International External Quality Assurance for Laboratory Diagnosis of Diphtheria[▽]

S. E. Neal* and A. Efstratiou on behalf of DIPNET and
International Diphtheria Reference Laboratories†

WHO Collaborating Centre for Diphtheria and Streptococcal Infections, Respiratory and Systemic Infection Department,
Health Protection Agency Centre for Infections, London, United Kingdom

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The diphtheria surveillance network (DIPNET) encompassing National Diphtheria Reference Centers from 25 European countries is a Dedicated Surveillance Network recognized by the European Commission. A key DIPNET objective is the quality assessment of microbiological procedures for diphtheria across the European Union and beyond. A detailed questionnaire on the level of reference laboratory services and an external quality assessment (EQA) panel comprising six simulated throat specimens were sent to 34 centers. Twenty-three centers are designated National Diphtheria Reference Centers, with the laboratory in the United Kingdom being the only WHO Collaborating Centre. A variety of screening and identification tests were used, including the cysteinase test (20/34 centers), pyrazinamidase test (17/34 centers), and commercial kits (25/34 centers). The classic Elek test for toxigenicity testing is mostly used (28/34 centers), with variations in serum sources and antitoxin concentrations. Many laboratories reported problems obtaining Elek reagents or media. Only six centers produced acceptable results for all six specimens. Overall, 21% of identification and 13% of toxigenicity reports were unacceptable. Many centers could not isolate the target organism, and most found difficulties with the specimens that contained *Corynebacterium striatum* as a commensal contaminant. Nineteen centers generated either false-positive or negative toxigenic results, which may have caused inappropriate medical management. The discrepancies in this diphtheria diagnostics EQA alarmingly reflect the urgent need to improve laboratory performance in diphtheria diagnostics in Europe, standardize feasible and robust microbiological methods, and build awareness among public health authorities. Therefore, DIPNET recommends that regular workshops and EQA distributions for diphtheria diagnostics should be supported and maintained.

Diphtheria is a potentially fatal disease caused by toxigenic *Corynebacterium diphtheriae* and *C. ulcerans*, controlled largely by vaccination. However, diphtheria re-emerged to epidemic levels in the Russian Federation and newly independent states (NIS) during the 1990s, peaking with more than 50,000 cases in 1995 (10). Although numbers

of cases have dropped significantly, there are still some countries affected by diphtheria, including countries within the European Region (Latvia, Russia, and Ukraine) and also Angola, India, Indonesia, and Nepal (from www.who.int/immunization_monitoring/en/globalsummary/timeseries/tsincidedip.htm, last accessed 23 June 2009). There has also been an increase in disease caused by *C. ulcerans* in some European countries and in others, such as Japan and the United States, which has been linked to domestic animals (4, 11, 21). Therefore, there is a continuing need to monitor the disease and to rapidly identify sporadic cases and outbreaks at both the local and international levels.

Laboratory data for diphtheria largely underpin and confirm the clinical and surveillance data. Therefore, it is essential to continually evaluate and ensure that laboratory results are of good quality and to maintain expertise in this specialized area of public health microbiology (5). It is also important to confirm the diagnosis using rapid and reliable laboratory methods (6). The 1994 WHO published guidelines recommending such procedures (7) are followed by many centers globally and include the differentiating tests for cysteinase and pyrazinamidase production and the key test to determine the toxigenicity status of the suspect organism, which has important public health and patient management implications (2).

An expansion of the European Laboratory Working Group on Diphtheria, the diphtheria surveillance network (DIPNET; agreement number 2005210) was awarded official status as a

* Corresponding author. Mailing address: WHO Collaborating Centre for Diphtheria and Streptococcal Infections, Health Protection Agency Centre for Infections, 61 Colindale Avenue, London NW9 5HT, United Kingdom. Phone: 44 (0)208 327 7160. Fax: 44 (0)208 205 6528. E-mail: shona.neal@hpa.org.uk.

† Contributing members of the International Diphtheria Reference Laboratories are as follows: S. Gabrielian (Armenia), U. Sagel (Austria), V. Kolodkina (Belarus), D. Pierard, I. Wybo (Belgium), A. Decheva (Bulgaria), K. Bernard (Canada), D. Pieridou-Bagatzouni (Cyprus), B. Kriz (Czech Republic), J. J. Christensen (Denmark), U. Joks (Estonia), Aila Soininen and J. Vuopio-Varkila (Finland), A. Le Fleche-Mateos (France), T. Gomelaury (Georgia), A. Sing (Germany), E. Alexandrou and A. Pangalis (Greece), M. Lynch and N. Wall (Ireland), M. Monaco, A. Pantosti, M. Pataracchia, and C. von Hunolstein (Italy), A. Kenbeilova (Kazakhstan), G. Djumalieva (Kyrgyzstan), R. Paberza (Latvia), S. Dauksiene (Lithuania), A. Halacu (Moldova), F. Reusaet (The Netherlands), A. Hoiby (Norway), A. A. Zasada (Poland), M. João Simões (Portugal), M. Damian and A. Diaconescu (Romania), D. Dovecar (Slovenia), L. Herrera, J. A. Sáez Nieto and S. Valdezate (Spain), C. Johansson, G. Möllerberg, and B. Henriques Normark (Sweden), E. Akbas (Turkey), T. Glushkevich (Ukraine), G. Mann and A. de Zoysa (United Kingdom), and P. Cassiday (United States).

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TABLE 1. Summary of results for each of the EQA specimens for the 34 participating laboratories

Specimen no.	Intended <i>Corynebacterium</i> sp. result (other organisms present)	Toxigenicity	No. of laboratories with:		
			Fully correct result	Acceptable result	Unacceptable result
EQA07-1	<i>C. diphtheriae</i> bv. mitis (<i>Streptococcus oralis</i> and <i>Neisseria lactamica</i>)	Nontoxigenic (Elek negative, PCR positive)	20	8	6 (1 unable to isolate target organism; 5 incorrect toxigenicity results by Elek)
EQA07-2	<i>C. diphtheriae</i> bv. gravis (<i>Streptococcus oralis</i> and <i>Neisseria lactamica</i>)	Toxigenic (Elek and PCR positive)	29	0	5 (2 unable to isolate target organism; 3 incorrect toxigenicity results: 2 Elek, 1 Vero cell assay)
EQA07-3	<i>C. diphtheriae</i> bv. belfanti (<i>Streptococcus oralis</i> , <i>Staphylococcus epidermidis</i> , and <i>Corynebacterium striatum</i>)	Nontoxigenic	10	2	22 (2 unable to isolate target organism; 18 incorrect identifications; 2 incorrect toxigenicity results by Elek)
EQA07-4	<i>C. ulcerans</i> (<i>Streptococcus oralis</i> , <i>Neisseria lactamica</i> , <i>Staphylococcus epidermidis</i> , and <i>Corynebacterium striatum</i>)	Weakly toxigenic	21	0	14 (7 incorrect identifications; 7 incorrect toxigenicity results: 3 Elek, 1 Vero cell, 1 Elek and passive hemagglutination, 1 Elek and PCR, and 1 PCR)
EQA07-5	<i>C. diphtheriae</i> bv. intermedius (<i>Streptococcus oralis</i> , <i>Neisseria lactamica</i> , <i>Staphylococcus capitis</i> , and <i>Corynebacterium striatum</i>)	Nontoxigenic (Elek negative, PCR positive)	6	10	18 (11 incorrect identifications; 7 incorrect toxigenicity results ^a : 6 Elek, 1 Vero cell and Elek)
EQA07-6	<i>C. pseudodiphtheriticum</i> (<i>Streptococcus oralis</i> and <i>Neisseria lactamica</i>)	Not applicable	29	4	1 (incorrect identification)

^a A further two centers reported a false-negative result using PCR; however, since this was an NTTB strain, these could be seen as acceptable results.

Dedicated Surveillance Network in November 2006 and currently includes 25 European countries (17). DIPNET's mission statement is "a collaborative and coordinated approach to the epidemiology and microbiology of diphtheria and related infections," which integrates key microbiologists from national reference centers and key epidemiologists from public health organizations. One major and specific DIPNET objective is to assess microbiological procedures for diphtheria in order to harmonize methods and laboratory performance across the European Union.

External quality assurance (EQA) exercises for diphtheria diagnostics have been conducted previously. They allow an assessment of each laboratory's capabilities and also identify areas for improvement. The last EQA exercise, in 2003, involved 18 European Union countries and revealed problems in the isolation of the target organism and the overall performance of the toxigenicity test in many centers (in the DIPNET Feasibility Study Final Report, submitted to European Commission, DG SANCO, 2003). This was compared to a previous EQA in 2000, where the majority of the 34 international centers produced accurate results for toxigenicity testing compared to biochemical identification (13). This echoed an earlier EQA distribution in 1998, where 6 specimens tested by 23 laboratories generated 121 (88%) correct toxigenicity reports, compared to 85 (62%) correct biochemical identification reports (9).

These previous EQAs have indicated that as a consequence of the decrease in diphtheria cases in Europe, laboratory expertise for isolation, identification, and toxigenicity testing has diminished in recent years. This EQA exercise was therefore performed to evaluate the current diphtheria diagnostic capa-

bilities of all DIPNET participants. In addition, other key NIS and international diphtheria reference centers beyond Europe were also invited to participate.

MATERIALS AND METHODS

Participants. The participants included all 25 DIPNET centers (<http://www.dipnet.org/contact.public.php>; last accessed 20 July 2009): Linz, Austria; Brussels, Belgium; Sofia, Bulgaria; Nicosia, Cyprus; Prague, Czech Republic; Copenhagen, Denmark; Tallinn, Estonia; Helsinki, Finland; Paris, France; Oberschleißheim, Germany; Athens, Greece; Dublin, Ireland; Rome, Italy; Riga, Latvia; Vilnius, Lithuania; Bilthoven, Netherlands; Oslo, Norway; Warsaw, Poland; Lisbon, Portugal; Bucharest, Romania; Ljubljana, Slovenia; Madrid, Spain; Stockholm, Sweden; Ankara, Turkey; and London, United Kingdom. A further nine diphtheria reference centers were invited to participate: Yerevan, Armenia; Minsk, Belarus; Winnipeg, Canada; Tbilisi, Georgia; Almaty, Kazakhstan; Bishkek, Kyrgyzstan; Chisinau, Moldova; Kiev, Ukraine; and Atlanta, GA. Reference centers unable to participate were in Azerbaijan, Russia (Moscow and St. Petersburg), and Uzbekistan, due to strict customs restrictions, and in Tajikistan, because of difficulties in acquiring media and reagents, and Turkmenistan, because a diphtheria laboratory contact could not be identified. The EQA was established and coordinated by the WHO Collaborating Centre for Diphtheria and Streptococcal Infections, based at the Centre for Infections (CFI), London, United Kingdom, which coordinated previous EQA studies.

Questionnaire assessment. A questionnaire requesting detailed information on the level of reference services offered, current methods used for identification and toxigenicity testing, the control strains used (if any), and difficulties in obtaining specialized reagents was sent to all participants before the specimens were dispatched.

Specimen preparation and dispatch. Six *Corynebacterium* sp. strains were selected based on their incidence and toxigenicity status from isolates referred to the WHO Collaborating Centre, London (Table 1). These were then 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, CFI, London. Quality control of the specimens was performed before and after freeze-drying to test for viability and retention of characteristic properties (9). The EQA panel was distributed to all participants with full instructions. Partic-

ipants were asked 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 using the enclosed form.

Evaluation of results. The results were evaluated on the basis of isolation, identification, and toxigenicity testing of any *Corynebacterium* spp. present in the specimens. Results from each center 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).

RESULTS

Completed questionnaires and results for the six simulated specimens were received from all 34 participants. Further information on the number of *C. diphtheriae*/*C. ulcerans*/*C. pseudotuberculosis* strains referred to the participants from 2000 to 2006 was requested to estimate the workload and experience in diphtheria laboratory diagnosis; nine countries received no isolates in this period, in contrast to Ukraine, which had received the most ($n = 4,756$); excluding Ukraine, the average number of isolates received in a participating country was 73.

Results from the questionnaire. (i) Laboratory diagnosis. A variety of tests were used for screening and identification of *Corynebacterium* spp. by all participating laboratories; these included Gram stain (32/34 centers), blood agar culture ($n = 32$), primary media containing tellurite ($n = 28$), selective media containing cysteinase ($n = 20$), pyrazinamidase activity ($n = 17$), nitrate reduction ($n = 21$), urease hydrolysis ($n = 25$), fermentation of Hiss serum water sugars ($n = 17$), and biochemical characterization using API Coryne kits (Biomérieux) ($n = 24$). A number of additional tests were used, including carbohydrate fermentation using Andrades peptone water sugars ($n = 2$), the modified Pizu medium for testing cysteinase activity ($n = 1$), the BBL Crystal identification system (BD) ($n = 1$), the RapID CB Plus system (Remel) ($n = 1$), fatty acid analysis (Sherlock microbial identification system; Midi Inc.) ($n = 2$), 16S rRNA gene sequencing ($n = 2$), and *rpoB* sequencing ($n = 3$).

In terms of the methodologies used for identification, all but one of the 34 centers had the capability to differentiate the three potentially toxigenic corynebacteria and biochemically characterize the four *C. diphtheriae* biotypes; the exceptional center did not perform a nitrate reduction test and thus could not differentiate between *C. diphtheriae* bv. belfanti and bv. mitis. Only 19/34 laboratories recorded the use of control strains for most of the tests and media; 7 of those used toxigenic reference strains. Some countries within the EU and beyond reported problems in obtaining culture media due to unavailability ($n = 2$), high costs ($n = 3$), or having to purchase a minimum amount of medium which was still too high for the numbers of isolates referred ($n = 2$).

(ii) Toxigenicity testing. All 34 centers performed the Elek test ($n = 28$) to detect phenotypic toxin production and/or a PCR-based assay to detect the gene encoding the toxin ($n = 19$); 14 centers performed both. Other assays used to detect toxin production included the in vivo test ($n = 4$), passive hemagglutination ($n = 2$), and Vero cell bioassay ($n = 2$). Fifteen laboratories prepared the specialized Elek medium in-house, and ten obtained it commercially, mostly from Russia and NIS. Manufacturers included BulBio-NCIPD (Bulgaria),

Becton, Dickinson and Company, Mikrogen (Russia), NPO Pitatel'nye Sredy (Russia), Toxagar (Russia), and the National Academy of Science of Ukraine, and three centers relied on CFI to send media to them. Although the WHO manual recommends the use of newborn bovine serum in the Elek medium (used by 10/28 centers), bovine ($n = 3$), fetal calf serum ($n = 1$), rabbit ($n = 1$), and equine ($n = 11$) sera were also used. The concentration of the antitoxin also varied between centers; the majority of centers used the WHO-recommended concentration of 500 IU/ml (17/28), and other concentrations used were 100 ($n = 1$), 750 ($n = 1$), and 1,000 IU/ml ($n = 3$). Antitoxin, derived mostly from an equine source, was obtained from Berna Biotech AG (Switzerland) ($n = 6$), Mikrogen ($n = 4$), Biomed (Russia) ($n = 3$), Pasteur Merieux/Aventis Pasteur (France) ($n = 2$), BulBio-NCIPD ($n = 1$), U.S. CDC ($n = 1$), Instituto Butantan (Brazil) ($n = 1$), or Refik Saydam National Hygiene Centre (Turkey) ($n = 1$). Ten countries reported problems in obtaining antitoxin, since many companies or institutes have discontinued production.

With the expansion of molecular techniques, various sets of PCR primers were used, targeting the fragment A of the toxin gene (11 centers), the B fragment ($n = 2$), or a fragment spanning the A-B toxin gene ($n = 7$); a few centers have also introduced real-time PCR assays ($n = 2$) (3, 12, 15, 16, 18, 19). Where stated, most centers used template DNA from the recommended Elek control NCTC strains, and at least five centers also included an internal positive control to detect possible PCR inhibition. Five centers used only a PCR-based assay for toxigenicity testing. At the time of the EQA, a further nine centers wished to implement a PCR-based assay to detect the diphtheria toxin gene.

Results from the EQA. The intended results, with a summary of the participants' findings, are shown in Table 1. Results were variable, largely according to the strain isolated. Four of the specimens generated acceptable or fully concordant results from at least 21/34 (62%) centers. The remaining two specimens generated poor concordant results from 22/34 (65%; EQA07-3) and 18/34 (53%; EQA07-5) centers. Results are detailed for each specimen, as follows.

(i) EQA07-1. Twenty centers reported the correct result on this specimen, which contained a phenotypically nontoxigenic *C. diphtheriae* bv. mitis strain. A further eight centers gave correct toxigenicity results but incorrect biotypes; four centers reported *C. diphtheriae* bv. gravis, two centers reported *C. diphtheriae* bv. mitis/belfanti, one center reported a "non-gravis" biotype, and one center did not identify the biotype. False-positive toxigenicity results were reported from five centers using the Elek test. One center did not isolate the target organism.

(ii) EQA07-2. The majority of centers submitted fully correct results for this strongly toxigenic *C. diphtheriae* bv. gravis strain (29/34 centers). No incorrect biotype results were reported; however, three centers gave negative toxigenicity results using either the Elek test or the Vero cell assay. Two centers were unable to isolate the target organism.

(iii) EQA07-3. Ten centers gave fully correct results for this specimen, containing a nontoxigenic *C. diphtheriae* bv. belfanti strain as the target organism and *C. striatum* as a commensal corynebacterium. Consequently, the majority of centers reported the presence of either the *C. striatum* commensal (10/22

unacceptable results) or other *Corynebacterium* spp.: *C. macginleyi* ($n = 2$), *C. xerosis* ($n = 2$), *C. accolens* ($n = 1$), *C. amycolatum* ($n = 1$), *C. bovis* ($n = 1$), and “Coryne group 1” ($n = 1$). Moreover, two centers submitted incorrect biotype results (one *C. diphtheriae* bv. *gravis* and one *C. diphtheriae* bv. *mitis/belfanti*). In addition, two centers were unable to isolate any corynebacteria, and two centers reported a false-positive toxigenic result using the Elek test.

(iv) **EQA07-4.** Twenty-one centers identified the target organism, a weakly toxigenic *C. ulcerans* strain; the specimen also contained *C. striatum* as a commensal contaminant. As a result, four centers missed the target organism and reported only *C. striatum*. *Corynebacterium accolens*, *C. pseudotuberculosis*, and *C. diphtheriae* bv. *intermedius* were misidentified from one center each. In addition, seven centers correctly identified the target organism but reported a false-negative toxigenic result using various methods, including the Elek test, the Vero cell assay, a passive hemagglutination assay, and a PCR assay.

(v) **EQA07-5.** Only six centers achieved fully correct results on this specimen, containing the target organism *C. diphtheriae* bv. *intermedius* and a commensal *C. striatum* strain. The *intermedius* biotype is rarely seen; thus, 10 centers reported the target organism as a *C. diphtheriae* bv. *mitis* strain. However, 11 centers did not isolate the target organism and reported other *Corynebacterium* spp. present (five centers reported *C. striatum*, two reported *C. accolens*, two reported *C. amycolatum*, and two reported *C. xerosis*). Seven centers correctly identified the correct organism at the species level but reported a false-positive toxigenicity result using the Elek test or the Vero Cell assay.

(vi) **EQA07-6.** The majority of centers (29/34) gave fully correct results for the *C. pseudodiphtheriticum* strain; a further 2 centers reported it as “*C. pseudodiphtheriae*,” one center as *Corynebacterium* spp., and another as “*C. pseudodiphtheriticum* probably.” However, one center reported toxigenic *C. pseudotuberculosis*, resulting in an unacceptable report.

(vii) **Performance of centers.** Performance varied between the 34 centers according to whether they achieved a full or acceptable result. Six centers produced acceptable results for all six specimens; this was in contrast to four centers that reported unacceptable results for four of six specimens and a further nine centers that reported unacceptable results for three of six specimens. From a total of 204 reports for toxigenicity and biochemical identification (six specimens tested by 34 labs), 26 (13%) toxigenicity reports were unacceptable, compared to 42 (21%) unacceptable biochemical identification reports. The turnaround time from plating out to achieving a final result for all six specimens varied between the centers from 2 to 24 days, generating an average of 6.9 days (median, 5 days). There was no obvious difference between the individual specimens.

DISCUSSION

EQA studies for laboratory diagnostics of diphtheria help to monitor the quality of service of the participating centers, to highlight problems in particular tests or specific laboratories, and to give assurance to those centers that performed well. This is the first EQA distribution in 5 years, from a scheme run under the auspices of DIPNET and the European Laboratory

Working Group on Diphtheria. However, there were limitations in this distribution, including the absence of six important centers from previous epidemic countries: two key centers in Russia and four NIS, Azerbaijan, Tajikistan, Turkmenistan, and Uzbekistan. These absences were due either to strict customs regulations making it extremely difficult to send infectious substances to these countries or due to a higher prioritization for infectious diseases other than diphtheria when targeting laboratory resources within the respective center. Two of the six simulated specimens were also difficult to identify due to the inclusion of a *C. striatum* strain as well as the target *Corynebacterium* spp. However, this highlights the importance of being able to rapidly identify a potentially toxigenic *Corynebacterium* strain to ensure appropriate case management and public health measures.

In this EQA exercise, there was a relatively high number of discrepancies for both the species identification and toxigenicity results. These errors could be inherent in the methods and practices used. However, an assessment of the methods did not identify any single test or assay, and there was no apparent influence of the workload of the laboratories—as an estimate of their experience in diphtheria diagnosis—on the level of discrepant results they produced. A few of the discrepancies were due to “transcriptional errors,” highlighting the importance of checking results before they are reported. However, 6 of the 13 centers that gave 3/6 unacceptable results were not recognized as National Diphtheria Reference Centers, and a further 2 centers had less than 2 years’ experience as a reference center. Therefore, there needs to be more support from ministries of health to achieve an adequate level for diphtheria diagnostics in key laboratories across the European Region.

Discrepancies due to the reporting of an incorrect biotype were considered acceptable, since the case management would not have been affected. In particular, some centers recorded “mitis/belfanti” for the intended strains in EQA07-1 and -3. These could be corrected by the knowledge that *C. diphtheriae* bv. *mitis* is nitrate positive and the belfanti biotype is nitrate negative; this information is not apparent when using the BioMerieux interpretation for the API Coryne identification system, which is widely used. Similarly, a number of centers reported “mitis” or “mitis/belfanti” for the *C. diphtheriae* bv. *intermedius* strain in EQA07-5. Although *intermedius* biotypes are biochemically similar to *mitis* biotypes, they can be distinguished by their smaller colony sizes (0.5 to 1 mm in diameter, cf. 1.5 to 2 mm, respectively) and ability to ferment dextrin. It was also noted by one center that the fatty acid profile of the biotype *intermedius* strain differed from those of the other biotypes.

Major identification discrepancies were considered unacceptable, since the inability to isolate or identify potentially toxigenic *Corynebacterium* spp. would have a great impact on the clinical and public health management of diphtheria cases. Alarming, four centers could not isolate one or two of the intended isolates; this reflects the lack of expertise for diphtheria diagnostics and the intolerable problem of acquiring basic media and reagents. This predicament was further highlighted since many centers experienced problems with the *C. striatum*-containing specimens, EQA07-3, -4 and -5. Twenty-one of 34 participating centers reported only *C. striatum* or one of seven other *Corynebacterium* spp., causing concern over the

screening capabilities of these diphtheria reference laboratories. Although these laboratories usually receive pure cultures from hospital and provincial laboratories, the skill needed to isolate and identify potential toxigenic *Corynebacterium* spp. is crucial for training purposes, initiation of screening practices, detection of sporadic isolates, and management of any large outbreak investigations. Furthermore, 11 centers that reported an incorrect identification result (i.e., did not identify the potential toxigenic strain) did not perform toxigenicity testing. It is paramount that in a clinical setting, if diphtheria is suspected and there are sufficient colonies on tellurite-containing medium, toxigenicity testing should not be delayed, particularly if waiting for the identification result. At least seven centers used toxigenic control strains for many of the screening and identification tests. A panel of control strains was sent with this EQA distribution to encourage participants to use the appropriate controls. A safer laboratory practice, especially in laboratories with minimum protection levels, can be established by using the recommended nontoxigenic control strains (9).

Toxigenicity testing is considered the most important test of diphtheria diagnostics, since false-positive and -negative toxigenic results could cause inappropriate clinical management and antitoxin treatment of cases. However, if the patient is severely ill and diphtheria is highly suspected, there should be no delay in appropriate treatment. In this EQA distribution, 19 of 34 centers generated 27 unacceptable phenotypic toxigenicity results; however, 3 results were most likely due to the incorrect isolation and testing of one of the commensals. A few discrepancies were produced via the Vero cell bioassay ($n = 3$), in vivo test ($n = 1$), or passive hemagglutination assay ($n = 1$), but the majority of centers used the phenotypic Elek test ($n = 21$). Although the Elek media used were either sourced commercially or made in-house, 10/17 centers used equine-derived serum and experienced either false-positive or -negative results. The WHO manual recommends the use of newborn bovine serum; rabbit, calf, and adult bovine serum also produce reasonable results (7). The use of equine serum should be discouraged, since the antitoxin is also derived predominantly from equine serum, thus possibly generating unwanted cross-reactions. Various concentrations of antitoxin can also affect the toxigenicity results; consequently a low or high concentration of antitoxin can cause either false-negative or -positive results, respectively. As a consequence, the three centers which used more than 500 IU/ml (i.e., 750 or 1,000 IU/ml) experienced false Elek positive results. Two centers recently introduced Elek toxigenicity testing as a result of their DIPNET participation. The Elek test results demonstrated the complexity of this highly specialized test, where interpretation can be problematic. An alternative and more rapid phenotypic method to detect diphtheria toxin was available in the late 1990s (8); however, production of the immunochromatic strips ceased. There have since been no successful contenders due to the low economic profit expectations hindering large-scale production of the immunochromatic strips. It would thus be fortuitous if a similar robust and low-tech diagnosis tool could be revisited for the phenotypic detection of diphtheria toxin.

A further seven unacceptable genotypic toxigenicity results were generated by a PCR-based method by four centers. All used different PCR assays, but two of these centers used only PCR for diphtheria toxigenicity testing. Some of the discrep-

ancies were also due to the failure in the isolation of the target organism. A general problem not assessed in this EQA is the detection of circulating nontoxigenic, *tox* gene-bearing strains (NTTB) (14), which should be reported as probable toxigenic strains if tested by PCR alone (20). Most centers reported correct positive PCR results for the NTTB strains in the EQA; two centers incorrectly reported negative PCR results for EQA07-5. Furthermore, there are no current control and management guidelines for when such strains are reported in a clinical setting. This will be considered when the 1994 WHO manual (1) is updated. Testing for the expression of diphtheria toxin (e.g., the Elek test) is always recommended. However, it is also acknowledged that acquiring the specialized media and reagents for the Elek test is increasingly difficult (17), and PCR methods are readily available, thus making the reporting of NTTB strains and patient management a problematic area.

Compared to previous EQA exercises, there has been no significant improvement in the isolation, identification, and toxigenicity testing of *Corynebacterium* spp. across Europe (9, 13). Since the fourth distribution in 1998, incorrect toxigenicity and identification reports have exceeded 10%, regardless of the composition of the EQA panel or the countries participating. Despite the poor results, most laboratories found the exercise useful and would participate again. Indeed, if there were yearly EQA distributions for laboratory diagnosis of diphtheria, laboratories would maintain expertise on these otherwise rare organisms. However, this level of discrepancy still reflects the lack of expertise, complacency, and minimal awareness that exist for diphtheria diagnostics. *Corynebacterium diphtheriae* has been shown to be an organism capable of generating significant epidemics, such as those seen in the 1990s, despite high vaccination levels and a low prevalence. This situation is hampered further by the difficulties some laboratories have in obtaining specialized media and reagents; under the DIPNET project, reagents have been distributed accordingly to some EU countries.

This EQA evaluation has highlighted the difficulties in the isolation and identification of potentially toxigenic corynebacteria. This can be improved by organization of workshops to train key diphtheria reference laboratory personnel; this has been undertaken under the auspices of DIPNET. The key test for laboratory diagnosis of diphtheria is phenotypic detection of the toxin, such as the Elek test. While the test may exhibit some inaccuracies in some centers, it is recommended that PCR toxin gene detection should be used only as an adjunct to the phenotypic test. In order to improve awareness and technical performance, as well as maintaining expertise in the laboratory diagnosis of diphtheria, DIPNET recommends the continuation of these EQA exercises and training workshops. The planned revised version of the 1994 WHO guidelines for the laboratory diagnosis of diphtheria will further recommend methods and processes to enable laboratories to improve their performance in this specialized area of microbiology.

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