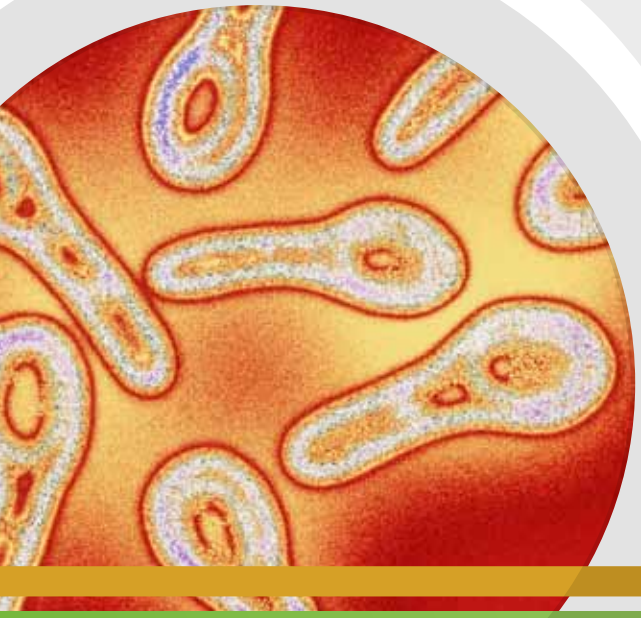

TECHNICAL REPORT

External quality assessment scheme for diphtheria diagnostics

2013

As part of the European Diphtheria Surveillance Network (EDSN)

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ECDC TECHNICAL REPORT

External quality assessment scheme for diphtheria diagnostics, 2013

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This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Dr Ida Czumbel, and produced by Dr Leonard Both and Prof Androulla Efstratiou (Public Health England, WHO Global Reference Centre for Diphtheria and Streptococcal Infections, London, UK), on behalf of the EU Diphtheria Laboratory Network consortium.

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Abbreviations

DIPNET	Diphtheria Surveillance Network
EDSN	European Diphtheria Surveillance Network
ELWGD	European Laboratory Working Group for Diphtheria
EQA	External Quality Assessment
eQAD	external Quality Assurance Department
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time of Flight
NEQAS	National External Quality Assessment Service
PCR	Polymerase chain reaction
PHE	Public Health England
WHO	World Health Organization

Executive summary

Diphtheria is an acute infectious disease affecting the upper respiratory tract and occasionally the skin, caused by the action of diphtheria toxin produced by toxigenic *Corynebacterium diphtheriae, Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*. Effective control of such an uncommon vaccine-preventable disease relies on prompt and early recognition and diagnosis. Diagnostic capacity is essential to prevent potential mass outbreaks, to achieve adequate vaccination coverage, and to assess the impact of control and elimination efforts. Because accurate microbiological diagnosis is crucial and complementary to clinical diagnosis, it is essential that all European countries have access to capacity for microbiological diagnosis of diphtheria caused by various potentially toxigenic strains of corynebacteria.

As part of ECDC's efforts to build and develop microbiology laboratory networks, the European Diphtheria Surveillance Network (EDSN) has been established. Since its creation in November 2009, the EDSN (initially termed 'DipNet') has accurately monitored the diphtheria burden across the European Union (EU) Member States and several associated countries. The EDSN regularly performs external quality assessments (EQAs) to continuously enhance and strengthen laboratory-based surveillance capacity. EQAs are an essential tool to enable laboratories to monitor, evaluate and improve their own performance. This report describes an EQA despatch in 2013 for the laboratory diagnosis of diphtheria under the auspices of EDSN. A total of 32 countries, including Croatia as a new member of the European Union, participated in this EQA and were asked to isolate, identify and perform toxigenicity testing on any *Corynebacterium* spp. present in the six simulated throat specimens sent. Key findings are reported below and a description of the work involved and the outcomes of these exercises are detailed further in this report.

Key findings

All EQA participants were required to undertake biochemical identification tests as well as toxigenicity tests for the six EQA specimens. Most of the 32 participants used the same methods as in the previous EQA in early 2012. However, the majority of reference centres reported problems in obtaining Elek test reagents including antitoxin and Elek base medium.

Overall, this EQA demonstrated that most EQA participants correctly identified the six specimens. However, many of the EQA participants struggled with the toxigenicity and a relatively large number of unacceptable toxigenicity reports were returned to the organising centre. These errors would have likely prevented induction of appropriate treatment of affected patients in a clinical setting.

A total of 95% of the identification reports for the six specimens were fully correct. The remaining 5% included minor errors, e.g. in *biovar* identification.

A total of 85% of the toxigenicity reports were correct. The remaining 15% of reports delivered unacceptable toxigenicity results. It would therefore be recommended that reference centres continue to use both current methods for toxigenicity alongside each other, i.e. both Elek test as well as polymerase chain reaction (PCR), in order to avoid major/unacceptable diagnostic errors which might lead to errors in patient treatment. Indeed, the EQA results reported hereafter confirmed that using only one of the two tests above, substantially increases the risk for incorrect toxigenicity reports, as observed with the weakly toxigenic EQA samples 1 and 5. These strains were both phenotypically and genotypically positive, requiring the participants to report positive Elek and positive PCR results. Whilst we noted several discrepancies between participants' reports for the Elek test and PCR results specifically with these two samples, the chances for both tests to fail simultaneously were decreased, emphasising the importance of using the two tests alongside each other.

In summary, the 30 EU/EEA countries plus Israel and Turkey submitted the following results:

- 9/32 reference centres submitted completely correct results for all six specimens. However, it should be mentioned that two of the nine centres did not perform toxigenicity testing; as such, only seven of 32 centres reported both correct identification and toxigenicity results for all six specimens. Six of seven centres are from EU/EEA Member States, i.e. only 20% of the 30 reference centres from EU/EEA Member States submitted fully correct results for all specimens.
- 31/32 countries, including the two non-EU/EEA countries, provided 100% correct species identification results for all *C. diphtheriae* specimens.
- 29/32 countries, including one of the two non-EU/EEA countries, provided 100% correct species identification results for all *C. ulcerans* specimens.
- 26/32 countries, including the two non-EU/EEA countries, provided 100% correct results to all identification tests, including the *C. pseudodiphtheriticum* isolate in specimen 4.

- 9/30 countries, including one of two non-EU/EEA countries, delivered fully correct toxigenicity results. Two centres (Malta, Iceland) did not perform any toxigenicity tests and would refer the isolate to an overseas laboratory. Amongst those EU/EEA Member States centres undertaking toxigenicity tests, only 20% obtained fully correct results for all specimens.
- 21/30 centres, including one of the two non-EU/EEA countries, perform both Elek test and PCR.

Overall, a decrease in performance was observed compared with the previous EQA in early 2012. Training activities and EQAs should therefore be continued to maintain the level of capability and quality of results in all Member States for laboratory diagnosis of diphtheria

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 to 'foster 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 and extend such cooperation and support the implementation of quality assurance schemes.' (Article 5.3, EC 851/2004[1]).

Laboratory-based surveillance capacity across a diagnostic network can be tested and trained in external quality assessment (EQA) schemes, in which laboratories are sent simulated clinical specimens or bacterial isolates for testing by routine and/or reference laboratory methods. EQAs are an invaluable tool to train and strengthen quality assurance systems and allow a laboratory performance to be assessed against reference methods and other peer laboratories. 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 the general standard of performance
- 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.

While this EQA assesses the standard of performance of many national reference laboratories, some countries do not have national reference laboratories recognised by their governments, but offer diphtheria diagnostic services.

The EQA process increases the probability of correct diagnosis, case management and an improved quality of surveillance data by training the participants regarding their laboratory performance.

Due to their epidemic patterns, the emergence of new strains, novel reservoirs and their dissemination to susceptible human and animal populations, *C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis* infections are usually difficult to detect [1]. Diphtheria is now largely under control in the WHO EURO region following mass immunisation campaigns and additional control measures and support from WHO, the European Laboratory Working Group on Diphtheria (ELWGD), and other agencies. However, a network of diphtheria reference laboratories and epidemiologists/public health specialists is still required, as there may be diphtheria resurgences in the future similar to the mass outbreak in the 1990s in the previous Soviet Union [2]. At the request of the WHO European Region, ELWGD was created in 1993 to help strengthen the diphtheria diagnostic capabilities of the many countries affected [3]. At the time, screening for diphtheria from routine throat swabs was adopted in many European countries; currently, no EU countries are screening for diphtheria, due to the low incidence of *C. diphtheriae* and *C. ulcerans* now observed.

The first EQA exercise for diphtheria diagnostics in Europe was performed in 1994 under the auspices of the ELWGD, and since then, eleven distributions for laboratory diagnostics have been performed (including two for serological immunity testing). Results from the last four distributions revealed that irrespective of the composition of the EQA panel or the countries participating, correct toxigenicity and identification reports have rarely exceeded 90% [4, 5]. Therefore, continued EQA programmes for diphtheria diagnostics need to be maintained.

[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.

Successful diphtheria prevention and control strategies rely on laboratory-based surveillance and rapid data reporting to a dedicated surveillance network. The European Diphtheria Surveillance Network (EDSN) was established in March 2010 and comprises the nominated epidemiologists and laboratory experts for diphtheria from the current 28 EU Member States and several 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 laboratory capacity at the 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 and focused on EQA and training. The key objective of this work, as described in this report is: to assess and improve laboratory performance, for standardised and appropriate methods to be used for the laboratory diagnosis of culture-confirmed diphtheria infections, for ensuring accurate and comparative diphtheria surveillance across Europe.

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.

The EQA distribution was aided by the availability of the large collection of corynebacteria isolates and expert knowledge at the WHO Global Reference Centre for Diphtheria at PHE. It was supported by the United Kingdom National External Quality Assessment Service (UK NEQAS) for Microbiology, and facilities in the external Quality Assurance Department Microbiology Services Division, London, UK. UK NEQAS for Microbiology undertake several international EQA schemes for other organisms that also require freeze-drying, distribution, results analysis and web-based reporting.

Participants

The list of the participating reference laboratories can be found in Annex 1. Croatia as a new member of the EU has also been included here. Israel and Turkey requested the EQA panel as they had participated in previous distributions. Both countries have recently isolated potentially toxigenic corynebacteria: In 2013, Israel isolated two *C. diphtheriae biovar gravis* strains which were shown to be non-toxigenic by PCR. Turkey isolated their most recent toxigenic corynebacteria strains in 2011; this was a toxigenic *Corynebacterium diphtheriae biovar gravis* isolate from one case and five toxigenic isolates from close contacts. The current epidemiological situation in Israel and Turkey, as well as migrants entering the EU from these countries, justified their inclusion in this European EQA. Also, Turkey was officially recognised as a candidate for EU membership in 1999 and accession negotiations were started in 2005, further justifying its inclusion in this EQA.

All participants were contacted before the EQA distribution to confirm the address and contact details for despatch of the potentially hazardous material. It was envisaged that some reference laboratories would wish to store the viable cultures and use them for their own quality processes. The distribution of the well-characterised material may become a resource within and between the reference laboratories.

Timeline

Table 1. Timeline for EQA exercise

Event	Date
Selection of EQA strains	July–August 2013
Building participants list	August–September 2013
Assessment of strains before freeze-drying	August–September 2013
Transfer of strains to eQAD UK NEQAS*	October 2013
Freeze-dry panel produced (eQAD UK NEQAS)	October 2013
Pre-despatch checks of freeze-dried panel	October 2013
Requests for specialised Elek media and antitoxin from participants	September–October 2013
EQA panel despatch (eQAD UK NEQAS)	25 November 2013
Additional Elek media and antitoxin despatch to various countries	20 November 2013
Reference laboratories testing EQA panel	November–December 2013
Final return of results	30 December 2013
Preliminary feedback to participants	Early January 2014
Intended results sent to participants	January 2014
Analysis and collation of results	January 2014
Production of report	January 2014

**eQAD UK NEQAS: External quality assurance department, United Kingdom National External Quality Assessment Service*

The EQA simulated specimen panel

Six *Corynebacterium* sp. strains were selected based on their variability and toxigenicity. The strains had been referred to the WHO Global Reference Centre, London, and were isolated from clinical cases. The panel contained three toxigenic and two non-toxigenic strains. The corynebacteria included were 2x *C. ulcerans, C. diphtheriae var gravis, C. diphtheriae var mitis, and C. pseudodiphtheriticum*. One of the specimens contained only background flora/commensal organisms and no target organism, to test whether the participants would name or report an isolate.

The strains were coded and prepared as simulated throat specimens by the addition of two or more commensal organisms and freeze-dried by the Quality Assurance Laboratory, PHE, London. Quality control of the specimens was undertaken by the WHO Global Reference Centre for Diphtheria and Streptococcal Infections both before and after freeze-drying to test for viability and retention of the organism's characteristic properties.

Full instructions were included in the despatch, asking participants to isolate, identify, and perform toxigenicity testing on any *Corynebacterium* sp. present and report their results, the time taken to achieve a final result, and any problems encountered. The EQA was distributed on 25 Nov 2013 to 30 EU/EEA countries plus Turkey and Israel. Full instructions and a result form were enclosed and also sent electronically, with results to be submitted by 30 Dec 2013. An accompanying questionnaire was circulated by email in Nov 2013 to investigate whether the participants had changed or introduced any methods for this EQA.

Additional dispatch of specialised media

Due to monetary restrictions, participants were not provided with a budget for laboratory consumables, reagents and media for the diagnosis of diphtheria. Therefore, all centres were generally expected to procure these items for this round of EQA. However, a total of ten countries still requested a shipment of Elek media and/or antitoxin strips or discs from the WHO Global Reference Centre for Diphtheria and Streptococcal Infections in London. Elek is the gold standard test for detecting toxigenicity, yet requires media which can be laborious to make, and antitoxin, which is hard to obtain. Therefore, eight countries each received a shipment of ten antitoxin strips, 50 antitoxin discs, and 15 bottles of Elek base. An additional two countries received 50 antitoxin discs and strips (and no media).

Data analysis

The intended results (Annex 2) were sent to all participants in late January 2014 for information and for each laboratory to rapidly assess how they performed. Participants' results were analysed against the intended results on the basis of isolation, identification, and toxigenicity testing of any *Corynebacterium* spp. present in the specimens. Results from each centre were evaluated as acceptable (fully correct results), acceptable with minor errors (e.g. incorrect biotyping results), or not acceptable (failure to isolate target *Corynebacterium* spp. and/or incorrect phenotypic toxigenicity result). If any participant experienced problems, or a method was identified as generating incorrect results, the WHO Global Reference Centre for Diphtheria and Streptococcal Infections in London offered direct advice and recommended repeating the specimens, following corrective action, in order to improve diphtheria diagnostics in EU/EEA countries.

Results

Laboratory capabilities

A total of 32 countries participated in this EQA and were asked to isolate, identify and perform toxigenicity testing on any *Corynebacterium* spp. present in the six simulated throat specimens sent. Of note, several national reference laboratories identified pathogenic corynebacteria during 2013 in their respective countries, e.g. in Belgium, Denmark, Latvia, the Netherlands, Sweden, Israel, Slovenia, Austria, France, Germany, and UK.

Croatia as a new member of the European Union (as of 1 July 2013) was included in the EQA scheme for the first time. Croatia's laboratory capabilities include both biochemical identification and toxigenicity testing using the Elek test (but not PCR).

Several national reference centres have recently implemented new tools and methods. Romania introduced the modified Elek test and molecular typing by MLST. Germany has established the MLST and the Vero cell assay (for serology). The Czech Republic has implemented a Real-time PCR for the toxin gene. France adopted the ISO 15185 standard for toxin gene detection by end-point PCR. Since the previous EQA in early 2012, Norway has further established Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) analysis for strain identification. In Austria, the Elek test is not performed anymore and Malta and Iceland do not currently perform any toxigenicity tests.

In this EQA, a total of 26/30 reference centres used the Elek test and a total of 25/30 reference centres used PCR for toxigenicity testing. Many of the reference centres (21/30) used a combination of both Elek test and PCR. Of note, France tested only specimen 1 using both methods, all others were tested only by PCR. Moreover, the Czech Republic used a tissue culture assay for phenotypic detection of the toxin instead of the Elek test.

Laboratory diagnostic EQA results

All 32 national reference centres submitted results. Malta and Iceland performed only the biochemical identification and did not submit any toxigenicity results. All intended results for the six specimens, with a summary of the participants' findings, are shown in Table 2a.

Identification: The corynebacteria included were 2x *C. ulcerans, C. diphtheriae var gravis, C. diphtheriae var mitis, and C. pseudodiphtheriticum*. A sixth specimen did not contain any target organisms. Most centres used the API Coryne as their standard identification method and some reference centres further analysed the specimens using Rosco tests, BBL crystal, 16S sequencing, MALDI-TOF, Gram-stain, O129 susceptibility testing, or cell culture tests.

If a closely related coryneform or another *biovar* was reported, this was considered only a minor identification error. However, if a potentially toxigenic strain was not identified, this was considered a major/unacceptable identification result. Failure of detecting the target organism would have delayed the treatment of the case, contact tracing and any other public health interventions.

Toxigenicity: Failure to detect the toxin was considered a major/unacceptable result, whether by Elek test or PCR, because this would have likely prevented induction of appropriate treatment of affected patients in a clinical setting.

Most reference centres correctly identified specimen 1 as *C. ulcerans*, with the exception of two centres who identified it as *C. pseudotuberculosis*. Thirteen centres encountered difficulties with the toxigenicity testing and submitted incorrect results using either Elek test or PCR. One centre generated an unacceptable result because the toxin was not detected using both Elek test and PCR. This could have led to inappropriate clinical and public health management.

All centres correctly identified specimen 2 as *C. diphtheriae var gravis*, with the exception of one centre who reported it as *C. diphtheriae biovar intermedius*. Two centres submitted incorrect toxigenicity results using either Elek test or PCR.

All centres correctly identified specimen 3 as *C. diphtheriae var mitis*, with the exception of one centre who reported it as *C. diphtheriae var intermedius (*as observed with specimen 2*)*. A total of six centres delivered incorrect toxigenicity results based on either Elek test or PCR.

Twenty-six reference centres identified specimen 4 as C*. pseudodiphtheriticum*. Another four centres delivered acceptable results by reporting 'None' (i.e. no potentially corynebacteria) or *C. urealyticum*, a closely related non-toxigenic coryneform. One centre incorrectly reported *C. diphtheriae var belfanti* which would have resulted in a diagnosis of clinical diphtheria and was therefore considered an unacceptable result. As *C. pseudodiphtheriticum* is not one of the potentially toxigenic coryneforms, most centres correctly reported the toxigenicity results either as non-toxigenic or 'N/A'. However, one centre that correctly identified this specimen as *C. pseudodiphtheriticum*, reported it nevertheless as toxigenic using both Elek test and PCR.

Specimen 5 was by far the most difficult specimen. This specimen contained an atypical strain which identifies as *C. ulcerans* (as previously confirmed by MALDI, RT-PCR and rpoB sequencing). However, some of the biochemical results were atypical for *C. ulcerans* (trehalose and glycogen negative which are characteristic of *C. pseudotuberculosis*). Both *C. ulcerans* and *C. pseudotuberculosis* were therefore evaluated as correct EQA results for this particular specimen. However, one centre delivered unacceptable results by reporting neither of these two and by not detecting any potentially toxigenic bacteria at all. The toxigenicity results for this specimen were alarming: A total of 17 centres made major errors in their toxigenicity reports. 14 centres delivered incorrect toxigenicity reports using either Elek test of PCR. Another three centres reported negative toxigenicity results using both methods; if this had been a real case, treatment and public health action might have been missed in 14 countries.

Specimen 6 contained no corynebacteria. This specimen contained only background flora/commensal organisms without any target organisms, to test whether the participants would name or report an isolate. All reference centres correctly reported this specimen.

Table 2a. Summary of identification and toxigenicity results for each of the EQA specimens*

			Result type		
Specimen Number	**Intended identification result**	**Toxigenicity (Elek and PCR)**	**Fully correct result (ID and tox)**	**Acceptable result**	**Unacceptable result**
DIPEQA-1	*C. ulcerans*	Weak toxigenic	19	0	13: 12 reported neg tox result with either Elek or PCR, 1 reported reported neg tox results using both methods; 2 of the 13 centres with incorrect tox results additionally made minor ID mistakes by reporting *C. pseudotuberculosis.*
DIPEQA-2	*C. diphtheriae biovar gravis*	Non-toxigenic	29	1:reported *biovar intermedius*	2: reported pos tox result with either Elek or PCR
DIPEQA-3	*C. diphtheriae biovar mitis*	Toxigenic	25	1:reported *biovar intermedius*	6: reported neg tox result with either Elek or PCR
DIPEQA-4	*C. pseudodiphtheriticum*	N/A (i.e. non-tox)	26	4:reported 'None' or *C. urealyticum*	2: 1 reported pos tox result using both Elek and PCR; 1 center reported isolate as *C. diphtheriae biovar belfanti* (i.e. clinical diphtheria)
DIPEQA-5**	*C. ulcerans*	Weak toxigenic	15	0	17: 14 reported neg tox results with either Elek or PCR, 3 reported neg tox results using both Elek and PCR; 1 centre with incorrect tox result also reported ID as 'None'
DIPEQA-6	*(only commensals)*	N/A	32	0	0

** The two reference centres performing only identification (no tox testing) achieved 100% correct results.*

*** DipEQA5 is specimen containing an atypical strain which identifies as C. ulcerans as previously confirmed by MALDI, RT-PCR and rpoB sequencing. However, some of the biochemical results were atypical for C. ulcerans (trehalose and glycogen negative which are characteristic of C. pseudotuberculosis).*

The performance of the participating centres divided into EU/EEA Member States (Table 2b) and non-EU/EEA countries (Table 2c):

Table 2b. Summary of identification and toxigenicity results for each of the EQA specimens for all EU/EEA Member States (30 countries)

			Result type		
Specimen Number	**Intended identification result**	**Toxigenicity (Elek and PCR)**	**Fully correct result (ID and tox)**	**Acceptable result**	**Unacceptable result**
DIPEQA-1	*C. ulcerans*	Weak toxigenic	18	0	12: 11 reported neg tox result with either Elek or PCR, 1 reported reported neg tox results using both methods; 1 of the 12 centres with incorrect tox results additionally made minor ID mistake by reporting *C. pseudotuberculosis.*
DIPEQA-2	*C. diphtheriae biovar gravis*	Non-toxigenic	27	1:reported *biovar intermedius*	2: reported pos tox result with either Elek or PCR
DIPEQA-3	*C. diphtheriae biovar mitis*	Toxigenic	23	1:reported *biovar intermedius*	6: reported neg tox result with either Elek or PCR
DIPEQA-4	*C. pseudodiphtheriticum*	N/A (i.e. non-tox)	24	4:reported 'None' or *C. urealyticum*	2: 1 reported pos tox result using both Elek and PCR; 1 center reported isolate as *C. diphtheriae biovar belfanti* (i.e. clinical diphtheria)
DIPEQA-5	*C. ulcerans*	Weak toxigenic	14	0	16: 13 reported neg tox results with either Elek or PCR, 3 reported neg tox results using both Elek and PCR; 1 centre with incorrect tox result also reported ID as 'None'
DIPEQA-6	*(only commensals)*	N/A	30	0	0

Table 2c. Summary of identification and toxigenicity results for each of the EQA specimens for the two non-EU/EEA countries

			Result type		
Specimen Number	**Intended identification result**	**Toxigenicity (Elek and PCR)**	**Fully correct result (ID and tox)**	**Acceptable result**	**Unacceptable result**
DIPEQA-1	*C. ulcerans*	Weak toxigenic	1	0	1: reported neg tox result with PCR (Elek N/D) plus minor ID mistake by reporting *C. pseudotuberculosis.*
DIPEQA-2	*C. diphtheriae biovar gravis*	Non-toxigenic	2	0	0
DIPEQA-3	*C. diphtheriae biovar mitis*	Toxigenic	2	0	0
DIPEQA-4	*C. pseudodiphtheriticum*	N/A (i.e. non-tox)	2	0	0
DIPEQA-5	*C. ulcerans*	Weak toxigenic	1	0	1: reported neg tox results with PCR
DIPEQA-6	*(only commensals)*	N/A	2	0	0

Taking all reports together, nine of 32 reference centres submitted completely correct results for all six specimens. It should however be mentioned that two of these nine centres did not perform any toxigenicity testing; as such, only seven of 32 centres reported the correct identification and toxigenicity results for all six specimens. Six out of seven centres are from EU/EEA Member States, i.e. only 20% of the 30 reference centres from EU/EEA Member States submitted fully correct results for all specimens.

A total of eight of 32 centres delivered incorrect identification results, and only one of these centres made two unacceptable errors. This centre delivered unacceptable identification results by reporting specimen 4 as *C. diphtheriae belfanti* and by reporting specimen 5 as 'none'. However, most identification errors by the other seven centres were minor ones, e.g. in *biovar* identification. This EQA included two different *C. diphtheriae biovars,* which one centre incorrectly reported both as *biovar intermedius*. All other centres correctly identified them as *biovars gravis* or *mitis*; the latter differs only in colony size (0.5–1 mm in diameter for *intermedius*, compared with 1.5 – 2 mm for *mitis*), and in *intermedius* strains fermenting dextrin.

A total of 21 of 30 centres delivered incorrect toxigenicity results. 21 centres performed both Elek test and PCR whereas four centres did not perform the Elek test and five centres did not perform any PCR. A total of 44 incorrect toxigenicity results for Elek and PCR were received; 12 of the centres made two or even more mistakes. Overall, these results indicate substantial problems with regards to capabilities in toxigenicity testing among the EU/EEA Member States. Of note, PCR was slightly less error prone than Elek test, highlighting the need for adequate laboratory skills for performing the Elek test.

The time taken to generate a final result ranged from one to 14 days. A total of 5% incorrect identification reports and 15% incorrect toxigenicity reports were submitted. This is similar to the 10% incorrect identification reports and 11% incorrect toxigenicity reports observed in the previous EQA in early 2012. Whereas minor errors have slightly decreased, it should however be noted that truly unacceptable results, i.e. incorrect toxigenicity reports which would have an immediate effect on patient treatment, have increased.

It should also be mentioned that only four of the specimens made toxigenicity testing absolutely necessary; no toxigenicity testing was necessary for specimens 4 and 6. Indeed, several reference centres did not submit any toxigenicity results for these two specimens after they had been correctly identified as *C. pseudodiphtheriticum* and 'Commensals only'. Taking this into account, the overall percentage of incorrect toxigenicity reports for the four specimens (excluding the reports for specimens 4 and 6) increases to 22%, which is a high figure.

Conclusions

This monitoring of performance through regular EQA despatches has ensured that key personnel in some EU countries have remained aware of diphtheria and maintained good standards in the use of specialised methods in diphtheria diagnostics.

More than 95% of the 192 identification reports for the six specimens included in this EQA were correct. A total of 8/32 reference centres submitted identification errors for at least one of the six specimens but most identification results submitted by the participants here only included minor mistakes, e.g. in *biovar* identification; the vast majority of results were therefore either fully correct or at least acceptable.

A total of 85% of the 307 toxigenicity reports for the six specimens were correct. In view of the 44 reports containing unacceptable toxigenicity results, it should be recommended that reference centres use both Elek test and PCR alongside each other to avoid major/unacceptable diagnostic errors which might lead to errors in patient treatment. If a single toxigenicity test is performed and delivers incorrect results in a clinical setting, this would almost certainly prevent induction of appropriate treatment of affected patients. The results for EQA specimens 1 and 5 confirmed that reliance on only one of the two tests significantly increases this risk for overall errors. Although PCR assays are rapid and relatively straightforward to implement, a PCR to detect the diphtheria toxin gene should be used with caution and only as an adjunct to the Elek test, which detects the actual expression of the toxin. However, in this EQA the Elek test was observed to be more prone to errors compared to PCR, pointing to the high requirements in laboratory skills for performing and reading the Elek test. It is also recommended that the classical Elek test is not read after 48 hours, as this can give false positives, due to non-specific agglutination lines between the antitoxin and other organism derivatives [6]. The modified Elek test should only be read between 18-24h.

Two of the specimens were to test the countries' ability to detect the target organism and almost all participating centres correctly reported the lack of any potentially toxigenic corynebacteria in specimens 4 and 6. Specimen 4 contained a *C. pseudodiphtheriticum* isolate which was mistakenly identified as a *C. urealyticum* by one centre. Of note, only potentially toxigenic corynebacteria were required to be tested for toxigenicity but this centre reported the *C. pseudodiphtheriticum* isolate to be toxigenic with both Elek test and PCR. Because this coryneform is always non-toxigenic, these results might point to 'transcriptional' errors when filling in the report form or alternatively to a certain lack of general knowledge about corynebacteria, highlighting the need for further training.

With regards to the high number of unacceptable reports, the EQA level of diphtheria diagnostics and capability among EU Member States has decreased compared to the previous EQA in early 2012. While there was simultaneously a decrease in minor errors, previous problems persist, including both phenotypic and genotypic toxigenicity testing.

Recommendations

- In view of numerous unacceptable toxigenicity results reported here, it should be recommended that reference centres use both current methods, i.e. Elek test and PCR, alongside each other in order to avoid diagnostic errors which might lead to inadequate patient treatment. As some Member States only perform PCR, they should ensure that the isolate is sent to another reference centre that can test the expression of the toxin using the Elek test. This should not, however, hamper the delay in clinical and public health management.
- Laboratory training workshops should be continued to maintain the level of capability and quality of results in all EU/EEA Member States, especially those who did not perform well in the present EQA. It is therefore essential to hold another diphtheria diagnostic training workshop for those countries with relatively poor performance and new countries.
- Ways to support countries that require specialised media and reagents for the Elek test through the EDSN should be investigated. A report [7] discussing the urgent issue of access to antitoxin has recently been submitted to a journal and should be of interest to the diphtheria scientific community.
- The laboratory counterparts at the annual network meetings should be brought together regularly to discuss diphtheria diagnostic issues face to face, and to discuss recent developments within this specialised field.
- EQA distributions for diphtheria diagnostics and serology should be undertaken at regular intervals.
- Cost-effective options to be explored, such as extending mutual assistance beyond Malta and Iceland for supranational reference testing within the network.

References

1. Bonnet JM, Begg NT. Control of diphtheria: guidance for consultants in communicable disease control. World Health Organization. Commun Dis Public Health. 1999 Dec;2(4):242–249.
2. 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 Jun 22;347(9017):1739–44.
3. Efstratiou A, Roure C. The European Laboratory Working Group on Diphtheria: A global microbiologic network. J. Infect. Dis. 2000 Feb;181(Suppl 1):S146–151.
4. Neal SE, Efstratiou A, and on behalf of DIPNET and International Diphtheria Reference Laboratories. International external quality assurance for laboratory diagnosis of diphtheria. J Clin Microbiol. 2009 Dec;47(12):4037–42.
5. Neal S, Efstratiou A. European Centre for Disease Prevention and Control. External quality assurance scheme for diphtheria diagnostics 2010. As part of the European Diphtheria Surveillance Network (EDSN). Stockholm: ECDC; 2011.
6. Efstratiou A, PAC Maple. Manual for the laboratory diagnosis of diphtheria. Copenhagen. WHO Europe; 1994.
7. Both L, White J, Mandal S, Efstratiou A. Access to diphtheria antitoxin for therapy and diagnostics. Eurosurveillance 2014. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20830

Annex 1. Participating reference laboratories

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Annex 2. Intended results for the EU diphtheria laboratory network 2013 EQA for the laboratory diagnostics of diphtheria

EQA code	Organism	Biovar	Elek toxigenicity	Original reference no
DIPEQA1	*C. ulcerans*	-	Tox	H085220173
DIPEQA2	*C. diphtheriae*	*gravis*	Non-tox	H133700001
DIPEQA3	*C. diphtheriae*	*mitis*	Tox	H131320904
DIPEQA4	*C. pseudodiphtheriticum*	-	Non-tox	H122700296
DIPEQA5	*C. ulcerans**	-	Tox	H081020547
DIPEQA6	*(only commensals)*	-	-	-

** DipEQA5 is specimen containing an atypical strain which identifies as C. ulcerans as previously confirmed by MALDI, RT-PCR and rpoB sequencing.*

However, some of the biochemical results were atypical for *C. ulcerans* (trehalose and glycogen negative which are characteristic of *C. pseudotuberculosis*).

Annex 3. Network questionnaire results

Apart from Denmark (no response), a total of 31 countries submitted answers to the questionnaire on the relevance of the European Diphtheria Surveillance Network. Several network members simply answered the questions with 'yes' while many others went into more detail (as shown in the selection below).

A) EQAs

1. Did you find this EQA exercise useful?

'Very helpful, very well organized and urgently needed for fulfilling national accreditation criteria; worldwide, there is no other diphtheria-related EQA available.'

'Yes. This exercise is absolutely needed to verify the performance of the lab (we only have 2-5 samples submitted annually from primary laboratories).'

'Yes, we consider that EQA give us the opportunity to exercise our capability to detect and characterise the Corynebacterium strains in the current situation when toxigenic strains are circulating and they could be spread in the areas with low vaccination coverage.'

2. In light of the future lack of EC funding: Do you generally believe that diagnostic EQAs are an important cornerstone of laboratory **diagnostic work for diphtheria**?

'I am working in the national reference lab for diphtheria, so the EQAs are important for my job.'

'EQAs in general are an important quality assurance tool required by many national (and international) accreditation laws for medical laboratories; the ECDC offered EQA is worldwide the only diphtheria-related EQA.'

'Yes, it's very important, since is a very rare disease, and in this way it's the only chance we have to practise the methodologies used for the diagnostic and characterization of Corynebacterium.'

3. Do you think that these EQAs should continue to be funded **in the future** and would you be willing to participate in another EQA?

(simply answered with 'yes' by all)

4. Within the remit of previous EQAs, diagnostic **reagents** (e.g. ELEK base and antitoxin) have been supplied to several countries, and **workshops** to enhance diagnostic skills have been undertaken – Do you generally consider these services useful?

'Yes very useful. Thanks very much for the ELEK base and antitoxin! I would like very much to participate in another workshop to better practise all the skills.'

'It is useful and we would like to improve our diagnostic skills in the future as well.'

'Both these services are very useful. Esp. without the support of the urgently required two reagents Elek base and most importantly antitoxin, at least our national consiliary laboratory would not be able to offer toxigenicity testing beside PCR. The issue of a shortage of antitoxin production facilities worldwide both for therapeutic and diagnostic purposes is well known, has been published by Wagner et al. in 2010 in Vaccine, and requires probably international public health action. Workshops are esp. important for standardizing purposes of laboratory methods (both for basic diagnostic procedures and also for typing methods, e.g. MLST) not only in the EU, but also internationally.'

5. In light of the (potential) future lack of EC funding: Do you generally believe that diphtheria diagnostic quality assessments play an important role in **European public health**?

'Since diphtheria still exists, I believe that it is very important to sustain preparedness, staff, knowledge, experience and reagents.'

'EQAs are a very important first step for standardizing laboratory methods within the EU and beyond, are required for fulfilling national accreditation criteria and are politically and in terms of European public health important due to globalization of travel, migration, etc. (e.g. still relatively high incidence in East Europe, neighbourhood to Syria, ex-pat communities from India and other endemic Asian countries in UK and other EU countries).'

'I think that diphtheria diagnostic quality assessments have a role in European public health, although diphtheria is rare. Diagnostic training is useful especially in countries such as Croatia, in order to be prepared for a diphtheria outbreak.'

B) Surveillance

1. Do you feel that we generally need **dedicated disease surveillance networks** in Europe (and associated countries)?

'Yes, the communication between the specialists in a specific disease is crucial for surveillance'

'Dedicated disease surveillance networks have been a success in the past. Esp. the combination of epidemiology and microbiology guaranteed a better interdisciplinary understanding of each other and esp. the needs and limitations of the respective epi and lab methods. This is also cost-effective, since epi questions and needs can be targeted more specifically towards sensible and useful public health goals when guided by microbiological expertise.'

'We need to have surveillance networks for such rare infections.'

2. In light of the (potential) future lack of EC funding: Do we specifically need a **diphtheria** surveillance network in Europe (and associated countries)?

'A diphtheria network can solve many problems in Europe. First of all, one of the most important points is the supply of reagents for toxigenicity tests; meetings and lab trainings are essential.'

'It is a disease that is vaccine preventable, but there are many people that aren't immunized. In this way, and because East Europe has cases and people are migrating, I think that we really need a surveillance network system.'

'A diphtheria surveillance network combining both epidemiology and microbiology might be even more important in relatively rare diseases needing highly specialized lab experience and reagents in order not to lose the respective expertise when public health crises might develop within or around the EC (Syria, Latvia, Eastern Europe, former Soviet Union).'

3. Do you generally believe that the European Diphtheria Surveillance Network plays an important role in **European public health?**

"The network plays a major role in Europe as we can share our lab methods; develop new ones, including standardisation and development of typing methods.'

'Yes, diphtheria cases are still reported and imported cases in areas free of diphtheria could be possible.'

'A diphtheria surveillance network combining both epidemiology and microbiology might be even more important in relatively rare diseases needing highly specialized lab experience and reagents in order not to lose the respective expertise when public health crises might develop within or around the EC (Syria, Latvia, Eastern Europe, former Soviet Union).'

4. Would the loss of the European Diphtheria Surveillance Network (due to absence of funding by EC) lead to a loss of know-how in diphtheria diagnostic skills?

'Yes, because the European Diphtheria Surveillance Network ensures the maintenance of the diagnostic capabilities of laboratories in Europe.'

'The most important point is scientific support (current information about the methods and reagents recommended should be regularly published). We are gratefully appreciated being provided with the reagents especially Elek test since the availability of these indeed is a problem.'

'I think it is possible to lose diphtheria diagnostic skills, especially in countries where there is no diphtheria testing in routine work.'

5. Would the loss of the European Diphtheria Surveillance Network ultimately be associated with **disadvantages for affected patients**, e.g. in a possible outbreak situation?

'Yes, it would be associated with serious disadvantages for affected patients and for the community.'

'Of course, the loss of European Diphtheria Surveillance Network will lead to misdiagnosis and eventually to inadequate treatment.'

'Absolutely. If we lose the expertise we have accumulated (in great part by the EQA), it will take much longer to verify an outbreak. In most labs there is a turn-over of staff and it is very difficult to educate all new personnel in this (for now) rare disease unless we are helped on the way (requirement to participate in this EQA).'