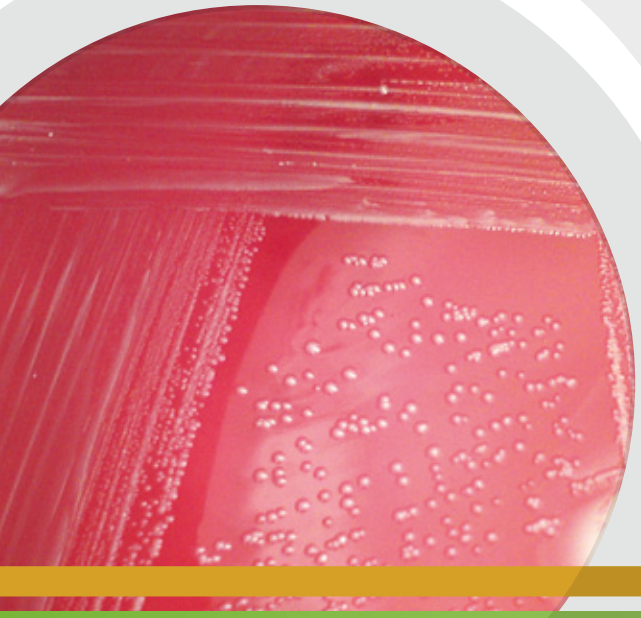

TECHNICAL REPORT

External quality assurance scheme for diphtheria diagnostics 2010

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ECDC TECHNICAL REPORT

External quality assurance scheme for diphtheria diagnostics 2010

As part of the European Diphtheria Surveillance Network (EDSN)

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Dr Ida Czumbel and produced by Dr Shona Neal and Dr Androulla Efstratiou (Health Protection Agency, Streptococcus and Diphtheria Reference Unit/WHO Global Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections, London, UK), on behalf of the EU DIP-LabNet consortium (referring to Specific Contract ECD.2083).

Suggested citation: 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.

Cover picture © Health Protection Agency, Colindale, London, UK.

Stockholm, August 2011

ISBN 978-92-9193-302-0

doi 10.2900/56088

Contents

Abbreviations

ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
DAE	Double-antigen enzyme-linked immunosorbent-assay
DELFIA	Dual double-antigen time-resolved fluorescence immunoassay
DIPNET	Diphtheria Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EDSN	European Diphtheria Surveillance Network
ELISA	Enzyme-linked immunosorbent assay
ELWGD	European Laboratory Working Group for Diphtheria
EQA	External quality assurance
eQAD	external Quality Assurance Department
EU DIP-LabNet	EU Diphtheria Laboratory Network
DSN	Dedicated surveillance network
HPA	Health Protection Agency (UK)
IU	International units
MALDI-TOF	Matrix assisted laser desorption ionization - time of flight
MLST	Multi locus sequence typing
MLVA	Multiple loci variable number tandem repeat analysis
NEQAS	National External Quality Assessment Service
NCTC	National Collection of Type Cultures
NTTB	Non-toxigenic, *tox*-gene bearing
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
SDRU	Streptococcus and Diphtheria Reference Unit (of the HPA, London)
WHO	World Health Organization

Executive summary

Effective control of an uncommon vaccine-preventable disease is dependent upon prompt and early recognition and diagnosis. It is often difficult to diagnose diphtheria clinically, particularly in those countries where the disease is rarely seen. 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. It is fundamental that EU Member States have the capacity and ability to undertake the procedures relating to the microbiological diagnosis of not only diphtheria but also the related infections caused by all potentially toxigenic strains of corynebacteria.

The European Centre for Disease Prevention and Control (ECDC) recognises the importance of international collaboration for sustaining high quality diagnostics within this specialised field across the EU and catalysing research to counteract this public health threat. ECDC promotes the performance of External Quality Assurance (EQA) schemes, in which laboratories are sent simulated clinical specimens or bacterial isolates for testing by routine and/or reference laboratory methods. These exercises strengthen quality assurance systems and enable a laboratory performance to be assessed against reference methods and other peer laboratories.

ECDC has a role in building and developing microbiology laboratory networks and has established the European Diphtheria Surveillance Network. One of the work packages of the laboratory aspects includes the organisation of a questionnaire assessment and an EQA dispatch for the laboratory diagnosis of diphtheria in Year One. Key findings are listed below; a description of the work involved and the outcomes of these exercises are detailed further in this report.

Key findings

- Questionnaire results indicated an increase in EU Member States' diphtheria diagnostic capacities.
- Overall, 86% of the identification reports were correct and 90% of the toxigenicity reports were correct, an improvement on the previous DIPNET EQA.
- However, at least nine centres had problems with the Elek test, which is the gold standard for detecting toxigenicity, reflecting the need for further training workshops and EQA studies.
- Specimen C revealed inconsistencies in species identification, highlighting that additional tests should be performed where necessary.
- Specific countries have been identified that would benefit from attending the next diphtheria diagnostics workshop in Autumn 2011, based on their average to poor performance in this EQA.
- In addition to the questionnaire and EQA dispatch, a number of countries received specialised Elek media and reagents, which are becoming increasingly difficult to obtain.
- These activities should be continued to maintain the level of capability and quality of results in all Member States.
- False negative toxigenic results, as observed in this EQA, would impact negatively on the speed of public health action and patient management.

Background

The European Centre for Disease Prevention and Control (ECDC) is a European Union (EU) agency with a mandate to operate dedicated surveillance networks (DSNs) and to identify, assess, and communicate current and emerging threats to human health from communicable diseases. Within its mission, ECDC shall '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]).

External quality assurance forms part of a quality management system and evaluates performance of laboratories by an outside agency on material that is supplied specially for the purpose. ECDC's disease-specific networks organise a series of EQA for EU/EEA countries. In some specific networks, non-EU/EEA countries are also involved in the EQA activities organised by ECDC. The aim of the EQA is to identify areas for improvement in laboratory diagnostic capacities relevant to surveillance of the diseases listed in Decision No 2119/98/EC[2] and to 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; and
- identification of needs for training activities.

Consequently, the EQA process motivates users to improve laboratory performance, which increases the probability of correct diagnosis, case management and an improved quality of surveillance data.

Infections caused by the potentially toxigenic corynebacteria (*Corynebacterium diphtheriae, C. ulcerans* and *C. pseudotuberculosis*) are usually difficult to track because of their epidemic patterns, the emergence of new strains, novel reservoirs and their dissemination to susceptible human and animal populations. *Corynebacterium diphtheriae* is the classic human pathogen that became uncommon in Europe following the introduction of mass immunisation programmes in the 1940s and 1950s. However, re-emergence 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. The European Laboratory Working Group for Diphtheria (ELWGD) was quickly established in 1993 to help strengthen the diphtheria diagnostic capabilities of many countries within the WHO European Region. Many European countries adopted enhanced practices, such as screening for diphtheria from routine throat swabs, but currently no EU countries are screening for diphtheria, due to the low incidence now observed. In addition, *C. diphtheriae* and *C. ulcerans* are now uncommon organisms rarely isolated in laboratories.

The EQA schemes for diphtheria diagnostics were first developed under the auspices of the ELWGD in 1994 and since then eight distributions for laboratory diagnostics have been performed (including one for serological immunity testing). Results from the last four distributions revealed that correct toxigenicity and identification reports have rarely exceeded 90%, irrespective of the composition of the EQA panel or the countries participating. However, EU Member State participation has been variable in these EQA distributions. Nonetheless, this clearly indicates the need for continued EQA programmes for diphtheria diagnostics.

Currently, there is a large variety of culture media and diagnostic tests in use across Europe, and although harmonisation of methods would be ideal, the recommendation of a set of standardised protocols, such as those developed under the remit of DIPNET, is more achievable. It is therefore important to periodically assess the methodologies used for diphtheria diagnostics in Europe, using initially a questionnaire-based approach. The last evaluation was performed under the remit of DIPNET in 2007, where no particular test posed a widespread problem in the EQA. Most centres performed the phenotypic toxigenicity test, the Elek test, which is regarded as the key test for microbiological diagnosis. However, several countries within the EU reported problems in obtaining culture media and antitoxin due to unavailability in 2007.

The European Diphtheria Surveillance Network (EDSN) was 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 of diphtheria and to strengthen and harmonise the laboratory capacity at national level. The network has two components:

[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.

epidemiological (conducted by ECDC and activities are focused on data collection and analysis) and laboratory (outsourced to the Health Protection Agency (HPA), London and focused on EQA, training and molecular typing). The key objective of this work, as part of Work Package 2 and 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

Year One contract requirements

Organisation of an EQA scheme for diphtheria identification and characterisation to be carried out in the national reference laboratories in the EU/EEA Member States, and in candidate and potential candidate countries (depending on budget availability and countries' interest).

Determine the current laboratory capabilities in terms of diagnostics and methods used. In the past, a questionnaire-based survey was carried out by DIPNET for determining the current laboratory capabilities. It is not necessary to repeat that work for countries that have already participated (unless there are new updates to consider) but it would need to be performed for those countries that were previously not members of the DIPNET network.

Study design

The previous questionnaire and EQA was distributed to 24/30 EU/EEA Member States and one candidate country in 2007. Therefore, within this programme all EU and EEA Member States liaising with ECDC as the nominated laboratory contacts for diphtheria were included. All the countries new to the scheme were initially assessed on their laboratory capabilities to detect diphtheria, by way of a detailed questionnaire, (based on the previous DIPNET questionnaire: see Annex 1) was sent to the nominated laboratory counterparts in all EEA countries that did not participate in the DIPNET project (Hungary, Iceland, Liechtenstein, Malta, Luxembourg and Slovakia). This included topics such as level of reference laboratory, details of services provided, laboratory diagnostics, toxigenicity testing, antibiotic sensitivity, epidemiological typing, culture collections, serological assays and EQA participation.

In addition, a table containing the results from the previous DIPNET questionnaire was sent to those countries that were surveyed in 2007 to rapidly check whether the details were still correct. Each country was asked to amend the table if any new tests/procedures had been implemented in their laboratory since 2007.

Results from the questionnaire-based survey were collected and analysed by the HPA on behalf of the EU DIP-LabNet consortium.

The questionnaire was undertaken so as to ascertain the laboratory capabilities before the EQA panel was sent and also to identify those laboratories most in need of training. This would also become more evident from the results of the EQA study.

The design of the study allowed individual reference laboratories to test the material using their routinely available techniques within the allocated time period. The reference laboratories were able then to compare their own submitted results with the intended results to determine differences, if any.

The EQA distribution utilised the availability of the large collection of corynebacteria isolates and expert knowledge at the HPA's Streptococcal and Diphtheria Reference Unit (SDRU, London, UK), with the specialised assistance of Nita Patel (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 2.

All participants were contacted at the time of the questionnaire dispatch and prior to the EQA distribution to confirm the address and contact details for dispatch of the potentially hazardous material. It was envisaged that the reference laboratories would wish to store the viable cultures and retain any unused material for their own quality processes. It was hoped that the distribution of the well-characterised material would become a resource within and between the reference laboratories.

Timelines

Table 1. Timelines for the EQA and questionnaire exercise

Event	Date
Building participants list	March – May 2010
Selection of EQA strains	April 2010
Send laboratory questionnaire and spreadsheet to participants	April 2010
Assessment of strains before freeze-drying	April 2010
Transfer of strains to eQAD UK NEQAS	April 2010
Deadline to submit laboratory questionnaire and/or spreadsheet	21 May 2010
Freeze-dry panel produced (eQAD UK NEQAS)	May 2010
Pre-dispatch checks of freeze-dried panel (SDRU)	May 2010
Requests for specialised Elek media and antitoxin from participants	May 2010
EQA panel dispatch (eQAD UK NEQAS)	11 June 2010
Additional Elek media and antitoxin dispatch to various countries	22 June 2010
Interim progress presentation at 1st EU Dip-LabNet meeting, Cyprus	29 June 2010
Reference laboratories testing EQA panel	June – September 2010
Final return of results	24 September 2010
Intended results sent to participants	29 September 2010
Analysis and collation of results	March – April 2011
Preliminary results presentation at EDSN meeting, Stockholm	17 March 2011
Producing report	April – May 2011

The EQA simulated specimen panel

Six *Corynebacterium* sp. strains were selected based on their variability and toxigenicity. The strains were referred to the WHO Collaborating Centre, London. Four strains were submitted recently from English laboratories (2009–10) and one other was a presumed *C. pseudotuberculosis* isolated from a patient in Ukraine in 1999. The remaining strain is the type strain for *C. striatum* (NCTC 764), a species which cannot exhibit toxigenicity, but was included to test the participants' ability to isolate this strain and also how they would report or name such an isolate.

The strains were coded and prepared as simulated throat specimens by the addition of one or more commensal throat flora and freeze-dried by the Quality Assurance Laboratory, HPA, London. Quality control of the specimens was undertaken by the WHO Collaborating Centre both before and after freeze-drying to test for viability and retention of the organism's characteristic properties.

Full instructions were included in the dispatch, 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 in June 2010 to 28/30 countries (no laboratory counterparts for Liechtenstein or Spain). Full instructions and a result form was enclosed and also sent electronically, with results to be submitted by 24 September 2010 (Annex 3).

Additional dispatch of specialised media

In order to complete the EQA effectively, all countries were provided with a maximum budget of €630 for laboratory consumables, reagents and media for the diagnosis of diphtheria. The gold standard for detecting toxigenicity, the Elek test, requires media which can be laborious to make, and antitoxin, which is hard to obtain. There are commercial products for this test, but these have not been as accurate as in-house preparations. Therefore, some countries requested a shipment of Elek media and/or antitoxin strips or discs from the WHO Collaborating Centre for Diphtheria and Streptococcal Infections in London. Fifteen countries received shipments, which in total comprised 316 vials of Elek media, 258 antitoxin strips and 110 antitoxin discs.

Results

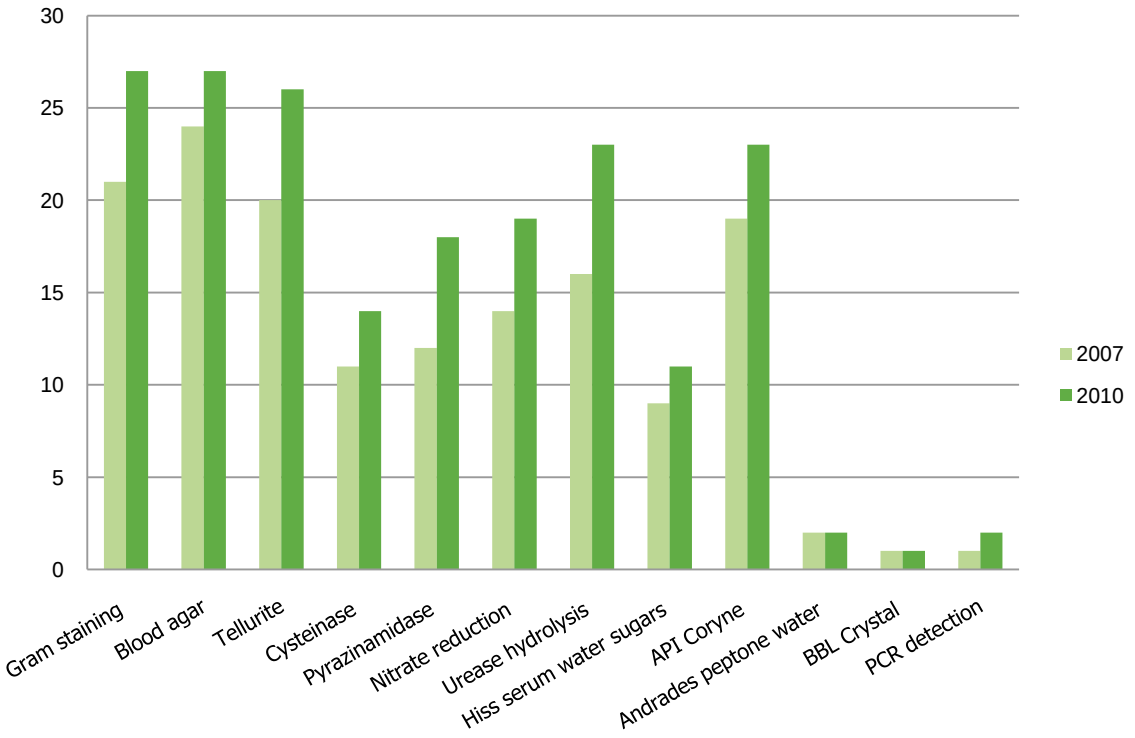
Questionnaire findings

Responses were received from 27/30 countries; there were no replies from Iceland, Liechtenstein or Spain. At the time of the questionnaire dispatch, laboratory counterpart nominations for Liechtenstein and Spain had not been received. However, results from the 2007 questionnaire were available for Spain, with the assumption that the same capabilities had not been changed.

1. Twenty-two centres are officially designated National Reference Centres for Diphtheria for their country. Centres in Cyprus, Greece, Malta, the Netherlands, Poland, Romania, Slovenia and Spain are not officially recognised as National Diphtheria Reference Laboratories by their Ministries of Health. The centre in Ireland has recently been recognised as such.

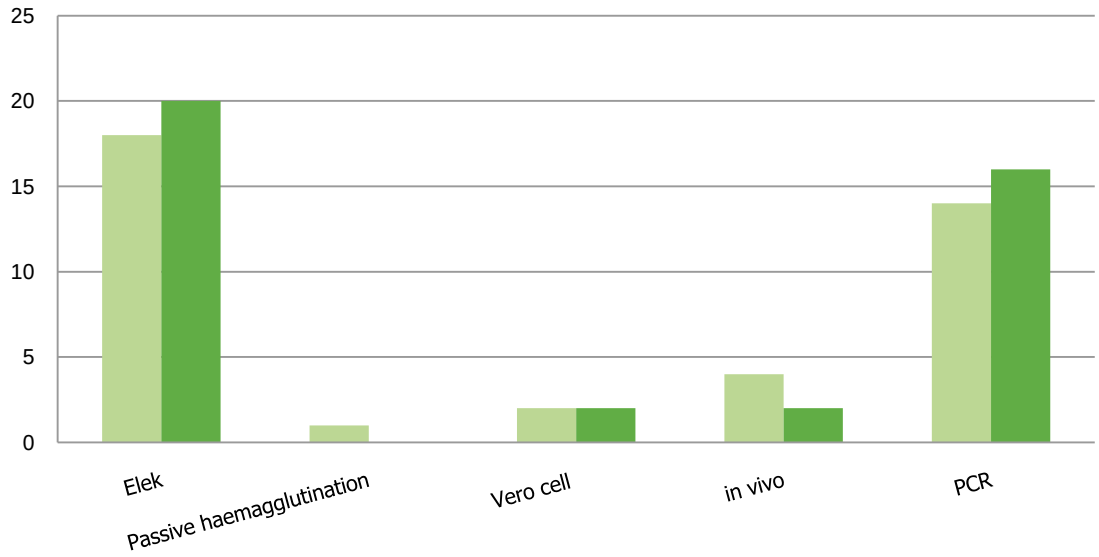
2. Centres have increased their laboratory capacities compared to the last exercise in 2007 and as a consequence of the DIPNET programme (Figure 1). The key primary isolation medium, containing tellurite, is used in 96% of centres (26/27), whilst the important presumptive identification tests (to differentiate *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* from other corynebacteria), pyrazinamidase, cysteinase, and urease are performed in 67% (18), 52% (14) and 85% (23) of centres, respectively. A commercial kit to identify and biotype corynebacteria (API Coryne, BioMerieux) is used in 85% (23/27) of centres.

Figure 1. Methods used for screening and detection of corynebacteria in 27 centres in EU and EEA countries, 2007 and 2010



3. The key diagnostic test for toxigenicity is the Elek; this is performed in 20/27 centres (Figure 2). Similar to findings from the DIPNET survey, there were many variations in the serum source (newborn bovine recommended, but bovine, rabbit and equine used) and antitoxin concentration (500 IU recommended), which could affect the final result. Furthermore, many countries were still experiencing problems in obtaining antitoxin and appropriate media.

Figure 2. Types of test used to determine toxigenicity of corynebacteria in 27 centres in EU and EEA countries, 2007 and 2010



4. The molecular technique PCR is used to detect the toxin gene. This is performed in 16/27 centres (Figure 2), of which three centres perform PCR as their only toxigenicity test. This could prove problematic, as detection of a non-toxigenic, *tox*-bearing (NTTB) *C. diphtheriae* would be reported as positive by PCR, but as a NTTB or negative organism using a phenotypic test, such as the Elek test. However, this scenario is relatively rare and a PCR-positive result is usually phenotypically positive.

5. Other rapid tests to identify species and detect the toxin gene include direct real-time PCR, multiplex PCR, 16S rDNA and rpoB (RNA polymerase beta subunit-encoding gene) sequencing, and MALDI-TOF.

6. At least 21 centres performed antibiotic sensitivity tests. Six would do these routinely and thirteen only if requested. A variety of assays are used, including the E-test (n=15), disc diffusion (13) and agar incorporation (1). Most centres used the CLSI guidelines for breakpoint data (15), and the corresponding ATCC control strains (13); five centres used the NCTC control strains.

7. Eight centres currently perform epidemiological typing for diphtheria using the following methods; ribotyping (6), MLST (2), phage typing (1), PFGE (1), and MLVA (1). A further nine centres would like to introduce epidemiological typing into their laboratories, but only five of these currently have the capability to do so.

8. Most countries would be willing to exchange isolates and twelve of these had culture collections, varying from nine isolates to hundreds, and spanning from 1930 to the present date.

9. Seventeen countries perform serological testing, using either the tissue neutralisation assay (6; gold standard for serological detection of diphtheria toxin), an ELISA (13), DAE (1), DELFIA (1), and the Luminex platform (1).

Laboratory diagnostic EQA results

The intended results were sent to all participants in September 2010 for information and for each laboratory to rapidly assess how they performed (Annex 4). 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 (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, EU DIP-LabNet offered direct advice and recommended repeating the specimens, following corrective action, in order to improve diphtheria diagnostics in EU/EEA countries. Those participants will also be offered training in the workshop scheduled for Year Two.

Twenty-six of 28 centres submitted results; Iceland and Portugal did not submit results because they did not have the laboratory expertise to undertake the work. The intended results, with a summary of the participants' findings, are shown in Table 2. The panel did not contain NTTB strains or diphtheroid commensals, and was therefore more straightforward than the previous DIPNET EQA. However, a couple of specimens in particular tested the centres' abilities to identify the species and detect the toxigenicity.

All bar one centre reported the correct species, biotype and toxigenicity for specimen A. This centre reported an incorrect biotype and a false positive toxigenic result (a weak positive Elek but a negative PCR result). The concentration of antitoxin that they used in the Elek was not in international units (2500 E) and the PCR primers were designed to amplify the B part of the toxin. Full details of their PCR protocol are unknown.

Specimen B was a weak toxigenic *C. ulcerans*, but three centres reported it as *C. pseudotuberculosis* and four as non-toxigenic. Twenty centres reported a fully correct result. The non-toxigenic results could have led to a misdiagnosis and would have missed treatment and public health action had this been a clinical case.

The initial intended results sent out to all participants for specimen C were initially incorrect. The intended API Coryne profile was 0111324 = 92.8% id for *C. pseudotuberculosis*. However, to exclude the possibility of *C. ulcerans*, further testing at the WHO Collaborating Centre revealed that it was a *C. ulcerans* (trehalose positive, gelatine liquefaction positive, MALDI-TOF, *rpo*B sequencing 99% identity). Therefore, as it was a different species, participants who reported *C. pseudotuberculosis* were graded as an unacceptable result. Only seven centres gave a fully correct result and sixteen centres incorrectly reported *C. pseudotuberculosis*. Eight centres reported non-toxigenic results, which, as with specimen B, would have led to missed or delayed treatment had the specimen been from a patient.

Table 2. Summary of results for each of the EQA specimens for the 28 participating laboratories

Specimen Number	Intended result	Toxigenicity (Elek and PCR)	Number of laboratories with		
			Fully correct result	Acceptable result	Unacceptable result
EQA WP2-10 **A**	*C. diphtheriae* var *gravis*	Non-toxigenic	25	0	1 (reported weak toxigenic result)
EQA WP2-10 **B**	*C. ulcerans*	Weak toxigenic	20	0	6 (3 reported *C. pseudotuberculosis*) (4 reported non-toxigenic results; 3 Elek, 1 PCR)
EQA WP2-10 **C**	*C. ulcerans*	Toxigenic	7	0	19 (8 reported non-toxigenic results; 7 Elek, 2 PCR) (16 reported *C. pseudotuberculosis,* 1 *C. pseudodiphtheriticum*)
EQA WP2-10 **D**	*C. striatum* *(C. amycolatum, C. striatum)*	Non-toxigenic	20	4*	2 (1 reported toxigenic result) (1 reported *C. pseudodiphtheriticum*)
EQA WP2-10 **E**	*C. diphtheriae* var *mitis*	Toxigenic	18	8	0
EQA WP2-10 **F**	*C. diphtheriae* var *belfanti*	Non-toxigenic	19	5	2 (reported toxigenic results; 1 Elek, 1 PCR)

*Four centres reported '*Corynebacterium* sp.'

Centres scored well with specimen D, where 20/26 centres gave a fully correct result for the *C. striatum* strain (non-toxigenic). Four centres reported a *Corynebacterium* sp.; this may be a reflection of how centres report corynebacteria that are not potentially toxigenic, and so it is marked as an acceptable result. Unacceptable results were a single toxigenic report and an incorrect identification of *C. pseudodiphtheriticum*. However, at least five centres stated they did not perform toxigenicity testing, which would have delayed public health action had it been a potentially toxigenic corynebacteria.

Specimen E resulted in all centres achieving either fully correct (18) or acceptable (8) results. Those centres reporting incorrect biotypes included *mitis/belfanti* (3), *intermedius* (2), *mitis/intermedius* (1), *gravis* (1) and a non-*gravis* (1). As reported in the last EQA, participants should take note that *intermedius* strains produce smaller

colonies after 16 to 24 hours (0.5 – 1 mm in diameter, compared with 1.5 – 2 mm for *mitis* biovars), and *mitis* is nitrate-positive and *belfanti* is nitrate-negative.

Most centres reported specimen F correctly as a non-toxigenic *C. diphtheriae* var *belfanti* (19/26). A further five centres gave acceptable results, reporting either *mitis/belfanti* (4) or non-*gravis* (1). Two centres reported toxigenic results; if this had been a true case, needless treatment and public health action would have occurred.

Overall, from the six specimens and 26 centres, there were 156 available reports, resulting in 16 (10%) incorrect toxigenicity reports and 21 (14%) incorrect identification reports. This was a slight improvement on the previous DIPNET EQA (13% incorrect toxigenicity and 21% incorrect identification from a panel of six specimens), although it included many other countries from the WHO European Region (n=32). Five countries managed to report acceptable or fully correct results for all six specimens: Denmark, France, Malta, Norway and the UK. These countries are either long-established National Diphtheria Reference Laboratories, have had recent toxigenic cases or attended the recent diphtheria workshop (work package 4). In addition, a further fourteen centres gave 5/6 fully or acceptable results, mostly in part due to the incorrect reporting of *C. pseudotuberculosis* for specimen C. Six countries determined 4/6 specimens correctly, whilst one centre only achieved 2/6 correct analyses, failing at both identification and toxigenicity. This centre had never sent a person to a diphtheria workshop or attended a network meeting for at least four years (under the remit of both DIPNET and ECDC).

Conclusions

Overall, there was an improvement in countries' capacities, with an increased range of methodologies and repertoire of diphtheria diagnostics. This was largely due to the continued awareness and training that the participants gained through the DIPNET programme. ECDC therefore acknowledged that regular EQA and laboratory training is key to maintaining good standards in the use of specialised methodologies relating to diphtheria diagnostics.

The EQA results received from 26 Member States were encouraging, as reflected in the lower number of incorrect reports, but more specific improvements are still necessary. Clarification of biotyping is detailed above, and at least nine participants had problems with the Elek test. Six of these procured Elek media and antitoxin strips and/or discs from the coordinating centre, and most were using newborn bovine serum, but gave a false negative toxigenic result for specimen C, the toxigenic *C. ulcerans*. All of these centres had also reported the strain as *C. pseudotuberculosis*, demonstrating that this isolate was difficult to characterise.

There were further lessons learnt from specimen C; all isolates need to be fully tested with additional differential tests where necessary, before preparation and dispatch. As a general rule, additional tests should be used if the API Coryne result shows an identification of less than 95%. Furthermore, a more detailed report form will be prepared, requesting the API profile, further identification tests performed, and with separate columns in which to enter Elek and PCR toxigenicity results.

A few centres reported 'not performed' or 'not done' for specimen D, a *C. striatum*. Although this was a non-toxigenic corynebacterium, if clinically suspected as diphtheria, toxigenicity tests should not be delayed whilst waiting for an identification result. The toxigenic result could further impact on the scale of public health action and screening contacts.

During Year One there were many requests for the specialised media and reagents for the Elek test. In total, 316 vials of Elek media, 258 antitoxin strips and 110 antitoxin discs were despatched to 15 countries, resourced from the Year One budget. This additional activity requires funding as the shipping and reagents have significant cost implications. In addition, commercial Elek media are non-specific and are thus inaccurate. Therefore, it is recommended that subsequent requests for Elek media and reagents from Member States are funded by ECDC through the European Diphtheria Surveillance Network.

Recommendations

To continue the EQA dispatches regularly (yearly or every two years), as this gives the Member States essential experience in handling toxigenic strains of corynebacteria, in order to improve and maintain quality in diphtheria diagnostics.

To train participants that did not perform well, by way of a training workshop (Work Package 4).

To fund further shipments of specialised media and reagents for the Elek test to countries in need.

To bring together the laboratory counterparts at the annual network meetings to discuss these issues face to face and to discuss recent developments within this specialised field.

Planned activities for Year Two

The EQA results identified the countries that require additional support and training. These countries will be invited to the second workshop scheduled for Year Two (Autumn 2011). During Year Three, a second EQA distribution for laboratory diagnostics will be conducted, so as to measure any improvements made since the previous EQA. This may be extended to other countries within the European Region and beyond but any such extension will not be funded by ECDC. A manuscript describing these activities and studies will be prepared and submitted to a scientific journal.

The next EQA for laboratory diagnostics is planned for summer 2012 under the third specific ECDC framework contract.

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Annex 1
Laboratory diagnostic questionnaire for EU DIP-Labnet
(contract number ECDC/10/002)

Your answers are important. Please complete the following questionnaire and return by email, fax or post (addresses on last page) by 21st May 2010.

Thank you for your co-operation.

Nominated laboratory counterpart for diphtheria

Name of Centre:

Address:

Telephone number:

Fax number:

E-mail address:

Director of Department/Establishment:

Completion Date of form. ...../...../.....

A. LEVEL OF REFERENCE

		YES	NO
1.	**Are you a designated Diphtheria National Reference Centre in your country?**		

If YES; since when? **YEAR** | | | | |

If NO, please indicate if you have applied for designation and the stage reached.

……

……

……

……

		YES	NO
2.	**Are you a designated WHO Diphtheria Reference Centre?**		

If YES, please indicate since when and *attach terms of reference*. **YEAR** | | | | |

B. DETAILS OF REFERENCE SERVICES PROVIDED

1. Do you provide a diphtheria reference service for your entire country? YES ☐ NO ☐

If NO,

a. What geographic area do you cover?

……

……

b. Please list below other diphtheria reference facilities you are aware of in your country:

……

……

2. In the area you cover, how many laboratories refer isolates/clinical specimens to you?

N°. labs ☐

3. Do you provide reference service for cultures sent from outside your own country? YES ☐ NO ☐

If YES, please list countries below:

……

……

4. Please indicate the number of isolates/specimen referrals received by your laboratory which were originally isolated from patients within your country.

	C. diphtheriae								*C. ulcerans*		*C. pseudotuberculosis*	
Biotype	Gravis		Mitis		Belfanti		Intermedius					
Year	Tox +	Tox -	Tox +	Tox -	Tox +	Tox -	Tox +	Tox -	Tox +	Tox -	Tox +	Tox -
2005												
2006												
2007												
2008												
2009												
2010 (to date)												

5. Do diagnostic laboratories within your country screen throat swabs for the presence of potentially toxigenic corynebacteria? YES ☐ NO ☐

If YES, please estimate the percentage of laboratories doing this: ☐ **%**

C. LABORATORY DIAGNOSIS

1. IDENTIFICATION PROCEDURES:

Briefly indicate your current methodologies for biotyping and identification practices in the table below. Please *include details of the primary selective media, screening tests used and method(s) for biochemical identification*.

	NO	YES	If yes, which controls do you use?
Primary culture:			
Gram stain			
Other stains (*please specify*) ……			
Blood agar			
Tellurite agar			
Screening, biotyping and identification tests:			
Cystinase			
Pyrazinamidase test			
Nitrate reduction			
Urease hydrolysis			
Hiss serum water sugars (Glucose, Maltose, Sucrose)			
APICoryne			
Identification via sequencing genes (*please specify which genes*) …… ……			
Other tests (*please specify*) …… …… ……			

2. CULTURE MEDIUM AVAILABILITY IN YOUR COUNTRY

	YES	NO
Do you experience problems with obtaining culture media?		

If YES, please comment:

……

……

……

3. TOXIGENICITY TESTING:

Please indicate method(s) currently used in your laboratory for toxin detection:

	NO	YES	If yes, which controls do you use?
Elek test			
PCR			
Passive haemagglutination			
Tissue culture (e.g. Vero cell bioassay)			
***In vivo* test**			
Other tests (*please specify*) **……** **……** **……**			

3.1 Elek test: If performed:

a. What is the source of your Elek medium?

Commercial ☐ **In house** ☐ **WHO/HPA** ☐

If the source is commercial, which manufacturer do you use?

……

……

b. What type of serum is used for the Elek medium?

Equine ☐ **Newborn bovine** ☐ **Bovine** ☐

Other (*please specify*)

……

……

c. What is the concentration of antitoxin used for the Elek test?

500 IU ☐ **1000 IU** ☐

Other (*please specify*)

……

……

d. What is the source of antitoxin used for the Elek test?

Equine ☐

Other (*please specify*)

……

……

Which manufacturer?

……

……

	YES	NO
Do you have problems in obtaining supplies of antitoxin?		

If YES, please give details

……

……

e. What controls do you use?

Standard NCTC controls		**ATCC controls**	

Other (*please specify*)

……

……

If PCR is performed in your laboratory, please go to 3.2 & 3.4
If PCR is NOT performed in your laboratory, please go to 3.3 & 3.4

3.2 (if performed) PCR test:

a. Which primer sets do you use (please give sequence)?

Fragment A of toxin gene	
Fragment A and B (entire gene)	

Which manufacturer and reference?

……

……

b. What controls do you use?

Standard NCTC controls		**None**	

Other (*please specify*)

……

……

	YES	NO
c. Do you use an internal template control? (e.g. artificial template which is amplified by the same primer pair as the template DNA, and is used to confirm the PCR reaction has not failed.)		

3.3 (if not performed) PCR:

	YES	NO
Are there relevant facilities (thermocycler, gel tanks, photographic equipment etc.) available in your laboratory/institute?		

	YES	NO
Would you like to introduce PCR toxin gene detection to your laboratory/institute?		

3.4. TOXIGENICITY TESTS OTHER THAN PCR AND ELEK TEST

Please describe your procedure or *attach methods and/or references if available*

……

……

……

……

……

……

……

……

……

4. ANTIBIOTIC SENSITIVITY

	YES	NO
a. Do you perform any antibiotic sensitivity tests in your laboratory?		

IF NO, GO TO SECTION D

IF YES:

	YES	NO
b. Do you routinely determine antibiotic sensitivities on all *C. diphtheriae* and *C. ulcerans* isolates received by your laboratory?		

	YES	NO
c. Do you only determine antibiotic sensitivities if specifically requested to do so?		

d. Which methods do you perform in your laboratory?

	NO	YES	If yes, which manufacturer are the reagents from?
E test			
Disc diffusion (please state method)			
Broth dilution			
Agar incorporation			
Commercially prepared MIC microtitre trays			
Other (*please specify*) …… ……			

e. Please list the range of antibiotics tested and the concentration or disk load

Antibiotic name	Concentration (mg/ml)

Antibiotic name (continued)	Concentration (mg/ml)

f. Which control strains do you use for antibiotic sensitivity testing?

Standard NCTC controls ☐ **ATCC controls** ☐

Other (*please specify*)

……

……

g. If breakpoint values are measured, what interpretation guidelines are used?

CLSI (formerly NCCLS) ☐ **BSAC** ☐

Other (*please specify*)

……

……

D. EPIDEMIOLOGICAL TYPING OF CORYNEBACTERIA

	YES	NO
1. Do you perform epidemiological typing of *C. diphtheriae* and/or *C. ulcerans*?		

2.1 If YES, which of these typing methods do you perform your laboratory?

	YES	NO
Ribotyping		
Phage typing		
Multilocus enzyme electrophoresis (MLEE)		
Pulsed-field gel electrophoresis (PFGE)		
SDS polyacrylamide gel electrophoresis		
Random amplified polymorphic DNA (RAPD) typing/PCR typing		
Amplified Fragment Length Polymorphism (AFLP)		
Multilocus sequence typing (MLST)		
Variable Number Tandem Repeats (VNTR)		
Other (*please specify*)		

2.2 Briefly describe your procedures (or *provide methodology or published references if available*).

.....

.....

.....

.....

.....

.....

	YES	NO
3.1 If NO, do you have the capabilities to perform epidemiological typing?		

	YES	NO
3.2 Would you like to introduce and perform epidemiological typing?		

4. Which epidemiological typing method do you/would you find most useful? Please state

.....

.....

5. Which analytical and/or database software do you use for this epidemiological typing/microbiological data?

Taxotron ☐ **BioNumerics** ☐

Other (*please specify*)

.....

.....

E. CULTURE COLLECTIONS OF *C. DIPHTHERIAE/C. ULCERANS*

One activity of EU DIP-LabNet is to maintain a database for European molecular surveillance based on a standard collection of isolates that have been subjected to different typing methods by the various centres.

		YES	NO
1.	**Are you willing to exchange representative isolates with other reference centres within EU DIP-LabNet for typing purposes?**		

		YES	NO
2.	**Do you have historic collections of isolates from epidemics, outbreaks and serious systemic cases?**		

If YES, please give details on any 'interesting' collections you may have.

……

……

……

……

……

……

		YES	NO
3.	**Have these isolates been characterised biochemically?**		

		YES	NO
4.	**Do these isolates have epidemiological and/or clinical information on the related case?**		

F. DIPHTHERIA POPULATION IMMUNITY SCREENING

	YES	NO
1. Do you perform any serological tests for the detection of antitoxin/antibody levels to diphtheria toxin?		

2.1 If YES, which of these serological methods do you perform in your laboratory?

	NO	YES	**If YES**, in-house or commercial? Please state manufacturer
***In vivo* toxin neutralisation**			
Tissue culture toxin neutralisation			
ELISA			
Passive haemagglutination			
Other (*please specify*)			

2.2 Briefly describe your procedures (or *provide methodology or published references if available*).

.....

.....

.....

.....

.....

3. What are your limits of normality in relation to immunity using your test criteria?

.....

.....

.....

4. Where do you obtain your standard reagents for the serological tests (which manufacturers)?

.....

.....

.....

G. PARTICIPATION IN THE EU DIP-LabNet DIAGNOSTICS EQA, 2010

Please comment on the following for the transportation of infectious substances (e.g. bacteria) and reagents into your country.

1. The EQA distribution to all countries will take place in July 2010.

	YES	NO
Will you be able to participate?		

If NO, please state your reasons below

.....

.....

2. Some countries require additional paperwork for allowing shipments of pathogens to be cleared through customs. Please indicate whether your country requires any of the following (if you are unsure, please make relevant inquiries in your institute/country):

	YES	NO
Shipper's declaration form		
Import Permit		
Commercial Invoice		
Quality Certificate		
Certificate of Conformity		
Analysis Bulletin		

3. Are there any customs regulations or specific clearance procedures in your country that we should know before sending the EQA?

……

……

……

4. Please complete the following details required for courier shipping:

Name of Recipient _____

Address for Shipment _____

_____

_____

_____

Telephone Number _____

Fax Number _____

Email _____

OTHER COMMENTS OR SUGGESTIONS
(Please continue on a separate sheet if necessary).

……

……

……

……

……

DEADLINE DATE FOR RETURN OF QUESTIONNAIRE: 21st MAY 2010

Annex 2
Participating reference laboratories

Country	Contact person	Institute
Austria	Ulrich Sagel*	Inst. f. med. Mikrobiologie und Hygiene Wahringerstr. 25a A-1096 Wien, Austria
Belgium	Denis Pierard	UZ Brussel Department of Microbiology Laarbeeklaan 101 B-1090 Brussel, Belgium
Bulgaria	Antoaneta Detcheva	National Centre of Infections and Parasitic Diseases Blvd. Yanko Sakazov, No26 Sofia 1504, Bulgaria
Cyprus	Despo Pieridou-Bagatzouni	Microbiology Department Nicosia General Hospital 215, Paleos Dromos Lefkosia-Lemesos Str. 2029 Strovolos Nicosia, Cyprus
Czech Republic	Jana Zavadilova	National Institute of Public Health National Reference Laboratory for Pertussis and Diphtheria Srobarova 48 100 42 Prague 10, Czech Republic
Denmark	Marianne Clausen	Statens Serum Institut Department of Bacteriology, Mycology and Parasitology 5 Artillerivej 2300 Copenhagen S, Denmark
Estonia	Rita Peetso	Central Laboratory of Microbiology Health Board Kotka st. 2 11315 Tallinn, Estonia
Finland	Jaana Vuopio-Varkila	National Institute for Health and Welfare (THL) Laboratory of Hospital Bacteria Mannerheimintie 166 PL 30 00270 Helsinki, Finland
France	Nicole Guiso	Institut Pasteur Unite de Prevention et Therapies Moleculaires des Maladies Humaines 25 rue du Dr. Roux 75724 Paris Cedex 15, France
Germany	Andreas Sing	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) Fachartz fur Mikrobiologie und Infektionsepidemiologie Dienststelle Oberschleißheim 85764 Oberschleißheim Bayern, Germany
Greece	Anastasia Pangalis	Athens Children's Hospital 'Aghia Sophia' Bacteriology Department Thivon & Papadiamantopolou Goudi Athens 11527, Greece
Hungary	Tamas Tirczka	National Center for Epidemiology Department of Bacteriology Gyali ut 2-6 H-1097 Budapest, Hungary
Iceland	Karl G Kristinsson	Landspitali University Hospital Dept of Microbiology V/Baronstig 101 Reykjavik, Iceland
Ireland	Phillip Murphy	The Adelaide & Meath Hospitals Dublin incorporating the National Children's Hospital Department of Microbiology Tallaght Dublin 24, Ireland
Italy	Monica Monaco	Instituto Superiore di Sanitȧ Department of Infectious, Parasitic and Immunomediated Diseases. Viale Regina Elena 299 00161 Rome, Italy

Country	Contact person	Institute
Latvia	Ruta Paberza	Infectology Center of Latvia Bacteriology Department 3 Linezera street Riga, LV 1006, Latvia
Lithuania	Snieguole Dauksiene	National Public Health Surveillance Laboratory Microbiological Department Zolyno str. 36 LT-10210 Vilnius, Lithuania
Luxembourg	Paul Reichert	Laboratoire National de Sante 42 rue du Laboratoire L-1911 Luxembourg, Luxembourg
Malta	Paul Cuschieri	Mater Dei Hospital Microbiology Laboratory Msida, Malta
Netherlands	Frans Reubsaet	RIVM LIS-BBD Antonie van Leeuwenhoeklaan 9 3721 MA Bilthoven, The Netherlands
Norway	Martin Steinbakk	Norwegian Institute of Public Health Division of Infectious Disease Control Department of Bacteriology and Immunology SMBI Lovisenberggata 8 0456 Oslo, Norway
Poland	Aleksandra Zasada	National Institute of Hygiene National Institute of Public Health Department of Bacteriology Chocimska 24 00-791 Warsaw, Poland
Portugal	Paula Bajanca Lavado	Instituto Nacional de Saude Dr. Ricardo Jorge Departamento de Doenças Infecciosas Avenida Padre Cruz 1649-016 Lisboa, Portugal
Romania	Maria Damian	INCDMI Cantacuzino Diphtheria Reference Laboratory & Molecular Epidemiology Laboratory 103 Splaiul Independentei C.P.1-525, 050096, Bucharest, Romania
Slovakia	Irena Mikova*	RÚVZ, Odbor lekárskej mikrobiológie National Reference Centre for Diphtheria Senný trh č.4 04011 KOŠICE, Slovakia
Slovenia	Verica Mioc	National Institute of Public Health Department for Medical Microbiology Grablovičeva 44 1000 Ljubljana, Slovenia
Sweden	Birgitta Henriques Normark	Swedish Institute for Infectious Disease Control (SMI) SE-171 82 Solna, Sweden
UK	Androulla Efstratiou	Health Protection Agency WHO Global Collaborating Centre for Diphtheria and Streptococcal Infections Respiratory & Systemic Infection Laboratory Microbiology Services Division: Colindale 61 Colindale Avenue, London, NW9 5EQ, United Kingdom

** At the time of the EQA; Rainer Hartl is the nominated laboratory expert for Austria and V. Lengyelova for Slovakia at the time of writing.*

Note: Iceland, Liechtenstein and Spain could not participate in the EQA.

Annex 3
Instructions and result form for the EQA dispatch

EXTERNAL QUALITY ASSURANCE STUDY FOR THE LABORATORY DIAGNOSIS OF DIPHTHERIA

EU DIP-LabNet (contract number ECDC/10/002)
EIGHTH DISTRIBUTION: JUNE 2010

INTRODUCTION

I am pleased to enclose a total of six freeze dried specimens for the microbiological diagnosis of diphtheria. To those new to the network, also enclosed is an additional five reference strains. Please read the attached information and instructions carefully before proceeding with the laboratory work. ***Ensure all results are appropriately recorded on the attached questionnaire and the questionnaire is fully completed before returning to the co-ordinating centre***.

Results from individual laboratories will be treated in strict confidence and will remain the property of EU DIP-LabNet and ECDC.

PLEASE SUBMIT YOUR RESULTS BY 24TH SEPTEMBER 2010. THE "INTENDED RESULTS" WILL BE SENT TO ALL PARTICIPANTS TWO WEEKS AFTER THE SUBMISSION DEADLINE.

Good luck!

Dr Shona Neal
EU DIP-LabNet Scientist
WHO COLLABORATING CENTRE FOR DIPHTHERIA & STREPTOCOCCAL INFECTIONS

A. INFORMATION AND INSTRUCTIONS FOR THE EIGHTH EXTERNAL QUALITY ASSURANCE STUDY

1. This distribution contains the following freeze-dried specimens in glass vials:-

Six simulated throat specimens, labelled EQA WP2-10A, EQA WP2-10B through to EQA WP2-10F.

Five control strains (**new countries only**), listed below:-

Strain number	Organism	Toxin status	Biotype	Strain used for…
10648	*C.diphtheriae*	POS	*gravis*	Toxin positive
3984	*C.diphtheriae*	weak POS	*gravis*	Toxin weak positive
10356	*C.diphtheriae*	neg	*belfanti*	Toxin neg, Cystinase pos, Urease neg, Nitrate neg
764	*C.xerosis*	neg	n/a	Cystinase neg, Pyrazinamidase pos, Hiss' serum water glycogen neg, Nitrate pos
12077	*C.ulcerans*	neg	n/a	Pyrazinamidase neg, Hiss' serum water glycogen pos, Urease positive

We hope the standard control strains will be useful to you - if you already have these, or have other appropriate control strains, please safely dispose of them.

2. The vials containing freeze-dried infectious material should be opened in an exhaust protective cabinet. Gloves should be worn during reconstitution and subsequent handling of the vials. For safe removal of the plastic tear-off seals, please proceed as follows:-

> With the arrow on the plastic flip top pointing away from you, carefully but deliberately pull the flip top up and away from you. When it reaches the far edge, pull downwards and to the right or to the left (depending on whether you are right or left-handed) until the seal separates; then still holding onto the plastic top, gently remove altogether and dispose into a sharps container.

Slowly remove the plug. Add 1mL of broth to the vial and allow 1 minute to reconstitute. Treat the resulting suspension as the simulated specimen **using a drop from a Pasteur pipette or dipped swab as the inoculum** before spreading.

3. Laboratories will achieve the maximum benefit from these specimens if they are treated as normal patient specimens without non-routine procedures or media being used. Pathogens, if isolated, should be identified only to the level normally attempted in your laboratory.

4. Examine the plate cultures for any potentially toxigenic corynebacteria that may be present. Perform and record the test results for characterising the organisms, (i.e.: biochemical tests, toxin tests, morphology, microscopy and other tests you may use).

5. Please record ALL your results on the form provided and check the form before sending.

NOTE: These specimens may contain toxigenic corynebacteria. It is suggested that you handle the specimens under your own local safety rules for toxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*.

EXTERNAL QUALITY ASSURANCE FOR THE LABORATORY DIAGNOSIS OF DIPHTHERIA:
EIGHTH DISTRIBUTION, JUNE 2010

REPORT FORM

Name of participant: .....

Address: .....

.....

.....

.....

Tel: .....

Fax: .....

E Mail: .....

RESULTS TABLE (return by post, fax, email; see details on next page)

SPECIMEN	*_CORYNEBACTERIUM_ SPECIES ISOLATED	BIOTYPE	†TOXIGENICITY STATUS (by Elek, PCR, other)	TIME TAKEN TO OBTAIN FINAL RESULT
EQA WP2-10A				
EQA WP2-10B				
EQA WP2-10C				
EQA WP2-10D				
EQA WP2-10E				
EQA WP2-10F				
Please state any change of method(s) from those reported in the 2010 Laboratory Diagnostics Questionnaire, if any				

* If *Corynebacterium* spp. is not isolated, record as none.
† Record as positive (+) or negative (-)

Date results sent to the organiser: ……

Signature of participant: ……

GENERAL COMMENTS

	Yes	No
Did you find this exercise useful?	☐	☐
Are you willing to participate in another exercise?	☐	☐

Any other comments/suggestions:

Please forward results by **24TH SEPTEMBER 2010**

Annex 4
Intended results for the EU DIP-LabNet 2010 EQA for the laboratory diagnostics of diphtheria

EQA code	Original ref	Organism	Biotype	Elek toxigenicity	PCR result
A	H10 139 0001	*C. diphtheriae*	gravis	non-tox	neg
B	H09 492 0187	*C. ulcerans*	n/a	wk tox	pos
C	CD 99/126	*C. pseudotuberculosis*	n/a	tox	pos
D	NCTC 764	*C. striatum*	n/a	non-tox	neg
E	H09 534 0738	*C. diphtheriae*	mitis	tox	pos
F	H10 116 0113	*C. diphtheriae*	belfanti	non-tox	neg