Regional workshop to build laboratory capacity on diphtheria diagnostics

Christian Medical College,
Vellore, India

18–21 June 2019
Regional workshop to build laboratory capacity on diphtheria diagnostics

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Suggested citation. Regional workshop to build laboratory capacity on diphtheria diagnostics: World Health Organization, Regional Office for South-East Asia; 2020. License: CC BY-NC-SA 3.0 IGO.

Cataloguing-in-Publication (CIP) data. CIP data are available at http://apps.who.int/iris.

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1. Brief overview

Background

Diphtheria and related infections caused by toxigenic corynebacteria continue to cause a lethal resurgent infectious disease. Diphtheria remains a serious public health problem, particularly in the Eastern Mediterranean, South-East Asia, South America and Africa regions.

The types of infections caused by *C. diphtheriae* and more recently *C. ulcerans* have changed over the past two decades. This was most clearly highlighted by the resurgence of diphtheria in the European Region in the mid-1990s, the emergence of toxigenic *C. ulcerans* and its possible correlation with domestic animals; and the significance of non-toxigenic strains causing systemic disease. In the wake of this resurgence, screening methodologies were reviewed in the 1990s and revived in many laboratories after having been discontinued years ago. However, more recently, because of the global decline of the disease, the laboratory screening situation has “reversed”, in that many countries once again have discontinued routine screening. This is a major cause for concern as it has considerable public health implications, particularly since toxigenic strains persist and clinical diphtheria cases are being reported from all WHO regions, with recent emergence of major epidemics in the Yemen and Bangladesh. The microbiological diagnosis of the disease, identification of contacts and carriers, and appropriate clinical management of patients are therefore crucial.

It is sometimes difficult to diagnose diphtheria clinically, particularly in those countries where the disease is rarely seen. Diphtheria can often be confused with illnesses that have similar presentations such as severe streptococcal sore throat, Vincent angina or glandular fever. Therefore, accurate microbiological diagnosis is crucial and is always regarded as being complementary to clinical diagnosis. The laboratory also aids the clinician by eliminating suspected cases or contacts from further clinical investigation, thus avoiding unnecessary treatment or control measures.

Lastly, it is essential that each country has a reliable case reporting system; in some countries, it is mandatory to report all toxigenic isolates. This is in accordance with the WHO case definitions, thus all toxigenic isolates of *C. diphtheriae* and *C. ulcerans* must be reported. It is also important for laboratories to have close liaison with the microbiologists and epidemiologists at the National Reference Centre. WHO highlights the importance of microbiological and epidemiological collaboration within and between countries.

At the request of the WHO Regional Office for Europe, the manual on laboratory diagnosis that was originally published in 1981 was rewritten and published in 1994. Not surprisingly, the overall contemporary approach to laboratory diagnosis was not very different from that used in earlier years. Since then, the manual has been extensively
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revised to take into account the developments within the field and the changing epidemiology of these infections. The content of the manual was covered during this four-day workshop.

This meeting was a collaborative effort between the WHO Global Collaborating Centre for Reference and Research on Diphtheria, at Public Health England, WHO headquarters and the WHO Regional Office for South-East Asia, US Centers for Disease Control and Prevention (CDC) and Christian Medical College (CMC), Vellore.

Microbiology and laboratory diagnosis of diphtheria

Respiratory or cutaneous diphtheria is caused by toxigenic strains of \textit{C. diphtheriae} and \textit{C. ulcerans} and, rarely, \textit{C. pseudotuberculosis}. \textit{C. diphtheriae} is a non-sporing, non-encapsulated, and non-motile Gram-positive bacillus. Four biovars of \textit{C. diphtheriae} can be distinguished biochemically: gravis, intermedius, mitis and belfanti. Both \textit{C. diphtheriae} and \textit{C. ulcerans} can produce an exotoxin that causes local tissue necrosis and, when absorbed into the bloodstream, causes toxaemia and systemic complications, including paralysis due to demyelinating peripheral neuritis and cardiac failure due to myocarditis. The structural gene of the diphtheria toxin (\textit{tox}) is carried by a family of corynebacteriophages. The toxin is a 535-amino acid 58-kDa exotoxin whose active form consists of two polypeptide chains linked by a disulphide bond. The clinical and epidemiological significance of non-toxigenic \textit{C. diphtheriae} and \textit{C. ulcerans} is unclear.

The diagnosis is by culture of an isolate of \textit{C. diphtheriae}, \textit{C. ulcerans} or \textit{C. pseudotuberculosis} in a clinical laboratory. The common detection methods in use in most laboratories are microbiological culture on standard blood agar (or tellurite-containing media) with Gram stain of a suspicious colony. Further identification of catalase-positive, Gram-positive coryneforms may be performed by conventional biochemical testing. An increasing number of laboratories now also use phenotypic methods such as matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). All these methods can have good specificity but the confirmation of identification, and determination of toxigenic strains usually requires submission to the National Reference Laboratory. Conventionally, the identification of a toxigenic strain is usually performed using the Elek test to detect toxin expression. The phenotypic Elek test takes \( \geq 24 \) h and confirmation of species identity of submitted isolates by traditional phenotypic methods can take \( \geq 48 \) h. Polymerase chain reaction (PCR) assays are available and can identify and detect the \textit{tox}-bearing gene or non-\textit{tox}-bearing \textit{C. diphtheriae}, \textit{C. ulcerans}/\textit{C. pseudotuberculosis} in DNA extracts from isolates within 4 h.

Swabs (nasopharyngeal, throat, wound or skin lesions) should be obtained for culture before starting treatment. Where a pseudomembrane or membrane is present, swabs should be taken, if possible, from underneath the pseudomembrane or a piece of the membrane should be removed. If antibiotics have already been commenced,
specimens for culture should still be taken. Clinicians should alert the local laboratory that diphtheria is suspected.

**Toxigenicity testing.** All isolates of potentially toxigenic corynebacteria (C. *diphtheriae*, C. *ulcerans* or C. *pseudotuberculosis*) should ideally be submitted to a reference/specialist laboratory for confirmation of identification and toxigenicity testing. Identification/confirmation and toxigenicity testing can be performed initially by conventional or real-time PCR (quantitative or qPCR) on a DNA extract of the isolate. Isolates that are qPCR positive for the toxin gene (*tox*) must be tested by the Elek test for toxin expression. Although PCR positivity of the toxin gene of C. *diphtheriae*, C. *ulcerans* and C. *pseudotuberculosis* will be confirmed by the Elek test, a toxin gene PCR-positive result should be acted upon without waiting for the Elek test result. A toxin gene PCR-negative result is final and no further toxigenicity testing is undertaken on these isolates.

Rarely, isolates of C. *diphtheriae* are tox-gene positive by PCR but do not express toxin, i.e. when tested by the Elek test they will give a negative result (non-toxigenic tox-gene bearing; NTTB). These will not cause diphtheria and so patients are not treated with antitoxin. If detected in symptomatic patients or asymptomatic carriers, they should, however, be eliminated using antibiotics in the same way as fully toxigenic strains. However, their presence/distribution in other some regions is unknown. It is important that awareness if these atypical isolates be highlighted.
2. Objectives

Aim of the workshop

The main aim of the meeting was to strengthen participants’ skills and build capacity in the laboratory diagnosis of diphtheria. The expected outcome of this workshop was to establish a formal coordinated approach to strengthen diphtheria surveillance and increase capacity for diagnosing diphtheria in the WHO South-East Asia (SEA) Region. Another outcome was to enhance participants’ knowledge on accreditation and quality systems and to update them in the diagnostics of vaccine-preventable bacterial infections, in particular, pertussis and meningitis.

Objectives of the workshop

The main objectives of this meeting were:

1. to train and update microbiology focal persons on the laboratory capabilities and needs (including quality assurance) of laboratories conducting diphtheria diagnostics;

2. to establish specialized methods for laboratory diagnosis of diphtheria and teach participants the isolation and identification of the causative organisms directly from clinical specimens;

3. to identify the immediate and long-term needs (over the following 12 months) in terms of reagents and diagnostic materials required for the laboratory diagnosis of diphtheria;

4. to undertake detailed discussions on various aspects of the microbiological diagnostics for diphtheria;

5. to provide participants with theoretical and practical information on epidemiological typing methods for Corynebacterium diphtheriae and other potentially toxigenic corynebacteria;

6. to discuss the current status of diphtheria and immediate and long-term needs among countries represented at the workshop (country presentations).
3. Structure of the workshop, key discussions and observations

Structure of the workshop

Plenary sessions were held on the morning of first day to inform participants about and discuss the current epidemiological status of diphtheria and the challenges that countries with low or undetected incidence have in performing laboratory diagnosis of diphtheria. This was complemented by additional lectures throughout the training.

Laboratory training with hands-on learning started on the morning of the first day and went on to the afternoon of the fifth day. The workshop comprised interactive lectures and laboratory sessions and had predominantly “hands on” practical sessions covering the following areas:

❖ preparation of special media for bacterial culture;
❖ processing of primary throat swab cultures;
❖ selection of colonies and colony morphology, microscopy, screening tests – pyrazinamidase and cystinase, biochemical identification tests by conventional and commercial methods;
❖ preparation for the Elek test for toxigenicity (conventional and modified);
❖ PCR for detection of the toxin gene (conventional and real-time PCR);
❖ discussions on antimicrobial susceptibility testing;
❖ discussions on novel molecular assays for diphtheria diagnostics;
❖ quality assurance for laboratory systems and diphtheria diagnostics.

A pre- and post-assessment questionnaire was provided to all participants. The average pre-workshop assessment score was 58%. The average post-assessment score was 92%, marking a significant improvement after the workshop.

Summary of presentations, discussions and observations

The number of globally reported cases of diphtheria fell rapidly from about 100 000 cases globally (50 000 from the SEA Region) in 1980 to about 21 000 cases in 1992. From that year, there was a sharp rise, peaking at 57 000 in 1995 with a fall to about 5000 cases in 1999. However, from 2000 to 2015, there was relative stability in the number of cases. Since 2016, the number of cases has been on the increase again to reach 16 616 cases globally with the SEA Region accounting for 10 299 cases.

In the background of the Immunization Technical Advisory Group (ITAG) 2018 recommendations that countries should institute case-based surveillance for diphtheria, by June 2019, four countries in the Region were carrying out case-based surveillance (Maldives, Sri Lanka, Thailand and Timor-Leste), India had a missed case-based and aggregate surveillance, and the other five carried out aggregate surveillance.
Between 2015 and 2018, the incidence of reported (suspected) diphtheria cases was on the rise in most countries that declared cases.

Fig. 1: Incidence of reported diphtheria cases from 2015–2018 in the WHO SEA Region.
Source: WHO/UNICEF JRF and SEAR Annual EPI Reporting forms 2015-2018

In 2018, the highest incidences were noted in Nepal and Indonesia. However, Bangladesh had an outbreak among displaced Myanmar nationals in Cox’s Bazar, which was not included in the calculation of incidence (Figure 1).

Fig. 2: Number of suspected diphtheria cases, laboratory-tested cases and percentage of cases tested that were laboratory confirmed in 2018

Source: WHO/UNICEF JRF 2018; WHO SEA Region Annual EPI reporting form 2018
As shown in figure 2, the percentage of laboratory-confirmed cases was 14% in 2018. This ranged from 13% in Indonesia to 19% in Thailand. There was no data on laboratory-confirmed cases for India, Nepal and Timor-Leste. In the absence of laboratory confirmation, the number of clinical/suspected cases was overestimated.

The workshop was organized in line with the SEAR ITAG 2018 recommendations to review the national laboratory needs for diphtheria diagnostics based on diphtheria epidemiology in the country.

From the presentation on WHO surveillance and laboratory networks and quality assurance systems, the way forward to improve diphtheria surveillance is as follows:

- WHO and partners would provide technical assistance to countries for building diphtheria surveillance and laboratory capacities.
- Leverage existing quality assurance systems to monitor laboratory performance globally (external quality assurance/quality control [EQA/QC]) and include diphtheria.
- Develop a global laboratory manual for diphtheria and other vaccine-preventable diseases (VPDs).
- Advocate for and enhance bacteriology laboratory capacities globally to sustain VPD surveillance (invasive bacterial [IB]-VPDs, diphtheria, pertussis, typhoid, etc.).

The main laboratory diagnostic tests for identification of diphtheria microorganisms include the following:

- **Culture** on blood agar, Hoyle’s tellurite, followed by **identification** by direct microscopy or cystinase production on Tinsdale medium;
- **Biochemical tests**: cystinase tests, pyrazinamidase tests, Hiss tests, API Coryne ID kits, MALDI-TOF;
- **Toxigenicity tests** (toxin detection) using:
  - **conventional tests** (Elek test, in vivo test, modified Elek test, enzyme immunoassay and immunochromatographic strips, and
  - **Molecular tests**: PCR, real-time PCR and multiplex PCR.

### Laboratory needs as expressed by country participants at the end of the workshop

**Bangladesh.** Laboratory diagnosis is in place. They culture to blood agar and Tinsdale medium, undertake microscopy (Gram) and perform E-tests. They require media for Elek testing and some control strains. The single screening tests demonstrated in the workshop are very useful for them. PCR capability is available.

**Myanmar.** Basic diagnostics are undertaken and include microscopy (Gram), blood agar culture, tellurite and possible Vitek 2 for bio typing. Toxin testing is not done. They need
media for the modified Elek test and control strains. Reagents for PCR have been requested from Christian Medical College.

**Nepal.** There are many suspected cases and requests for microbiological diagnosis. They only undertake microscopy (Gram and Albert stains) and subculture to blood agar. There is a need for media and control strains. There is no molecular testing at all. This is gradually being introduced in the National Public Health Laboratory.

**Thailand.** Laboratory diagnosis is in place, including blood agar and tellurite agar culture, microscopy (Gram), biotyping and MALDI-ToF facilities. They undertake PCR for the tox gene and have also performed Elek testing. They urgently require Tinsdale medium, and newborn bovine serum for the Elek test (they use horse serum). They require methods for the preparation of antitoxin discs and Tinsdale medium.

**Timor-Leste.** There is (practically) no capability at all for diphtheria diagnostics. The country requires both media and strains. Biosafety level (BSL)2 cabinets are available. There is also a real-time PCR machine within the National Laboratory. They have many suspected cases and there is an urgent need to establish laboratory diagnostics.
Recommendations of the diphtheria training workshop

a. Specific recommendations for laboratories
   1. Countries should estimate and express their needs in order to reinforce diphtheria diagnostic procedures in national laboratories to meet the WHO technical standards, as taught in this workshop.
   2. Laboratory capacity should be strengthened in the SEA Region to ensure the quality of laboratory diagnosis of diphtheria (including procurement of media, reagents, laboratory supplies and control strains needed for laboratory diagnosis of diphtheria);
   3. Countries should be encouraged to designate at least one laboratory as the national reference laboratory for diphtheria and maintain capacity for toxigenicity testing.
   4. Cascade training should be organized in the country for laboratory or field staff in specimen-taking and transportation.

b. Recommendations for WHO and partners
   1. WHO and partners should support countries to put in place/improve case-based surveillance, including laboratory and field line lists and investigation forms.
   2. Technical assistance needs to be provided to countries for building diphtheria surveillance and laboratory capacity where necessary.
   3. Existing quality assurance systems should be leveraged to monitor laboratory performance globally (EQA/QC) and include diphtheria. An EQA scheme for diphtheria diagnostics should be developed in South-East Asia.
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Agenda

Tuesday, 18 June 2019
09.00 – 09.30 : Registration
09.30 – 10.00 : Welcome Addresses
10.00 – 10.30 : Introduction of the participants
10.30 – 11.00 : Pre-workshop evaluation for participants
11.30 – 11.50 : Diphtheria disease epidemiology and control in the South East Asia Region
11.50 – 12.45 : Diphtheria: The disease and its epidemiology, WHO surveillance and laboratory networks, quality assurance systems
13.30 – 14.00 : Laboratory diagnosis of diphtheria and related infections
14.00 – 14.45 : Molecular diagnostics and typing of pathogenic Corynebacterium diphtheriae and C.ulcerans

PRACTICAL SESSION 1
14.45 – 17.30 : Safety in the teaching laboratory
Examination of primary cultures and toxigenicity tests

Wednesday, 19 June 2019
PRACTICAL SESSION 2
09.00 – 16.00 : Primary cultures and toxigenicity tests (continued)
16.30 – 17.30: Discussion

Thursday, 20, June 2019
PRACTICAL SESSION 2
09.00 – 10.30 : Primary reading from phenotypic tests and molecular testing
11.00 – 12.30: Practical Session 2 continued
13.30 – 15.00: Practical Session 2 continued
15.30 – 17.00: Practical Session 2 continued
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Friday, 21 June 2019
PRACTICAL SESSION 3

09.00 – 11.00 : Laboratory practical work continued from Thursday
11.30 – 13.00 : Practical Session 3 continued
14.00 – 15.00 : Practical Session 3 continued
15.30 – 16.30 : Completion of work sheets by participants and grading of results
16.30 – 17.00 : Final Discussion and Wrap Up

Concluding remarks and presentation of certificates
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