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Determination of low tetanus or diphtheria antitoxin titers in sera by a toxin neutralization assay and a modified toxin-binding inhibition test

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Abstract

A method for the screening of tetanus and diphtheria antibodies in serum using anatoxin (inactivated toxin) instead of toxin was developed as an alternative to the *in vivo* toxin neutralization assay based on the toxin-binding inhibition test (TOBI test). In this study, the serum titers (values between 1.0 and 19.5 IU) measured by a modified TOBI test (Modi-TOBI test) and toxin neutralization assays were correlated ($P < 0.0001$). Titers of tetanus or diphtheria antibodies were evaluated in serum samples from guinea pigs immunized with tetanus toxoid, diphtheria-tetanus or triple vaccine. For the Modi-TOBI test, after blocking the microtiter plates, standard tetanus or diphtheria antitoxin and different concentrations of guinea pig sera were incubated with the respective anatoxin. Twelve hours later, these samples were transferred to a plate previously coated with tetanus or diphtheria antitoxin to bind the remaining anatoxin. The anatoxin was then detected using a peroxidase-labeled tetanus or diphtheria antitoxin. Serum titers were calculated using a linear regression plot of the results for the corresponding standard antitoxin. For the toxin neutralization assay, L+/10/50 doses of either toxin combined with different concentrations of serum samples were inoculated into mice for anti-tetanus detection, or in guinea pigs for anti-diphtheria detection. Both assays were suitable for determining wide ranges of antitoxin levels. The linear regression plots showed high correlation coefficients for tetanus ($r^2 = 0.95$, $P < 0.0001$) and for diphtheria ($r^2 = 0.93$, $P < 0.0001$) between the *in vitro* and the *in vivo* assays. The standardized method is appropriate for evaluating titers of neutralizing antibodies, thus permitting the *in vitro* control of serum antitoxin levels.

Key words
- Tetanus
- Diphtheria
- Toxin-binding inhibition test
- Toxin neutralization

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Introduction

In vivo and *in vitro* tests for measuring tetanus and diphtheria antitoxin levels in serum from different species have been standardized and executed for many years. Among the *in vivo* protocols is the classical toxin neutralization (TN) assay in mice or guinea pigs as described by the World Health Organization (1). For diphtheria antitoxin, there is also the neutralization test in microcell culture using VERO (green monkey renal epithelium) cells (2,3). Several *in vitro* serologic methods have been described, such as passive hemagglutination (1,4-6), enzyme-linked immunosorbent assay (ELISA) (1,7-9) and the toxin-binding inhibition (TOBI) test (10,11).

The toxin neutralization assay has been identified as a precise and sensitive test able to detect antitoxin levels as low as 0.001 IU/mL (1,12). On the other hand, this test involves high costs, is time-consuming, and requires specialized personnel (trained to work with toxins), as well as large numbers of animals and significant amounts of serum. The same situation applies to the VERO cell assay, since cell culture tests also are expensive and require specialized personnel as well as special laboratory equipment. Consequently, *in vitro* serologic methods have been developed as alternative approaches to reduce costs and to improve animal welfare.

One of the major goals of controlling the quality of the production of vaccines against tetanus toxoid (TT), diphtheria and tetanus (DT, for infants under 6 years), diphtheria and tetanus (dT, for adults), diphtheria, tetanus and pertussis (DTP, for infants under 6 years) and DTP plus *Haemophilus influenzae* type b (DTP-Hib, for infants under 6 years) is to determine the immunogenicity of the tetanus and diphtheria components. This control is usually performed by TN tests in mice or guinea pigs, as cited above. We have described a modification of the TOBI test for high levels of tetanus antitoxin in serum from horses immunized to obtain antitoxin for therapeutic use (11).

In the present study, we describe another modification of the TOBI test (Modi-TOBI) for detecting low levels of tetanus and diphtheria antibodies in serum as an alternative protocol to TN tests for the control of DT, dT, DTP, and DTP-Hib vaccines. This Modi-TOBI test utilizes inactivated tetanus or diphtheria toxins (anatoxins) instead of the toxins employed in the original method (10), resulting in significant safety improvement for the laboratory staff.

Material and Methods

Animals

Guinea pigs (250-300 g) and Swiss NIH mice (18-22 g) of both sexes were maintained under standard conditions at the Butantan Institute.

Antibodies, anatoxins and vaccines

Standard anti-tetanus and anti-diphtheria antibodies were purchased from the National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK.

Tetanus and diphtheria anatoxins and purified horse polyclonal anti-diphtheria and anti-tetanus IgG were produced at the Butantan Institute (São Paulo, SP, Brazil) as recommended by WHO (13,14). Peroxidase-labeled antibodies were prepared from purified horse polyclonal antibodies (Butantan Institute) according to the protocol described by O'Sullivan and Marks (15) and Nakane and Kawaoi (16).

Samples were analyzed from 17 lots of TT containing 15 Lf/mL, adult use-dT containing 4 Lf/mL diphtheria toxoid, and 15 Lf/mL TT or DTP vaccine containing 15 Lf/mL diphtheria toxoid and 15 Lf/mL TT. The TT, dT, and DTP vaccines were adsorbed onto 0.5 mg/mL aluminum hydroxide. All

samples were taken from the routine production process at the Butantan Institute. All chemicals and reagents used were of analytical grade or equivalent.

Immunization protocol

Guinea pigs (6 per group) were inoculated subcutaneously with half a human immunizing dose of TT, DTP, or dT. The animals were bled 4 weeks later to determine anti-diphtheria antibody titers, and 6 weeks later for anti-tetanus antibody titers. The sera were inactivated and stored at -20ºC. Seventeen samples of sera from guinea pigs inoculated with different lots of vaccine were analyzed.

Modi-TOBI test for the determination of anti-tetanus antibody titers

Flat bottom (Corning Costar, NY, USA) microplates (plate 1, P1) were blocked for 90 min at 37ºC with 200 µL/well PBS containing 0.5% bovine serum albumin (PBS-BSA; Sigma, St. Louis, MO, USA). After washing with PBS containing 0.05% Tween 80 (PBS-T), a 2-fold dilution range of guinea pig serum samples was prepared in duplicate in 100-µL quantities. On each plate, 2.5 to 0.019 IU/mL standard tetanus antitoxin (NIBSC) was titrated to quantitate the unknown serum samples. Subsequently, 100 µL tetanus anatoxin (0.1 Lf/mL diluted in PBS 1% peptone) instead of toxin, as described previously (10), was added to all wells, with the exception of the blank column, according to our previously described modifications (11). These microplates were incubated for 60 min at 37ºC and overnight at 4ºC. A parallel series of plates (Maxisorp® Nunc, Roskilde, Denmark) (plate 2, P2) were coated with 12 µg/mL purified polyclonal horse antitetanus serum (diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4ºC. The next day, these plates were blocked for 90 min at 37ºC with 200 µL per well of PBS-BSA. After washing with PBS-T, 100 µL serum-anatoxin mixtures of P1 were transferred to the corresponding wells of P2 and incubated for 90 min at 37ºC. After a second washing step, 100 µL peroxidase-labeled horse anti-tetanus IgG was added to the wells (10 µg/mL diluted in PBS) and incubated at 37ºC for 90 min. After the incubation step and washing with PBS-T, 100 µL o-phenylenediamine tablets (Sigma) substrate was added in 25 mL 0.15 M citrate-phosphate buffer, pH 5.0, and 5 µL H_2O_2 30% and incubated in the dark for 15 min. The reaction was stopped by the addition of 100 µL 4 N H_2SO_4.

The determination of anti-diphtheria antibodies by the Modi-TOBI test was performed as described for the anti-tetanus assay, but using 10 to 0.078 IU/mL standard diphtheria antitoxin (NIBSC) and 1.0 Lf/mL diphtheria anatoxin on plate P1. Plate P2 was coated with 10 µg/mL purified horse anti-diphtheria serum and assayed with 10 µg/mL peroxidase-labeled horse anti-diphtheria IgG.

Absorbance was measured at 492 nm using a Multiscan EX automatic plate reader (Labsystems Inc., Helsinki, Finland). To obtain maximum absorbance, only anatoxin and PBS were added to a row of wells. The absorbances of the serum sample dilutions with values near 50% of maximum absorbance were plotted on the curve obtained from standard serum antitoxins in order to calculate the titers, using a statistical calculation based on linear regression analysis.

Toxin neutralization (TN assay)

The *in vivo* TN assay was performed according to the protocol described by the WHO (1) at the L+/10/50 level. To determine anti-tetanus antibody titers, different dilutions of guinea pig serum samples were mixed with the L+/10/50 dose of tetanus toxin, incubated for 45 min at 37ºC, and inoculated subcutaneously into Swiss NIH

mice. The L+/10/50 test dose was defined as the amount of tetanus toxin that, combined with 0.1 IU standard tetanus antitoxin (NIBSC), caused death of 18-20 g mice 96 h after injection. Anti-diphtheria antibody titers were determined by mixing different dilutions of guinea pig serum samples with L+/10/50 doses of diphtheria toxin, incubating for 45 min at 37ºC, and subcutaneously injecting them into 250-300 g guinea pigs. The L+/10/50 test dose was defined as the amount of diphtheria toxin that, combined with 0.1 IU standard diphtheria antitoxin (NIBSC), caused the death of 250-300 g guinea pigs 96 h after injection. The mice and guinea pigs were observed for 96 h and all deaths were recorded. Parallel control tests with L+/10/50 doses of toxins mixed with three dilutions of the standard antitoxins (NIBSC) were also performed.

Statistical analysis

Pearson's correlation was calculated for the results obtained by the TN assay and by the Modi-TOBI test, with the level of significance set at 5%.

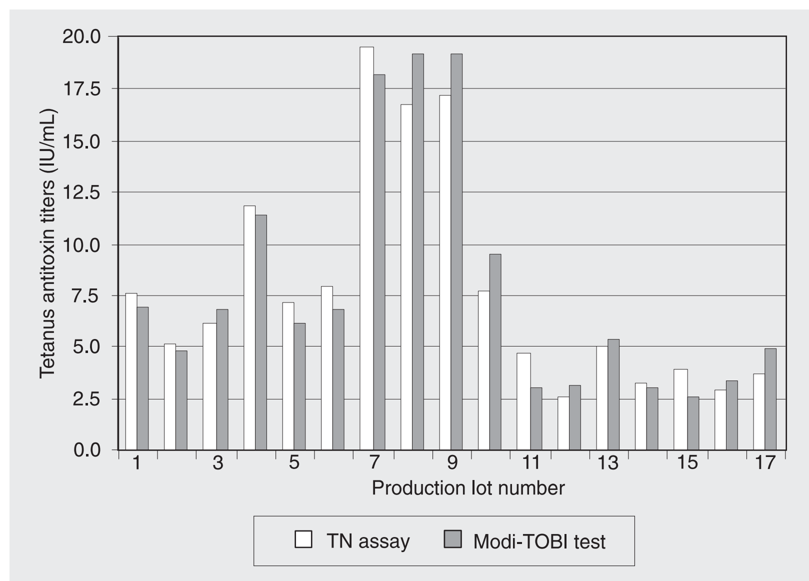

Figure 1. Comparison of the levels of tetanus antitoxin determined by the toxin neutralization (TN) and modified-toxin-binding inhibition (Modi-TOBI) tests. Lots of tetanus toxoid (TT), diphtheria and tetanus (dT) vaccine, and diphtheria, tetanus and pertussis (DTP vaccine were inoculated into guinea pigs that were bled after 6 weeks. The levels of anti-tetanus toxin antibodies (IU/mL) were measured in serum by the *in vitro* Modi-TOBI test (filled columns) and by the *in vivo* TN assay (open columns).

Results

The TOBI test reaction is based on the capacity of the coated antitoxin in the second step (P2) to bind any anatoxin that did not interact with the antibodies of the sera tested in the first step (P1). The anatoxin in P2 is assayed by peroxidase-labeled IgG. In the TN test, the toxin not inactivated by the serum tested provokes death when inoculated into mice.

In the present study, serum anti-tetanus and anti-diphtheria antibodies were measured using both the Modi-TOBI test and TN assays. The results obtained by the two methods were analyzed and compared. Figure 1 presents the results for the tetanus component of different lots of tetanus TT, dT, and DTP. The results of the Modi-TOBI test ranged from 2.6 to 19.2 IU/mL for the tetanus component, while the results of the TN assay ranged from 2.6 to 19.5 IU/mL. The lowest concentration of antitetanus antibodies detected by the two methods was 2.6 IU/mL.

The titers of the diphtheria component of the different lots of dT and DTP are shown in Figure 2. The results of the Modi-TOBI test ranged from 1.5 to 7.6 IU/mL, while the results of the TN assay ranged from 1.0 to 7.8 IU/mL.

As shown in Figure 3, positive and significant correlations were observed between the results obtained by the TN assay and the Modi-TOBI for anti-tetanus antibody titers (A) and for anti-diphtheria antibody titers (B). A very good correlation ($r = 0.98$, $P < 0.0001$) was observed between the tetanus TN assay and the Modi-TOBI test. The correlation coefficient had an r^2 of 0.95. A high correlation was also observed ($r = 0.96$, $P < 0.0001$) for the diphtheria TN assay and the Modi-TOBI test. The correlation coefficient (r^2) was 0.93, $P < 0.0001$.

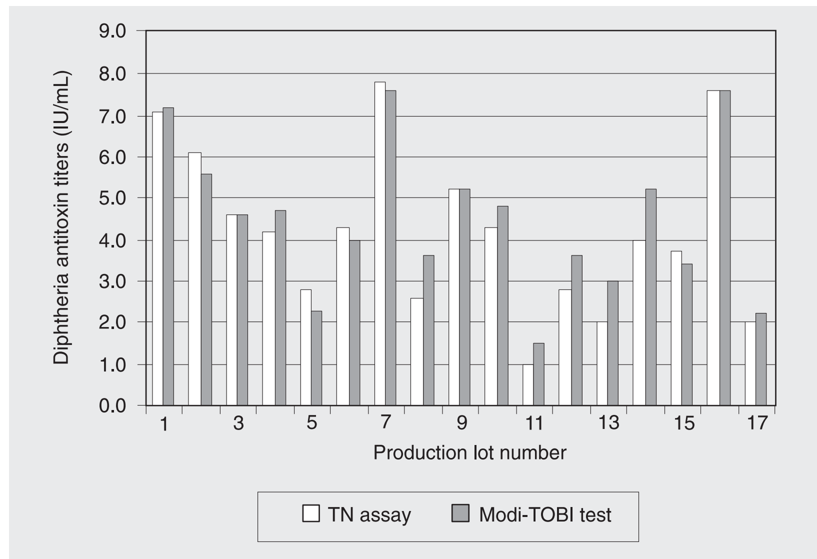

Figure 2. Comparison of the levels of diphtheria antitoxin determined by the toxin neutralization (TN) and modified toxin-binding inhibition (Modi-TOBI) tests. Lots of diphtheria and tetanus (dT) vaccine and diphtheria, tetanus and pertussis (DTP) vaccine were inoculated into guinea pigs that were bled after 4 weeks. The levels of anti-diphtheria toxin antibodies (IU/mL) were measured in serum by the *in vitro* Modi-TOBI test (filled columns) and by the *in vivo* TN assay (open columns).

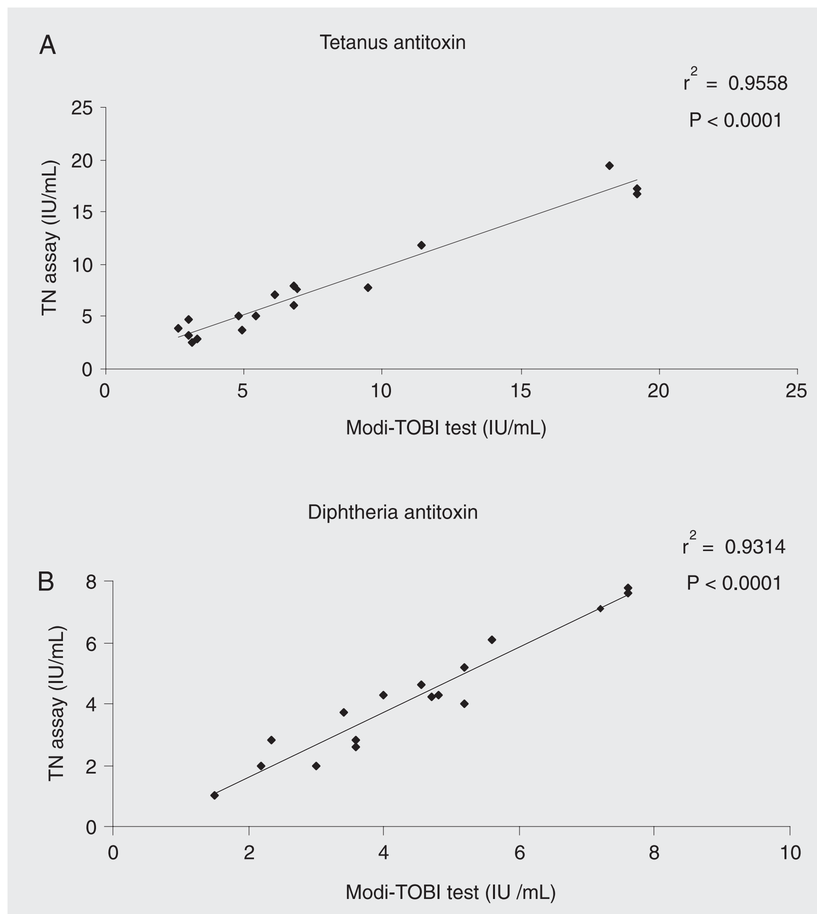

Figure 3. Correlation between the modified toxin-binding inhibition (Modi-TOBI) test and the toxin neutralization (TN) assay. *A*, Correlation analysis of the tetanus antitoxin titers measured by the Modi-TOBI test and by the TN assay of 17 serum samples from guinea pigs immunized with tetanus toxoid (TT), diphtheria-tetanus vaccine for adults (dT), or diphtheria-tetanus-pertussis vaccine for infants (DTP). *B*, Correlation analysis of diphtheria antitoxin titers measured by the Modi-TOBI test and by the TN assay for 17 serum samples from guinea pigs immunized with dT or DTP.

Discussion

We describe here a second modification of the TOBI test protocol (10,11), denominated Modi-TOBI test, in order to detect low levels of tetanus and diphtheria antibodies in serum. By the earlier modified assay (11), it was only possible to detect titers higher than 1,000 IU/mL in the serum of immunized horses. In the present study, tetanus and diphtheria antibodies reached a maximum level of approximately 20 IU/mL in the serum of guinea pigs immunized to test the potency of vaccines with tetanus and diphtheria fractions produced at the Butantan Institute.

If the *in vitro* techniques cited in the Introduction were applied as alternative protocols, the passive hemagglutination test could be performed in much less time (1). However, two principal disadvantages are: first, passive hemagglutination is preferentially sensitive to IgM antibodies (unable to neutralize tetanus toxin) (17), and second, a poor correlation (r = 0.34) was observed between passive hemagglutination and the reference VERO cell tests for the diphtheria antitoxin (18). There are many different ELISA protocols. The toxoid-ELISA and TN tests demonstrate a relatively good correlation (r = 0.74) for tetanus antitoxin (10), and a better correlation with VERO cell tests (TN VERO) for the diphtheria antitoxin (r = 0.81) (18). However, the ELISA methods have a very poor correlation at concentrations below 0.2 IU/mL (1,8,9). A comparative evaluation of ELISA and TN tests was performed to quantify antibodies against tetanus toxin in the sera of guinea pigs and mice immunized with TT. ELISA determination on sera collected after less than 6 weeks of immunization and tested against a hyperimmune serum did not correlate with TN levels. The antibody levels determined by ELISA were 35-40 times higher than those determined in TN tests (19) in samples taken 2 weeks after the mice had been injected.

A very good correlation between the TOBI and VERO cell tests was described for diphtheria antitoxin (r = 0.93) (1,18). The correlation between the TN and TOBI tests applied to samples of human sera was also very high (r = 0.95) (10) for tetanus antitoxin. Another advantage of the TOBI test is that it is based on pharmacodynamic interactions of TT. The toxin (or anatoxin) reacts in solution with the corresponding antitoxin in reactions related to the binding of the tetanus toxin to neuronal membrane components at the beginning of its mechanism of action. No toxicity was detected when this binding was prevented by tetanus antitoxin (17,20). In the present analysis, the choice of the TOBI test as an alternative protocol for TN tests in the quality control of vaccine production was based on the fact that this test can detect neutralizing antibodies against tetanus and diphtheria toxins. This characteristic is essential for the control of vaccine potency.

A very good correlation was obtained here between the TOBI and TN tests both for tetanus antitoxin (r = 0.98, r^2 = 0.95) and diphtheria antitoxin (r = 0.96, r^2 = 0.93) in terms of determining titers of these antitoxins in guinea pigs. Our results agree with the data obtained by Hendriksen et al. (10) for tetanus antitoxin (r = 0.95) and by Walory et al. (18) for diphtheria antitoxin, although the latter investigators studied the correlation between the TOBI and VERO cell tests.

The present results further indicate that the Modi-TOBI test can be performed as a precise alternative to the TN test for the control of TT, DT, dT, DTP, and DTP-Hib vaccines as well as tetanus and diphtheria anatoxins. Although the WHO determines that measurements of the potency of these vaccines must be determined by TN tests, international studies have shown the competence of the TOBI/ELISA assays in determining the potency of tetanus toxoid vaccines (21,22). The alternative protocol validated for diphtheria toxoid vaccines would be the VERO cell assay (2,3,23), but our

results indicate that the Modi-TOBI test also can serve as an alternative assay for diphtheria toxoid. Additionally, the Modi-TOBI test could be used for the determination of the titers of tetanus and diphtheria antitoxins in intermediate steps of the production process in order to reduce the number of animals utilized.

The reduction of animal use in humane vaccine quality control is a goal for both production institutions and regulatory agencies like the WHO. Initiated with the publication of "The Principles of Humane Experimental Technique" in 1959 (24), the concept of 3R was developed, i.e., replacement, reduction and refinement of animal tests. The establishment and validation of alternative *in vitro* methods for *in vivo* tests in the quality control of vaccines, such as the Modi-TOBI test for potency determination, may greatly contribute to this goal. Various other alternative *in vitro* methods have been developed to reduce animal use (for a review, see Ref. 21). *In vivo* TN tests utilize live animals, and the VERO cell tests require a cell line, both of which imply the use of biological material. Such tests generate data that often require subjective interpretation (for example, diagnosing the symptoms of tetanus). The Modi-TOBI test has the advantage (as does ELISA) of automatically analyzing results with an ELISA reader that can then be expressed numerically. This technique could refine the quality control of the vaccines and make it possible to compare and share data between vaccine producers worldwide.

Acknowledgments

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