Study on the use of an enzyme-linked immunosorbent assay in determining human antibodies to diphtheria toxin as compared with a reference toxin neutralization assay

L. Skoura\textsuperscript{a}, A. Efstratiou\textsuperscript{b}, A. Tsakris\textsuperscript{c}, S. Pournaras\textsuperscript{a}, R.C. George\textsuperscript{b}, J. Douboyas\textsuperscript{a, *}

\textsuperscript{a}Department of Microbiology, AHEPA University Hospital, 1 Kyriakidis Street, Thessaloniki 54 636, Greece
\textsuperscript{b}Respiratory and Systemic Infection Laboratory, Central Public Health Laboratory, London NW9 5HT, UK
\textsuperscript{c}Department of Microbiology, Medical School, Aristotelian University of Thessaloniki, Thessaloniki 54 006, Greece

Abstract

Serum samples from 156 Greek persons were assessed by an IgG-specific enzyme-linked immunosorbent assay (ELISA) and a reference tissue culture toxin-neutralization (TN) assay for the quantitation of diphtheria toxin antibodies. By the reference method, 7.7% of the persons were susceptible to diphtheria (antitoxin < 0.01 IU/ml), 28.8% had basic protection (antitoxin 0.01–0.09 IU/ml) and 63.5% were fully protective (antitoxin $\geq$ 0.1 IU/ml), while the corresponding figures were 17.9, 36.5 and 45.5% when they were tested by the immunoassay. None of the samples been susceptible by the TN assay were found to have some protection when tested by ELISA. However, three (6.7%) of the 45 samples showing a basic protection with TN, were fully protective when titrated by the immunoassay. In addition, 31 (31.3%) of the 99 samples been fully protective by the bioassay, were found to be either basically protective or susceptible by means of the ELISA. Overall, validity features of the immunoassay were: sensitivity 68.7%, specificity 94.7%, positive predictive value 95.8% and negative predictive value 63.5%. The ELISA tested in our study could be used to determine diphtheria antitoxin in individuals needed a booster immunization (susceptible or basic protective samples), although it might falsely
include in the above categories samples that are within the fully protective levels of antibodies. © 1999 Elsevier Science Ltd. All rights reserved.

**Keywords:** ELISA; Diphtheria; Toxin-neutralization assay; E.L.I.S.A; Neutralisation de toxine diphtérique

**Résumé**

Les anticorps neutralisants anti-diphértiques ont été titrés dans le sérum de 156 sujets Grecs selon d’une part, une technique E.L.I.S.A. IgG et d’autre part par la technique de référence de séraneutralisation (NT) en culture cellulaire.

Selon la technique de référence, 7.7% des sujets ont été classés comme potentiellement réceptifs à la diphtérie (titre en antitoxine < 0.001 UI/ml), 28% avaient un titre protecteur bas (titre compris entre 0.01 et 0.09 UI/ml) et 63.5% une protection avérée avec titre élevé. En technique E.L.I.S.A. IgG, et selon cette même classification, les valeurs étaient respectivement de 17.9, 36 et 45.5%. Parmi les sujets classés potentiellement réceptifs à la diphtérie d’après la technique de référence (NT), aucun d’entre eux ne présentaient de titre protecteur bas ou élevé en technique E.L.I.S.A. IgG.

Parmi les 45 sérum montrant un titre protecteur bas, trois d’entre eux (soit 6.7%) montraient un titre protecteur élevé en E.L.I.S.A. IgG. Trente et un (soit 31.3%) des 99 sérums présentant un titre protecteur élevé selon la technique de référence (NT) montraient, eux, soit un titre non protecteur, soit un titre limite bas en technique E.L.I.S.A. IgG.

Les critères de validation de la méthode E.L.I.S.A. IgG par rapport à la technique de référence (NT) avaient respectivement, une sensibilité de 68.7%, une spécificité de 94.7% et une valeur prédictive de positivité de 95.8% contre une valeur de négativité de 63.5%.

La technique E.L.I.S.A. IgG étudiée lors de notre étude serait susceptible de classer les sujets selon leur niveau de titre en antitoxine diphtérique. Ceci permettrait d’identifier les candidats à une immunisation de rappel (titres non protecteurs et protecteurs limites) en dépit du fait que cette technique E.L.I.S.A. IgG amènerait à revacciner des sujets ayant un titre élevé en anticorps protecteur en séraneutralisation. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Nowadays, diphtheria is a rare disease in Greece as in most other European countries, due to widespread immunization with diphtheria toxoid. However, it has been shown that in some circumstances, initial courses of immunization may be ineffective in preventing sporadic cases of diphtheria among adults [1]. So, the current diphtheria epidemic in the Newly Independent States (NIS) of the former USSR together with the increasing travelling and migration from the neighbouring eastern European countries in Greece highlight the need for reliable seroepidemiological surveys [2, 3]. Thus, meaningful in vitro assays evaluating the
precise immune status and monitoring the efficacy of the immunization program within our region are required.

Previous data have been suggested that a tissue-culture toxin-neutralization (TN) assay was the most appropriate test for measuring diphtheria antitoxin concentrations in large numbers of human serum samples [4]. On the other hand, there is a considerable interest in the validity of immunoassays due to their rapidity and convenience, particularly when limited culture facilities exist. However, there is not adequate evidence that such assays could accurately estimate individuals needed immunization [5–7].

In this report we are trying to study whether ELISA could be a reliable predictor of immunity in a Greek population, comparing results deriving from a commercial IgG-specific ELISA with those derived from the reference Vero cell neutralization-assay.

2. Materials and methods

In our study serum samples were collected from 156 individuals from Greece (89 male, 67 female). Newborns, children, adults and elderly persons over 60 years were included (age ranged 1 to 67, mean age 27.8). Until tested sera were stored at −20°C.

The tissue-culture TN assay used to estimate serum diphtheria antitoxin was a modified version of the monkey cell assay [8]. In a microtitre plate, doubling dilutions (final volume 50 μl) of serum and WHO reference antitoxin were made in cell-culture medium. 50 μl of a 2.5×10⁻³ Lf/ml toxin-solution in culture medium was added to the serum and reference antitoxin solutions. After 1 h incubation in the dark at 37°C, toxin neutralization occurred. Then 50 μl of a suspension (2.5×10⁵ cells/ml) of Vero cells in cell culture medium was added to each well. After a 3-day incubation in the dark (10% CO₂) at 37°C, cell viability was tested with an inverted microscope. Antitoxin concentrations in IU/ml were calculated, by taking the last dilution of serum at which cells are viable, and then multiply the dilution factor by the lowest concentration of reference antitoxin that neutralised the added toxin.

The ELISA test used for the quantitative determination of serum antidiphtheria antibody title was developed by LMD Laboratories, Carlsbad, CA. In a microwell plate, 100 μl of serum samples and standard sera were added. The antibody to be detected in human serum formed an immune complex with the antigen fixed on the test strips, during the first incubation. The conjugate enzyme (protein-A-peroxidase) is attached to this complex during the next incubation. Few washings are provided to remove the unbound enzyme, and then a substrate (citric acid and peroxide) and chromogen (TMB) was added, resulting in a blue colour in the presence of the enzyme complex and peroxide. The stop solution ended the reaction and turned the blue colour to yellow. Antitoxin concentrations in IU/ml
Validity features of the ELISA method were calculated as appropriate.

### 3. Results

Degrees of diphtheria immunity found by the two methods (TN and ELISA), are shown in Table 1. Antitoxin concentration less than 0.01 IU/ml are deemed to indicate susceptibility to diphtheria, 0.01–0.09 IU/ml to provide basic protection against the toxic manifestations of disease and ≥0.1 IU/ml to be fully protective [4]. By these criteria, 7.7% of the subjects were susceptible to diphtheria, 28.8% had basic protection and 63.5% were fully protective when they were analyzed by the bioassay, while the corresponding figures were 17.9, 36.5 and 45.5% when tested by the immunoassay. None of the samples with a value of less than 0.01 IU/ml in the TN had a higher value when tested by ELISA. However, three (6.7%) of the 45 samples showing a basic protection with the TN assay, were fully protective tested by ELISA. In addition, 31 (31.3%) of 99 samples been fully protective by the bioassay, were found to be either basic protective (29) or susceptible (2) by means of the ELISA. The validity features of the ELISA were estimated when susceptible and basic protection categories were unified. Thus, the immunoassay had 68.7% sensitivity, 94.7% specificity, 95.8% positive predictive value and 63.5% negative predictive value.

### 4. Discussion

It has been proposed that TN assay in cell cultures is the reference method to detect diphtheria antitoxin levels in sera because it measures functional antibodies that neutralize diphtheria toxin [4, 9]. Other tests, such as ELISA, can also be used to estimate antibodies to diphtheria toxin [7]. However, few studies have compared tissue culture TN assays with enzyme-linked immunoabsorbent tests for determination of diphtheria antitoxin in human sera [5–7]. All these previous
reports conclude that ELISA have a poor correlation with TN tests for sera containing $<0.1$ IU/ml, with a significant risk of false positive interpretations of immunity. It has been considered that ELISA false positive reactions are due to the binding of nonneutralizing antibodies [9]. Furthermore, it has been reported that even for sera with antitoxin concentrations more than $0.1$ IU/ml, ELISA may not be a reliable predictor of immunity [6]. However, in a recent study, immunoassays were found promising for detection and quantitation of antitoxin antibodies and good correlations were reported with established TN assays [10].

In our study the results of the ELISA for samples with a value of antitoxin concentration $<0.01$ IU/ml by the tissue-culture method, have fully agreed with the reference method. However, the sensitivity of the immunoassay was low. The reason for this statement is that a large proportion of samples with protective levels of antitoxin when tested by TN, were found to have basic protection and more rarely susceptibility by the ELISA method. On the other hand, when assumed that a value of $0.1$ IU/ml or more is necessary to confer protection, only a small proportion (1.9%) of persons tested by the ELISA method would be wrongly not included in those needed immunization. This is in contrast to other studies, where due to the low specificity of the immunoassays, up to 17% of individuals would not have been identified as needing immunization on the basis of their results [5, 11]. It seems, that in our immunoassay, antigen coating the wells is more specific from those used previously, containing antigens who respond to neutralizing and nonneutralizing antibodies.

Hence, the ELISA tested could be a valuable tool to determine diphtheria antitoxin in individuals at risk for exposure to diphtheria and for our region its results could be used to determine whether or not reimmunization of the population with the adult diphtheria (and tetanus) toxoids is required. This will ensure a high level of protection against diphtheria. However, it might falsely reimmunize some individuals with fully protective levels of antibodies. It seems that continuous monitoring of immunoassays, containing antigens from individual fragments or epitope of fragments, is needed in order to assess the most appropriate assays for the precise immune status of the community.

References


