

Diphtheria (*Corynebacterium diphtheriae*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *Corynebacterium diphtheriae*.

Authorisation: PHLN

Consensus date: N/A

1 PHLN SUMMARY LABORATORY DEFINITION

1.1 Condition:

Diphtheria

1.1.1 Definitive Criteria

- Isolation of toxigenic *Corynebacterium diphtheriae* or *C. ulcerans* (confirmed by specialist laboratory).

1.1.2 Suggestive Criteria

- Isolation of *C. diphtheriae* (toxin production unknown).

1.1.3 Guide for Use

Nucleic acid tests have been developed in house for the detection of diphtheria toxin genes in clinical specimens but are not acceptable for the purpose of notification in Australia.

Serological assays, including EIA for diphtheria antitoxin antibodies are available in Australia but are used for immune status studies not for diagnosis of diphtheria.

2 Introduction

Classical diphtheria is an acute infectious disease of the upper respiratory tract caused by toxigenic *Corynebacterium diphtheriae*. The bacterium multiplies in the pharynx and produces an exotoxin, diphtheria toxin, which is encoded on a lysogenic bacteriophage. Diphtheria toxin is readily absorbed through mucous membranes and disseminated through the body, targeting the heart and peripheral nervous system. *C. diphtheriae* may also cause skin infections, particularly in tropical climates, and invasive disease including endocarditis¹⁷, bacteraemia, septic arthritis and splenic abscesses¹. Most strains causing skin and invasive disease are non-toxigenic unless associated with an outbreak of pharyngeal diphtheria.

The highly effective diphtheria toxoid was licensed, and school based immunisation campaigns commenced, in the early 1930's in Australia and had an immediate impact on disease rates. Small foci of classical diphtheria persisted in disadvantaged areas of Australian capital cities and rural areas until the early seventies. The last known outbreak in Australia occurred in Alice Springs and Katherine in NT during 1991–1992^{9,15}, involving 23 cases with one death. Since then occasional cases have occurred in travelers entering Australia from endemic areas overseas.

Since immunisation with diphtheria toxoid does not prevent carriage of *C. diphtheriae*, it remains endemic in Australia and is occasionally cultured from upper respiratory and wound swabs, particularly from Aboriginal people. These isolates and those causing endocarditis, which also occurs infrequently in Australia, are nearly always non-toxigenic.

Toxigenic strains of *C. diphtheriae* have been isolated from respiratory and skin infections of horses and cattle, particularly when outbreaks of human disease are occurring but it is unknown whether the cattle are infected by humans or whether animals may be a reservoir for human disease¹¹.

The veterinary pathogens *C. ulcerans* and *C. pseudotuberculosis* can be lysogenised by the *C. diphtheriae* bacteriophage and thus produce diphtheria toxin. Toxigenic *C. ulcerans* can cause classical diphtheria. The majority of human isolates of *C. ulcerans* referred to the Health Protection Agency (formerly PHLS) in UK between 1986 and 2002 were toxigenic¹⁸. Ribotypes of *C. ulcerans* strains isolated from cats were indistinguishable from those of human strains¹⁸.

The diagnosis of pharyngeal diphtheria usually depends on clinical suspicion and a request for throat swab culture on appropriate media. In Australia, where doctors are unfamiliar with the clinical characteristics of diphtheria and few, if any, laboratories routinely culture throat swabs on selective media for *C. diphtheriae*, the diagnosis is likely to be delayed. If there is a strong clinical suspicion of diphtheria treatment should not wait for laboratory confirmation. Infections with toxigenic strains of both *C. diphtheriae* and *C. ulcerans* are notifiable in Australia.

3 Tests

3.1 Culture

Although *C. diphtheriae* and *C. ulcerans* are not fastidious bacteria and grow well on media routinely used in a medical microbiology laboratory, special media containing tellurite are used to aid in distinguishing them from other flora present in swabs from non-sterile sites. Pai (egg based) or Loeffler (serum based) slants should also be inoculated. Smears made from growth after 18 hr incubation at 35 oC on these lipid rich media will show the distinctive cell morphology of *C. diphtheriae* when stained with Loeffler's alkaline methylene blue or Neisser's stain. Respiratory specimens should also

be inoculated onto blood agar to detect haemolytic streptococci which can cause symptoms which may be confused with diphtheria.

3.1.1 Suitable and unsuitable specimens

Routine throat swabs, nasopharyngeal aspirates or swabs, membrane tissue, wound swabs sterile site tissue. Swabs may be placed in bacterial transport media such as Stuart's if transport is likely to be delayed although the organism is not fastidious and has been shown to survive on fomites for up to 6 months. However, special media are usually required for the culture of *C. diphtheriae* and most microbiologists have had little or no experience in the diagnosis of diphtheria. Therefore the laboratory should be alerted that a specimen for diphtheria is on its way so that media can be prepared or the specimen referred to a reference laboratory.

3.1.2 Media

Two formulations of tellurite media are commonly used for the primary isolation of *C. diphtheriae*: Tinsdale's which contains tellurite and cystine and Hoyle's which contains tellurite without cystine. Both media should be freshly prepared and have a shelf life of approximately 7 days. Colonies of *C. diphtheriae* are greyish black (gunmetal grey) and range in size from 1–3mm on cystine tellurite agar after 24–48 hours incubation.

3.1.3 Test sensitivity

Unknown, but *C. diphtheriae* and *C. ulcerans* are readily cultured on solid media from throat swabs and membrane, despite some inhibition of *C. diphtheriae* by tellurite, albeit less than of normal flora. Sensitivity may be improved by culture on Loeffler's or Pai media.

3.1.4 Test specificity

Although tellurite is inhibitory for much of the normal upper respiratory tract flora, some non-corynebacteria will grow on media containing tellurite. Suspect colonies should be Gram stained to confirm diphtheroid morphology and subcultured to Loeffler's or Pai slants to demonstrate the presence of the distinctive metachromatic granules and to provide inoculum for biochemical identification tests and toxin detection.

3.1.5 Predictive values and relevant populations

High during outbreaks but may be missed in sporadic cases if the laboratory is not prepared for investigating a case of diphtheria. Although *C. diphtheriae* grows well on routine laboratory media it may be difficult to distinguish among normal respiratory flora and especially by inexperienced microbiologists. The laboratory must be notified and given a full clinical history if diphtheria is suspected clinically.

3.1.6 Suitable test acceptance criteria

Non-motile, Gram positive rods. *C. diphtheriae* colonies show typical morphology on tellurite media. Typical cell morphology in methylene blue stained smears of growth on lipid rich media. Ferment glucose and maltose, reduce nitrate, urease negative, do not hydrolyse arginine. *C. ulcerans* has similar colony morphology but does not produce metachromatic granules, is urease positive and does not reduce nitrate.

3.1.7 Suitable internal controls

A control strain of *C. diphtheriae* must be cultured along with the unknown strain.

3.1.8 Suitable external QAP programme

RCPA QAP (rarely).

3.1.9 Special considerations

Most microbiologists will not have investigated a case of diphtheria although they may have isolated *C. diphtheriae* or *C. ulcerans* from a cutaneous lesion or heart valve. If a clinician suspects diphtheria the laboratory must be notified so they can prepare special isolation media and obtain a control organism if possible. The specimens may need to be referred to a more specialized laboratory.

Presumptive isolates must be tested for diphtheria toxin production by phenotypic and/or molecular methods. Multiple colonies should be subcultured to Loeffler's or Pai slants for further testing since toxigenic and non-toxigenic colonies can be isolated from the same specimen

3.2 Diphtheria Toxin Detection

Traditional methods for toxigenicity testing are the in vitro plate method (Elek Plate), cell culture method in Vero cells and in vivo method in guinea pigs. All rely on a supply of high quality antitoxin, usually raised in rabbits, and are tedious and slow to perform, usually taking 1–4 days for a result. Only tertiary laboratories in Australia and elsewhere are likely to have the experienced staff and facilities required to obtain reliable results.

The guinea pig method remains the gold standard assay for toxigenicity and relies on the ability of diphtheria antitoxin to protect a guinea pig from a lethal injection of the test isolate which should kill an unprotected guinea pig if the isolate is toxigenic. If both challenged animals survive the isolate is non-toxigenic; if both die within 1–4 days the isolate may not be *C. diphtheriae*. This test is rarely used these days.

Rapid serological methods including EIA have been developed in house for detecting toxin in isolates (Engler et al 00). An immunochromatographic strip test method (ICT), introduced in 2002 ⁸, is sensitive and specific for detecting toxin in specimens and isolates. These methods are not available commercially.

The Elek test is the most commonly performed phenotypic test for toxin detection and is discussed below. Further information about other tests is available ³ and WHO Manual for Laboratory Diagnosis of Diphtheria http://whqlibdoc.who.int/euro/1994-97/ICP_EPI_038_.pdf - C

In 1991 Pallen ¹⁴ described a PCR based assay for detecting diphtheria toxin genes in isolates. PCR has now largely superseded phenotypic testing for toxigenicity and has been adapted to direct detection in specimens to provide a rapid presumptive laboratory diagnosis ^{13,12}.

3.2.1 In vitro toxin test (Elek Plate)

The original assay was first described by Elek in 1948 ⁴ and modified in 1949 ⁵. It virtually replaced the guinea pig in vivo test in most laboratories, but has itself now been largely replaced by molecular testing except in countries with a relatively high rate of diphtheria. More recent modifications to the conventional Elek test have reduced the time required to perform the assay from 48 hours to 16–24 hours ³.

3.2.1.1 Suitable and unsuitable specimens

Single colonies should be used as inocula, although earlier protocols state that mixed cultures and the original swab can be used to inoculate an Elek plate. Several colonies from each primary isolation plate should be tested as both toxigenic and non-toxigenic isolates can be present in a single specimen.

3.2.1.2 Test details

The assay is an immunoprecipitation test performed in clear peptone agar containing foetal calf serum in Petri dishes. In the original assay a strip of filter paper impregnated with diphtheria antitoxin is laid onto the centre of the dish and the test and control isolates are inoculated in single streaks up to the antitoxin filter paper strips (WHO manual). A modification ^{3,6} uses filter discs impregnated with antitoxin and single point inocula 7mm from them. Diphtheria toxin produced by toxigenic strains diffuses into the agar and forms a line of precipitation when it meets antitoxin diffusing from the impregnated strips.

3.2.1.3 Test sensitivity

Highly variable and is influenced by the experience of the staff, the test protocol and the quality of the reagents. The components of the Elek base medium must be standardised, prepared with extreme care and tested with toxigenic controls. The optimal dilution of each batch of antitoxin must be determined. The depth of the agar and distance of inocula from filter paper impregnated with antisera will influence the results ³.

3.2.1.4 Test specificity

Purity of the antitoxin will influence the number of non-specific precipitin lines and must be obtained from a reliable source.

3.2.1.5 Predictive values and relevant populations

UK surveys in 1993 and 1995 showed that laboratories were more likely to obtain correct results for toxigenic survey strains than for non-toxigenic strains – possibly owing to misinterpretation of non-specific precipitation lines ³.

3.2.1.6 Suitable test acceptance criteria

Toxigenic control strains produce expected lines of precipitation.

3.2.1.7 Suitable internal controls

Each plate must be inoculated with known toxigenic and non-toxigenic control strains.

3.2.1.8 Suitable original test validation criteria

Fully referenced method.

3.2.1.9 Suitable External QAP Programme

RCPA QAP (rarely).

3.2.2 Nucleic acid detection

PCR assays were introduced originally to confirm the toxigenic status of isolates ¹⁴. In 1997 Nakao and Popovic ¹³ described a PCR assay to amplify toxin A and B subunit fragments directly from clinical

specimens with the products being visualized on agarose electrophoresis gels. In 2002 Mothershed et al 12 adapted the assay to real time PCR technology to provide a more rapid result.

Most primers to confirm toxigenicity status in isolates have been designed to target the biologically active (fragment A) subunit although other targets have been used successfully. For real time direct detection assays primers should be designed to target at least 2 gene fragments (usually A and B subunits of the toxin gene).

There are no commercial PCR kits available.

3.2.2.1 Suitable and unsuitable specimens

Clinical specimens include throat and nasopharyngeal swabs and membrane tissue for direct detection assays. Several colonies of *C. diphtheriae* / *C. ulcerans* / *C. pseudotuberculosis* from each isolation plate should be tested to confirm their toxigenicity status since both toxigenic and non-toxigenic isolates may be present.

3.2.2.2 Test details

Refer to published literature for appropriate protocols.

3.2.2.3 Test sensitivity

Very high correlation with the gold standard Elek plate assay for toxigenic isolates of *C. diphtheriae*. Real time PCR assays may not detect the presence of toxigenic *C. ulcerans* owing to variability in toxin genes (*tox*) of this species. Standard PCR assays which do not rely on probe hybridization for detection are recommended for *C. ulcerans* ^{2,16}.

The efficiency of the nucleic acid extraction method is important for direct detection of tox in specimens.

3.2.2.4 Test specificity

Highly specific for diphtheria toxin genes – however, toxin gene positive strains may not produce toxin.

3.2.2.5 Predictive values and relevant populations

Negative predictive value for non-toxin producing strains of *C. diphtheriae* is very high, lower for strains of *C. ulcerans* which produce toxin but whose toxin genes may not be detected by the particular PCR assay.

Positive predictive value is influenced by the ability of the tox positive strains to produce detectable biologically active toxin.

3.2.2.6 Suitable test acceptance criteria

All controls within expected ranges.

3.2.2.7 Suitable internal controls

As recommended in NPAAC guidelines: Laboratory Accreditation Standards and Guidelines for Nucleic Acid Detection and Analysis.

Controls should be designed to detect sample inhibitory activity.

Control strains of toxigenic and non- toxigenic *C. diphtheriae* and *C. ulcerans* should be included.

3.2.2.8 Suitable original test validation criteria

In accordance with NPAAC guidelines: Requirements for the Validation of In House In vitro Diagnostic Devices. Refer to published papers for specific data.

3.2.2.9 Special considerations

PCR is a useful test to screen isolates for toxigenicity but *tox* positive strains should be referred to a specialist laboratory for confirmation by molecular and biological tests and for subtyping.

PCR for direct detection of *tox* in clinical specimens should only be used by experienced laboratories and, preferably, only during outbreaks when the assay has been shown to detect the causal strain/s.

Diagnosis of diphtheria by direct PCR on specimen is not notifiable in Australia although a positive result should be communicated by telephone to public health authorities if diagnosis by culture techniques is delayed.

4 Typing and Subtyping Methods

There are four biotypes of *C diphtheriae* (var. *gravis*, var. *mitis*, var. *belfanti* and var. *intermedius*) classified on morphologic and biochemical features. Laboratories rarely report biotype data these days as the information is of limited use for epidemiological investigations and experienced staff are rarely available to determine the biotype.

Ribotyping has now replaced biotyping and provides good discrimination for epidemiological studies. An international nomenclature for *C diphtheriae* ribotypes has been published ¹⁰.

5 References

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