Despite the success of mass immunization in many countries, diphtheria continues to play a major role as a potentially lethal resurgent infectious disease. Early, accurate diagnosis is imperative since delay in specific therapy may result in death. The microbiologic diagnosis of the disease, the identification of contacts and carriers, and the appropriate clinical management of these patients are therefore crucial. The epidemiology of diseases caused by *Corynebacterium diphtheriae* has changed dramatically over the decades, a situation that is highlighted by the resurgence of infections in the European region. These factors have strengthened the need for laboratories to screen for *C. diphtheriae*. Many modified and new methodologies are now used widely within laboratories for diphtheria diagnosis. Recent developments have focused upon methods for detection of the lethal and potent exotoxin produced by the causative organism, *C. diphtheriae*; this detection is the definitive test for the microbiologic diagnosis of diphtheria.

Since the introduction of mass immunization and the resulting decline in diphtheria incidence, there are mixed views in many countries concerning the need and necessity for laboratories to screen routinely for *Corynebacterium diphtheriae*, the causative organism of diphtheria. The rarity of cases and the expense and complexity associated with laboratory diagnosis provided many countries with the indication to cease screening throat specimens for *C. diphtheriae*; therefore, expertise and recognition of the organism declined. Despite the success of mass immunization in many countries, diphtheria continues to play a major role as a lethal resurgent infectious disease [1, 2]. Therefore, diphtheria remains a serious health problem within many regions of the world (e.g., Eastern Europe, Southeast Asia, South America, and the Indian subcontinent) and consequently poses a potential threat to other countries [1]. The microbiologic diagnosis of the disease, the identification of contacts and carriers, and the appropriate clinical management of patients are therefore crucial [3].

The type of infections caused by *C. diphtheriae* have changed over the decades, as is most clearly highlighted by the dramatic resurgence of disease in the European region and also by the emergence of nontoxicogenic strains of *C. diphtheriae* causing atypical diseases, such as endocarditis, septic arthritis, and, more commonly, severe and recurrent episodes of sore throat [4]. All these important factors have strengthened the need for laboratories to screen for *C. diphtheriae*. In many countries within Europe and in the United States, Australia, and Southeast Asia, the methodologies for laboratory diagnosis of diphtheria have been reviewed and revived after having been discontinued years ago.

**Role of the Laboratory in the Diagnosis of Diphtheria**

The major role of the laboratory is the provision of simple, rapid, and reliable methods to assist clinicians in confirming a clinical diagnosis. In many advanced cases of the disease, the clinical diagnosis would normally precede microbiologic diagnosis. However, it is sometimes 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 microbiologic diagnosis is crucial and is always regarded as being complementary to clinical diagnosis. The laboratory may also aid the clinician by eliminating suspected cases or contacts from further clinical investigation, thus avoiding unnecessary treatment or control measures, such as isolation [3].
Review of Methodologies

At the request of the World Health Organization (WHO) Regional Office for Europe (Copenhagen), the manual on laboratory diagnosis that was originally published in 1981 was rewritten and updated in 1994 [3, 5]. The manual was intended for use globally, although it was written with the aim of promoting and developing laboratory technologies within the European region, in particular Eastern Europe, and also to promote the formation of an international network of diphtheria reference centers in the European region and beyond [6]. Laboratory diagnostics within the Newly Independent States (NIS) of the former Union of Soviet Socialist Republics are based upon the work of several generations of microbiologists and are in accordance with the WHO recommendations.

Not surprisingly, the overall, contemporary approach to the laboratory diagnosis of diphtheria is not very different from that used in the 1920s. The main areas are the collection and transportation of specimens, primary culture, microscopy, screening tests, biotyping, and, in specialized reference centers, epidemiologic typing and antimicrobial susceptibility testing[3].

Methods for the Laboratory Diagnosis of Diphtheria

Speed coupled with accuracy is of utmost importance in the diagnosis of diphtheria, but the range of investigations will depend upon the availability of reagents, experience of laboratory staff, and financial resources. The recommended microbiologic procedure for the examination of specimens is outlined in figure 1. The first step in laboratory diagnosis is to obtain appropriate clinical specimens from the patient. Guidelines for the collection of specimens on swabs from suspected diphtheria cases and for the transportation of the swabs have been published [3, 7]. In cases of suspected respiratory diphtheria, samples should be obtained from the throat or nasopharynx (or both). In cases of cutaneous disease, samples should be obtained from any wound or skin lesions. If present, membranous material should also be examined. In addition, care should be taken to obtain material beneath the membrane [7]. Specimens must be transported to the laboratory immediately because rapid inoculation of special culture media is essential.

For microbiologic and epidemiologic surveillance, it is crucial that the laboratory receive the following information for each specimen from suspected cases, contacts, and carriers: name, age, and sex of patient; hospital to which the patient was admitted; name of physician caring for the patient; laboratory details (source of specimen, date collected); clinical details (symptoms, onset date, treatment regime); epidemiologic information (case, contact, or carrier); and immunization and travel histories.

Screening tests for presumptive identification. Unless a clinical diagnosis of diphtheria is suspected, laboratory diagnosis may be difficult because C. diphtheriae is not easily identified...
on blood agar. In addition, swabs with specimens from asymptomatic carriers or contacts may contain only small numbers of organisms, which may be obscured by the overgrowth of normal throat flora. For this reason, it is recommended that all throat swabs should be routinely screened for \textit{C. diphtheriae}, using a tellurite-containing medium.

Clinical specimens should ideally be cultured onto blood agar and selective tellurite media; Hoyle’s tellurite medium is recommended [3]. Tellurite-containing media inhibit the growth of normal oral flora; however, \textit{C. diphtheriae} (and some other corynebacteria, staphylococci, and yeasts) reduce the tellurite salts, producing characteristic black colonies [7, 8]. The diagnosis of diphtheria on the basis of direct microscopy of a smear is unreliable because both false-positive and false-negative results may occur. Rapid screening tests recommended for the differentiation of potentially toxigenic species of corynebacteria (\textit{C. diphtheriae}, \textit{Corynebacterium ulcerans}, and \textit{Corynebacterium pseudotuberculosis}) are the presence of cystinase (using either Tinsdale or Pizu media) and the absence of pyrazinamidase activity [3, 9].

\textbf{Significance of biotyping \textit{C. diphtheriae}.} Pathogenic strains are biochemically identified by use of simple tests (the key reactions are outlined in table 1); commercial kits, such as the API CORYNE (API bioMérieux, Marcy l’etoile, France) and Rosco Diagnostica (Taastrup, Denmark) tests, are readily available. All conventional and commercial methods are described fully in the WHO manual [3].

There are four biotypes of \textit{C. diphtheriae} (var. \textit{gravis}, var. \textit{mitis}, var. \textit{belfanti}, and var. \textit{intermedius}), and they are classified on the basis of their morphologic and biochemical properties (table 1).

Biotyping is of limited use in epidemiologic investigations because discrimination is poor. Developments are currently under way within the UK Public Health Laboratory Service (PHLS) Reference Laboratory to establish a range of tests (i.e., screening tests for cystinase, urease, nitrate, and pyrazinamidase) as a “single kit system” for the rapid (<3 h) identification of \textit{C. diphtheriae}.

The minimum laboratory information required to report a specimen as positive for \textit{C. diphtheriae} is as follows: Most biotypes are catalase positive, urea negative, nitrate positive (except the biotype \textit{belfanti}), pyrazinamidase negative, and cystinase positive. They will also ferment glucose, maltose, and starch (var. \textit{gravis} only) (table 1).

Within the NIS, the percentage of biotype var. \textit{gravis} and var. \textit{mitis} isolates differed during various phases of the epidemic. During the peak epidemic periods, isolates that belonged to only one predominant biotype circulated at specific periods of time: In the first peak (1984–1985), biotype \textit{mitis} predominated (80.3% and 83.1% of the total for each year), and during the second peak (1993–1995), biotype \textit{gravis} predominated (71.5%, 78.6%, 79.3%, respectively). However, in 1989, before the second upsurge, there was an almost equal distribution of \textit{mitis} and \textit{gravis} strains (48% and 52%, respectively). A similar distribution was observed in 1980 (\textit{mitis}, 49.1%; \textit{gravis}, 50.9%). The predominance of a particular biotype in a country could be related to the immunity status of the population to that particular biotype, which minimizes the circulation of such strains (of a given biotype) and perhaps favors the circulation and dissemination of the other biotypes.

\textbf{Detection of Toxigenicity.} The test for toxigenicity, which detects the potent exotoxin, a phage-encoded protein, is the most important test and should be done without delay on any suspect isolate that is found by routine screening or while investigating a possible case of diphtheria. The potentially toxigenic species (\textit{C. diphtheriae}, \textit{C. ulcerans}, and \textit{C. pseudotuberculosis}) acquire this characteristic when infected by the family of \textit{β}-phages or other families of corynephages [10].

\textit{The Elek immunoprecipitation test.} The Elek test was first described in 1949 [11] and replaced the in vivo virulence test in guinea pigs, a test that was used by many countries at that time. Prior to the introduction of tissue culture cytotoxicity assays, the in vivo test was the only method available to detect biologic activity of the toxin, and as such, it was considered the reference standard test [12]. The expense, slowness, risk of accidental self-inoculation, and the increasing unacceptability of in vivo tests in many countries led to a decline in the use of this test, which is now performed rarely, if at all, in the majority of countries. The Elek test, therefore, replaced the in vivo test as the first-line test for toxigenicity in most laboratories. However, even the Elek test is far from ideal; it is technically de-

\begin{table}[h]
\centering
\caption{Biochemical identification of pathogenic corynebacteria.}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Species} & \textbf{CYS} & \textbf{PYZ} & \textbf{Nitrate} & \textbf{Urea} & \textbf{Glucose} & \textbf{Maltose} & \textbf{Sucrose} & \textbf{Starch} & \textbf{Trehalose} & \textbf{Gelatin liquefaction} \\
\hline
\textit{C. diphtheriae} & & & & & & & & & & \\
\hline
\end{tabular}
\footnotesize{NOTE. CYS, cystinase production on Tinsdale medium; PYZ, pyrazinamidase activity.}
\end{table}
manding, greatly prone to misinterpretation, and can lack sensitivity, particularly when used by inexperienced technicians. In addition, some laboratories can take 2–3 days to produce results with the test. Consequently, the number of laboratories within nonepidemic or nonendemic areas using this test unfortunately has declined over the years.

In the early 1990s, a considerable amount of work was done to improve the Elek test; that is, the basal medium was modified and standardized so as to improve the clarity and accuracy of the test [9]. However, misinterpretation still remains a problem. In the UK National External Quality Assessment Scheme exercises, which were conducted between 1993 and 1995, a small number of laboratories (<25% of participating laboratories) reported toxin results. Of those, only 78% and 88% obtained a correct result on two occasions when nontoxigenic strains were distributed, compared with >90% when toxigenic strains were distributed. From a distribution in 1997, similar results were obtained with only 20% of laboratories performing toxin tests (Snell J.J.S. [PHLS], personal communication).

Various modifications to the original Elek test have been described and are shown in figures 2 and 3. The modified Elek test used within the United Kingdom (figure 3) has been used in Russia and other NIS laboratories since 1966 (option 2, figure 2) and 1988 (option 3, figure 2). The value of these modifications is that the test may be undertaken directly from the primary isolation plates. This is important when only a few organisms are present; to perform the test by the “dot method,” one colony is adequate. Modification 2 in figure 2 allows the examination of a large number of individual colonies [13]. Simultaneous examination of several colonies (at least 6–10) from the patient’s sample is crucial because it has been shown that toxigenic and nontoxigenic organisms can be present at the same time.

These methods are, therefore, particularly applicable to epidemic situations. These test methods were the basis for the development of the modified tests in the United Kingdom and United States that provide results within 16–24 h, compared with 48 h for the conventional Elek test [14, 15]. Irrespective of plate design, quality control of reagents and standardization of the procedure is essential. Variability in the Elek basal media and serum supplement can affect the clarity and specificity of the test [9]; details of recommended media are described in the WHO manual. The purity of the antitoxin used for the preparation of strips and disks used in the Elek tests is important. If it is impure, nonspecific precipitin lines and false-positive results can occur. Antitoxin should be obtained from a reliable source (Pasteur Méribux, Lyons, France; CNG, Peyrm, Russia; Wyeth Laboratories, Marietta, PA; Connaught Laboratories, Swiftwater, PA).

In the modified tests described in the United Kingdom and the United States, the distance between the inocula and the antitoxin was found to be one of the most critical factors in the test design [14, 15]. In the method described by Reinhardt et al. [15], a distance of 7 mm was shown to be the best membrane (containing the antitoxin)–to-inoculum distance, resulting in the appearance of specific precipitin bands after 24 h of incubation, even for the weakly toxigenic control strain. However, when the antitoxin was placed in a central 5-mm-diameter well that was filled with 9 µL of antitoxin (4.5 IU), precipitation bands were detected at 24 h when the test strains were inoculated at a distance of 10 mm [15]. In the modification described by Engler et al. [14], a distance of 9 mm between the inoculum and antitoxin disk (10 IU) enabled a result to be determined after 16 h of incubation at 37°C.

Use of polymerase chain reaction (PCR) for the laboratory diagnosis of diphtheria. In recent years, the use of PCR for the detection of the diphtheria toxin structural gene (tox) has been described [16–21]. Most assays that use pure bacterial cultures have focused on the detection of sequences that code for the biologically active (fragment A) subunit of the toxin [16, 18–21]. However, primers specific for other regions of the gene have also been used successfully [12, 17, 22]. The detection of the tox gene by PCR directly from clinical material has also been described [12]. A PCR assay positive for the presence of the diphtheria toxin gene in clinical material suggests that toxigenic C. diphtheriae may be the causative agent for illness; such

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**Figure 2.** Diagrammatic representation of conventional Elek test (1) and modifications used within former Union of Soviet Socialist Republics (2 and 3). +, toxin-positive control strain; ±, weak toxigenic control strain; −, nontoxigenic control; T, test strain.
results can be obtained when suspected diphtheria case-patients received antibiotics prior to specimen collection and had negative culture results [8, 23]. However, it must be emphasized that although PCR for detection of the diphtheria toxin provides supportive evidence for the diagnosis, data are not yet sufficient for PCR to be accepted as a criterion for laboratory confirmation [23]. At present, a patient should be regarded as a probable case if PCR testing is positive but the organism has not been isolated, histopathologic diagnosis has not been made, and there is no epidemiologic linkage to a laboratory-confirmed case [23].

PCR has contributed significantly in the development of modern molecular approaches to the laboratory diagnosis of diphtheria because it allows for preliminary presumptive results on toxigenicity within a few hours of specimen collection. A valid negative tox PCR test on suspect isolates will exclude the diagnosis and prevent further control measures. In addition to the speed of the test, interpretation of results is simple, and PCR facilities are becoming increasingly available in many laboratories throughout the world. Data from some studies has shown 100% correlation with phenotypic or biological assays (or both) [17, 18]. However, other studies have identified isolates of *C. diphtheriae* that possess toxin genes but do not express a biologically active protein and are, therefore, for diagnostic purposes, nontoxicogenic [12, 20, 21]. In the 1970s, such isolates were found in the mid United States, Canada, and the West Indies; more recently, they have been found in Eastern Europe [12, 21, 24]. In these instances, most of the patients presented with mild pharyngitis. Although such isolates may be uncommon, PCR alone cannot provide a definitive result for epidemiologic and clinical purposes [3, 8, 12, 23].

Recent reports from Russia and Ukraine suggest an increase in the number of nontoxicogenic strains that are positive by PCR [25, 26]. Until more is learned about these strains and their frequency of isolation in other parts of the world, caution is needed in interpreting a positive PCR assay as a definitive diagnosis of diphtheria when it is performed as the sole diagnostic procedure and not supported by the isolation of toxigenic *C. diphtheriae*. The only exception would be in a situation where clinical samples were held in transport media for an extended period of time, as demonstrated by Kobaidze et al. [27]. Swab collections of nose and throat specimens from patients with clinically defined diphtheria were PCR positive even after prolonged storage (5–12 months) in silica gel packages at 4°C, while the culture, as expected, was negative [27].

**Other methods for the detection of toxigenicity.** In view of the problems associated with the current tests available for the detection of diphtheria toxin, a rapid phenotypic enzyme immunoassay (EIA) has been developed [28]. The EIA uses equine polyclonal antitoxin as the capture antibody and an alkaline phosphatase–labeled monoclonal as the detecting antibody, specific to fragment A of the diphtheria toxin molecule. It takes ~3 h to obtain a result from selection of colonies; the limits of detection are 100 pg/mL, and toxigenicity can be detected using bacterial isolates grown on a variety of media (including selective agars). In a comparative study of 220 isolates, the EIA showed 100% correlation with the Elek test [28]. The EIA is a simple, rapid, accurate, and specific phenotypic method for the detection of toxigenicity.

Other methods for detecting toxin have also been described; these are primarily phenotypic tests based on complex techniques, such as immunoblotting, other EIAs, Vero cell bioassays, passive hemagglutination, immunofluorescence, and the use of latex immunologic techniques, but they have not found widespread use [12, 29].

The immunofluorescence technique has been used for large-
scale screening in areas where the organism is endemic or epidemic, particularly in South America [29]. However, the technique should be used in conjunction with other phenotypic tests. A major advantage of the immunofluorescence technique is that pure cultures are apparently unnecessary; however, this test must be used with caution because false-positive results are likely. The incidence of false-positives results is largely dependent upon the purity and labeling of the reagents [29].

Proposed Recommendations to Diagnostic Laboratories

Given the immense public health significance attached to the isolation of C. diphtheriae, the delay between isolation of a suspicious organism and the results of toxigenicity tests can provoke great anxiety among laboratory staff, clinicians, and public health officials. Therefore, a rapid and accurate result is essential. In the United Kingdom, it is recommended that all suspect isolates be referred to the Reference Laboratory without delay, and this system seems to be working extremely well [30, 31]. In the United States, all isolates, whether toxigenic or non-toxigenic, from any body site should be referred to the Centers for Disease Control and Prevention (CDC) Diphtheria Laboratory [23].

It is recommended that diagnostic laboratories send isolates to a Reference Laboratory. This highlights the importance of reference facilities within countries. The rapid EIA or the modified Elek test are ideal tests for toxigenicity, and PCR is an excellent adjunct to the Elek test, but it is essential that it be used only in conjunction with a phenotypic test.

Conclusions

With the increase in global travel and the emergence of epidemic strains, the existence of diphtheria anywhere in the world represents a threat to unimmunized persons and those with low levels of immunity. These problems highlight the importance of strengthening microbiologic and epidemiologic surveillance in addition to accessible and reliable laboratory screening. The situation for clinical and laboratory diagnosis has been strengthened in some countries but needs to be strengthened in others.

Within the United Kingdom, Finland, and the United States, microbiologists, infectious disease physicians, and physicians in communicable disease control have a strong network of disease-reporting systems, which include official disease reports, the WHO manual and other manuals, courses, and seminars. And, as an example of accessible laboratory screening, the PHLS and CDC Reference Laboratories provide a 24-h, 7-days-a-week service for the laboratory diagnosis of diphtheria.

During 1997, 3 cases of toxigenic C. diphtheriae infection occurred within the United Kingdom. The first case was in a 40-year-old man who acquired a cutaneous infection with C. diphtheriae var. mitis in Indonesia. The patient had been fully immunized in childhood and had received a booster prior to traveling in 1996. One household contact had a positive throat culture even though he was asymptomatic. Neither the case nor the contact had signs of toxicity or presence of throat infection (Efstratiou A., unpublished data).

The second incident occurred in an unimmunized 71-year-old woman who developed a sore throat during a cruise in the Baltics. The cruise lasted 12 days and included visits to Oslo, Copenhagen, St. Petersburg, Tallinn (Estonia), Stockholm, Bornholm (Denmark), and Amsterdam. The ship’s physician, who prescribed penicillin, saw the patient 2 days after onset. The patient returned to the United Kingdom 5 days after onset and was admitted to hospital. A grey, strongly adherent membrane covered the tonsillar region and extended onto the uvula. The area bled when the membrane was removed. The patient was given diphtheria antitoxin and clarithromycin. Swabs were used to obtain nose and throat specimens from close family contacts, who were given antibiotic prophylaxis and low-dose diphtheria boosters. Nose and throat specimens were obtained also from hospital staff. The local laboratory liaised with the Diphtheria Reference Centre (PHLS), and to reduce the delay, samples of the membrane were sent directly to the Reference Laboratory. A toxigenic strain of C. diphtheriae var. gravis was isolated from the membrane within 24 h of receipt by the Reference Laboratory. Further characterization of the isolate by ribotyping demonstrated clearly that the pattern produced was indistinguishable from the “epidemic pattern” D1 currently circulating within Eastern Europe. This was the first importation of D1 to the United Kingdom from the Eastern European Region [32].

The third incident was a laboratory-acquired C. diphtheriae infection contracted by a hospital laboratory worker, who handled a control toxigenic organism on the “open bench.” In the United Kingdom, it is strongly recommended that when staff handle suspected C. diphtheriae isolates in procedures likely to generate aerosols, it should be done in a safety cabinet [33]. It is also advisable that people who may be exposed to diphtheria in the course of their work be fully immunized and that their levels of antibody be examined at least 3 months after completion of immunization. Booster doses should be given at intervals of 10 years. Appropriate working practices are, therefore, required to protect staff from acquiring infection in the laboratory and subsequently transmitting it to others. These three incidents of diphtheria infection strongly emphasize the importance of collaboration between clinicians, hospital microbiologists, epidemiologists, and the reference laboratory.

Importation of toxigenic diphtheria strains to the United Kingdom, United States, Finland, and other countries also continues to be problematic. In 1994, for example, 2 US citizens contracted diphtheria while living in Russia and Ukraine. Both patients were treated in Europe [8]. As a result of these dangers and the likelihood that toxigenic strains of C. diphtheriae may still be circulating in some US communities, various strategies...
have been undertaken to enhance surveillance for respiratory diphtheria [8]. It is recommended that local health departments within the United States should assure the availability of laboratory capabilities for isolation and (at the state level) reference laboratory capacity for biotyping and toxigenicity testing [8]. Similarly, within the United Kingdom, an enhanced surveillance of all infections caused by C. diphtheriae commenced in 1995. Such strategies are now being applied to other countries within the European region. The need for enhanced surveillance of C. diphtheriae infections is crucial within epidemic areas. This is particularly applicable to specific mid-US areas that had endemic C. diphtheriae infections in the 1970s and 1990s [8]. High-risk populations should be defined on the basis of the current status of C. diphtheriae infections within those areas [8].

It is essential that a reliable case reporting system be available in each country. In the United Kingdom, other parts of Europe, the United States, and the NIS, it is mandatory to report all toxigenic C. diphtheriae isolates. It is also important for laboratories to have close liaison with the Reference Center microbiologists and epidemiologists. Through the European Laboratory Working Group on Diphtheria, international links have been strengthened, and the establishment of national and international laboratory networks highlights the importance of microbiologic collaboration within and between countries [6].

References

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