_{50} of the tested serum pool, B was the effective dose 50 (ED_{50}) of the standard antitoxin and C was the IU/mL of the standard antitoxin.

2.6. Modified ToBI test

This test was performed as described by Hendriksen et al. [4] with the difference of using toxoid instead of toxin [5,9]. Briefly, the test comprised two steps: (1) seroneutralization and (2) ELISA.

(1) Seroneutralization – plates (96 wells, round bottom) were blocked (overnight, 0.1% bovine serum albumin – BSA). After washing, twofold serial dilution of sera samples (1:4 to 1:512) and reference antitoxin (1.25 IU/mL to 0.00977 IU/mL) (tetanus – anti-TTxd, or diphtheria – anti-DTxd) standards were applied. Toxoid solution at 0.1 flocculating units (Lf)/mL was added, except in negative control wells. The plates were incubated for 1 h at 37 °C.
(2) ELISA - ELISA plates (96 wells, flat bottom, NUNC Maxisorb®) were coated with 0.1 IU/well of anti-TTxd or anti-DTxd equine serum (50 mM carbonate buffer, pH 9.6), incubated overnight at 4 °C and then washed and blocked (0.1% BSA, 1 h, 37 °C). After a second washing, 100 μl of the serum–toxoid mixtures from the seroneutralization step were transferred to the ELISA plates. The plates were incubated (90 min, 37 °C), washed, and then the appropriate dilution of horseradish peroxidase-labelled equine anti-TTxd or anti-DTxd was added. Plates were incubated for 2 h at room temperature, washed, and peroxidase substrate (3,3′,5,5′-Tetramethylbenzidine 0.025 M in 0.11 M acetate buffer containing 3 mM H_2O_2) was added to the wells. The reaction was stopped (sulfuric acid 1 M) and the absorbance (450 nm) recorded in a microplate reader. The antibody titers were calculated by interpolation in MS–Excel or by sigmoid curve model in Combistats© software version 4.0 (EDQM). The average of duplicates was expressed in IU/mL relative to the reference antiserum.

2.7. Statistical analysis

For statistical analysis, the data were divided in three groups according to *in vivo* titer: less than 2 IU/mL (<2 IU/mL), from 2 IU/