ORIGINAL ARTICLE

Detection of diphtheria antitoxin by four different methods

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Objective: To investigate the reliability of the different methods used in Norway and Russia for detection of diphtheria antitoxin.

Methods: One hundred and twenty-two sera were selected among Russian serum samples previously collected for seroepidemiologic studies of diphtheria antitoxin. The sera were selected to cover the total antitoxin range and were analyzed by four different antidiphtheria toxin assays: an in vitro toxin neutralization test using Vero cells (in vitro NT), an in vivo neutralization test using rabbit skin inoculation (in vivo NT), an indirect enzyme immunoassay (EIA) and a passive hemagglutination assay (PHA). The results were expressed according to the international standard as: not protected (<0.01 IU/mL), relatively protected (0.01–0.1 IU/mL) or protected (≥0.1 IU/mL). The sensitivity, specificity and inter-rater agreement (K or Kw) of each method were related to the in vitro NT selected as the reference method.

Results: The in vivo NT test corresponded very well with the in vitro NT in its ability to differentiate between protection/relative protection and no protection (sensitivity 97%, specificity 87% and K=0.84). The EIA test showed a high sensitivity (96%), but since many sera were categorized as protected rather than not protected, the specificity (30%) and inter-rater agreement (K=0.29) were low. The PHA test had a very high specificity (100%) but a low sensitivity (86%).

Conclusions: The agreement between the two neutralization tests was high. If none of the neutralization assays is routinely available, the PHA test can be used to predict the need for vaccination on an individual basis but should not be used for seroepidemiologic studies, since the protection rate for diphtheria would be falsely too low, due to the lower sensitivity. The indirect EIA test used in this study should not be used routinely.

Key words: *Corynebacterium diphtheriae*, diphtheria antitoxin, serologic assays, protection

INTRODUCTION

Diphtheria has become a rare disease in developed countries during the last decades. However, it re-emerged in Russia in 1990, and an epidemic spread to Ukraine the following year [1]. By 1994, all the New Independent States (NIS) formerly belonging to the Soviet Union had an ongoing epidemic, and neigh-

boring countries of the NIS, Norway included, have experienced sporadic cases of diphtheria [1–3].

The re-emergence of diphtheria has demonstrated the importance of infection control and immunity surveillance. Seroepidemiologic surveillance is dependent on reliable methods for measuring protective levels of diphtheria antitoxin. The earliest method for detecting serum antitoxin levels was developed by Behring, Ehrlich and Roux (1892–1895). In a sensitive assay using skin inoculation of guinea pigs, the ability of serum to neutralize the local effect of diphtheria toxin was measured by titration [4]. However, despite modifications of this technique by Romer and Somogyi [5] and Jensen [6], methods requiring animals are not easily applied as screening tests. Therefore, different in vitro methods have been developed in order to measure the degree of protection against diphtheria [7,8]. A toxin neutralization test based on Vero cells is now well

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established [9] and indirect enzyme immunoassays (EIA) have been tried. In Russia, a passive hemagglutination test has been the routine test for many years, using the in vivo toxin neutralization assay as the reference test.

In this study we wanted to compare the agreement of these four methods, in order to investigate the reliability of detecting the true protection level of diphtheria antitoxin.

MATERIALS AND METHODS

Serum samples

One hundred and twenty-two serum samples were included in the study. The sera belonged to a collection of Russian samples used in a previously reported and still continuing seroepidemiologic study of diphtheria protection [10]. The sera were selected to cover the total normal diphtheria antitoxin range. The male/female ratio was 1.27, and the mean age of the subjects was 39.6 years (range 2–85 years). The sera were all stored at −20°C, before being analyzed blindly.

Serologic tests

Four different diphtheria antitoxin assays were used in the study. An in vitro toxin neutralization test (in vitro NT) and an indirect EIA were performed at the Department of Bacteriology, National Institute of Public Health, Oslo, Norway. An in vivo toxin neutralization test (in vivo NT) and a passive hemagglutination (PHA) assay were performed at the Laboratory of Epidemiologic Surveillance for Diphtheria, G.N. Gabrichevsky Institute for Epidemiology and Microbiology, Moscow, Russia.

In vivo toxin neutralization test (in vivo NT)

The in vivo NT was performed with minor modifications according to the method described by Jensen [6] and according to the L.A. Tarasevich State Institute for Standardization and Control of Medical Bioproducts, Moscow, Russia. The amount of toxin used in the test corresponded to the 1/3000 IU reactive dose (LR/3000). LR is the minimal amount of toxin that, when mixed with 1/3000 IU antitoxin in a volume of 0.1 mL, produces a 10-mm-diameter non-necrotic erythematous area after 48 h when inoculated intracutaneously in rabbits (≥3 kg). For the biological assay, serum dilutions were incubated with the toxin for 2 h at 37°C, and 0.1 mL of the mixture (containing LR/3000) was then inoculated intracutaneously. The results were read after 48 h, and the serum antitoxin titer was calculated by comparison to standard serum inoculated at the same time. The results were expressed as IU/mL.

In vitro toxin neutralization test (in vitro NT)

The in vitro toxin neutralization assay was based on the method described by Miyamura et al. [9] and performed as described earlier [10]. In brief, two-fold dilutions of serum were mixed with four times the minimal cytotoxic dose of diphtheria toxin (Statens Seruminstitut, Copenhagen, Denmark) and incubated at 37°C for 1 h before Vero cells (African green monkey kidney) were added. An antitoxin-positive control serum was run for every 17th serum sample. The pH-mediated color of the culture medium, which changed from red to yellow, as a result of growing Vero cells, was recorded after 5 days of incubation at 37°C. The antitoxin level of each sample was determined by comparing the color change breakpoint to that of a WHO standard (Statens Seruminstitut) analyzed simultaneously [10]. The results were expressed as IU/mL.

Enzyme immunoassay

Flat-bottomed immunoplates (Nunc-immunosorp, code 439454, Nunc, Denmark) were coated with 100 μL (2.5 μg/mL) of diphtheria toxoid (Statens Seruminstitut) in phosphate-buffered saline (PBS), pH 7.2. Following overnight incubation at 37°C, the plates were washed three times with PBS containing 0.05% Tween-20 (PBST), pH 7.4. One hundred microliters of PBST with 0.1% bovine serum albumin was then added to each well, and the plates were incubated at 37°C for 30 min to block unspecific binding sites, and then washed three times with PBST. The serum samples were diluted 1:50 in PBST, and 100 μL was added to duplicate wells. The samples were then incubated at 37°C for 2 h, followed by washing three times. One hundred microliters of swine antihuman IgG conjugated to alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) diluted 1:200 in PBST was then added to each well. Following incubation for 2 h at 37°C, 100 μL (1 mg/mL) of dinitrophenylphosphate (Sigma 104, St Louis, MO, USA) in diethanolamine buffer, pH 9.8, was added. The color development was stopped after 45 min at room temperature by adding 50 μL of 4M NaOH, and the plates were read in a microplate reader (340 ATC, SLT Laboratory Instruments, Salzburg, Austria) at 405 nm with 492 nm as the reference wavelength. The OD result of each serum was transformed to IU/mL by comparing the result to a standard curve, obtained by analyzing fourfold dilutions of a human reference serum (Statens Seruminstitut), using a curve-fit computer program (Soft 2000, SLT Laboratory Instruments) connected to the reader. Optimization of the test was determined by preliminary experiments. The lowest detectable antibody amount was 0.02 IU/mL.

Passive hemagglutination test (PHA)

The passive hemagglutination test (I. I. Mechnikov/ Biomed, Moscow, Russia) was performed according to the standard instructions given by the Russian Health Authorities [11]. Each serum, diluted 1:5 in isotonic saline, was adsorbed with non-sensitized sheep erythrocytes for 24 h at 4°C to remove non-specific agglutinins. Each serum was then diluted two-fold in U-bottom microtiter wells starting at 1:10, using the diluent buffer provided by the manufacturer. To each well, containing 100 μL of diluted serum, 25 μL of a suspension of sheep erythrocytes sensitized with diphtheria toxoid was added. The plates were stirred gently and stored at room temperature for 60 min before the agglutination pattern was read. The results were expressed as titers. A serum control for the presence of non-specific agglutinins was included for each serum, and positive controls were included in each run.

Protection levels

The antibody amounts detected for the in vivo NT, the in vitro NT and the EIA were grouped according to the protection level against diphtheria [12]: no protection, <0.01 IU/mL (EIA <0.02 IU/mL); relative protection, 0.01–0.1 IU/mL (EIA 0.02–0.1 IU/mL); or protection, ≥0.1 IU/mL. For the PHA test, the protection levels related to titer values were based on recommendations given by the Russian Health Authorities: no protection titer ≤10, relative protection titer 20–80, and protection titer ≥160 [11].

Statistical methods

Sensitivity and specificity for each test were calculated according to Galen [13], and inter-rater agreement was estimated by kappa (K) and weighted kappa (Kw) according to Altman [14], with the in vitro NT as the reference test (SPSS for Windows, version 8.0, Chicago SPSS, Illinois, USA). The inter-rater agreement (K and Kw) was classified as poor (<0.20), fair (0.21–0.40),

moderate (0.41–0.60), good (0.61–0.80) or very good (0.81–1.00), according to the guidelines given by Landis and Koch [15] and Altman [14]. The agreement was calculated for two different cut-off levels: between no protection and relative protection, and between relative protection and protection.

RESULTS

The results achieved by the different assays grouped according to the degree of protection are shown in Tables 1 and 2. By the in vitro NT, 23 sera (18.9%) had non-protective levels of antitoxin, 47 (38.5%) had relatively protective levels, and 52 (42.6%) protective levels. By the in vivo NT, the same number of sera showing no protection (18.9%) was found, while a higher number showed protection (54.9%). Only 10.7% of the sera showed non-protective levels of antitoxin by the EIA, while as many as 69.7% showed protective levels. The highest proportion of sera classified as having no protection (30.3%) was found by the PHA. By this test, only 38.5% of the sera were classified as protected. The agreement between the in vitro NT and the in vivo NT expressed by weighted kappa (Kw) was good (Kw=0.71, 95% confidence interval (CI), 0.57–0.85) (Table 2). For the EIA, the Kw was moderate (Kw=0.43, 95% CI, 0.30–0.55), and for the PHA the Kw was good (Kw=0.76, 95% CI, 0.63–0.90).

The highest agreement (K=0.84) was found when comparing the two toxin neutralization tests using a cut-off of 0.01 IU/mL (Table 3) while the lowest was found between the EIA test and the in vitro NT using a 0.01 IU/mL cut-off. Also, at a cut-off of 0.1 IU/mL, the agreement was lowest between the in vitro NT and the EIA. Although the sensitivity of the EIA was 100%, the specificity was low (53%) (Table 3). The PHA test showed the highest specificity (100%) but the lowest sensitivity (86%) (Table 3).

Table 1 Antibodies against diphtheria toxin for 122 sera examined by four different methods according to level of protection against diphtheria [12]

Method	No protection, <0.01 IU/mL[a]			Relative protection, 0.01 to <0.1 IU/mL[a]			Protection, ≥0.1 IU/mL		
	n	%	95% CI	n	%	95% CI	n	%	95% CI
In vitro NT[a]	23	18.9	11.9–25.8	47	38.5	29.9–47.2	52	42.6	33.8–51.4
In vivo NT	23	18.9	11.9–25.8	32	26.2	18.4–34.0	67	54.9	46.1–63.7
EIA	13	10.7	5.2–16.1	24	19.7	12.6–26.7	85	69.7	61.5–77.8
PHA	37	30.3	22.2–38.5	38	31.1	22.9–39.4	47	38.5	29.9–47.2

[a]For the EIA test, the cut-off between not protected and relatively protected was 0.02, in accordance with the detection limit of the test (see text).

NT, diphtheria toxin neutralization test; EIA, enzyme immunoassay (indirect); PHA, passive hemagglutination assay.

Table 2 Comparison of four different assays for determination of antibodies against diphtheria toxin[a] with in vitro NT as reference assay

		In vitro NT					
Method		No protection[b] ($n=23$)	Relative protection[b] ($n=47$)	Protection ($n=52$)	Total ($n=122$)	Kw[c]	95% CI[d]
In vivo NT	No protection	20	3	0	23	0.71	0.57–0.85
	Relative protection	3	25	4	32		
	Protection	0	19	48	67		
EIA	No protection	7	6	0	13	0.43	0.30–0.55
	Relative protection	10	14	0	24		
	Protection	6	27	52	85		
PHA	No protection	23	13	1	37	0.76	0.63–0.90
	Relative protection	0	31	7	38		
	Protection	0	3	44	47		

[a]No protection, <0.01 IU/mL, relative protection, 0.01 to <0.1 IU/mL; protection, ≥0.1 IU/mL [12].
[b]For the EIA test, the cut-off between not protected and relatively protected was 0.02, in accordance with the detection limit of the test (see text).
[c]Weighted Kappa [14].
[d]95% confidence interval.
NT, diphtheria toxin neutralization test; EIA, enzyme immunoassay (indirect); PHA, passive hemagglutination assay.

Table 3 Sensitivity, specificity and inter-rater agreement for 122 selected sera using four different antidiphtheria toxin assays, with in vitro NT as reference test

	Sensitivity (%)	Specificity (%)	Agreement (K)[a]	95% CI[b] for K
Cut-off=0.01 IU/mL[c]				
In vivo NT	97	87	0.84	0.66–1.00
EIA	96	30	0.29	0.13–0.46
PHA	86	100	0.70	0.53–0.87
Cut-off=0.1 IU/mL[c]				
In vivo NT	92	73	0.63	0.46–0.80
EIA	100	53	0.49	0.34–0.64
PHA	85	96	0.81	0.64–0.99

[a]Kappa [14].
[b]95% confidence interval.
[c]No protection, <0.01 IU/mL, relative protection, 0.01 to <0.1 IU/mL; protection; ≥0.1 IU/mL [12].
NT, diphtheria toxin neutralization test; EIA, enzyme immunoassay (indirect); PHA, passive hemagglutination assay.

DISCUSSION

A reliable test for the detection of immunity to diphtheria is important both when performing sero-epidemiologic studies and on an individual basis in order to investigate the need for diphtheria vaccination or for the detection of clinical diphtheria.

The in vivo NT was the original standard method [4]. However, the need for animals makes this test difficult to use as a standard routine reference assay. Therefore, we decided to use the toxin neutralization test on Vero cells in microculture (in vitro NT) as the reference method. This is in accordance with the recommendation given by the First International Meeting of the WHO Laboratory Working Group on Diphtheria in 1994 [4]. The agreement (Kw) between the two neutralization assays was classified as good. The main discrepancy was that 19 of 47 sera (40%) grouped as relatively protected by the in vitro NT were grouped as protected by the in vivo NT. A high correlation between the in vivo and the in vitro NT has also been shown by Miyamura et al [9,16], Kriz [17] and Kjeldsen et al [18].

The EIA test used in this study produced a large number of false-positive results. Used in a sero-epidemiologic study, this test would give the impression that the population was better protected against diphtheria than is actually the case. If this test were used

to evaluate the need for either basic or booster vaccination, many individuals who need immunization would be falsely judged as protected and thus escape vaccination.

The serum samples included in this study were selected to be equally distributed over the total antibody range. In a population with a high vaccine coverage against diphtheria, the proportion of protected individuals will normally dominate. Although the sensitivities and the specificities of the different tests in the study are independent of the prevalence of antitoxin in the population, the inter-rater agreement as measured by K is dependent on the proportions of sera classified as no protection, relative protection and protection, respectively. Thus, the EIA test, which had a high agreement with the reference test in the upper part of the antibody range (protection) (Table 2), would have a higher inter-rater agreement with the reference test than measured in our study, when used in a seroepidemiologic study on a highly vaccinated population. Despite this fact, we cannot recommend this indirect EIA test. The tendency towards false-positive results by EIA has also been shown in previous studies by Knight et al [7] and Mellville-Smith and Balfour [8]. However, Hendriksen et al [19] have described a modified version of the EIA test which has shown higher correlation with the neutralization test, and recently Kristiansen et al [20] have described an EIA test using the double antigen principle for detection of diphtheria antibodies that corresponds well with an in vitro NT.

Due to rapidity, low cost and no need for special equipment, the PHA is the most frequently used routine assay in Russia, both in the pre-epidemic period and during the epidemic, where it is used for monitoring immunity. Seroepidemiologic studies conducted in the Soviet Union during the 1980s and the beginning of the 1990s, using the PHA as the test assay, showed a high prevalence of people lacking immunity to the diphtheria toxin [21–24]. These studies supplied evidence for some of the reasons for the resurgence of diphtheria, such as low vaccine coverage among children and adults and waning of vaccine-induced immunity.

The overall strength of agreement between the PHA assay and the in vitro NT was classified as good (Kw=0.76), and the strength of the agreement using cut-off 0.01 IU/mL and 0.1 IU/mL was good (K=0.70) and very good (K=0.81), respectively. However, the main limitation of the PHA was the low sensitivity. Of the 47 sera belonging to the relative protection group by the in vitro NT, 13 sera (28%) showed no protection by the PHA, and one of 52 sera (2%) in the protection group also tested negative by the PHA. This tendency to underestimate low concentrations of diphtheria

antibodies by the PHA has also been shown by Simonsen [25] and Galazka and Abgarowicz [26]. In seroepidemiologic studies based on the PHA test, the proportion of individuals found to be unprotected against diphtheria would be falsely too high, giving the impression that the population is more susceptible to the spread of diphtheria than is actually the case. In a previous study comparing antibody prevalences among Norwegians and Russians, the protection levels among Russians was not significantly different from that detected among Norwegians [10]. Nevertheless, it is a fact that the diphtheria epidemic spread in Russia and not in Norway. Therefore, lack of protection against diphtheria seems to be only one factor necessary for the spread of the epidemic.

When one of the neutralization tests is not routinely available, the PHA can be used for routine testing bearing in mind that when it is used for seroepidemiologic purposes the protection rate will be higher than what is actually measured. When the PHA is used to predict the need for individual vaccination, almost all subjects with insufficient immunity, i.e. subjects who would have been identified as relatively protected or non-protected by the in vitro NT, will be recommended for vaccination.

Acknowledgments

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