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EXPERIMENTAL / LABORATORY STUDIES

Development of a Diagnostic and Screening Elisa System for Measuring Tetanus Antitoxoid Levels

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Abstract: Tetanus is a vaccine-preventable disease of global importance. When acquired it has a high mortality rate. Screenings of special subpopulations such as women of childbearing age provide important clues about tetanus vaccination coverage, and tools for mass protective measures, e.g, protective measures for neonatal tetanus. In addition to its reliability and sensitivity, the main advantage of a mass-screening tool should be its cost-effectiveness. We aimed to develop a cost-effective and sensitive enzyme-linked immunosorbent assay (ELISA) that can be used for diagnostic research as well as mass screening purposes for antitetanus antibodies. Our in-house antitetanus ELISA was validated and tested for its sensitivity and specificity and then compared with a commercially available kit. The analytical sensitivity of the in-house ELISA was less than 0.008 IU/ml. For the tested concentrations the intra- and inter-assay coefficients of variation were between 0.8 and 4.1% and between 2.2 and 8.6% respectively. The results demonstrated that our in-house ELISA is quite sensitive and has a high performance with human sera similar to that of a commercial kit in determining the antitetanus antibody concentrations and may be used for diagnostic purposes as well as in mass screenings for tetanus vaccination coverage.

Key Words: Antitetanus antibodies, Tetanus toxoid, Tetanus diagnosis, ELISA

Introduction

Tetanus is a disease of global importance produced by the toxin of *Clostridium tetani*. Its pathogenesis involves the absorption of preformed toxin or invasion of toxin-producing organisms from contaminated wounds. The risk of acquiring tetanus increases in certain age groups and especially in countries where poor sanitary conditions predispose to umbilical stump contamination (1). Despite intentions to eradicate it, tetanus remains endemic in developing countries (2).

Although its diagnosis is made clinically, the measurement of antitetanus antibodies is of value in assessing the immune status of individuals at risk of tetanus infection (3). The determination of antitetanus antibodies is also of great importance in monitoring the efficiency of mass vaccination programs (2,4). Techniques for measuring antitoxin levels vary. The toxin neutralization (TN) test, performed in vivo and regarded as the gold standard, is a sensitive test that can detect very low levels of antitoxin, but it is expensive and requires well trained personnel. The TN test directly measures the biological activity of tetanus antitoxin. The passive hemagglutination (HA) test, ELISA, and the radioimmunoassay are the in vitro techniques applied for the detection of antitoxin levels. The HA test has a simple design and can be conducted in a moderately equipped laboratory. It shows a relative high correlation with the TN test but its main disadvantage is its preferential sensitivity for IgM (4). The ELISA test is commonly used to determine tetanus antibody titers, and several modifications have been developed to overcome the unreliability it may show, especially at low antitoxin concentrations. Discrepancies between tetanus ELISA and the TN test have been reported in incomplete vaccination cases and it has been postulated that the antibodies during the early course of tetanus immunization may be of low affinity and low avidity and so are responsible for binding in ELISA but are not able to neutralize tetanus toxin (5,6). Besides the indirect ELISA test, competition

ELISA and the toxin-binding inhibition tests have been proposed for the evaluation of tetanus immunity (5,7,8). Time resolved fluorometric immunoassays have also been developed and applied for the quantitation of antitetanus toxin levels (9,10). Especially in mass-screening procedures, a simple, cheap and rapid assay for the quantitative determination of antitetanus levels is preferable. For diagnostic purposes the assay should be sensitive at low concentrations of antibodies.

The present study describes the development of a biotin-streptavidine ELISA for the sensitive determination of antitetanus toxin antibody levels in human sera.

Materials and Methods

Tetanus toxoid. Tetanus toxoid at a protein content of 996 µg/ml was obtained from the Refik Saydam Hıfzısıhha Institute Vaccine Development Center, Ankara.

Diphtheria toxin. Diphtheria toxin at 440 Limes flocculation (Lf)/ml (protein content was 1.272 mg/ml) was obtained from the Refik Saydam Hıfzısıhha Institute Vaccine Development Center, Ankara.

Tetanus antitoxin. The tetanus antitoxin used in this study was standard tetanus antitoxin IgG reagent supplied in a commercially available antitetanus ELISA kit (Tetanus ELISA, Genzyme Virotech GmbH, Rüsselsheim, Germany).

Human sera. Human sera were kept at -75 ºC until the day of study. Forty randomly taken human sera were used for comparing the commercially available kit with the in-house antitetanus toxoid ELISA.

Labelling of tetanus toxoid with biotin. Tetanus toxoid was dialyzed against 0.1 M $NaHCO_3$ pH 7.4 and then labeled with biotin (Sigma, St Louis, MO, USA) as described elsewhere (11).

Enzyme-linked immunosorbent assay (ELISA). High binding capacity ELISA plates (Costar, No: 3590, Corning Incorporated, Corning, NY, USA) were coated with 100 µl of tetanus toxoid at 2 µg/ml in 0.05 M carbonate-bicarbonate buffer pH 9.6 (CBB) by incubating at +4 ºC overnight. After washing 3 times with distilled water, blocking was performed with 200 µl of PBS containing 3% bovine serum albumin (BSA) by incubating at +4 ºC overnight, followed by washing 3 times with PBS containing 0.5% Tween 20 (PBS-T). To each well was added 100 µl of samples diluted to 1/100 in PBS containing 1%BSA (PBS-1%BSA) or standards of the commercially available tetanus antitoxin ELISA kit, followed by incubation at 37 ºC for 1 h. After incubation, the plates were washed 3 times with PBS-T and then to each well was added 100 µl of tetanus toxoid-biotin diluted at 1/5000 in PBS-1%BSA. The plates were incubated for 1 h at 37 ºC and then washed 3 times with PBS-T. To each well was added 100 µl of streptavidine-horseradish peroxidase (Sigma), followed by incubation for 30 min at 37 ºC. After incubation, the plates were washed 3 times with PBS-T and then the reaction was revealed with 100 µl of 3,3',5,5' tetramethylbenzidine (TMB) solution for 15 min at room temperature. After stopping the reaction with 50 µl of 1 M H_2SO_4, the plates were read with an ELISA reader (LP400, Diagnostics Pasteur, France) at 450 nm with or without the reference at 620 nm. The commercially available diagnostic kit was studied according to the manufacturer's instructions. For testing the specificity of the reaction, an inhibition test was carried out in 2 steps using antitetanus toxoid standards and human sera with predefined antitetanus toxoid concentrations. The predefinition of these sera was performed by the in-house antitetanus ELISA after its validation experiment was regarded as successful. Various concentrations of tetanus toxoid and diphtheria toxoid were prepared in PBS-1%BSA and then added to appropriate wells at a volume of 50 µl. Except for the blank, to the wells was added 50 µl of antitetanus toxoid standard at 2 IU/ml. The subsequent steps of the ELISA were performed as described above. For evaluation of inhibition, 4 different human sera with predefined antitetanus toxoid concentrations were also used. After adding tetanus or diphtheria toxoids as mentioned above, the human sera, diluted finally to 1/100 in PBS-1%BSA, were added to appropriate wells at a volume of 50 µl. The subsequent steps of the ELISA were performed as described above.

Statistical analysis. Regression-correlation analysis was performed for the evaluation of the in-house ELISA. Inter- or intra-assay coefficients of variation (CV) were calculated and used for the quality control of the in-house ELISA. Calculations were performed with SPSS for Windows version 10.0.

Results

In the first step, 5 different standards (2, 1, 0.5, 0.2 and 0.1 IU/ml) of the tetanus antitoxoid (supplied as ready-to-use standards within the Virotech kit) and blanks were assayed in parallel using both the in-house ELISA system and the commercial ELISA kit. Both systems revealed a statistically significant and high linearity between OD 450/620 nm values and the antitoxoid concentrations (r^2 = 0.956, P = 0.001 and r^2 = 0.960, P = 0.001 respectively). In the second step, tetanus antitoxoid was prepared at various concentrations in PBS-1%BSA by 2-fold dilutions from 1 to 0.0078 IU/ml using the 5 IU/ml ready-to-use tetanus anti toxoid standard. A statistically significant and high correlation (P = 0.003, r^2 = 0.958) between antitetanus toxoid concentrations and OD 450/620 nm values was observed (Figure 1). The analytical sensitivity of the in-house ELISA was less than 0.008 IU/ml because 0.008 IU/ml produced an OD value (0.172) higher than the cut-off value (0.162). The cut-off value was determined by adding 3 standard deviations (3 x 0.023) to the mean optical density (0.093) obtained when the zero standard was assayed 4 times. The quality control of the in-house ELISA was checked by analyzing its intra- and inter-assay coefficients of variation (CV). For the tested concentrations the intra- and inter-assay CV values were between 0.8 and 4.1% and between 2.2 and 8.6% respectively (Table).

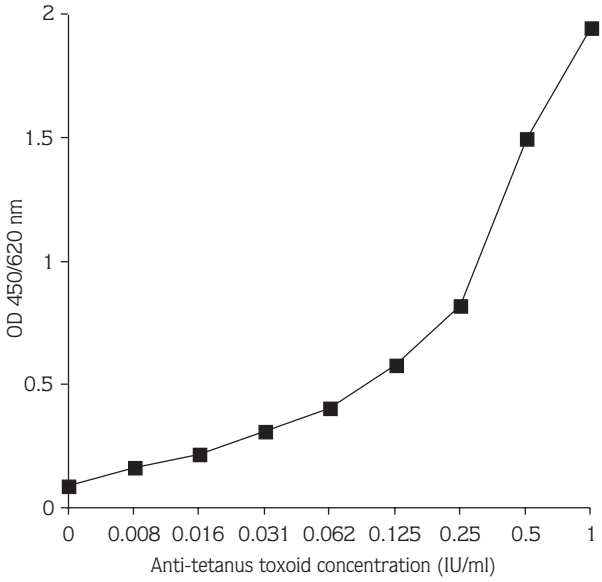

Figure 1. Analytical sensitivity of the in-house antitetanus toxoid ELISA. There is a statistically significant and high correlation between the antitetanus toxoid concentrations and OD 450/620 nm values obtained (P = 0.003, r^2 = 0.958). Data are the means of duplicate wells.

The specificity of the in-house ELISA was analyzed by testing the inhibitory effect of the tetanus toxoid both on the antitetanus toxoid standards and on human sera with predefined antitetanus toxoid concentrations. Tetanus toxoid inhibited the binding activity of antitetanus toxoid standard at 2 IU/ml in a concentration-dependent manner (Figure 2a). The OD read for the dilutions of tetanus toxoid varied significantly (r^2 = 0.7861, P = 0.024) and pointed to an inversely proportional relationship. However, an inhibitory effect was not observed (r^2 = 0.2398, P > 0.05) with the irrelevant antigen, diphtheria toxoid, even at a concentration of 5 µg/ml (Figure 2a). To determine whether this specificity was also applicable to human sera, a similar assay procedure was performed using 4 different sera, which were then tested in duplicate. Although tetanus toxoid inhibited the binding activity of antitoxoid antibodies in sera in a concentration-dependent manner, no effect could be detected for the diphtheria toxoid, indicating the high specificity of the in-house ELISA (Figure 2b).

Two ELISA systems were studied simultaneously with 40 different human sera diluted to 1/100 in order to compare the performances of both ELISA systems with human sera. A significant correlation (Pearson correlation coefficient, r = 0.93; P < 0.01) was observed between the antitetanus levels measured with 2 different ELISA systems (Figure 3).

Table. Intra- and inter-assay coefficients of variation (CV) of the in-house antitetanus toxoid ELISA.

Standard (IU/ml)*	Intra-assay CV% (n = 4)	Inter-assay CV% (n = 4)
1	1.6	3.6
0.5	0.8	2.2
0.125	4.1	8.6
0.0625	1.9	4.5

* Antitetanus toxoid standard as supplied by a commercial kit (Virotech)

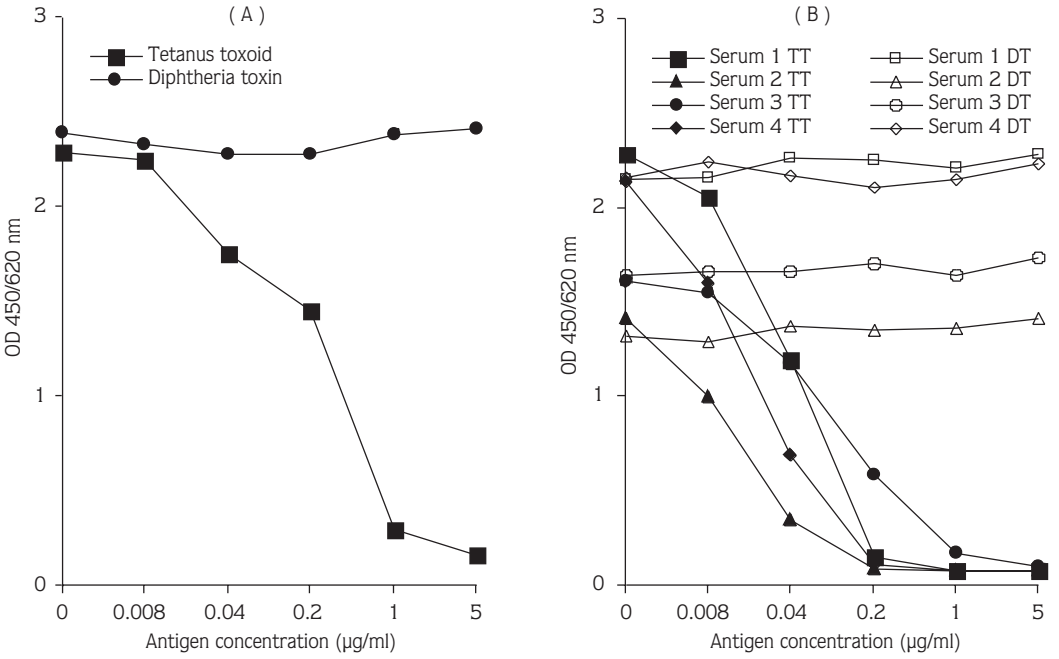

Figure 2. Specifity testing of the in-house ELISA by inhibitory reaction. Tetanus toxoid inhibited the binding activity of 2 IU/ml antitetanus toxoid standard (A) and various human sera containing different levels of antitetanus toxoid antibodies (B) in a concentration-dependent manner. However, inhibition was not observed when a nonspecific antigen, diphtheria toxin, was used, even at a high concentration of 5 μg/ml. Data are the means of duplicate wells (TT: tetanus toxoid, DT: diphtheria toxoid).

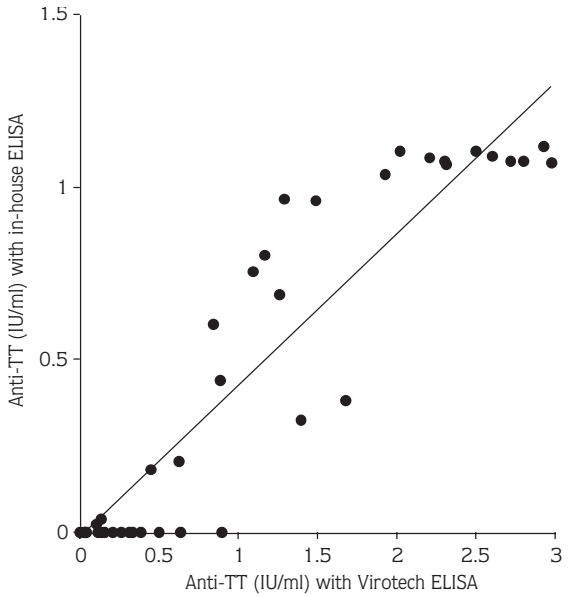

Figure 3. Comparison of the performances of both the Virotech tetanus ELISA and the in-house tetanus ELISA systems using human sera. Forty different serum samples were measured for their antitetanus toxoid concentrations simultaneously with both systems. A significant correlation ($r^2 = 0.86$, $P < 0.01$) was observed between 2 different types of assays. Given values for both ELISA systems are antitetanus toxoid concentrations expressed in IU/ml.

Discussion

Infection caused by *C. tetani* does not confer immunity, and recovery from clinical tetanus does not result in protection against further attacks. Vaccination with the formaldehyde treated tetanus toxin is the only means of protection. It is well known that the level of protective antitetanus antibodies decreases with age (12,13) and hence population-based screenings for tetanus toxoid vaccination coverage according to age or sex may serve as important clues for population-based studies. In regard to large-scale population-based screenings, the test used for the detection of antitetanus antibodies should be easily and rapidly performed under simple laboratory conditions, it should be commonly available and cost-effective (2,4). Although several methods for the detection of tetanus-toxoid antibodies are available many are not suitable for population screenings. ELISA due to its simplicity, ease of automation and availability is widely used for the determination of antitetanus antibodies (14-19).

Diagnostic accuracy and comparisons between our in-house ELISA and the Virotech kit are based on Pearson coefficients of correlation, and intra- and inter-assay

coefficients of variation. These statistical parameters are well defined and generally accepted (20).

In this study we developed an in-house ELISA that can be used for research as well as population-screening studies for antitetanus antibodies. After the establishment of the in-house ELISA we performed several comparison steps with a commercially available diagnostic antitetanus kit (Virotech). The validation of our in-house ELISA was performed using ready-to-use standards with known concentrations of antitetanus toxoid antibodies of the Virotech kit. The sensitivity of the in-house ELISA was less than 0.008 IU/ml (Figure 1). The level of circulating antibodies against tetanus toxoid, i.e. "protective level", is somewhat controversial and is closely related to the method used for the determination of antitetanus toxoid antibodies. The "protective level" of 0.01 IU/ml is defined for in vivo, i.e. neutralization, tests. This level does not reflect the "protective level" determined with in vitro tests including ELISA. The use of a level of antibody determined in vitro that is equivalent to 0.01 IU/ml determined in vivo is advised; taking this into account, 0.1 IU/ml is usually suggested as safe (2). When the protective cut-off value of antitetanus toxoid antibodies (>0.1 IU/ml) is considered the critical demarcation point for serological evaluation, the tested concentration range around this cut-off value and their respective ODs displayed a higher correlation coefficient at a statistically significant level for the Virotech kit ($r = 0.999$, $P = 0.001$) and for the in-house ELISA ($r = 0.998$, $P = 0.002$). According to these results, we assume that the in-house ELISA is as precise as the Virotech kit in diagnosing the cut-off value for the antitetanus immunity.

In order to assess the quality, i.e. reproducibility, of our in-house ELISA, both intra- and inter-assay CV values were determined under the same experimental conditions. As shown in Table, we obtained quite satisfactory levels of CV for the tested concentrations. Even the highest CV value was less than 10%, which indicated that our in-house ELISA is highly reproducible at significant levels. The low CV values we obtained may be regarded as a good reflection of assay precision (21).

The specificity of the in-house ELISA was analyzed by an inhibition test using specific antigen. In addition, diphtheria toxoid was used in order to determine whether any inhibition by an irrelevant antigen for the experimental designs occurs. It was clearly demonstrated that an inhibitory effect was observed only with the specific antigen (Figure 2). Therefore, the reaction obtained in this assay format was directly related and proportional to the amount of anti toxoid antibodies present in samples, favoring a specific reaction that could be inhibited only by tetanus toxoid.

In order to evaluate the performance of our in-house ELISA with human sera we performed a comparison test with the Virotech kit by measuring the antitetanus toxoid antibody levels of 40 different human sera (Figure 3). The measured antibody levels with both systems revealed a statistically significant ($P < 0.01$) correlation ($r = 0.93$).

Our study results about the in-house developed antitetanus toxoid ELISA demonstrate an important data collection that has been verified at each step with a well-known and commonly used commercially available kit (Virotech).

The ELISA developed and validated for its specificity, sensitivity and analytical measuring range is a simple method for the determination of antitetanus toxoid antibodies. It is much more satisfactory and displays a correlation coefficient close to one at the demarcation value for the antitetanus toxoid that is referred to as the protective level, thus providing an important and cost-effective tool for mass screenings of certain age groups or genders.

In summary, the results suggested that the in-house developed antitetanus toxoid ELISA is as reliable and precise as the well-known and commercially available Virotech kit for determining antitetanus toxoid antibodies in human sera. The main advantage of our in-house ELISA is its reliability and cost-effectiveness. In addition, according to the working principle of the in-house ELISA system developed, it can measure antitetanus toxoid without being affected by the species or isotype of the specific antibody because the specific antibody in the sample binds the antigen (tetanus toxoid) on the solid phase with one of its Fab fragments while binding biotinylated specific antigen with the other. Therefore, the system developed in our laboratory enabled us not only to measure antitetanus toxoid from human sera but also antitetanus toxoid from other species, such as mice, successfully.

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