

Laboratory guidelines for the diagnosis of infections caused by *Corynebacterium diphtheriae* and *C. ulcerans*

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Summary: *These guidelines represent an application of the World Health Organization European Region's manual for the laboratory diagnosis of diphtheria for laboratories in the United Kingdom (UK), but they could be applied to laboratories overseas. The manual was rewritten in response to the re-emergence of diphtheria in eastern Europe and the emergence of other infections caused by Corynebacterium diphtheriae and C. ulcerans in the UK and overseas. The guidelines summarise our current recommendations and procedures for the microbiological diagnosis of infections caused by toxigenic and non-toxigenic isolates of corynebacteria, with particular reference to C. diphtheriae and C. ulcerans.*

Key words:
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Introduction

Guidelines for the laboratory diagnosis of diphtheria in the World Health Organization (WHO) European Region¹ were written in response to the re-emergence of diphtheria in eastern Europe¹. This document applies the guidelines for use in laboratories in the United Kingdom (UK).

The guidelines present current recommendations for the microbiological diagnosis of infections caused by potentially toxigenic isolates of corynebacteria, with particular reference to *Corynebacterium diphtheriae* and *C. ulcerans*. They cover the following main areas:

- laboratory safety issues
- the role of the diagnostic laboratory
- the role of, and interaction with, the reference laboratory
- procedures for presumptive identification of *C. diphtheriae* and *C. ulcerans*
- importance of toxigenicity testing
- laboratory responsibility for reporting toxigenic *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*
- susceptibility testing of coryneform bacteria
- serological immunity testing
- specialised testing; molecular typing
- enhancing microbiological surveillance

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Background

Microbiology of infections caused by *C. diphtheriae* and *C. ulcerans*

Diphtheria is a rare disease caused by toxigenic strains of *C. diphtheriae* and, less often, *C. ulcerans*. *C. diphtheriae* is a Gram positive, fermentative, pleomorphic rod, which comprises four biotypes, var *gravis*, var *mitis*, var *intermedius*, and var *belfanti*. All biotypes, with the exception of the biotype *belfanti*, may produce the lethal diphtheria exotoxin. Diphtheria is usually classified according to its site of manifestation; most cases are either

TABLE 1 Toxigenic *C. diphtheriae* isolates referred to SDRU: 1993 to 1998

Year	Number of isolates	Country of origin	Source	Biotype (var)
1993	4	Africa	skin	<i>mitis</i>
		Pakistan	skin	<i>gravis</i>
		Tunisia	throat	<i>mitis</i>
		no travel [*]	throat	<i>mitis</i>
1994	4	Bangladesh	skin	<i>mitis</i>
		Bangladesh	throat	<i>mitis</i>
		India	throat	<i>mitis</i>
		Pakistan [†]	throat	<i>gravis</i>
1995	1	Thailand	skin	<i>mitis</i>
1996	3	Nepal	skin	<i>intermedius</i>
		Thailand no travel [*]	throat throat	<i>mitis</i> <i>mitis</i>
1997	3	eastern Europe	throat	<i>gravis</i>
		Indonesia no travel [‡]	skin throat	<i>mitis</i> <i>gravis</i>
1998	3	Tanzania	skin	<i>mitis</i>
		Tanzania	throat	<i>mitis</i>
		Tanzania	nose	<i>mitis</i>

* contact with overseas person

† fatal case of diphtheria

‡ laboratory acquired infection

TABLE 2 Isolates of toxigenic *C. ulcerans* referred to SDRU: 1993 to 1998

Year	Number of isolates	Country of origin	Age range of patients	Source
1993	4	UK	9 to 54 years	skin, throat (3)
1994	4*	Italy	16 to 67 years	nose, skin (2), throat
1995	4†	Turkey	16 to 74 years	throat
1996	5	UK	16 to 75 years	skin, throat (4)
1997	4	UK	5 to 51 years	throat
1998	1	UK	35 years	throat

* one case associated with ingestion of unpasteurised dairy products in Italy
 † foreign travel in Turkey

respiratory or cutaneous. Most cases in the UK are travellers returning from areas where the disease is either endemic or epidemic². Toxin-producing strains of *C. ulcerans* as well as *C. diphtheriae* can cause respiratory diphtheria, but this is rare^{2,3}.

Eighteen isolates of toxigenic *C. diphtheriae* were referred to the PHLS Streptococcus and Diphtheria Reference Unit (SDRU) from within the UK between 1993 and 1998 (table 1). One was from a 14 year old boy who had travelled from Pakistan to the UK and died of diphtheria⁴. Twenty-two isolates of toxigenic *C. ulcerans* were also referred to SDRU, some from patients noted to have a 'diphtheritic membrane'. Four patients presented with infected skin lesions (table 2).

From 1989 to 1992, SDRU received a total of 95 non-toxicogenic isolates of *C. diphtheriae*, most from cases of severe and often recurrent throat infections^{2,5}. Non-toxicogenic strains of *C. diphtheriae* have subsequently emerged as potential pathogens in the UK. The numbers of non-toxicogenic *C. diphtheriae* isolates referred to SDRU (particularly the *gravis* biotype) rose from 51 in 1993 to 178 in 1997. In 1998, 147 out of 163 non-toxicogenic isolates were of the biotype var *gravis*, 15 var *mitis*, and one var *belfanti*. Var *gravis* is the predominant biotype; isolates of var *mitis* are commonly isolated from cases of cutaneous infection, as seen elsewhere in Europe, in particular, in areas of eastern Europe where the epidemic of diphtheria is declining (T Glushkevich, I Mazurova, personal communication). This observation has also been made in other parts of Europe. It is not clear whether immunisation selectively inhibits growth of toxigenic

TABLE 3 Biochemical identification of clinically significant corynebacteria

Species	CYS	PYZ	Nitrate	Urea	Glucose	Maltose	Sucrose	Glycogen
<i>C. diphtheriae</i>								
var <i>gravis</i>	+	-	+	-	+	+	-	+
var <i>mitis</i>	+	-	+	-	+	+	-	-
var <i>intermedius</i>	+	-	+	-	+	+	-	-
var <i>belfanti</i>	+	-	-	-	+	+	-	-
<i>C. ulcerans</i>	+	-	-	+	+	+	-	+
<i>C. pseudotuberculosis</i>	+	-	-	+	+	+	-	-
<i>C. amycolatum</i>	-	+	v	v	+	v	v	-
<i>C. imitans</i>	-	±	-	-	+	+	±	-
<i>C. pseudodiphtheriticum</i>	-	v	+	+	-	-	-	-
<i>C. striatum</i>	-	+	+	-	+	-	v	-

±: weak reaction
 v: variable reaction
 CYS: cystinase production on Tinsdale medium
 PYZ: pyrazinamidase activity

strains or whether laboratories are increasingly identifying corynebacteria in diseases other than respiratory diphtheria. It has been postulated that non-toxicogenic strains occur more frequently in people who have been immunised⁶. Non-toxicogenic strains have been reported (albeit rarely) to cause systemic disease, both in the UK and overseas^{2,7,8}, which makes the apparent increase in non-toxicogenic biotypes of concern. The global incidence of infections caused by these organisms is unknown but it is worth recording. The isolation of a non-toxicogenic isolate from a patient with pharyngitis without the presence of a membrane is not defined as a case of diphtheria^{1,5}.

Laboratory based surveillance of non-toxicogenic *C. diphtheriae* in England and Wales was enhanced in 1995 and 1996⁵. Isolates came from patients in different geographic locations and without any suggestion of importation from overseas. The increased number of infections since 1990 could be due to improved ascertainment, but there has been no upward trend in the numbers of toxigenic strains in the UK. This suggests, therefore, that the increase in non-toxicogenic strains is genuine².

Characteristics of potentially toxigenic corynebacteria

The minimal characteristics for presumptive identification include positive reactions for catalase, nitrate reduction (except the biotype *belfanti*), cystinase production, and glucose and maltose fermentation and negative reactions for urease, sucrose, xylose, and pyrazinamidase, which distinguish the biotypes and the other potentially toxigenic species (table 3). The four biotypes of *C. diphtheriae* differ biochemically: in particular, var *intermedius* is lipophilic, requiring lipids (for example, hydrolysis on tween 80 medium) for optimal growth, as it grows as a tiny translucent colony on routine media. *C. diphtheriae* will grow on nutrient agar, but the presence of serum or blood will improve growth⁶.

Significance of *Corynebacterium* sp isolates

Standard operating procedures for the identification of corynebacteria in diagnostic laboratories are being established by the PHLS⁹. It is advisable for laboratories to establish protocols to ensure that appropriate action is taken, however, if corynebacteria are isolated in the following instances:

- from a respiratory specimen, notably throat and nose swabs
- in a wound or skin swab/specimen from a person who has recently travelled abroad
- as the predominant organism isolated from a normally sterile site, or from a wound, abscess, or purulent sputum
- from more than two sets of blood cultures
- at a level $\geq 10^4$ colony forming units/mL in pure culture from urine
- from multiple specimens
- as significant growth of 'coryneforms' with white blood cells in the original Gram stain

Laboratory safety issues

Laboratory acquired diphtheria has been reported at least twice in the UK^{10,11}. To minimise risks to staff, all cultures of suspected *C. diphtheriae* and *C. ulcerans* should be handled in a Class 1 safety cabinet. This is particularly important when preparing and handling fluid cultures for biochemical tests. The production of aerosols should be minimised at all times and inhalation of aerosols should be avoided to reduce the risk of droplet transmission. It is sometimes forgotten that the strong (NCTC 10648) and weak (NCTC 3984) toxin producing control strains used in the Elek test are toxigenic, and are as likely to cause infection as clinical isolates.

In addition to safe handling in the laboratory national guidelines recommend that all laboratory workers who may be exposed to diphtheria in the course of their work should be protected by immunisation¹². A minimum protective level of 0.01 International Units per mL (IU/mL), as determined by a toxin neutralisation assay, is recommended. Laboratory staff who handle toxigenic strains regularly are recommended to maintain the higher level of 0.1 IU/mL. For most adults in the UK (with and without laboratory exposure), who are expected to have been fully immunised in childhood, it may be more appropriate to boost immunity and examine antibody levels at least three months after vaccination, rather than test, boost if necessary, and retest (as is the case among laboratory staff at the PHLS Central Public Health Laboratory (CPHL)). Exceptions to this general recommendation would include those who have received a booster within the past ten years or have had an adverse reaction to immunisation. Additional boosters should be given according to the results of serological testing and individual risks of encountering toxigenic strains.

Role of the diagnostic laboratory

The success of immunisation in eradicating indigenous diphtheria in the UK and most developed countries has led to debate about the need to screen throat specimens for *C. diphtheriae*. The rarity of cases in the UK, the complex procedures associated with laboratory investigation, and the 'streamlining' of laboratory procedures has led many laboratories to cease screening. Laboratory expertise in the identification of potentially toxigenic corynebacteria has therefore diminished. Since the resurgence of the disease in the Eastern European Region, however, both clinical and microbiological awareness has increased in many countries and the number of public health laboratories (PHLs) in England and Wales screening for *C. diphtheriae* has increased since 1997. The PHLS standard operating procedures (SOPs) for the examination of throat specimens recommend that all throat specimens are screened for *C. diphtheriae*¹³. Some PHLs have always done this. In a few laboratories, wound swabs - particularly from patients who have recently returned from overseas - are also screened routinely.

In many cases of respiratory diphtheria, clinical diagnosis precedes microbiological diagnosis, but a

microbiological report of *C. diphtheriae* (and rarely, *C. ulcerans*) in routine throat and other swabs taken from the respiratory tract may be the first indication that the disease should be considered. In countries like the UK, where the disease is uncommon, the clinical diagnosis may be difficult and may be confused with other conditions such as severe streptococcal sore throat, infectious mononucleosis, and Vincent's angina^{2,14}. The clinician should therefore be encouraged to exclude these conditions. This highlights the role of the diagnostic laboratory in providing simple, rapid, and reliable methods to help clinicians make the correct diagnosis and to eliminate suspected cases or contacts of patients from further control measures and isolation.

Role of, and interaction with, the reference laboratory

SDRU was designated a WHO Collaborating Centre for Diphtheria in 1998. The laboratory has close links with all international reference centres, WHO, and other national and international agencies. The main national role of SDRU is to provide an 'on demand' reference service to all UK laboratories for the microbiology of infections caused by *C. diphtheriae* and *C. ulcerans* (table 4).

Laboratory testing

Diagnostic tests used to confirm infection include the isolation of *C. diphtheriae* or *C. ulcerans* on primary culture and toxigenicity testing. There are no definitive commercial tests for the diagnosis of diphtheria, but there are rapid tests for detection of toxigenicity; SDRU offers a direct polymerase chain reaction (PCR) and a rapid enzyme immunoassay (EIA) on pure cultures to confirm toxigenicity in response to urgent referrals of isolates. The use of PCR as a primary test on clinical specimens is still being evaluated and is not currently recommended. Further details on the interpretation and use of toxigenicity tests are given below (see *Importance of toxigenicity testing*).

Collection of clinical specimens

Microbiological culture is essential for confirming diphtheria. A clinical specimen should be obtained as soon as possible when any form of diphtheria is suspected, even if treatment with antibiotics has already begun. Specimens should be taken from the nose and

TABLE 4 PHLS diphtheria reference service

- Identification and toxigenicity testing of *C. diphtheriae* and other potentially toxigenic corynebacteria (*C. ulcerans*, *C. pseudotuberculosis*)
- Diphtheria immunity/vaccination studies
- Antimicrobial susceptibility testing
- Molecular typing
- Training laboratory personnel in laboratory diagnostics for diphtheria
- Microbiological surveillance of diphtheria worldwide

TABLE 5 Collection of clinical specimens for the isolation of *C. diphtheriae* and *C. ulcerans*

<p>Throat specimens</p> <ul style="list-style-type: none"> • The pharynx should be clearly visible and well illuminated • Depress the tongue with an applicator and swab the throat without touching the tongue or inside the cheeks • Rub vigorously over any membrane, white spots, or inflamed areas; slight pressure with rotating movement must be applied to the swab • If any membrane is present, lift the edge and swab beneath it to reach the deeply located organisms • Transport the swab immediately to the laboratory for culture <p>Nasopharyngeal specimens</p> <ul style="list-style-type: none"> • Insert the swab into the nose through one nostril beyond the anterior nares • Gently introduce the swab along the floor of the nasal cavity, under the middle turbinate, until the pharyngeal wall is reached • Force must not be used to overcome any obstruction • Transportation to the laboratory immediately <p>Skin lesions</p> <ul style="list-style-type: none"> • Lesions should be cleansed with sterile normal saline and crusted material removed • Press the swab firmly into the lesion • Transport to the laboratory without delay
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throat, and from the membrane, if present. Multiple site sampling should always be considered in a suspected case as this may increase the organism recovery rate and hasten laboratory results. If possible, a specimen should be taken from under the membrane, where bacteria are concentrated^{6,14}. Removal of sections of membrane for microscopy and culture is also of value. Nose and throat specimens (usually swabs) should be taken from all suspected cases and their close contacts for culture. Isolation of toxigenic *C. diphtheriae* from close contacts may help to confirm the diagnosis of the case if the patient's culture is negative.

After collection, the swabs should be sent to the local laboratory immediately, as rapid inoculation of special culture media is important. At the same time, the clinician should inform the laboratory that diphtheria is suspected.

The isolation of *C. diphtheriae* (usually non-toxigenic) from blood cultures of patients with endocarditis from Europe and Australia also highlights the importance of correct isolation and screening procedures for these organisms from normally sterile sites^{7,8}.

Procedures for the collection of specimens are described fully in the WHO Manual for laboratory diagnosis of diphtheria¹ and are summarised in table 5.

Transportation of specimens to the diagnostic laboratory

If the transportation of specimens is likely to be delayed, the use of an appropriate transport medium – such as

the gel medium described by Amie's – should be considered¹. If the transit time will exceed 24 hours, the swab should be 'preserved' in a special pack containing a desiccant such as silica gel. It is reported that *C. diphtheriae* can be isolated from such swabs after delays of up to nine weeks^{6,15}.

Minimum data required to accompany specimens

The following data are important clinically and epidemiologically and should be collected in the course of a case investigation.

- Patient details
 - name, age, sex
 - hospital where being treated
 - attending physician
 - general practitioner's name and contact details
- Laboratory details
 - source of specimen(s)
 - date(s) collected
 - time(s) of collection
- Clinical details
 - symptoms
 - onset date
 - treatment (antibiotics, antitoxin)
 - date treatment began
 - dose and planned duration of regimen
- Epidemiology
 - suspected case, contact, or carrier
 - immunisation history
 - travel history
 - details of any contact with farms, farm animals, or unpasteurised milk or milk products if *C. ulcerans* or *C. pseudotuberculosis* infection is suspected
 - contact list

Submission of *C. diphtheriae* isolates to the reference laboratory

All UK isolates of potentially toxigenic corynebacteria (*C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*) from any body site (respiratory or cutaneous) should be referred promptly to SDRU (see address for correspondence, page 250) for confirmation and toxigenicity testing. The unit offers diagnostic services 24 hours a day, seven days a week, and may be contacted at any time through the CPHL switchboard (0208 200 4400). It is important to contact the unit before isolates arrive to ensure that they receive appropriate priority. Isolates should ideally be submitted as pure cultures on heavily inoculated blood agar or Loeffler's agar slopes; submission should not be delayed while the slope is incubated. In all cases of suspected diphtheria it is advisable to transfer isolates to the unit by a courier.

Primary diagnostic specimens are not usually examined by SDRU, but this is possible in some circumstances. For example, in the first imported case of diphtheria from the Eastern European Region, in May 1997, the diagnostic laboratory referred membrane samples to the unit, because they had not at that time isolated the causative organism from nasopharyngeal cultures¹⁶.

Primary culture

Specimens should ideally be inoculated onto culture media without delay. The diagnosis of diphtheria based upon direct microscopy of a smear is unadvisable as false positives and false negatives may occur. The use of selective media, such as Hoyle's Tellurite, is advised¹. The primary culture procedure is described fully in the PHLS SOP for the culture of throat specimens¹³ and in the WHO Manual¹⁶.

Some laboratories occasionally use Tinsdale's medium for primary culture, but its high selectivity increases the likelihood of false negatives, particularly with specimens that contain small numbers of organisms and it is therefore not recommended¹. This cystinase-containing medium is, however, ideal for the identification of presumptive corynebacteria. Many diagnostic laboratories do not stock such special selective media as routine primary culture on Tinsdale's medium for *C. diphtheriae* is rarely undertaken. In addition, the stability and shelf life of Tinsdale's medium is short. *C. diphtheriae* and *C. ulcerans* will also grow on standard blood agar and other primary culture media – for example, chocolate agar.

The laboratory diagnosis of 'unsuspected diphtheria' infection can be difficult as colonies of *C. diphtheriae* are not distinct upon the standard agar media used for routine throat or wound cultures. In addition, the presence of 'coryneforms' in mixed culture with other throat or skin flora may not excite interest unless the laboratory is aware that the patient recently travelled overseas. Furthermore, the detection of *C. diphtheriae* as the causative organism of cutaneous diphtheria or other systemic disease (for example, endocarditis) is primarily related to the full identification of the 'coryneforms' isolated. Once isolated it is easy to identify *C. diphtheriae* or *C. ulcerans*.

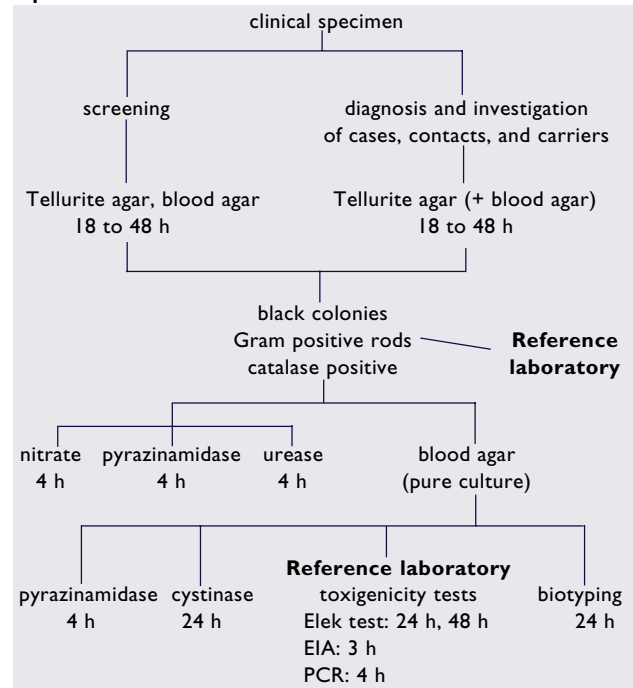
Procedures for the presumptive identification of *C. diphtheriae* and *C. ulcerans*

All identification procedures are described fully in the WHO Manual and the PHLS SOP on the identification of corynebacteria^{1,9}. The procedure for laboratory diagnosis is outlined in the figure. The minimal culture media required are blood agar, tellurite blood agar, Tinsdale agar medium, and biochemical tests for pyrazinamidase and, perhaps, urease hydrolysis and nitrate reduction. The minimal laboratory criteria required to presumptively report an isolate as *C. diphtheriae* and *C. ulcerans* are as follows:

- catalase positive
- urea: negative for *C. diphtheriae*, positive for *C. ulcerans*
- nitrate positive (except the biotype var *belfanti*)
- pyrazinamidase negative
- cystinase negative

The tests for pyrazinamidase (PYZ) and cystinase (CYS) are useful for screening in order to distinguish between the potentially toxigenic species and other coryneforms. If screening tests are unavailable then conventional biochemical tests should be employed – for example, urease hydrolysis, nitrate reduction, and fermentation tests for glucose, maltose, glycogen, or

FIGURE Algorithm for the laboratory diagnosis of diphtheria



starch. The biochemical characteristics of the potentially toxigenic corynebacteria (four biotypes of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*) and other coryneforms that may be encountered in throat or wound specimens are described in table 3. The commonest non-toxin producing *Corynebacteria spp.* that may be encountered are *C. amycolatum*, *C. pseudodiphtheriticum*, and *C. striatum*¹⁷.

Commercial identification kits identify *C. diphtheriae* and *C. ulcerans* with a good degree of accuracy. The API Coryne (bioMérieux, France) was the first commercial kit. It was launched in 1991 and is being used increasingly by laboratories in the UK and overseas. Although reliable for the identification of *C. diphtheriae*, the kit is slightly costly but is an excellent alternative to conventional methods.

Importance of toxigenicity testing

The most important test in the microbiological diagnosis of diphtheria is the detection of toxin-producing strains. Toxigenicity tests are not readily available in most diagnostic laboratories; it is strongly recommended that all isolates be referred promptly to SDRU, which is proficient in performing these tests. Several in vitro methods are available at SDRU, including the conventional Elek test, modified Elek test, a rapid EIA, and a genotypic test based on PCR for detection of the 'A' portion of the toxin gene¹⁸⁻²⁰. The modified and conventional Elek tests are used routinely in conjunction with the EIA and usually yield results in between three and 24 hours. Because of the delays in obtaining results, the severity limit upon animals, and the complex nature of the test in vivo tests are no longer available from SDRU. A suitable 'gold standard' alternative for the in vivo assay is the in vitro Vero cell assay, which is based on the cytotoxicity of diphtheria toxin to cultured Vero cells¹⁹.

All the methods (apart from the newly developed EIA) for toxigenicity have been fully documented^{19,20}. Genotypic methods, based on PCR, offer many advantages over standard phenotypic tests: they are rapid, simple, easy to interpret, and becoming more widely available in laboratories. These methods, however, do not show whether the organism can express fully functional diphtheria toxin. PCR must therefore be used cautiously, because some isolates of *C. diphtheriae* possess the toxin gene but do not express biologically active toxin¹⁹. The geographical distribution of such strains is unknown. It is therefore advisable to use PCR only as an adjunct to a phenotypic test, such as the Elek. *C. ulcerans* and *C. pseudotuberculosis* may also possess the diphtheria toxin gene and can produce diphtheria toxin, but an unequivocal negative PCR result with appropriate internal controls will exclude the diagnosis of diphtheria.

Given the immense public health implications associated with the isolation of a toxigenic strain 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, public health officials, and patients and their families. It is important that all isolates of presumptive *C. diphtheriae* and *C. ulcerans* be examined for toxin production, because of the resurgence of diphtheria in the Eastern European Region and the endemicity of the disease in many parts of the world²¹.

Laboratory responsibility for reporting toxigenic *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*

When a presumptive toxigenic strain is isolated, the following must be informed immediately: the clinician responsible for the case, the local consultant in communicable disease control, SDRU, and the PHLS Communicable Disease Surveillance Centre (CDSC). The isolate must be sent to SDRU for confirmation.

Susceptibility testing of coryneforms

Neither the British Society for Antimicrobial Chemotherapy (BSAC) nor the National Committee for Clinical Laboratory Standards (NCCLS) of the United States (US) has published specific guidelines for susceptibility testing of potentially toxigenic corynebacteria. This may reflect the relative rarity of their isolation from clinical specimens in the UK and US. It is not clear at present which medium should be used for growing the culture for susceptibility testing, nor which medium should be used for broth microdilution or agar dilution techniques. Interpretive criteria should probably resemble those recommended for other Gram positive species such as streptococci and staphylococci. The potentially toxigenic corynebacteria are usually susceptible to penicillin and macrolide antimicrobials; these are the agents recommended by WHO for antimicrobial treatment of cases and carriers⁵. All strains isolated from epidemic areas in eastern Europe were susceptible to erythromycin, penicillin, ampicillin, cefuroxime, chloramphenicol, ciprofloxacin, gentamicin,

and tetracycline. Two isolates exhibited reduced susceptibilities to rifampicin, an antibiotic often used in eastern Europe²². In more recent studies of a global collection of isolates, all strains were fully sensitive to the antibiotics examined (penicillin, levofloxacin, ofloxacin), but five isolates from epidemic areas in south east Asia and sporadic isolates from Australia showed intermediate resistance to erythromycin²³. These studies demonstrated the diverse range of antimicrobial agents that may be used as alternatives to penicillin or erythromycin should the need arise.

Serological immunity testing

Serological testing is undertaken for several reasons, which include helping to confirm a clinical diagnosis, the assessment of individual and population immunity, and investigation of responses to immunisation in selected individuals.

Measurement of serum antibody levels to diphtheria toxin in a suspected clinical case, before administration of antitoxin, may help to confirm the diagnosis, particularly when cultures are negative. If antibody levels are low or undetectable, the diagnosis cannot be eliminated. If high, toxigenic *C. diphtheriae* should not cause systemic disease. In either case, determination of antibody level should not be regarded as a truly diagnostic test for diphtheria and therapeutic intervention(s) should never be delayed pending the results of such tests.

Several methods for assessing antibody levels have been described¹, among which the *in vivo* toxin neutralisation test is regarded as the gold standard. Fortunately, alternative tests using tissue culture cells have been developed as reliable alternatives. These tests measure the ability of serum from an individual to neutralise the cytopathic effects of pure diphtheria toxin in a tissue culture system, and thus measure functional antibodies. Neutralisation assays are particularly useful in assessing the necessity or otherwise of boosting immunity through immunisation. Criteria for interpretation of serum antitoxin levels are given in table 6.

ELISA and passive haemagglutination tests have also been developed and used for measuring serum antitoxin levels. The attraction of both these methods is their speed and convenience, but several reports have suggested that they may be less reliable than tissue culture neutralisation assays. Passive haemagglutination assays have been found to lack sensitivity for serum specimens containing <0.1 IU/mL antitoxin, with an ensuing risk of false negative interpretations. Similarly, ELISA tests have been shown to correlate poorly with tissue culture and *in vivo* neutralisation tests for specimens containing <0.1 IU/mL antitoxin, and therefore carry a significant risk of giving false positive results. The ELISA false positive reactions are believed to be due to the binding of non-neutralising antibodies. ELISA tests have been used for preliminary screening of serum specimens; these are re-tested by tissue culture/*in vivo* neutralisation assays if their antitoxin titres are <0.1 IU/mL. Our

laboratory has not adopted this approach because it has been reported that ELISA may not be a reliable predictor of immunity even for specimens with antitoxin concentrations > 0.1 IU/mL.

Specialised testing; molecular typing

Several methods have been developed and evaluated by SDRU and the members of the European Laboratory Working Group on Diphtheria (ELWGD) to investigate the molecular epidemiology of *C. diphtheriae*. These include ribotyping, pulsed field gel electrophoresis, PCR typing, single stranded confirmational polymorphisms (SSCP) of the *tox* gene, and more recently amplified fragment length polymorphisms (AFLP)²⁴⁻²⁷. The most thoroughly evaluated and discriminatory method thus far appears to be ribotyping and databases as described below have been established using this technique²⁸.

The 'DipIdent' database of molecular typing patterns of *C. diphtheriae* has been established among members of ELWGD. Its central database is at the Institut Pasteur in Paris and a sub-database ('DipRibo UK') is held at the SDRU. The database contains data from the examination of over 600 isolates of *C. diphtheriae* from epidemic and non-epidemic areas worldwide, using the standardised ribotyping scheme agreed by ELWGD. In brief, this consists of a ribotype protocol, which includes DNA restriction with *PvuII* and *BstEII* and hybridisation with a mixture of five digoxigenin-labelled oligonucleotides targeting universally conserved regions of 16S and 23S rRNA. The patterns are scanned using a 256-grey scanner and interpreted using the *Taxotron*® programme²⁸. Thus far, more than 70 different patterns have been identified, with the predominant patterns from the Eastern European Region being provisionally designated as 'D1' and 'D4' (D representing diphtheria, based upon provisional nomenclature of these genotypes). The nomenclature is currently being finalised by ELWGD. This database allows us to identify the most likely geographical origins of particular isolates and their relationship to endemic and epidemic strains worldwide. From this we have confirmed that heterogeneity between strains from different regions exists and that database storage allows the recognition, confirmation, and identification of apparent clones worldwide.

Clinical application

These methods were used in the investigation of three cases of toxigenic *C. diphtheriae* infection that arose in the UK in 1997.

The first case was a 40 year old man with cutaneous infection with toxigenic *C. diphtheriae* var *mitis* who had recently returned from Indonesia. He had been fully immunised in childhood and had received a booster before travelling in 1996. One asymptomatic household contact was found to have a positive throat culture. Neither the case nor the contact had signs of toxicity nor apparent throat infection. Isolates from the index case and the contact were genotypically

TABLE 6 Interpretation of measured antitoxin levels as defined by the in vitro neutralisation assay

Antitoxin level (IU/mL)	Interpretation
<0.01	Individual is susceptible
0.01	Lowest level of circulating antitoxin giving some degree of protection
0.01 - 0.09	Levels of antitoxin giving some degree of protection
0.1	A protective level of circulating antitoxin
>1.0	A level of antitoxin giving long term protection

indistinguishable by ribotyping and resembled previous isolates from Indonesia.

In the second incident an unimmunised woman aged 72 years developed a sore throat during a Baltic cruise. The local laboratory liaised with SDRU and, to reduce any potential delay in confirming the clinical diagnosis, samples of the membrane were sent directly to the unit. A toxigenic strain of *C. diphtheriae* var *gravis* was isolated from the membrane within 24 hours of receipt. Further characterisation of the isolate by ribotyping showed that the pattern produced was indistinguishable from the 'epidemic pattern' D1 then circulating in eastern Europe. This was the first importation of D1 to the UK from the Eastern European Region¹⁶.

The third incident was a *C. diphtheriae* infection acquired at work by a hospital laboratory worker who had handled a toxigenic organism in a sample distributed by the National External Quality Assurance Scheme (NEQAS) for microbiology in November 1997. Information supplied with the sample said that it came from a patient with severe sore throat who had recently returned from Russia. The clinical isolate, the isolate used in the NEQAS sample, and the original source culture provided by SDRU to NEQAS were indistinguishable by ribotyping and were found to belong to ribotype 'D20', an uncommon pattern, seen only once before, in a patient from Kazakhstan¹⁰.

Enhancing microbiological surveillance

Further improvements to proficiency in laboratory diagnosis are being addressed in the PHLS by the organisation of specialised workshops on the diagnosis of diphtheria for PHLS and NHS laboratory personnel. Such workshops have been held once or twice yearly for UK workers since 1994. Workshops held in conjunction with WHO for personnel from countries in Europe (as part of the European Commission funded programme on the 'Microbiological Surveillance of Diphtheria in Europe') have also taken place. Through such workshops, the WHO Manuals, the PHLS SOPs, reports in *CDR Weekly*, and regular liaison with international agencies (for example, WHO) both clinical and microbiological awareness has improved in the past few years. Laboratory skills in the recognition of these pathogens should not be allowed to decline as the disease is far from eradicated worldwide^{29,30}.

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