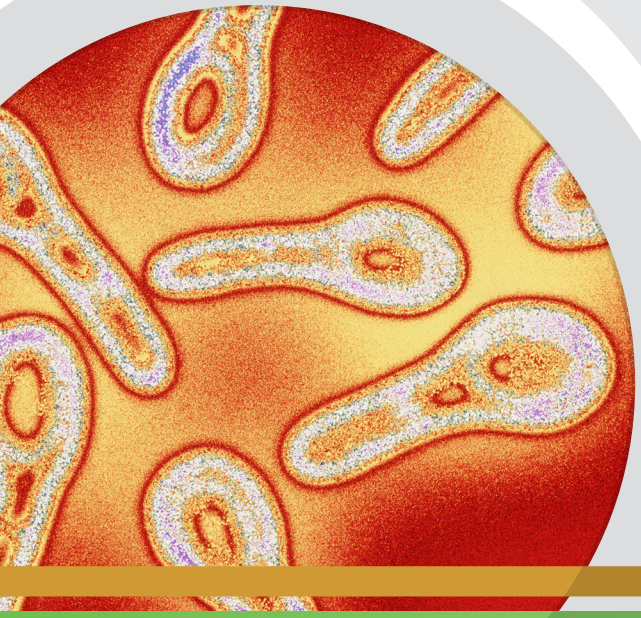
ecdc
EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL



TECHNICAL REPORT

Evaluation and assessment of serological immunity methods and external quality assessment scheme of diphtheria

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

Evaluation and assessment of serological immunity methods and external quality assessment scheme of diphtheria

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Dr Ida Czumbel and produced by Christina von Hunolstein, Luisa Ralli, Antonella Pinto, Andrea Gaggioli (Istituto Superiore di Sanità – Rome – Italy *Subcontractor* of Public Health England WHO Global Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections, London, UK), with the collaboration of P. Stickings (NIBSC South Mimms, Potters Bar -UK), Professor Androulla Efstratiou, Dr Shona Neal on behalf of the EU DIP-LabNet consortium (referring to Specific Sub Contract ECD 2796).

Acknowledgements
We are grateful to all participants who took part in the collaborative study and follow up external quality assessment study.

This work was in part supported by the European Centre for Disease Prevention and Control (ECDC), Programme on European Diphtheria Surveillance Network, ECD.2796, implementing Framework Contract No. ECDC/10/002.

We are extremely grateful to the blood donors of the UOC di Immunoematologia e Medicina Trasfusionale, Università degli Studi "La Sapienza", Rome, Italy, to Prof. G. Girelli, Responsible of the UOC and to her collaborators Dr A. Fakeri and M. Santonicola.

Suggested citation: European Centre for Disease Prevention and Control. Evaluation and assessment of serological immunity methods and EQA scheme of diphtheria. Stockholm: ECDC; 2014.

Stockholm, September 2014

ISBN 978-92-9193-584-0

doi 10.2900/31454

Catalogue number TQ-04-14-649-EN-N

Contents

Abbreviations

dDA-DELFIA	Dual Double-Antigen Time-Resolved Fluorescence Immunoassay
DIPNET	Diphtheria Surveillance Network
EDSN	European Diphtheria Surveillance Network
ELISA	Indirect Enzyme-Linked Immunosorbent Assays
ELWGD	European Laboratory Working Group for Diphtheria
EQA	External Quality Assessment
EU DIP-LabNet	EU Diphtheria Laboratory Network
IU	International Units
MIA	Fluorescent Bead-Based Multiplex Immunoassay
NIBSC	National Institute for Biological Standards and Control
PHE	Public Health England
TNT	VERO cell Toxin Neutralization Test
ToBI	Toxin Binding Inhibition Test
WHO	World Health Organization

Executive summary

Diphtheria has become a well-controlled disease and is therefore uncommon in many western countries. Subsequently, recognition and diagnosis of a case is often difficult. Diphtheria is often confused with other conditions, such as severe streptococcal sore throat, Vincent's angina, or glandular fever. Therefore, accurate microbiological diagnosis is crucial and complementary to clinical diagnosis.

Effective control of a vaccine-preventable disease is dependent upon adequate vaccination programmes; therefore measuring the immunisation status of the population is essential. Accurate determination of diphtheria toxin antibodies is of value in assessing responses to vaccination and immunisation schedule efficacy, determining the rates of immunity within broad populations, as well as the immune status of individuals who may be at risk of infection (i.e. travellers, physicians, lab personnel).

It is important that EU Member States have the capacity and ability to undertake the procedures relating to the microbiological/serological diagnosis of diphtheria, as well as to all the related infections caused by all potentially toxigenic strains of corynebacteria. Moreover, diphtheria is still endemic in many parts of the world.

This report comprises of the findings of the external quality assessment (EQA) dispatch for the laboratory diagnosis of diphtheria serology under the auspices of the European Diphtheria Surveillance Network (EDSN). A total of fifteen countries participated in this EQA and were asked to determine the antibodies to diphtheria toxin in human sera. Key findings are listed below; a description of the work involved and the outcomes of these exercises are detailed further in this report.

The key objective of the work, as described in this report is:

- To assess and improve laboratory performance for standardised and appropriate methods to be used for the laboratory serological immunity testing for ensuring accurate and comparative diphtheria surveillance data across Europe.

Key findings

- Performance of laboratories using the VERO cell toxin neutralisation test was generally very good.
- Laboratories' performance using *in vitro* methods such as Dual Double-Antigen Time-Resolved Fluorescence Immunoassay (dDA-DELFIA) or Fluorescent Bead-Based Multiplex Immunoassay (MIA) was also good.
- Laboratories using in-house Indirect Enzyme-Linked Immunosorbent Assays (ELISA) performed differently, probably dependent upon the validation of the assay.
- The use of commercial ELISA kits does not guarantee that the laboratory will perform well.

Key recommendation

- An EQA is essential for monitoring performance amongst laboratories that use *in vitro* non standardised methods or commercial ELISA kits. It is also crucial for accurate determination of serological immunity levels amongst populations and measuring vaccine effectiveness within and between countries.

Background

The European Centre for Disease Prevention and Control (ECDC) was formed as a European Union (EU) agency to identify, assess, and communicate current and emerging threats to human health from communicable diseases. Part of ECDC's mandate includes fostering the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The Centre shall maintain such cooperation, and support the implementation of quality assessment schemes. (Article 5.3, EC 851/2004)[1].

An external quality assessment (EQA) exercise evaluates the performance of laboratories by an outside agency, which provides material especially for that purpose; this can be used as part of a quality management system.

Thus, EQAs may identify areas for improvement in laboratory diagnostic capacities. ECDC support EQA schemes as they impact on the surveillance of the diseases listed in Decision No 2119/98/EC[2] and ensure comparability of results across laboratories from all EU/EEA countries. The main purposes of EQA schemes include: assessment of performance of national reference laboratories and of laboratories offering diphtheria laboratory service; assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration):

- evaluation of individual laboratory performance
- identification and justification of problem areas
- provision of continuing education
- identification of needs for training activities.

Thus, the EQA process can increase the probability of correct sero-diagnosis, and improve the quality of sero-epidemiology surveillance data.

Clinical diphtheria is caused by toxin-producing corynebacteria. Three species, *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, and *Corynebacterium pseudotuberculosis*, have the potential to produce diphtheria toxin and hence can cause classic respiratory diphtheria.

It is worthy to note that *C. ulcerans* infections have exceeded *C. diphtheriae* infections in recent years in France, Germany, Italy, Netherlands, Sweden and the UK, and fatal infections have been recorded [1, 2]. As the morbidity, as well as the mortality of diphtheria is almost entirely due to diphtheria toxin, protection against the disease is dependent on antibodies against the toxin.

Today diphtheria is a marginal problem in western countries as only sporadic cases are reported [1] but is endemic in many other regions of the world, particularly Asia (India, Indonesia, Nepal, Philippines), Africa (Sudan) and South America (Brazil), and cases occurred recently in New Zealand (20 in 2011) where it had never been found [3]. The sudden explosion of diphtheria to epidemic proportions within Russia and surrounding countries in the 1990s highlighted the continuing potential of this disease to cause morbidity and mortality in areas where few cases had previously been seen [4].

In 1993, the European Laboratory Working Group on Diphtheria (ELWGD) was established [5] to strengthen the diphtheria diagnostic capabilities in the European region and beyond. During this time many European countries adopted enhanced practices, such as screening for diphtheria from routine throat swabs, and participated in serosurveillance studies to assess seroepidemiology of diphtheria in Europe (ESEN 1 and ESEN2) [6, 7]. The European Diphtheria Surveillance Network (EDSN) was subsequently established in March 2010 and comprises the nominated epidemiologists and laboratory experts for diphtheria from the 27 EU Member States and the three other EEA countries. The purpose of the EDSN is to establish a system of expertise for the prevention and control of diphtheria and to strengthen and harmonise the laboratory capacity at national level. The network has two components: epidemiological (conducted by ECDC and focused on data collection and analysis) and laboratory (outsourced to Public Health England (PHE), London, since April 2013, and focused on EQA and training).

Accurate determination of diphtheria toxin antibodies is essential to establish susceptibility of clinical laboratory workers, to obtain reliable information on the diphtheria immune status of a person or a given population, to evaluate the immunogenicity of diphtheria vaccines in clinical trials, as well as to monitor long-term immunity and thus to provide recommendations for vaccination policy. Therefore, it is of crucial importance to have serological methods that are accurate, reliable, specific and sensitive.

---

[1] Regulation (EC) No 851/2004 of the European Parliament and of the Council of 21 April 2004 establishing a European Centre for Disease Prevention and Control.

[2] Decision No 2119/98/EC of the European Parliament and of the Council of 24 September 1998 setting up a network for the epidemiological surveillance and control of communicable diseases in the Community.

Currently, there are a large variety of serological diagnostic tests for diphtheria in use across Europe and therefore it is important to periodically assess the methodologies used for diphtheria against the *in vitro* gold standard.

The *in vivo* toxin neutralisation test using guinea pigs or rabbits is regarded as the gold standard method for determining protective levels of serum antitoxin [8]. However, as it requires animals and specialised facilities, it is labour intensive, expensive and requires relatively large volumes of test serum and is thus not practical for use in serological diagnosis and seroepidemiological studies. Tests using cells in culture have been developed as reliable alternatives to the *in vivo* test for detection of diphtheria toxin and for toxin neutralisation [9]. The VERO cell toxin neutralisation test (TNT) is also recommended by the WHO and European Pharmacopeia for the *in vitro* analysis of immune sera for potency testing of diphtheria vaccines, giving comparable information to protection models in guinea pigs [10–14]. However, because these assays are time consuming and require cell culture facilities, diagnostic laboratories prefer to use simple format indirect enzyme-linked immunosorbent assays (ELISA), that offer significant advantages in terms of cost, speed, ease of use and adaptability to automation. Other *in vitro* methods are available, such as the double antigen, time-resolved fluorescence immunoassay (dDA-DELFIA) [15], and the fluorescent bead-based Multiplex immunoassay (MIA) [16], but none of these are easily performed as an indirect ELISA.

The first EQA for diphtheria serology in Europe was performed in 1994 under the auspices of the ELWGD, and since then two other distributions for laboratory serological immunity testing have been performed. The last diphtheria serology EQA was performed in 2008 under the remit of the Diphtheria Surveillance Network, officially recognised by the European Commission, where inconsistent results were observed using ELISAs and Passive Haemagglutination Assay [17].

Materials and methods

EQA design

The design of the EQA scheme allowed the material to be tested by the individual reference laboratories, using their routinely available techniques within the allocated time period. All participating laboratories were able to compare their own submitted results with the intended results to determine any differences and implement any improvements, if required.

Each of the participating laboratories received, from the Istituto Superiore di Sanità (ISS), Rome, Italy, a panel of 150 human serum samples to be tested for diphtheria antitoxin antibodies using an assay of their choice (Annex 1). Each laboratory used its own standard curve and included a positive and a negative control sample normally used in the assay.

The standard panel was required to be tested twice by each participant.

The results of specific diphtheria antitoxin concentration, expressed in IU/ml, were calculated by each laboratory according to their standard operating procedures and sent by e-mail to ISS.

The EQA panel preparation was approved by the ethical committee of the ISS, Rome, Italy (CE/11/314) and by the ethical committee of the UOC Immunoematologia e Medicina Trasfusionale, Università degli Studi "la Sapienza", Rome, Italy (C.E Prot. 122/11).

Participants

The list of the participating reference laboratories can be found in Annex 1.

All participants were contacted before the EQA distribution to confirm the address and contact details for dispatch of the sera panel and to send them the assigned code number.

Standard panel preparation

The standard panel was prepared using sera donated by blood donors of the UOC Immunoematologia e Medicina Trasfusionale, Università degli Studi "la Sapienza", Rome, Italy.

The panel comprised 148 serum samples (coded 1-231); 120 of these were native human serum samples, while 28/148 sera were pooled samples, prepared from up to three individual serum samples with comparable titres (this became necessary to provide sufficient volumes to all participants).

In addition to the human serum samples, the panel also included aliquots from two diphtheria antitoxin standard preparations. These standard preparations, 00/496 (NIBSC Diphtheria Antitoxin Human Serum, working standard, panel code 230) and 10/262 (NIBSC Diphtheria Antitoxin Human IgG, candidate WHO International Standard, panel code 231), were prepared at NIBSC by reconstituting 1 ampoule with 1 ml of sterile water. For each standard, the contents of multiple ampoules were pooled to provide sufficient volume for the study. Pooled standards were divided into aliquots and shipped with the rest of the panel to all participants. The results obtained for these two samples (in comparison to results obtained for the rest of the panel) were used in an extension of the EQA to determine the commutability of the candidate WHO International Standard (IS 10/262) as part of a WHO collaborative study, the results of which are published elsewhere [18].

The panel samples were selected based on the results obtained in two separate assays performed using an in-house immunoassay (dDA-DELFIA) at the ISS, Rome and likely to be classified as negative, equivocal and positive respect to anti toxin antibodies.

Aliquots of all samples were stored at –20°C and shipped by courier to all participants.

Participants received aliquots of 200 or 150 µl, depending on whether they were using VERO cell TNT or ELISA and MIA, respectively.

Due to casual events, Lab II and XV tested 149 samples, Lab IX tested 148 samples.

Assays

In the present EQA study, various assays were used to measure specific human diphtheria toxin antibodies. These included VERO cell TNT, dDA-DELFIA, MIA, and in-house or commercial ELISA methods. The commercial ELISA kits specific for the determination of diphtheria antitoxin antibodies were: Serion ELISA classic, Diphtheria IgG (Serion), Diphtheria ELISA IgG Testkit (Sekisui Virotech GmbH), anti-Diphtheria toxoid ELISA IgG (Euroimmun), Diphtheria toxoid IgG (The Binding Site Group, product code MK014), NovaLisa™ *Corynebacterium diphtheriae* toxin IgG ELISA (NovaTec Immunodiagnostica GmbH), Novagnost Diphtheria toxin 5S IgG (Siemens Novagnost), and Diphtheria Ab ELISA (IBL International GmBH). Commercial ELISAs were performed according to the manufacturers' specifications, using reagents that were supplied with the kits. Details of the assays used by each participating laboratory, including toxin/toxoid and reference antitoxin used and the reported limit of detection are listed in Annex 2.

Reference assay

The assay selected as a reference to evaluate the performance of the other assays was the VERO cell TNT from Lab I. Using this assay, diphtheria antitoxin levels in individual serum samples were classified as follows, based on WHO guidelines [19], and the work performed by Ipsen (20): positive, i.e. the full protective level of circulating antitoxin ($\geq$ 0.1 IU/ml); equivocal, partial protective level of circulating antitoxin (0.01 and 0.09 IU/ml), or negative, providing no protection (< 0.01 IU/ml). Results obtained by the reference Lab I showed that of the panel of 150 sera, 63 samples were positive, 54 samples were equivocal, 33 samples were negative (Annex 3).

Data analysis

Raw data comprised estimates for anti-diphtheria concentration in IU/ml for each sample in the panel. For those laboratories reporting two results for the same serum sample, the geometric mean was used for all subsequent analyses. In the case of ELISA kits, for serum samples with concentrations reported as 0 IU/ml an arbitrary value of 0.009 IU/ml, that is considered a negative value of diphtheria antitoxin, was imputed (Lab XI, n. of sera 10; Lab. XV, n. of sera 25). For values reported as 'lower than' or 'higher than' the imputed value was decreased or increased by 0.01 IU/ml accordingly (Lab XI, XII, XIII).

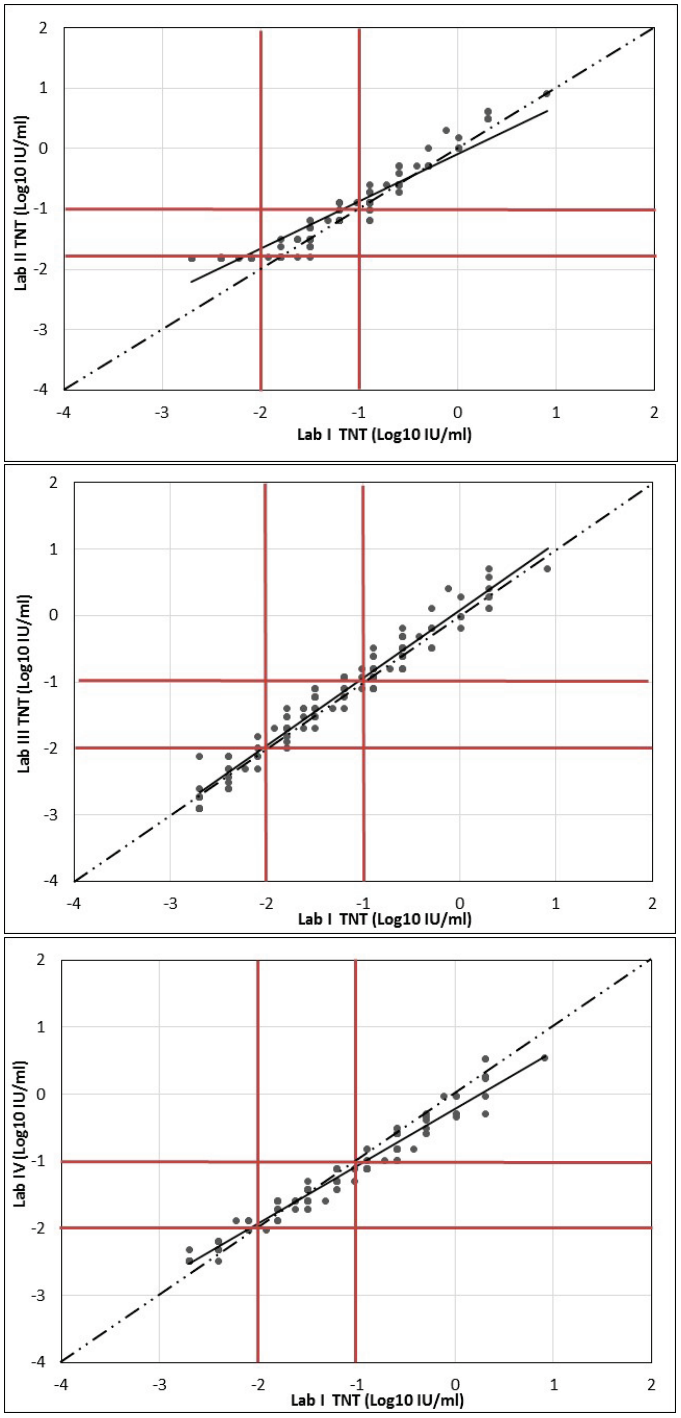
Participants' results were compared on a quantitative and qualitative basis. Data were analysed using IBM SPSS Statistics Version 20.0 and Microsoft Excel 2007.

To assess the extent of quantitative diagnostic agreement, the TNT-derived values from Lab I and the results obtained with all of the other tests were compared using a scatter plot of antibody measurements on a $\log_{10}$ scale. Pearson's correlation coefficient R was calculated for each scatter plot.

Results

Four laboratories (II, III, IV and reference Lab I) performed the VERO cell TNT assay. The inter-laboratory comparison of this method showed a regression line close to the line of identity for all laboratories (Fig. 1).

Figure 1. Interlaboratory comparison of the diphtheria antitoxin levels (IU/ml) of the standard panel tested by TNT in Lab II, III and IV *vs* Lab I.



Regression line (solid), and line of identity (dashed) are shown. Vertical and horizontal lines indicate the cutoffs used by to determine negative (<0.01 IU/ml), equivocal (0.01-0.09 IU/ml) and positive (≥0.1 IU/ml) sera. Lab II used different cutoffs for negative (<0.016 IU/ml) and equivocal (0.016-0.09 IU/ml).

Lab II had used a lower limit of detection of 0.016 IU/ml and considered negative all those sera that are below this value. Thus, their quantitative correlation (R=0.94) (Table 1) with the Lab I TNT is slightly lower than TNT of the other laboratories. However, the quantitative agreement as well as the qualitative agreement between lab I TNT and all the other labs TNT was very good (Table 2).

Table 1. Pearson's R correlation coefficient

Laboratory	Test	R
Lab II	TNT	0.94
Lab III	TNT	0.98
Lab IV	TNT	0.98
Lab V	dDA-DELFIA	0.92
LabVI	MIA	0.95
Lab VII	ELISA In-house	0.55
Lab VIII	ELISA	0.77
Lab IX	ELISA - A	0.74
Lab X	ELISA -B	0.92
Lab XI	ELISA	0.85
Lab XII	ELISA	0.92
Lab XIII	ELISA	0.83
Lab XIV	ELISA -A	0.91
Lab XV	ELISA	0.82
Lab XVI	ELISA – B	0.93
Lab I-E	ELISA In-house	0.93

Table 2. Qualitative (diagnostic) agreement between the reference TNT *vs* TNT, dDA-DELFIA, MIA

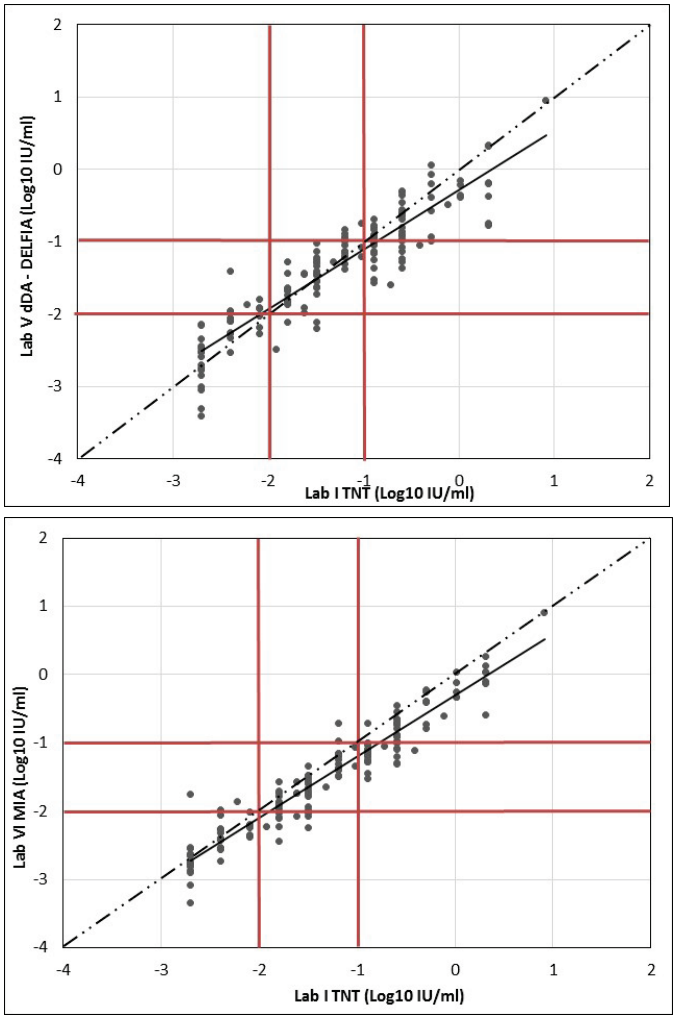
Laboratory test	TNT			Laboratory	TNT		
	Positive	Equivocal	Negative	Test	Positive	Equivocal	Negative
	≥0.1	0.01–0.09	<0.01		≥0.1	0.01–0.09	<0.01
Lab. II TNT				**Lab. III TNT**			
P [a] ≥0.1	58	6	0	P [a] > 0.1	58	10	0
E [b] 0.016-0.09	5	48	2	E [b] 0.01-0.1	5	44	3
N [c] < 0.016	0	0	30	N [c] < 0.01	0	0	30
Lab. IV TNT							
P [a] ≥0.1	48	0	0				
E [b] 0.01-0.09	15	53	3				
N [c] < 0.01	0	1	30				
Lab. V dDA-DELFIA				**Lab. VI MIA**			
P [a] ≥0.1	47	7	0	P [a] ≥0.1	39	2	0
E [b] 0.015-0.09	16	39	2	E [b] 0.01-0.09	24	42	3
N [c] < 0.015	0	8	31	N [c] < 0.01	0	10	30

According to the reference Lab I TNT, 63/150 samples were positive, 54 samples were equivocal and 33 samples were negative. Lab II tested 149 samples. [a] P, Positive; [b] N, negative; [c] E, Equivocal

It is worth noting that with TNT, there was a high correlation when concentration of antibodies were determined using the human (Lab I) or the equine antitoxin (Lab II, III and IV) as the reference preparation (R> 0.90).

The correlation between the Lab V dDA-DELFIA and Lab I TNT was R=0.92 (Table 1 and Fig. 2). Lab V, from previous studies (17) had set the cutoff for negative sera at < 0.015 IU/ml; equivocal sera were therefore those included in the range 0.015–0.09 IU/ml. Amongst a total of 150 samples, the dDA-DELFIA test identified 47 out of 63 samples as positive, 39 out of 54 samples as equivocal, and 31 out of 33 as negative (Table 2). Thus, 2 TNT negative sera were identified as equivocal, and 16 TNT positive as equivocal. Fifteen TNT equivocal sera were classified as positive or negative.

Figure 2. Interlaboratory comparison of the diphtheria antitoxin levels (IU/ml) of the standard panel tested by TNT and dDA-DELFIA and MIA



Regression line (solid) and line of identity (dashed) are shown. Vertical and horizontal dotted lines indicate the cutoffs used by the laboratories to determine negative (<0.01 IU/ml), equivocal (0.01-0.09 IU/ml) and positive (≥0.1 IU/ml) sera. dDA-DELFIA used a different cutoff for negative (<0.015 IU/ml) and equivocal (0.015-0.09 IU/ml).

The correlation between the Lab VI MIA and Lab I TNT was R=0.95 (Table 1 and Fig. 2). Cutoffs used by MIA are the same as those of the TNT. Amongst a total of 150 samples, the MIA identified as positive 39 out of 63 samples as positive, 42 out of 54 samples as equivocal, and 30 out of 33 as negative (Table 2). Thus, three TNT negative sera were identified as equivocal, and 24 TNT positive as equivocal. Twelve TNT equivocal sera were classified as positive or negative.

Two laboratories used an in-house validated ELISA (Annex 1). Lab I-E validated the in-house against the TNT VERO cell assay using the same human reference as calibrator serum, i.e. NIBSC 00/496; Lab VII used an in-house human serum calibrated against WHO IS (equine).

Testing the standard panel by the two in-house methods, Lab I-E and Lab VII obtained an R=0.93 and R=0.55, respectively (Table 1). For the two in-house ELISAs, the qualitative agreement with TNT test was determined using a diagnostic threshold cutoff value of 0.1 IU/ml. Thus, the sera were divided only in two categories: negative (<0.1 IU/ml) and positive (≥0.1 IU/ml). The ELISA performed by Lab VII showed poor agreement with the reference TNT assay: 10 samples that were categorized as negative by TNT were positive in the ELISA and 5 positive sera resulted false negative (Table 3). The ELISA performed by Lab I-E categorised one TNT positive serum as negative in ELISA, but no TNT negative samples were categorized as positive in the ELISA (Table 3). Samples that were categorised as equivocal in the TNT assay (i.e. likely to offer some degree of protection) were mostly reported as negative (45/54 samples) by Lab I-E and positive (42/54) by Lab VII.

Lab VII categorised the sera also according to the cutoffs that are usually used for TNT (Table 3). In this case, the qualitative agreement with TNT did not improve.

Table 3. Qualitative (diagnostic) agreement for the reference TNT and in-house or commercial ELISAs kits[a].

Laboratory test	TNT			Laboratory	TNT		
	Positive	Equivocal	Negative	Test	Positive	Equivocal	Negative
	≥0.1	0.01-0.09	<0.01		≥0.1	0.01-0.09	<0.01
Lab. I-E in house ELISA				**Lab. VII in house ELISA**			
P [b]≥0.1	62	9	0	P [b]≥0.1	58	42	10
N [c]<0.1	1	45	33	N [c]<0.1	5	12	23
Lab. VII in house ELISA				**Lab. XII**			
P [b]≥0.1	58	42	10	P [b]>1.0	8	0	0
E [d]0.01–0.09	2	6	8	E [d]0.1-1.0	55	25	2
N [c]< 0.01	3	6	15	N [c]< 0.1	0	29	31
Lab. VIII				**Lab. XIII**			
P [b]>0.149	59	35	12	P [b]>1.0	17	0	0
E [d]0.1–0.149	4	11	11	E [d] 0.1–1.0	43	18	0
N [c]< 0.1	0	8	10	N [c]< 0.1	3	36	33
Lab. IX ELISA-A				**Lab. XIV ELISA-A**			
P [b]> 1	7	0	0	P [b]> 1	14	0	0
E [d]0.1–1.0	47	19	1	E [d]0.1–1.0	49	27	2
N [c]< 0. 1	8	34	32	N [c]< 0. 1	0	27	31
Lab. X ELISA-B				**Lab. XV**			
P [b]≥0.1	63	28	2	P [b]> 1	6	0	0
E [d]0.01–0.09	0	26	30	E [d]0.1–1.0	48	14	1
N [c]< 0.01	0	0	1	N [c]< 0. 1	9	39	32
Lab. XI				**Lab. XVI ELISA-B**			
P [b]≥0.1	48	4	0	P [b]≥0.1	51	3	0
E [d]0.01–0.09	15	46	8	E [c]0.01–0.09	12	49	18
N [c]< 0.01	0	4	25	N [c]< 0.01	0	2	15

[a] According to the reference Lab I TNT, 63/150 samples were positive, 54 samples were equivocal and 33 samples were negative. Lab IX and XV tested 148 and 149 samples, respectively.

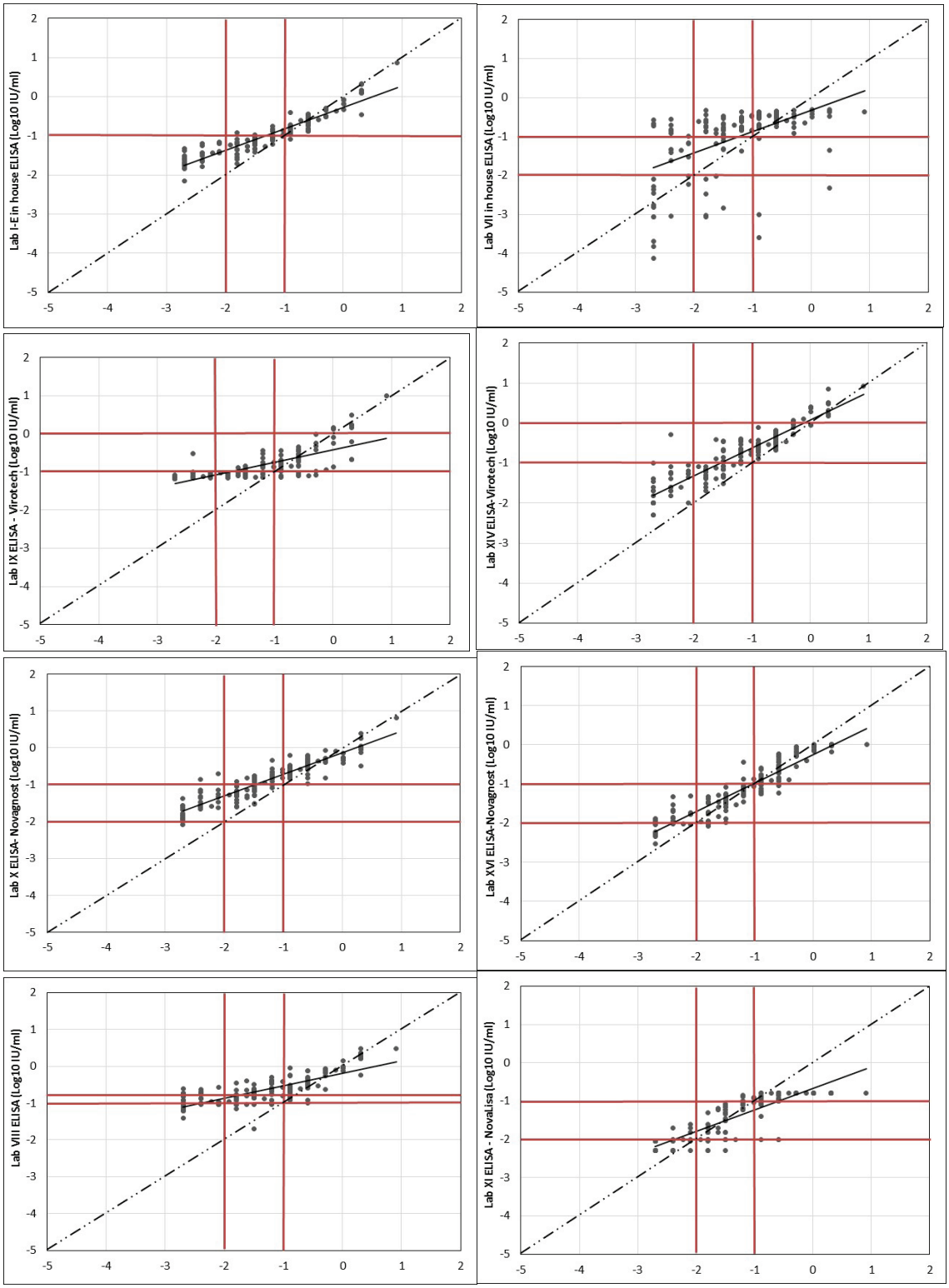
[b] P, Positive; [c] N, negative; [d]E, Equivocal.

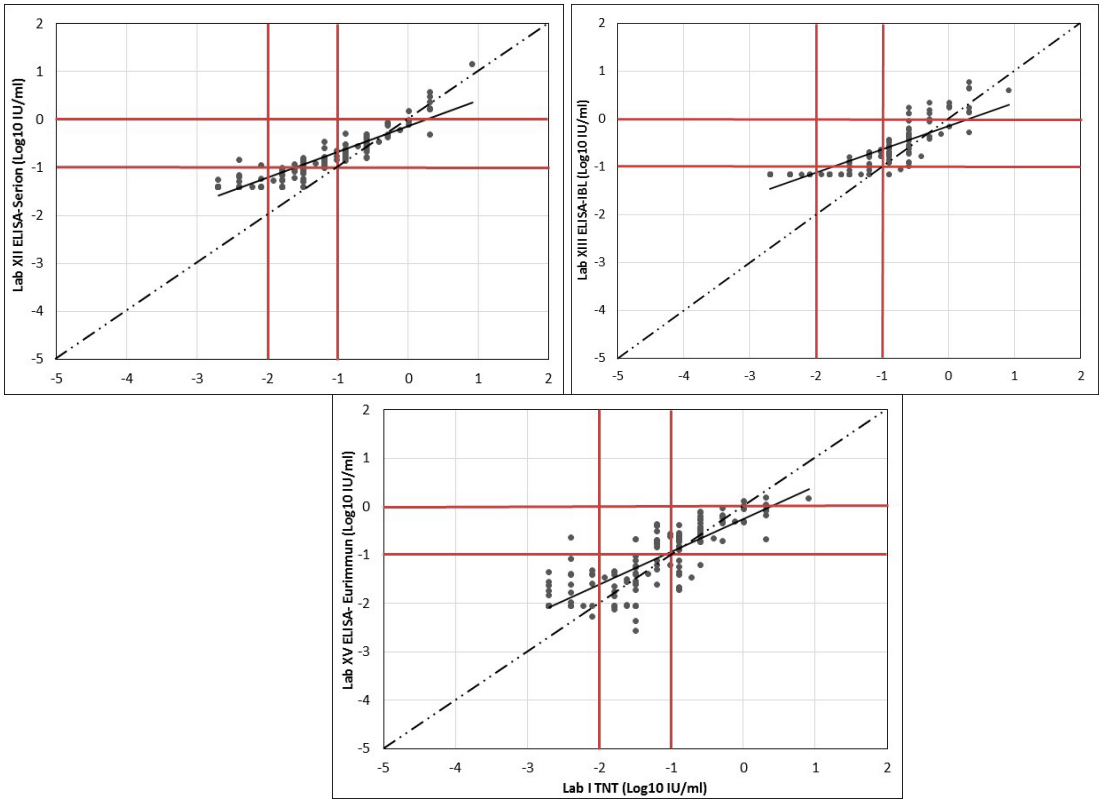
Nine laboratories used commercial ELISA kits from different manufacturers. The results are shown in Fig. 3. At a glance, all showed a great deviation from the identity line, usually overestimating the content at low levels of antibodies (< 0.1 IU/ml). Correlation with TNT ranges from R= 0.55 to R=0.93 (Table 1).

Lab VIII tested the panel only once and the test showed a fair correlation with TNT (R=0.77). The diagnostic agreement was poor: 12/33 TNT negative sera were classified as positive, i.e. false positive and 11/33 as equivocal.

Two laboratories, IX and XIV, tested the standard panel by the same kit (ELISA-A). The interassay precision between the two laboratories was low (data not shown); the correlation obtained by comparing the results from Lab IX and Lab XIV vs Lab I TNT corresponded to R=0.74 and R=0.91, respectively (Table 1). Both laboratories classified the negative sera almost in agreement with the TNT test (Table 3 and Fig. 3); the concordance for positive and equivocal sera was poor due to the majority of TNT positive sera being classified as equivocal and TNT equivocal as negative (Table 3).

Figure 3. Inter-laboratory comparison of the diphtheria antitoxin levels (IU/ml) of the standard panel tested by TNT and eleven different ELISA tests.





Regression line (solid) and line of identity (dashed) are shown. Vertical and horizontal lines indicate the cutoffs used by the laboratories to determine negative, intermediate and positive sera.

Labs X and XVI tested the standard panel serum by using the same ELISA kit (B), but in different situations, i.e. Lab XVI used an automated system. Comparing the results from Lab X and Lab XVI vs Lab I TNT, the correlation corresponded to R=0.92 and R=0.93, respectively (Table 1). Regarding the diagnostic agreement with TNT, it is evident that the assays carried out by the two laboratories classified the sera differently (Table 3); Lab X identified only one TNT negative serum correctly.

Lab XI did not further process all sera with a value of IU/ml >0.16. The correlation with Lab I TNT corresponded R=0.85 (Table 1, Fig. 3). The cutoffs indicated by this kit to classify the sera in terms of diagnostic interpretation are equivalent of those used for TNT (Table 3). The laboratory classified eight negative serums as equivalent.

Lab XII test showed a correlation with TNT of R=0.92 (Table 1, Fig. 3). Applying the criteria of results interpretation reported in the kit instruction and shown in Table 3, negative sera were identified correctly, while positive were considered equivocal and many equivocal identified as negative.

Lab XIII test showed a correlation with TNT of R=0.83 (Table 1, Fig. 3). The laboratory classified the negative sera in agreement with TNT test (Fig. 3 and Table 3); the concordance for positive and equivocal sera was poor due to the majority of TNT positive sera being classified as equivocal and TNT equivocal as negative (Table 3).

Lab XV test showed a correlation of R=0.82 in respect to Lab I TNT (Fig. 3). Even if the laboratory classified 97% of the negative sera in agreement with the TNT test (Fig. 3 and Table 3) there is no qualitative agreement between Lab I TNT and Lab XV ELISA. Many of the TNT positive sera were classified as negative (14%) or equivocal (76%) (Table 4). Seventy-four percent of TNT equivocal sera were categorised as negative.

Conclusions

The organisation of an EQA for serology required precise and intense work. In order to achieve a panel of 150 sera, representing, according to WHO classifications [19, 20] negative, equivocal, and positive sera for diphtheria antitoxin, at least 300 sera needed to be tested. It was preferable to obtain sera from blood donors, as these are regularly checked for the absence of infectious agents. This safety aspect is relevant both for the analyst (although the samples still have to be handled as potentially infective) as well as for shipping purposes. In addition, when collecting human sera, the consensus of the donor must be acquired and all administrative, legal and ethical issues must be respected. Furthermore, as pooling of sera is not recommended, in general, a large volume of blood is required from each donor, as the number of EQA participants will affect the number of serum aliquots that need to be prepared.

In this EQA study, serology for diphtheria was performed by selected laboratories using different methods. The VERO cell TNT is considered as the in vitro gold standard assay, and this assay was chosen as the reference test.

Lab I, II, V, VI, VII and XII participated within previous EQAs or seroepidemiological studies [17,21]. Lab I previously participated using TNT and the in-house ELISA; results were consistent. Lab II previously used the TNT test and correlation was above R=0.9; the correlation R of Lab V dDA-DELFIA with Lab TNT was 0.92, 0.89 and 0.92 in ESEN 1, Diphtheria Surveillance Network (DIPNET) and Eu-DIP-LabNet (this study), respectively; Lab VI participated within this EQA using the recently implemented MIA. Results were similar to when a Toxin Binding Inhibition Test (ToBI) was used previously (correlation R of ToBI 0.96 and 0.92 in ESEN1 and DIPNET, and correlation of MIA 0.95 in EU-DIP-LabNet) (22). Lab VII did improve slightly with their in-house ELISA (R=0.22 in DIPNET vs R=0.55 in Eu-DIP-LabNet, this study), but the assay needs to be further validated. Lab XII participated to the current and DIPNET EQA study using different ELISA kits and therefore the performance could not be compared.

The comparable correlation over time for Lab I, II and V indicated that changes in critical reagents, as well as analysts, affects only minimally the methods that are properly validated and supports the use of Lab I TNT assay data as the reference.

The inter-laboratory comparisons showed that the quantitative correlation between the TNT performed in the different laboratories is high, even when different protocols and key reference reagents, such as reference antiserum and toxin, are used. However, qualitative comparison revealed that some equivocal sera were classified differently by TNT from each laboratory, but no sera were considered as false negative or as false positive. Lab II, could consider performing the assay at a different toxin dose level to obtain a lower limit of detection in order to be more precise in the classification of serum samples with low levels of diphtheria antibody.

Only a few laboratories used alternative in vitro tests such as dDA-DELFIA or MIA which are not commercial methods and therefore required establishment in-house and validation. These methods are more laborious as some reagents need to be prepared, but the assays themselves are as easy as the ELISA.

The majority of laboratories (12/16) participating in the study performed an indirect ELISA. While the quality of an in-house ELISA is dependent on the validation performed by the individual laboratory, the commercial ELISA kits are sold as supposedly validated methods inclusive of all key reagents and reference sera to allow calculation of diphtheria antitoxin concentration in human serum samples. Participants using the same kit unexpectedly showed less reproducible results between laboratories (Lab IX and XIV; Lab X and XVI, respectively). These results are in contrast with what was observed in the DIPNET EQA where highly reproducible data were obtained by laboratories using the same ELISA kits [17].

Diagnostic agreement between ELISAs and TNT is commonly evaluated using different cutoffs, typically ten times higher than those applied for TNT. Selection of cutoffs has a direct influence in terms of diagnostic interpretations of the immune status of a person, and consequent decisions as to whether or not to re-immunize and assess potential deficiencies in humoral immunity. Usually, in the ELISA, sera with antibodies levels < 0.1 IU/ml are considered negative and those with antibodies ≥0.1 IU/ml are considered to be positive [23, 24].

However, for some of the ELISA kits used by the participants in this EQA, manufacturers' recommended division of test sera into the three categories (positive, equivocal and negative), but with titres set 10-fold higher compared with those used in the TNT. In this EQA study, for qualitative agreement analysis, only three categories were used, i.e. negative, equivocal and positive, with no differentiation between positive sera containing antibodies levels that confer a short or a long term protection.

On the basis of this qualitative classification, it is evident from the EQA study that laboratories using ELISA have underestimated the immune status of a subject, or even overestimated it. The clinical implication of over- or under- estimating diphtheria antibody titres would be that some subjects may be wrongly assumed to need or not need immunisation.

Recommendations

Vero TNT is still recommended as the assay of choice. Other *in vitro* tests such as dDA-DELFIA, ELISA or MIA are valid alternatives – provided they are suitably validated (against the TNT) and are performed using relevant criteria to define the cutoffs negative and positive. The need for appropriate validation is highlighted by the results obtained in this EQA. It is important that assays are standardised such that results are expressed in IU/ml and traceable to an appropriate International Standard for Diphtheria Antitoxin. In addition, the availability of serum classified as negative, equivocal and positive (by TNT) as run controls may help in assay validation and would also be useful in routine use for internal monitoring purposes.

It is important to maintain the EQA activity as it provides a good opportunity to compare and monitor assay performance, thus maintaining confidence in results generated for clinical and surveillance purposes. However, as the preparation of standard panels as the one used in this study is a very complex procedure, future EQAs may need to be performed using a smaller panel of samples that cover the full range of negative to positive.

Laboratories using ELISA must be very prudent in using this method to establish the protection against diphtheria in the case of laboratory personnel as false negative or false positive might be obtained.

References

1. Wagner KS, White JM, Lucenko I, Mercer D, Crowcroft NS, Neal S et al; Diphtheria Surveillance Network. Diphtheria in the postepidemic period, Europe, 2000-2009. Emerg Infect Dis 2012;18:217–25.
2. ECDC Surveillance Report. Annual epidemiological report. Reporting on 2011 surveillance data and 2012 epidemic intelligence data. 2013. http://www.ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf. Accessed 9 March 2014
3. World Health Organization. 2014. Diphtheria reported cases. World Health Organization, Geneva, Switzerland. http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tsincidencediphtheria.html. Accessed 9 March 2014
4. Hardy IR, Dittmann S, Sutter RW. Current situation and control strategies for resurgence of diphtheria in newly independent states of the former Soviet Union. Lancet 1996; 347:1739–1744.
5. Efstratiou A, Roure C. The European Laboratory Working Group on Diphtheria: A global microbiologic network. Journal Infect 2000; 181 Suppl 1:S146–S151.
6. Edmunds WJ, Pebody RG, Aggerbeck H, Berber G, Conyn van Spaendonk MAE, Hallander HO et al. The seroepidemiology of diphtheria in western Europe. Epidemiol Infect 2000; 125:115–125 and relative corrigendum in Epidemiol Infect 2001; 126:331.
7. Di Giovine P, Kafatos G, Nardone A, Andrews N, Ölander RM, Alfarone G et al. Comparative seroepidemiology of diphtheria in six European countries and Israel. Epidemiol Infect 2012; doi: 10.1017/S0950268812000210.
8. Jensen C. Die intrakutane Kaninchenmethode zur Auswertung von Diphtherie-Toxin und Antitoxin. Acta Pathologica et Microbiologica Scandinavica 1933; 14: 1–211.
9. Miyamura K, Nishio S, Ito A, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and anti toxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. J Biol Stand 1974; 2:189–201.
10. WHO Technical Report Series, No. 927: Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003). Annex 5 in: *WHO Expert Committee on Biological Standardization. Fifty-forth report.* Geneva, World Health Organization, 2005.
11. European Pharmacopoeia 7th ed., Assay of Diphtheria Vaccine Adsorbed (2.7.6), Strasbourg, Council of Europe, 01/2008
12. Gommer AM. Vero cell assay validation as an alternative to the diphtheria potency tests. Dev Biol Stand 1996; 86: 217–224.
13. Sheifele DW, JJ Ochnio. 2009. The immunological basis for immunization series. Module 2: Diphtheria update 2009. World Health Organization, Geneva, Switzerland. http://whqlibdoc.who.int/publications/2009/9789241597869_eng.pdf Accessed 9 March 2014
14. Winsnes R, Sesardic T, Daas A and Behr-Gross ME. Collaborative study for validation of serological methods for potency testing of diphtheria toxoid vaccines. Pharmeuropa Bio 2003; 2: 35–68.
15. Aggerbeck H, Norgaard-Pedersen B, Heron I. Simultaneous quantitation of diphtheria and tetanus antibodies by double antigen, time-resolved Fluorescence immunoassay. J Immunol Methods 1996; 190: 171–183.
16. van Gageldonk PGM, von Hunolstein C, van der Klis FRM, Berbers GAM. Improved Specificity of a Multiplex Immunoassay for the Quantitation of anti-Diphtheria Toxin Antibodies with the use of Diphtheria Toxoid, Clin Vaccine Immunol 2011; 18:1183–6.
17. Di Giovine P, Pinto A, Ölander RM, Sesardic D, Sticking P, Berbers Get al. External quality assessment for the determination of diphtheria antitoxin in human serum. Clin Vacc Immunol 2010; 17:1282–1290.
18. Sticking P, Rigsby P, Coombes l, von Hunolstein C, Ralli L, Pinto A et al. Collaborative study for the calibration and commutability assessment of the proposed 1st International Standard for Diphtheria Antitoxin Human. Biologicals 2013; 41 (6): 384–392.
19. Efstratiou A, Maple PAC. Diphtheria. Laboratory diagnosis of diphtheria. The expanded programme on immunization in the European region of WHO. 1994. WHO ICP/EPI 038.
20. Ipsen J. Circulating antitoxin at the onset of diphtheria in 425 patients. J Immunol 1946; 54: 325–347.
21. von Hunolstein C, Aggerbeck H, Andrews N, Berbers G, Fievet-Groyne F, Maple PA et al. European sero-epidemiology network: standardisation of the results of diphtheria antitoxin assays. Vaccine 2000; 18:3287–96.
22. van Gageldonk PGM, van Schaijk FG, van der Klis FRM, Berbers GAM. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to *Bordetella pertussis*, *diphtheria* and *tetanus*. J Immunol Methods 2008; 1(2):79–89.

23. Galazka, A. 1996. Immunological Basis for immunization, Module 2. Geneva, World Health Organization, WHO/EPI/GEN/93.12.

24. Sesardic D, Corbel MJ. Testing for neutralizing potential of serum antibodies to tetanus and diphtheria toxin. The Lancet 1992; 340:737–738.

Annex 1. Participating reference laboratories

Country	Contact persons	Institute
Bulgaria	A. Decheva, R. Alexiev	National Centre of Infections and Parasitic Diseases Blvd. Yanko Sakazov, No26 Sofia 1504, Bulgaria
Cyprus	T. Genneou, G.Mitis	Nicosia General Hospital Microbiology Department 215, Paleos Dromos Lefkosia-Lemesos Str. 2029 Strovolos Nicosia, Cyprus
France	N. Guiso, E. Badell	Institut Pasteur Unite de Prevention et Therapies Moleculaires des Maladies Humaines 25 rue du Dr. Roux 75724 Paris Cedex 15, France
Germany	A. Sing	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) Fachartz fur Mikrobiologie und Infektionsepidemiologie Dienststelle Oberschleißheim 85764 Oberschleißheim Bayern, Germany
Greece	A. Pangalis, H. Alexandrou- Athanassoulis	Athens Children's Hospital 'Aghia Sophia' Bacteriology Department Thivon & M. Asias Goudi Athens 11527, Greece
Italy	A. Pinto, L. Ralli C. von Hunolstein,	Istituto Superiore di Sanità Viale Regina Elena,299 00161 Roma , Italy
Latvia	R. Paberza	Riga East Clinical University Hospital Infectology Center of Latvia Bacteriological Department of Laboratory 3 Linezera street Riga, LV 1006, Latvia
Netherlands	G. Berbers, P. van Gageldonk	RIVM Centre for Infectious Disease Control Antonie van Leeuwenhoeklaan 9 3721 MA Bilthoven, The Netherlands
Norway	M. Steinbakk, A. Aase	Norwegian Institute of Public Health Division of Infectious Disease Control Department of Bacteriology and Immunology SMBI Lovisenberggata 8 0456 Oslo, Norway
Poland	A. Zasada	National Institute of Hygiene National Institute of Public Health Department of Bacteriology Chocimska 24 00-791 Warsaw, Poland
Portugal	M. A. Santos	Instituto Nacional de Saude Dr. Ricardo Jorge Departamento de Doenças Infecciosas Avenida Padre Cruz 1649-016 Lisboa, Portugal
Romania	M. Damian, C. Dragomirescu	INCDMI Cantacuzino Diphtheria Reference Laboratory & Molecular Epidemiology Laboratory 103 Splaiul Independentei C.P.1-525, 050096, Bucharest, Romania

Country	Contact persons	Institute
Slovakia	V. Lengyelová, A. Belyovà	RÚVZ, Odbor lekárskej mikrobiológie National Reference Centre for Diphtheria Senný trh č.4 04011 KOŠICE, Slovakia
Slovenia	V. Mioč , M. Paragi	National Institute of Public Health Department for Medical Microbiology Grablovičeva 44 1000 Ljubljana, Slovenia
United Kingdom	K. Broughton, A. Efstratiou, S.Neal	Public Health England WHO Global Collaborating Centre for Diphtheria and Streptococcal Infections Bacteriology Reference Department Microbiology Services Division: Colindale 61 Colindale Avenue, London, NW9 5EQ, United Kingdom
United Kingdom	P. Stickings, L. Coombes, D. Sesardic	National Institute for Biological Standards and Control, Health Protection Agency Potters Bar, Hertfordshire (UK)

Annex 2. Serological assays and reference materials used among participating laboratories

Laboratory	Assay	Lowest level of detection (IU/ml)	Diphtheria toxin or toxoid/producer/Lf	Diphtheria reference serum (antitoxin) [a]
I	Vero cell (TNT)	0.0008	Toxin/EDQM/1 Lf/ml	NIBSC-batch 00/496 (human)
II	Vero cell (TNT)	0.016	Toxin/ RIVM/ 1000Lf/Ampoule	NIBSC-batch 66/153 (equine)
III	Vero cell (TNT)	0.00125	Toxin/List Biological Laboratories/GI50 0.94 ng/ml	WHO IS, DI (equine)
IV	Vero cell (TNT)	0.0016	Toxin/ RIVM/ 1000Lf/Ampoule	NIBSC-batch 66/153 (equine)
V	dDA-DELFIA	0.0004	Toxoid/SSI/1911 Lf/ml	WHO, batch DI09-204 (equine)
VI	Multiplex Immunoassay (MIA)	0.0001	Microspheres conjugated with Toxoid/Netherlands Vaccine Institute, Bilthoven/4500 Lf original stock	In-house reference serum (human) calibrated against WHO IS batch DI07 (equine)
VII	ELISA (in-house method)	0.0009	Toxoid/BulBio-NCIPD, Bulgaria/1000 Lf	In-house reference serum (human) calibrated against WHO IS (equine)
VIII	ELISA	0.004	Toxoid/Binding Site	NIBSC-batch 00/496 (human)
IX	ELISA - A	0.065	Toxoid/Virotech	NIBSC-batch 00/496 (human)
X	ELISA -B	<0.01	Toxin	NIBSC-batch 91/534 (human)
XI	ELISA	0.01	Toxoid	NIBSC-batch 00/496 (human)
XII	ELISA	0.05	Toxoid	1st International standard, Statens Serum Institute Copenhagen, Denmark
XIII	ELISA	0.08	Toxoid/IBL international GmbH	NIBSC-batch 00/496 (human)
XIV	ELISA - A	0.04	Toxoid/Virotech	NIBSC-batch 00/496 (human)
XV	ELISA	0.0004	Toxoid	NIBSC-batch 00/496 (human)
XVI	ELISA – B	< 0.01	Toxin	NIBSC-batch 91/534 (human)
I-E	ELISA (in-house method)	0.015	Toxoid/NIBSC/1100 Lf/ml	NIBSC-batch 00/496 (human)

[a] ELISA kits contain internal calibrators (human sera) calibrated against the standard shown in the tables

Annex 3. Intended results for the EU DIP LabNet 2012 EQA for serological immunity

Samples	IU/ml (Value by TNT)	Interpretation
1/2011	0.064	Equivocal
3/2011	0.064	Equivocal
4/2011	0.032	Equivocal
5/2011	2.048	Positive
6/2011	0.128	Positive
8/2011	0.096	Equivocal
9/2011	0.024	Equivocal
10/2011	0.256	Positive
11/2011	0.256	Positive
16/2011	0.064	Equivocal
18/2011	2.048	Positive
20/2011	0.512	Positive
22/2011	0.128	Positive
23/2011	0.002	Negative
25/2011	0.512	Positive
26/2011	0.016	Equivocal
28/2011	0.002	Negative
29/2011	0.024	Equivocal
31/2011	0.128	Positive
32/2011	0.256	Positive
33/2011	0.064	Equivocal
36/2011	1.024	Positive
37/2011	0.128	Positive
39/2011	0.002	Negative
42/2011	0.064	Equivocal
43/2011	0.128	Positive
44/2011	0.032	Equivocal
46/2011	0.256	Positive
47/2011	0.128	Positive
50/2011	0.256	Positive
51/2011	0.004	Negative
52/2011	0.004	Negative
53/2011	0.032	Equivocal
54/2011	0.064	Equivocal
55/2011	0.002	Negative
56/2011	0.016	Equivocal
57/2011	2.048	Positive
58/2011	0.008	Negative
59/2011	0.032	Equivocal
60/2011	0.192	Positive
62/2011	0.016	Equivocal
63/2011	0.002	Negative
64/2011	0.096	Equivocal
66/2011	0.004	Negative
67/2011	0.002	Negative
68/2011	0.128	Positive
70/2011	1.024	Positive
71/2011	0.128	Positive
72/2011	0.032	Equivocal
73/2011	0.064	Equivocal
74/2011	0.002	Negative
75/2011	0.016	Equivocal

Samples	IU/ml (Value by TNT)	Interpretation
77/2011	0.128	Positive
78/2011	0.064	Equivocal
79/2011	0.016	Equivocal
80/2011	0.006	Negative
81/2011	0.064	Equivocal
82/2011	0.032	Equivocal
85/2011	0.002	Negative
87/2011	0.032	Equivocal
88/2011	0.002	Negative
90/2011	0.002	Negative
92/2011	0.256	Positive
93/2011	0.016	Equivocal
94/2011	0.032	Equivocal
95/2011	0.016	Equivocal
96/2011	0.016	Equivocal
101/2011	0.512	Positive
103/2011	0.128	Positive
104/2011	0.032	Equivocal
105/2011	1.024	Positive
106/2011	0.016	Equivocal
108/2011	0.016	Equivocal
109/2011	0.002	Negative
111/2011	0.016	Equivocal
112/2011	2.048	Positive
113/2011	0.004	Negative
114/2011	0.256	Positive
115/2011	0.008	Negative
117/2011	0.256	Positive
120/2011	0.256	Positive
124/2011	0.128	Positive
125/2011	0.256	Positive
127/2011	0.512	Positive
128/2011	0.064	Equivocal
129/2011	0.002	Negative
131/2011	0.002	Negative
132/2011	0.128	Positive
136/2011	1.024	Positive
138/2011	0.002	Negative
151/2011	0.064	Equivocal
153/2011	0.512	Positive
156/2011	0.064	Equivocal
157/2011	0.256	Positive
162/2011	0.004	Negative
164/2011	0.256	Positive
165/2011	0.032	Equivocal
166/2011	0.016	Equivocal
167/2011	0.256	Positive
168/2011	2.048	Positive
169/2011	8.192	Positive
170/2011	0.004	Negative
171/2011	0.096	Equivocal
172/2011	0.256	Positive
175/2011	0.128	Positive
176/2011	0.256	Positive
177/2011	0.032	Equivocal
178/2011	0.128	Positive

Samples	IU/ml (Value by TNT)	Interpretation
179/2011	0.032	Equivocal
180/2011	0.256	Positive
181/2011	0.032	Equivocal
182/2011	0.004	Negative
185/2011	0.004	Negative
186/2011	0.016	Equivocal
188/2011	0.128	Positive
189/2011	0.004	Negative
192/2011	0.002	Negative
195/2011	0.256	Positive
196/2011	0.004	Negative
200/2011	0.012	Equivocal
201/2011	0.002	Negative
202/2011	0.008	Negative
203/2011	0.008	Negative
204/2011	0.024	Equivocal
205/2011	0.008	Negative
206/2011	0.008	Negative
207/2011	0.032	Equivocal
208/2011	0.128	Positive
209/2011	0.128	Positive
210/2011	0.024	Equivocal
211/2011	0.048	Equivocal
212/2011	0.032	Equivocal
213/2011	0.032	Equivocal
214/2011	0.032	Equivocal
215/2011	0.128	Positive
216/2011	0.032	Equivocal
217/2011	0.128	Positive
218/2011	0.256	Positive
219/2011	0.064	Equivocal
220/2011	0.384	Positive
221/2011	0.256	Positive
222/2011	0.512	Positive
223/2011	0.256	Positive
224/2011	0.064	Equivocal
225/2011	0.768	Positive
227/2011	0.512	Positive
228/2011	0.256	Positive
229/2011	2.048	Positive
230/2011	1.024	Positive
231/2011	2.048	Positive