DEVELOPMENT OF A DIAGNOSTIC AND SCREENING ELISA SYSTEM FOR MEASURING DIPHTHERIA ANTI-TOXIN LEVELS

DİFTERİ ANTİ-TOKSİN SEVİYELERİNİN ÖLÇÜLMESİ İÇİN TANI VE TARAMA AMAÇLI BİR ELISA SİSTEMİ GELİŞTİRİLMESİ

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ABSTRACT

Purpose: *It had been thought that diphtheria was a very rare disease because of routine immunization programs conducted worldwide. However, the epidemic seen in the newly independent states of the former Soviet Union prompted worries that public immunity levels might not be sufficient and therefore the disease might spread. In order to conduct vaccination programs to eliminate the disease, first of all, the serum antibody levels of all age groups to diphtheria toxin (DT) should be measured with a standardized method. However, the cost of commercial anti-DT ELISA kits is very high. In this study we aimed to develop an anti-DT ELISA system that could be used for both diagnosis and screening, with high specificity and sensitivity but low cost.* ***Methods:*** *Solid phase was coated with diphtheria toxin while enzyme-labeled DT was used as conjugate. Since the anti-DT antibodies in serum or other samples to be measured will bind DT in the solid phase and conjugate with its two Fab ends, the system can be used for sera from other species as well. This in-house ELISA was also compared with a commercially available anti-DT ELISA kit.* ***Results:*** *Although the commercial anti-DT ELISA kit had restrictions for measuring antibody levels below 0.1 IU/ml, the in-house ELISA system managed to measure anti-DT antibody levels below 0.01 IU/ml with a dynamic analytical measuring range between 0.01 and 1 IU/ml.* ***Conclusion:*** *The in-house anti-DT ELISA system is a highly sensitive, reliable and low-cost test technique that can be used in the diagnosis and screening of immunity to diphtheria. In addition, it can be used in many different in vivo and in vitro investigations of humoral immune responses.*

Key Words: *Diphtheria, Anti-Toxin, ELISA, Humoral Immune Response.*

ÖZET

Amaç: *Tüm dünyada ve Türkiye'de uygulanan rutin aşılama programları nedeni ile difterinin çok nadiren görülen bir hastalık olduğu düşünülüyordu. Ancak, eski Sovyetler Birliği'nden ayrılan Yeni Bağımsız Devletler'de görülen epidemi toplumsal bağışıklığın yeterli düzeyde olmayabileceği ve bu nedenle hastalığın yayılabileceği kaygısını uyandırdı. Hastalığı ortadan kaldırmaya yönelik aşılama programlarını başlatabilmek için ilk önce tüm yaş gruplarında difteri toksinine (DT) karşı serum antikor seviyeleri standardize edilmiş bir metod ile ölçülmelidir. Ancak ticari anti-DT ELISA kitlerinin maliyeti çok yüksektir. Bu çalışmada hem tanı hem de tarama amaçlı kullanılabilecek yüksek duyarlılıkta ve özgüllükte, ancak düşük maliyetli bir anti-DT ELISA sisteminin yapılması amaçlanmıştır.* ***Yöntem:*** *Katı faz antijen olarak kullanılan difteri toksini ile kaplanırken konjugat olarak da enzimle işaretli DT kullanıldı. Serum ya da diğer örneklerdeki anti-DT antikorlar katı fazdaki ve konjugattaki DT'ye iki adet Fab ucları ile bağlandığından, oluşturulan sistem farklı türlere ait serum örneklerindeki özgül antikorları da ölçebilmektedir. Laboratuvarda hazırlanan bu ELISA sistemi ayrıca ticari bir anti-DT ELISA kiti ile de karşılaştırıldı.* ***Bulgular:*** *Ticari anti-DT ELISA kiti 0.1 IU/ml'nin altındaki antikor seviyelerini ölçemediği halde, geliştirilen sistemin 0.01 IU/ml altındaki antikor seviyelerini ölçebildiği ve dinamik ölçüm aralığının 0.01-1 IU/ml arasında olduğu saptandı.* ***Sonuç:*** *Hazırlanan anti-DT ELISA çok duyarlı, güvenilir ve düşük maliyette bir sistem olup, difteriye karşı bağışıklık durumunun değerlendirilmesinde kullanılabilecektir. Ayrıca, hümoral immün yanıtla ilgili çeşitli in vivo ve in vitro araştırma çalışmalarında da kullanılabilecektir.*

Anahtar Kelimeler: *Difteri, Anti-Toksin, ELISA, Hümoral İmmün Yanıt.*

INTRODUCTION

Diphtheria was one of the most important causes of death in the first half of the twentieth century (1). The introduction of formalin-treated toxin (toxoid) as a vaccine was a real revolution in the prevention of the disease. Because of routine immunization programs conducted around the world, it had been thought that diphtheria was on the wane. However, the diagnosis of occasional individual cases has urged medical professionals to monitor anti-toxin levels in different age groups (2). In addition, the epidemic seen in the newly independent states of the former Soviet Union has sparked off concerns that public immunity might not be sufficient and therefore the disease might spread (3-6). This concern caused a revision of studies on immunization programs in some countries such as Norway (1). In order to conduct vaccination programs to eliminate the disease, first of all, the serum antibody levels of all age groups should be measured using a standardized method (7-10).

In Turkey, the immunization program for diphtheria involves childhood vaccination, though no routine booster dose is given after 12 years of age (11). There is insufficient data to assess the immune status of different age groups for diphtheria in Turkey, other than one study where 497 people of various ages were screened. Overall, 35.8% of the study group showed insufficient immunity (anti-toxin level <0.1 IU/ml) to diphtheria (12). Therefore, close contact with Russia and other newly independent states of the former Soviet Union is a very important risk factor for the possible outbreak of a diphtheria epidemic in Turkey. In order to start new immunization programs, it is necessary to carry out widespread public screening for diphtheria anti-toxin levels. However, the cost of such a program would likely be very high if commercially available anti-DT ELISA kits were used. Therefore, the primary aim of this study was to develop a reliable, sensitive and specific ELISA system to measure diphtheria anti-toxin levels for diagnostic and screening purposes at low cost.

One of the other aims of this study can be summarized as developing a screening method for the status of humoral immunity, since specific antibodies cannot develop after immunization in humoral immunodeficiencies (13). In addition, the developed system can also be used to assess humoral immune response both in vivo (in experimental animals) and in vitro (in cell cultures) for research purposes at very low cost.

MATERIALS AND METHODS

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.

Diphtheria anti-toxin: Immunized horse serum containing antitoxin at 650 IU/ml was obtained from the Refik Saydam Hıfzısıhha Institute Vaccine Development Center, Ankara.

Human sera: The properties of human sera used in the experiments are given in Table 1.

SDS-PAGE Analysis of Diphtheria Toxin: A mixture of human albumin and goat immunoglobulin (Ig) was prepared as standard proteins. This standard protein mixture or diphtheria toxin (DT) was mixed with 2X sample buffer containing 2-mercaptoethanol and then boiled for 10 min. Each sample was then added to a corresponding well at a volume of 50 µl. Samples were run at 10 mA constant flow rate for 5 h on 10% SDS-PAGE gel and then stained with Coomassie blue.

Labeling of diphtheria toxin with horseradish peroxidase: Diphtheria toxin was labeled with horseradish peroxidase (HRP) enzyme (Type VI-A, Sigma, USA) using the periodate method (14). In order to eliminate free HRP from HRP-DT conjugate, the sample was passed through a Sephacryl S-300 (Pharmacia, Sweden) column (0.9x30 cm) connected to a HPLC system. During the purification process via chromatography, the fractions between 14 and 24 min where the first peak was observed at 280 and 403 nm were pooled (5 ml). Bovine serum albumin (BSA) was added to the HRP-DT conjugate for a final concentration of 1%. HRP-DT conjugate for use in ELISA studies was divided into aliquots and shock-frozen with liquid nitrogen and then kept at -74°C until the day of study.

Enzyme-linked immunoabsorbent assay (ELISA): High-binding capacity ELISA plates (Costar, No: 3590, Corning Incorporated, Corning, NY, USA) were coated with 100 µl of

DT at 1 μg/ml in 0.05 M carbonate-bicarbonate buffer (CBB) pH 9.6 by incubation at +4°C overnight. After washing three times with distilled water, blocking was performed with 200 μl of PBS containing 1% BSA by incubating at 37°C for 1 h followed by washing three times with PBS containing 0.05% Tween 20 (PBS-T). To each well was added 100 μl of diluted samples or standards of a commercially available anti-DT ELISA kit (Diphtheria ELISA for detection of IgG anti-toxin antibodies in human serum, Genzyme Virotech GmbH, Rüsselheim, Germany) and then incubated at 37°C for 1.5 h. After incubation, the plates were washed five times with PBS-T and then incubated with 100 μl of HRP-DT conjugate, diluted to 1/250 in PBS containing 5% fetal calf serum (FCS) and 0.05% Tween 20 (PBS-FT), at 37°C for 1 h. After incubation, the plates were washed five 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 100 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 a reference at 620 nm. A commercially available diagnostic kit (Virotech, Genzyme Virotech GmbH, Rüsselheim, Germany) was also tested according to the manufacturer's instructions.

Statistical analysis: Regression-correlation analysis was performed. Inter- or intra-assay coefficient of variation (CV) was measured according to the following formula:

$$C.V. = S \bullet \frac{100\%}{\bar{X}} = \sqrt{\frac{\sum (X - \bar{X})^2}{N - 1}} \bullet \frac{100\%}{\bar{X}}$$

where: S = standard deviation of optical density (OD) readings,

$\bar{X}$ = mean of OD readings,

X = individual OD readings,

N = number of readings (wells)

RESULTS

SDS-PAGE analysis of DT: A DT preparation obtained from the Refik Saydam Hıfzısıhha Institute Vaccine Development Center was used for both coating and preparing the conjugate. When DT was analyzed with 10% SDS-PAGE, it revealed a band between the bands of the immunoglobulin heavy chain (50 kDa) and BSA (66 kDa) as expected since the mol wt of DT is 62 kDa (Fig. 1). DT preparation also revealed two minor bands corresponding to its sub-units A and B with molecular weights of 22 kDa and 38 kDa respectively (15). It was thought that the A and B sub-units might have formed due to fragmentation in some steps of the experimental study. Since no other bands were seen, the DT preparation used in this study was regarded as being a sufficiently pure antigen.

Purification of HRP-labeled DT with size-exclusion-based liquid chromatography: When monitoring is performed both at 280 nm (for protein imaging) and at 403 nm (appropriate for HRP) simultaneously, it indicates the performance of labeling. As seen in Fig. 2, the labeling of DT with HRP was obtained at an adequate level and no important amount of HRP was left free behind. The HRP-DT conjugate to

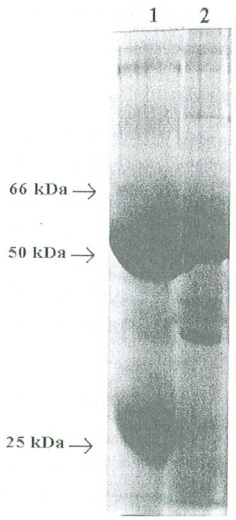

Fig. 1: SDS-PAGE analysis of diphtheria toxin used in the experiments. Each sample, at a volume of 50 μl, was loaded on 10% SDS-PAGE under reducing conditions and then run for 5 h at 10 mA. The gel was stained with Coomassie blue. Lane 1 contained standard proteins of known molecular weights (66 kDa: bovine serum albumin 10 μg; 50 kDa: immunoglobulin heavy chain 140 μg; 25 kDa: immunoglobulin light chain 70 μg). Lane 2 contained 50 μg of diphtheria toxin. Diphtheria toxin with a mol wt of 62 kDa revealed a major band in the appropriate region. Although trypsin was not used, minor bands that might correspond to diphtheria toxin subunits A (22 kDa) and B (38 kDa) were also visualized.

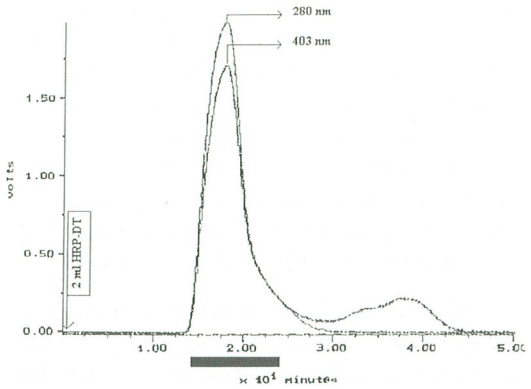

Fig. 2: Purification of HRP-labeled diphtheria toxin (HRP-DT) using Sephacryl S-300. Two milliliters of HRP-DT conjugate solution was loaded on a PBS-equilibrated Sephacryl S-300 column (0.9x30 cm) at a constant flow rate (0.5 ml/min). To prepare for use in ELISA studies, 5 ml of HRP-DT conjugate fractions corresponding to 14-24 min (shown with bold horizontal line) were pooled and BSA was added to a final concentration of 1%.

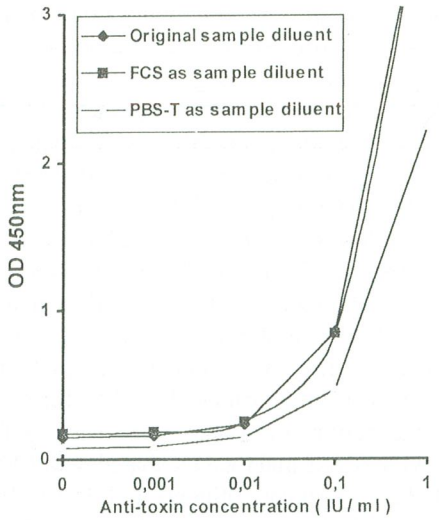

Fig. 3: Evaluation of the analytical sensitivity of the in-house anti-DT ELISA system using different sample diluents. There is a statistically significant and high correlation between the diphtheria anti-toxin concentration and OD 450 nm values obtained ($p<0.0005$, $r=0.999$ for FCS and kit diluent, and $p=0.00021$, $r=0.9968$ for PBS-T as sample diluent). Different concentrations of diphtheria anti-toxin produced similar OD 450 nm values with both the original ready-to-use sample diluent of the commercially available kit and FCS. The representative data are the mean of OD 450 nm values of each sample studied in triplicate.

be used in ELISA studies was pooled as 5 ml fractions between 14 and 24 min. When the study concentration of HRP-labeled DT conjugate was determined in preliminary experiments, it was found that 100 ml of conjugate, diluted at 1/250, per well resulted in good performance. It was calculated that 5 ml of conjugate, prepared at one time, was sufficient for 12,500 tests.

Evaluation of the analytical sensitivity of in-house anti-DT ELISA system using different sample diluents: By using the original sample diluent of the Virotech kit or FCS or PBS-T as sample diluent, different concentrations (0.001 IU/ml, 0.01 IU/ml, 0.1 IU/ml, and 1 IU/ml) of diphtheria anti-toxin were prepared from 5 IU/ml of ready-to-use diphtheria anti-toxin standard of the commercial anti-DT ELISA kit (Virotech). With the in-house anti-DT ELISA system, diphtheria anti-toxin caused a statistically significant increase ($p<0.0005$, $r=0.99$) in OD 450 nm values in correlation with the anti-toxin concentrations (Fig. 3). This data suggested that different sample diluents did not cause the matrix effect on OD values with the in-house ELISA system. When PBS-T was used as the sample diluent, the intra- and inter-assay CV values at 0.1 IU/ml were measured as 2.4% and 7.6% respectively (n=5).

Comparison of the analytical sensitivities of two different anti-DT ELISA systems: Different concentrations (0.1, 0.2, 0.5, and 1 IU/ml) of diphtheria anti-toxin, supplied by the ready-to-use standards of the Virotech kit, were studied with both the in-house ELISA system and the commercial ELISA kit as described above. Both the in-house and commercial ELISA systems revealed a statistically significant and high correlation between OD 450 nm values and anti-toxin concentrations ($r=0.9704$, $p=0.00609$ and $r=0.9808$, $p=0.00055$, respectively). Although the commercial ELISA kit produced OD 450 nm values very near to blank at low anti-toxin concentrations, up to 0.2 IU/ml, the in-house system managed to measure diphtheria anti-toxin levels as low as 0.01 IU/ml as OD 450 nm=0.148, which is a much higher value compared to that of the blank value (0.081) (Fig. 4). In addition, the

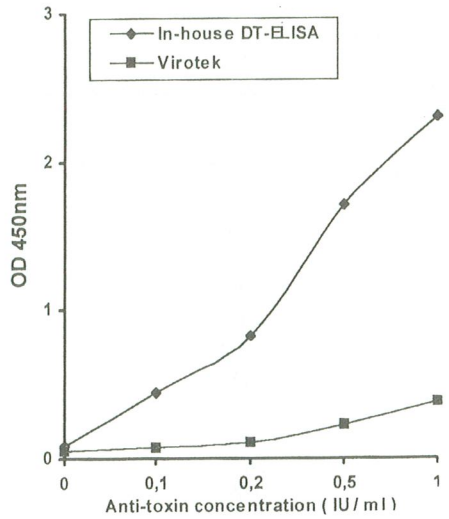

Fig. 4: Comparison of the assay performances of the in-house and the commercial (Virotech) anti-DT ELISA systems. Both the in-house and commercial anti-DT ELISA systems revealed statistically significant and high correlations between the diphtheria anti-toxin concentrations and OD 450 nm values obtained (r=0.9704, p=0.00609, and r=0.9808, p=0.00055 respectively). Anti-toxin concentrations up to 0.2 IU/ml gave OD 450 nm values very close to that of blank with the commercial kit. However, diphtheria anti-toxin concentrations less than 0.1 IU/ml produced a much higher value with the in-house system. The representative data are the mean of OD 450 nm values of each sample studied in triplicate.

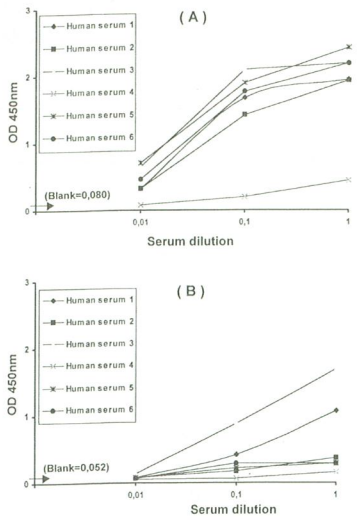

Fig. 5: Performances of the in-house (A) and commercial (Virotech) (B) anti-DT ELISA systems with human serum samples of different dilutions. The OD values obtained with the in-house ELISA system are higher compared to those obtained with the Virotech kit. The serum samples were studied in triplicate and the data are represented as the mean of triplicate wells.

in-house system again proved its ability to make linear measurements of anti-toxin concentrations between 0.1 and 1 IU/ml. These data implicated that the analytical sensitivity of the in-house ELISA system was at least 20-50 times higher than the Virotech ELISA kit.

Comparison of the performances of two different anti-DT ELISA systems using human sera: When the two ELISA systems were compared by using human sera of different dilutions, the in-house ELISA system revealed itself to be of better sensitivity (Fig. 5). When six different human sera were studied to measure anti-toxin levels by using both of the systems undiluted as well as diluted to 1/10 and 1/100 with PBS-T, OD 450 nm values changed in relation to dilution in both systems. However, the OD 450 nm values obtained with undiluted forms of human sera by using the Virotech ELISA kit were lower than those obtained with 1/10 diluted forms of the same sera for the in-house ELISA system. In addition, when sera were diluted before the study, anti-toxin levels of 1/100 diluted forms gave negative results with the Virotech kit. Two ELISA systems were also studied simultaneously with 31 different human sera (at 1/100 dilution) of various ages (Table 1). With the Virotech kit, diphtheria anti-toxin levels were found to be measurable in only seven people. These seven people were relatively younger compared to the others we used for testing. On the other hand, the levels were within measurable limits in most (24 out of 31) of the cases when the in-house ELISA system was used to measure diphtheria anti-toxin levels.

DISCUSSION

The exotoxin of toxigenic strains of Corynebacterium diphtheriae is responsible for local and systemic toxicity seen during diphtheria (16). Immunity to diphtheria is mainly provided by IgG antibodies made against the exotoxin, which is a powerful antigen (17). The status of immunity to diphtheria is described according to the serum level of the specific IgG against the toxin: If the level is below 0.01 IU/ml it is regarded as "insufficient immunity", between 0.01 and 0.09 IU/ml as "basic immunity", and the

Table 1: Comparison of the anti-DT levels of different human serum samples measured with the commercially available anti-DT ELISA kit (Virotech) and the in-house anti-DT ELISA system.

Serum samples	Age (Years)	Antitoxin levels with the Virotech anti-DT ELISA (IU/ml)	Antitoxin levels with the in-house anti-DT ELISA (IU/ml)
1	38	ND*	0.092
2	35	ND	0.0908
3	25	0.134	0.244
4	30	ND	ND
5	41	ND	0.269
6	27	ND	0.153
7	53	ND	0.482
8	53	ND	ND
9	31	0.161	0.421
10	38	ND	ND
11	29	ND	ND
12	32	ND	0.387
13	63	ND	0.06
14	49	0.067	0.418
15	44	ND	0.122
16	40	ND	0.045
17	30	2.637	1.01
18	53	ND	0.358
19	31	0.099	0.489
20	38	ND	ND
21	46	ND	0.236
22	34	ND	0.192
23	34	ND	ND
24	13	0.385	0.795
25	56	ND	0.067
26	38	ND	0.019
27	57	ND	0.053
28	49	ND	0.195
29	3	4.34	1.925
30	58	ND	0.116
31	35	ND	ND

*ND (Non-detectable): Antitoxin level lower than the measurable limit of the ELISA system.

level equal or above 0.1 IU/ml as "complete immunity" to diphtheria (18).

Many different laboratory methods have been used to measure diphtheria anti-toxin levels (19-33). The in vivo neutralization tests performed in experimental animals and the in vitro neutralization tests performed on Vero cells are both time-consuming and expensive methods; therefore, they are not practically applicable for routine laboratory usage. The most recommended method to measure diphtheria anti-toxin levels is the ELISA system (19-25).

In this study, we aimed to develop an ELISA system to measure antibodies specific for DT. According to the working principle of the in-house ELISA system developed, it can make measurements of diphtheria anti-toxin without being affected by the species or isotype of the specific antibody because the antibody (anti-DT) in the sample binds the specific antigen (diphtheria toxin: DT) on the solid phase with one of its Fab terminals while binding HRP-labeled specific antigens (HRP-DT conjugate) with the other. Therefore, the system developed in our laboratory enabled us to not only measure anti-DT from human sera but also from other species such as horses or mice successfully. With the in-house ELISA, the serum of non-immunized mice gave OD values close to that of the blank even at 1/10 dilution. In addition, diphtheria anti-toxin from immunized horse

serum, which is used for treatment, caused an OD increase dependent on concentration with the in-house ELISA (data not shown). These findings suggest that the in-house anti-DT ELISA system is also usable on different species for research purposes.

Various methods have been used to measure human serum antibody levels to DT (19-33). For example, Walory et al. (26) compared four different serological methods for the detection of diphtheria anti-toxin IgG antibodies: Passive hemagglutination, latex agglutination, toxoid ELISA and toxin-binding inhibition ELISA; the toxin neutralization test by Vero cells was used as a reference method. They found the validity features of these four methods to be 14, 10, 94 and 96% for sensitivity and 86, 76, 94 and 90% for specificity, respectively for both. Toxin-binding inhibition ELISA was also tested as an in vitro alternative to the toxin neutralization test by Hendriksen et al. (21), and they found a high correlation between the two methods without any false positive results in low titer sera. Skoura et al. (10) compared the sensitivity of an anti-DT ELISA system with a reference toxin neutralization (TN) assay. They found that the validity features of immunoassay were: sensitivity 68.7%, specificity 94.7% because TN assay had measured the susceptibility to diphtheria (non-immunity) in 7%, basic protection in 28.8% and full protection in 63.5% while their ELISA measured the same immune status as 17.9%, 36.5% and 45.5% respectively (10). In order to test the analytical sensitivity of the in-house anti-DT ELISA system, the standards of a commercially available anti-DT ELISA kit (Virotech) in their ready-to-use forms (1, 0.5, 0.2 and 0.1 IU/ml) and at various dilutions prepared in PBS-T (0.001-1 IU/ml) were studied as samples. It was found that there was a significant correlation between anti-toxin concentration and OD 450 nm values obtained with both ready-to-use forms ($r=0.9704$ and $p=0.00609$) (Fig. 4) and PBS-T-diluted forms ($r=0.9968$ and $p=0.00021$) (Fig. 3). Different anti-DT concentrations diluted in the original sample diluent of the commercial anti-DT ELISA kit or FCS gave similar OD 450 nm values indicating that different sample diluents did not cause any matrix effect in the ELISA system prepared (Fig. 3). In the study performed with diluted standards, it was predicted that human serum samples could be studied in a 1/100 diluted form (in PBS-T) with the in-house system (Fig. 3). When the performances of both the in-house and commercial anti-DT ELISA systems were compared using ready-to-use standards (Fig. 4), it was observed that the in-house system was able to measure very low serum anti-DT levels (a minimum of 0.01 IU/ml) that gave overtly higher OD 450 nm values compared to those of the blank. Additionally, the in-house system managed to make linear measurements at anti-DT concentrations of 0.1-1 IU/ml. On the other hand, while a statistically significant and high correlation with concentration was detected using ready-to-use standards of the commercially available kit ($r=0.9808$ and $p=0.00055$), the minimum anti-DT levels of protective immunity (0.01-0.009 IU/ml) gave OD 450 nm values very close to the OD values of blank. The lowest level of standard anti-DT causing a significant increase in OD values was between 0.2 and 0.5 IU/ml. Therefore, the analytical sensitivity of the in-house ELISA system was estimated to be at least 20-50 times higher than that of the commercially available ELISA kit.

The in-house and commercial anti-DT ELISA systems were also compared using different human sera of various dilutions (Fig. 5). In all dilutions, the OD 450 nm values obtained with the in-house ELISA were higher than those obtained with the commercial kit. Thirty-one different human serum samples (diluted to 1/100) of various ages were studied with both systems and their anti-toxin levels were detected according to standard curves (Table 1). Only seven samples revealed measurable levels of anti-toxin with the commercial kit, and these seven people were relatively younger than the others. When the in-house system was used, anti-toxin levels could be measured in most (24 out of 31) of the samples. When data related to the analytical sensitivities of these two measuring systems were considered, it was predicted that the commercial ELISA kit (Virotech) could provide false negatives and that the in-house ELISA system could measure lower levels of anti-toxin; therefore, it was considered more sensitive (Table 1).

These data showed that the developed ELISA system was more sensitive compared to the expensive commercially available anti-DT

ELISA kit.

In addition, the developed system can be used for other purposes. Humoral immunodeficiencies constitute an important group of immunodeficiency diseases. Diseases due to antibody insufficiency can be seen both during childhood and in adult life (34, 35). Most primary immunodeficiencies are acquired and usually diagnosed clinically after 10 years of age (36). The diagnosis of humoral immunodeficiency can be suspected when expected protective antibodies cannot be detected, while recurrent infections start to be seen between 4 months and 2 years of age. Laboratory measurement methods are very important for performing diagnoses. Investigating a specific antibody response to a known antigen is a far safer method than measuring total immunoglobulin levels in serum for the diagnosis of humoral immunodeficiency and detecting sensitivity to infections. Although total serum immunoglobulin levels are normal in some cases, specific antibodies cannot develop after vaccinations. Measuring specific antibody responses to diphtheria or tetanus toxoid, which are included in the DPT vaccine that forms a part of the routine immunization program, can help in the early diagnosis of humoral immunodeficiency (13). A sensitive, low-cost screening test system like the one developed in our laboratory will readily be of use for this purpose.

The developed in-house anti-DT ELISA system can be used not only for clinical purposes but also for in vivo and in vitro research into humoral immune responses. In the immunological sciences, the investigation of humoral immune responses in experimental animals and/or cell cultures is a broad topic area. As mentioned above, the in-house anti-DT ELISA system was designed to measure anti-DT antibodies without being affected by the species or isotype of a specific antibody. When diphtheria toxoid is used as the antigen, in order to investigate the in vitro antibody response developed or to study the different factors affecting this antibody response, a highly sensitive antibody measurement system is needed. The chosen measurement system should be able to measure very low levels of antibody in order to be able to investigate in vitro antibody response. Similarly, an antibody measurement system of high sensitivity and specificity is necessary to investigate the effectiveness of vaccination routes or of the adjuvants used in vaccine preparation. Therefore, in immunology research centers where different studies are planned on humoral immune response, having such systems to measure specific antibody response, especially without needing to worry about cost, will be of great assistance.

As mentioned above, a commercially available anti-DT ELISA kit (Virotech, Germany) was studied in comparison with the laboratory-made system. The ability of the commercial kit was limited to measuring antibody levels below 0.1 IU/ml; moreover, the cost of 88 tests (except for the standards) was about 200 USD. However, the analytical sensitivity of the laboratory-made ELISA system was below 0.001 IU/ml, and the 500 USD spent on this project in 2001 was enough to prepare dozens of ELISA plates. From the results of the preliminary experiments it was calculated that 5 ml of conjugate prepared at one time was sufficient for about 12,500 tests.

Consequently, a new anti-DT ELISA system of high specificity and sensitivity but very low cost was developed in this project. It can be used with great success in many different areas such as for the screening of immunity to diphtheria, the screening of humoral immunodeficiency, and for in vivo and in vitro research into humoral immune responses.

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