Diphtheria

WHO laboratory manual for the

Diagnosis of diphtheria and other related infections

THIRD REVISION

World Health Organization

Diphtheria

Laboratory diagnosis of diphtheria and other related infections

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FOREWORD

Diphtheria and related infections caused by toxigenic strains continue to be reported and are lethal resurgent infectious diseases. The rarity of cases, as well as the expense and complexity associated with laboratory diagnosis, mean that many countries ceased to screen throat specimens, and therefore, expertise and recognition of the organism have declined. Public health management cannot be effective without the appropriate microbiological diagnosis of the disease. Diphtheria is re-emerging in areas where population immunity through vaccination has not been maintained. Therefore, both clinicians and laboratory personnel should always maintain a high index of suspicion in patients presenting with signs and symptoms of respiratory or cutaneous diphtheria, particularly after being in countries endemic for the disease.

Diphtheria cases are persisting in many areas in the world with several thousand being reported annually, but the numbers are underestimated due to the lack of surveillance infrastructure in many areas.

(http://www.who.int/entity/immunization/monitoring_surveillance/data/incidence_series.xls)

Over the decades and since the last edition of this manual, many developments in the field of diphtheria have occurred:

- Case definitions and surveillance strategies have been revised
- Atypical and unusual manifestations of the disease caused by other potentially toxigenic corynebacteria have been reported
- Novel reservoirs of the disease have been identified in many countries, for example, *Corynebacterium ulcerans* as a potential reservoir in companion animals
- New methodologies for laboratory diagnostics, molecular epidemiology and serological immunity have been developed, and new insights into the pathogenesis are emerging due to fast-moving genomic technologies

This revision of the manual considers all the developments listed above and incorporates extensive sections on laboratory diagnostics, molecular technologies and serology. It extends the repertoire of infections to all the potentially toxigenic corynebacteria; *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*. The manual is aimed for global use and will hopefully fulfil the needs of all laboratories including those with minimal resources.

ACRONYMS AND ABBREVIATIONS

ACDP	Advisory Committee on Dangerous Pathogens
API	Analytical profile index
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
COSHH	Control of Substances Hazardous to Health
DAE	Double-antigen enzyme-linked immunosorbent assay
DATS	Diphtheria antitoxin serums
DELFIA	Dual double-antigen time-resolved fluorescence immunoassay
DIPNET	Diphtheria Surveillance Network
DLVs	Double-locus variant
DTP3	Diphtheria-tetanus-pertussis
ECDC	European Centre for Disease Prevention and Control
EDSN	European Diphtheria Surveillance Network
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ELWGD	European Laboratory Working Group for Diphtheria
EQA	External quality assurance
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EU DIP-LabNet	EU Diphtheria Laboratory Network
HPA	Health Protection Agency (UK)
ICS	Immunochromatographic strip
IQA	Internal Quality Assurance
IU	International units
LQA	Laboratory quality assurance
MALDI-TOF MS	Matrix-assisted laser desorption ionization – time of flight mass spectrometry
MCD	Minimum cytotoxic dose
MIA	Multiplex immunoassay
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multiple loci variable number tandem repeat analysis
MSDS	Material Safety Data Sheet
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt
NCTC	National Collection of Type Cultures
NCT	Non-template control
NGS	Next-generation sequence
NIBSC	National Institute for Biological Standards and Control
NIS	Newly Independent States of the Former Soviet Union
NTTB	Non-toxigenic, *tox* gene bearing
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHA	Passive haemagglutination assay

PHE	Public Health England
PYZ	Pyrazinamidase
QC	Quality control
RAPD	Random amplification of polymorphic data
RFLPs	Restriction fragment length polymorphism
RT-PCR	Real-time-PCR
SBM	Sequence-based methods
SLV	Single-locus variant
SNP	Single nucleotide polymorphism
SOP	Standard Operation Procedures
ST	Sequence types
STGG	Skimmed milk tryptone glucose glycerol
TLVs	Triple-locus variants
TNT	Toxin neutralization test
ToBI	Toxin-binding inhibition
TPP	Target Product Profile
WGS	Whole-genome sequencing
WHO EURO	World Health Organization Regional Office for Europe

CHAPTER 1 Introduction

1.1 Aims and objectives

The aims and objectives of this manual are to describe the microbiological procedures to isolate, identify and confirm the toxigenicity of *Corynebacterium diphtheriae, Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* (shortened versions will be used hereon). This laboratory manual will assist the laboratory worker in the correct procedures to diagnose diphtheria cases and will guide the treating clinician in treatment options. This manual includes a veterinary component that considers the increasing reports of human infection mainly due to *C. ulcerans* and occasionally *C. pseudotuberculosis* zoonotic infections. The manual will address serological procedures for assessing immunity and molecular epidemiological typing of potentially toxigenic corynebacteria. The manual is intended for global use; therefore, methodologies which may not be applicable in low-income countries will be included with the aim of promoting and developing laboratory technologies within the international network of diphtheria reference centres and beyond. The manual is arranged such that step-by-step methods for each assay are described in detail in the corresponding appendices.

1.2 Microbiology and clinical aspects of diphtheria and other related infections

Despite the success of mass immunisation, diphtheria and other infections caused by potentially toxigenic corynebacteria continue to play major roles as a lethal resurgent infectious disease. Epidemiology is driven by the success of the vaccination program in a given country; countries with higher diphtheria-tetanus-pertussis (DTP3) coverage have fewer cases and a higher proportion of persons infected that are ≥15 years of age. However, there are more cases with a higher proportion of persons infected ≤15 years of age in those with lower DTP3 coverage (Clarke *et al.* 2019). Diphtheria remains a serious health problem within many regions of the world. In addition, diagnosis of diphtheria may be delayed in countries with low or no incidence; consequently, fatality rates in non-endemic countries are similar to levels seen before mass immunisation, at approximately 16% (Wagner *et al.* 2012). Thus, correct microbiological diagnosis of the disease, identification of contacts and carriers, and clinical management of patients are crucial. The type of infections caused by potentially toxigenic corynebacteria notably, *C. diphtheriae* in humans has changed over the decades. This is highlighted by the recent dramatic resurgences in many WHO global regions and the emergence of non-toxigenic strains of *C. diphtheriae* causing atypical diseases and systemic complications, such as endocarditis, myocarditis, septic arthritis and more commonly, severe and recurrent episodes of sore throat.

Additionally, the past three decades have shown a significant increase in the global number of migrants. In 2019, more than one-third of the world's migrants lived in Europe (about 90 million, which is about 15% of the area's population). Screening for certain infectious diseases, particularly those where vaccines are available, is important in view of the breakdown of vaccination programmes in many 'immigrant countries' and specifically in those countries where war and violence predominates.

This is highlighted by the resurgence of 'old diseases' notably, diphtheria where the numbers of cases have increased dramatically in all global regions. During 2015-2018, diphtheria outbreaks occurred in Haiti, Venezuela, Yemen and Bangladesh owing to poor socio-economic crisis and/or war that resulted in poor access to health care systems. These outbreaks affected all age groups (Sharma *et al.* 2019).

The other recent major change has been the isolation of toxigenic *C. ulcerans* from human cases as well as from domestic animals (Bonmarin *et al.* 2009; Dias *et al.* 2010; Katsukawa *et al.* 2016, Konrad *et al.* 2015; Schuhegger *et al.* 2009; Wagner *et al.* 2010). All these important factors have strengthened the need for laboratories to screen for potentially toxigenic corynebacteria, particularly in high-risk areas.

Diphtheria is generally an uncommon disease caused by potentially toxigenic *Corynebacterium* species, namely, *C. diphtheriae* and less often *C. ulcerans* and *C. pseudotuberculosis*. The latter two species are zoonoses and are discussed below. All three organisms are Gram-positive, pleomorphic, aerobic rods. Among *C. diphtheriae*, there are four main biovars; gravis, mitis, intermedius and belfanti. Recently, taxonomic studies have shown that the biovar belfanti represents a branch that is clearly demarcated from *C. diphtheriae* biovar mitis and gravis and a new species has been proposed based upon these findings, *Corynebacterium belfantii* sp. nov (Dazas *et al.* 2018). We will refer to these strains as biovar belfanti

1.2.1 *C. diphtheriae*

The major virulence determinant of *C. diphtheriae* is diphtheria toxin, which is a bacteriophage-encoded protein. Clinical complications are more severe when this toxin is produced, causing respiratory obstruction, myocarditis and neurological damage. The three potentially toxigenic species (*C. diphtheriae, C. ulcerans*, and *C. pseudotuberculosis*) can also produce diphtheria toxin. The detection of diphtheria toxin is therefore the most important test for the microbiological diagnosis of diphtheria. This should be done without delay on any suspect isolate found during routine screening or while investigating a possible case of diphtheria. It is essential to contain any possible spread of the disease by identifying contacts who may be or become carriers. Novel methods using polymerase chain reaction (PCR) directly on clinical specimens speeds up the process of laboratory diagnosis. However, any samples testing positive for the toxin gene should be cultured, and the isolate tested for diphtheria toxin production.

1.2.2 *C. ulcerans*

C. ulcerans can produce a diphtheria toxin that is immunologically similar to that produced by *C. diphtheriae* because it harbours a lysogenic bacteriophage carrying a *tox* gene. The organism can also produce an exotoxin, phospholipase D. *C. ulcerans* can produce both toxins simultaneously and in varying proportions (Barksdale *et al.,* 1981).

C. ulcerans is a bacterium with a worldwide distribution and broad host range. It has been identified as an infrequent cause of bovine mastitis (Hommez *et al.* 1999), and raw milk has been recognized as a source of human infection (Bostock *et al.* 1984; Galbraith & Barrett 1986; Hart 1984). Udder infection may be prolonged, but excretion

in the milk may be intermittent. Epidemiological evidence from human disease investigations suggests that occasionally goat's milk may be contaminated with the organism (Barrett 1986). *C. ulcerans* has also been recovered from clinically-affected and healthy wild and domesticated animal species including dogs (chronic labial ulceration, sneezing and rhinorrhoea) (Sykes *et al.* 2010), cats (chronic nasal discharge), horses (nasopharynx), goats (pyogranulomatous meningoencephalitis), camel (caseous lymphadenitis), red fox, roe deer (caseous abscess), otters (lungs), pigs and wild boars (caseous abscesses), Richardson ground squirrels (gangrenous dermatitis), owls' feed (shrew-moles), lions (sepsis), killer whales (purulent pneumonia/bacteraemia), and non-human primates (respiratory infections, mastitis, bite wounds and cervical abscesses). Demographic distributions have been reported in various recent publications (Meinel *et al.* 2014, Meinel *et al.* 2015) from France (Bonmarin *et al.* 2009, Vandentorren *et al.* 2014), Japan (Yasuda *et al.* 2018; Hatanaka *et al.* 2003; Seto *et al.* 2008), Belgium (Detemmerman *et al.* 2013), and the UK (Wagner *et al.* 2010).

1.2.3 *C. pseudotuberculosis*

C. pseudotuberculosis has a cytotoxic surface lipid coat containing mycolic acids that mediate resistance to killing by phagocytes and appear to facilitate intracellular survival and abscess formation. The organism also produces a phospholipase exotoxin that increases vascular permeability, has an inhibitory effect on phagocytes, and may facilitate the spread of infection in the host (McNamara *et al.* 1995; Dorella *et al.* 2006; McKean *et al.* 2007).

C. pseudotuberculosis has been classified into two biotypes according to their ability to break down nitrate. Both biotypes produce an exotoxin, phospholipase D (Cuevas *et al.* 1993), which functions as a sphingomyelinase and acts on the vascular endothelial cell, this may explain its ability to increase vascular permeability and facilitate the spread of infection through the lymphatic system. Variations in toxin production between the strains may relate to differences in pathogenicity. *C. pseudotuberculosis* is similar to *C. diphtheriae* and *C. ulcerans* in that it can harbour the phage-borne diphtheria toxin gene (Ciancotto *et al.* 1986). However, *C. pseudotuberculosis* rarely produces diphtheria toxin and tests for diphtheria toxin production are rarely performed or even reported in many countries when this organism is isolated from animal samples.

C. pseudotuberculosis causes caseous lymphadenitis in sheep and goats (usually presenting as grossly evident superficial abscesses affecting lymph nodes, but in cases with internal lesions, the infection may only be detectable at necropsy) (Domenis *et al.* 2018), ulcerative lymphangitis in cattle and horses and also external and internal abscesses in horses. Most reported infections in the rural setting have been among rural sheepherders (House *et al.* 1986) or in butchers and have presented with either acute or chronic lymphadenitis (Lester *et al.* 1997; Peel *et al.* 1997). Several other clinical forms of the disease have been described in cattle; pyogranulomatous reactions, abscess formation and mastitic and visceral forms plus recently ulcerative and necrotic dermatitis of the heel of the foot. The organism has been recovered from bovine and caprine milk and has been reported rarely in purulent foci in deer, swine, hedgehogs, laboratory mice, camels and alpacas.

1.3 Transmission and carriage

The incubation period for diphtheria is usually two to five days, occasionally longer, and the most common mode of transmission is by infected droplet spread through contact with an infected person. Sources of infection include respiratory discharges from the pharynx and nose or occasionally from the skin and conjunctiva in the case of cutaneous diphtheria. Historically, especially in publications from the beginning of the twentieth century, poor hygiene and overcrowding conditions were reported to be associated with diphtheria outbreaks due to environmental conditions leading to infections, e.g. via inhaling corynebacteria-containing dust. Asymptomatic carriage of potentially toxigenic corynebacteria (*C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*) may occur during the incubation period, during convalescence or in healthy individuals. In countries where diphtheria is endemic, between 3% and 5% of healthy persons may carry the organism in the nasopharynx (even higher in some countries). Based on recent carriage studies within Europe, in non-endemic countries, isolation of potentially toxigenic corynebacteria is relatively uncommon (Wagner *et al.* 2011). Other manifestations of the disease, particularly cutaneous diphtheria, are problematic in tropical countries and the lesions may act as reservoirs for transmission and spread of pharyngeal diphtheria. Some travellers, returning from diphtheria-endemic countries with wound infections, have been found to have toxigenic cutaneous diphtheria (Griffith *et al.*, 2019.)

In recent years, diphtheria due to *C. ulcerans* has been reported in developed countries where *C. diphtheriae* incidence is low (Blue *et al.* 2011). There have also been numerous reports of transmission between humans and companion pets who may be potential sources of infection (De Zoysa *et al.* 2005; Lartigue *et al.* 2005). Zoonotic transmission was proven by molecular typing of identical strains isolated from both humans and their respective companion pets or livestock animals in several reports (Hogg *et al.* 2009, Schuhegger *et al.* 2009, Vandentorren *et al.* 2014, Yasuda *et al.* 2018, Berger *et al.* 2011, Meinel *et al.* 2014, Meinel *et al.* 2015). Although there is no direct evidence of person-to-person transmission of *C. ulcerans*, this route of transmission cannot be ruled out; Konrad *et al.* 2015 reviewed the current knowledge on human-to-human transmissions of toxigenic *C. ulcerans* with examples from the UK and Germany.

Human infection of any sort with *C. pseudotuberculosis* is rare and usually produces a localized suppurative granulomatous lymphadenitis with a long and recurrent course. It is an occupational disease of shepherds, shearers, abattoir workers and butchers with skin cuts being a potential route of infection. Consumption of infected non-pasteurised milk can also be a risk for human infection.

1.4 Serological testing for population and individual immunity/susceptibility to diphtheria

Since the early 1980s, diphtheria has increased globally, particularly within Eastern European, South-East Asia, India, Africa, South America and Western Pacific regions, and more recently in the Middle East and Western Pacific. Several factors contribute to the rise and continuation of these epidemics, including low immunisation coverage rates in some areas, lack of immunity among adults and the general unavailability of

vaccines in some countries, especially due to war and man-made disasters resulting in the breakdown of public health services. Given the evidence from these epidemics, it is apparent that adults are a high-risk group for the disease. Population immunity studies using tissue culture toxin neutralisation test (TNT) on Vero cells, Enzyme-Linked Immunosorbent Assay (ELISA), dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA), toxin-binding inhibition test (ToBI) or passive haemagglutination, have been performed in some European countries (Edmunds *et al.* 2000; von Hunolstein *et al.* 2000). Because of their ease of use, immunoassays are most often the preferred method to define population immunity. All these methods are highly dependent upon critical reagents and are not currently harmonized between laboratories and countries, leading to diverse information on identical sets of samples (Di Giovine *et al.* 2010, von Hunolstein *et al.* 2014). Because these methods are all surrogate models for the TNT, which detect functional toxin neutralisation antibodies, and validation studies are limited, assigning protective levels in a population using these methods can be problematic. Thus, it is essential to understand the limitations of methods currently used in population immunity studies. The use of a common protocol or at least well-defined standardised reagents, for example, the WHO International Standard for diphtheria antitoxin has been established and confirmed as suitable for use in population immunity studies and should help considerably in understanding assay performance (Stickings *et al.* 2013). This manual addresses the tests currently recommended and used by many centres where facilities are available.

1.5 Role of the laboratory in the diagnosis of diphtheria

1.5.1 Roles of reference laboratories

The importance of laboratory diagnostics has been highlighted in the WHO new surveillance standards for vaccine-preventable diseases:

https://www.who.int/immunization/monitoring_surveillance/burden/vpd/WHO_SurveillanceVaccinePreventable_04_Diphtheria_R2.pdf?ua=1

The final case classification is always dependent upon laboratory confirmation. This is almost always undertaken by the National Reference Laboratory within the country. The key objectives of a reference centre are to strengthen laboratory collaboration and support, particularly to those in greater need, to increase current knowledge and develop and implement new technology relating to the laboratory diagnosis and epidemiological surveillance of diphtheria. Due to the need for specialised media and the scarce availability of antitoxin, toxigenicity testing is usually only performed by National Reference Laboratories. Therefore, diagnostic laboratories are recommended to submit suspect isolates and, in some instances, the original specimen to the National Reference Laboratory, highlighting the importance of reference facilities within countries. Additionally, reference laboratories play a role in teaching and training scientists on the laboratory diagnosis of diphtheria, both within their own laboratories as well as those from other hospitals and regional laboratories in-country and beyond. The recent gap analysis initiated by the European Centre for Disease Prevention and Control (ECDC) and expanded by the WHO undertaken within

many regions demonstrated that there are significant gaps in diphtheria diagnostic capacity:

https://www.who.int/immunization/monitoring_surveillance/burden/vpd/WHO_SurveillanceVaccinePreventable_04_Diphtheria_R2.pdf?ua=1

The areas with the greatest gaps are related to training and surveillance for all three potentially toxigenic pathogens. Therefore, a programme for laboratory training workshops has been developed, and training has been conducted in many WHO global regions in collaboration with international partners namely the Public Health England (PHE) (WHO Collaborating Centre for Diphtheria), ECDC and CDC. It is essential to maintain an ongoing programme of laboratory training workshops to maintain awareness and ensure specialised expertise on a global level.

1.5.2 Specialised testing

1.5.2.1 Molecular epidemiological studies

Conventional epidemiological approaches such as molecular typing (ribotyping) and monitoring of the Newly Independent States (NIS) epidemic clone 'Sankt Petersburg' was developed and implemented by the European Laboratory Working Group for Diphtheria (ELWGD) and ECDC Diphtheria Surveillance Network (DIPNET) and used in reference centres globally (France, UK, Finland, Romania, USA) (Efstratiou & Roure 2000; Grimont *et al.* 2004). These methods have been superseded by more modern technologies, such as multilocus sequence typing (MLST) and next-generation sequencing (NGS). A more detailed discussion of current molecular technologies can be found in **Chapter 7**.

1.5.2.2 Clinical diagnostic microbiology laboratories

Since the introduction of mass immunisation and the resulting decline in diphtheria incidence, there are mixed views in many developed countries concerning the need and necessity for laboratories to screen routinely for potential toxigenic corynebacteria. The uncommon occurrence of cases in some countries and the expense and complexity associated with laboratory diagnosis means many countries cease to routinely screen throat specimens; therefore, over the years, expertise and recognition of these organisms have declined.

In many advanced cases, a clinical diagnosis of diphtheria would normally precede the microbiological diagnosis. However, the first indication of the disease is often given by the microbiology laboratory reporting the presence of the causative organism usually as *C. diphtheriae* or *C. ulcerans* in respiratory tract samples. Since the recent widespread availability of matrix-assisted laser desorption ionization – time of flight mass spectrophotometry (MALDI-TOF MS), species can be identified rapidly (see **Chapter 5**). Rapid and accurate diagnosis is of utmost importance. Clinical diagnosis, particularly in countries where the disease is uncommon, is not easy and often confused with other infections, such as tonsillitis and streptococcal pharyngitis (strep throat). This highlights the important role of the diagnostic laboratory in providing simple, rapid and reliable methods to assist clinicians in achieving the correct diagnosis. However, a bacteriological diagnosis must be regarded as complementary to, and not as a substitute for clinical

diagnosis. The laboratory may also aid the clinician by eliminating suspected cases or contacts of diphtheria and thus avoiding treatment and isolation of these cases. The diagnostic laboratory should refer any presumptive *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* isolates to their National Reference Laboratory for confirmation and toxigenicity testing. If such a laboratory does not exist, arrangements need to be in place for urgent referrals to another reference laboratory in a neighbouring country or the WHO Collaborating Centre for Diphtheria in the UK.

See https://apps.who.int/iris/handle/10665/108108 (Efstratiou and Maple 1994).

Conventional phenotypic methods are time-consuming and result in delayed reporting. These methods have recently been augmented by real-time multiplex polymerase chain reaction (RT-PCR), which detects toxin gene bearing corynebacteria within a few hours (Pacheco *et al.* 2007).

1.5.3 Notification of potentially toxigenic strains of *C. diphtheriae, C. ulcerans* or *C. pseudotuberculosis*

Each country should have in place a formal disease notification system. All suspected cases should have at least two specimens collected (a nasal and pharyngeal /nasopharyngeal swab). The reference laboratory or diagnostic laboratory (where laboratory diagnosis of diphtheria is undertaken) should notify the public health officials/epidemiologists as soon as a presumptive result is available (potentially toxigenic strain). However, in some countries, it is the laboratory that will make the notification. An immediate alert is crucial to initiate prompt public health action. However, clinically suspected cases should be treated promptly without waiting for laboratory confirmation. Further guidance is available in the WHO Surveillance Standards document for vaccine-preventable diseases.

https://www.who.int/immunization/monitoring_surveillance/burden/vpd/WHO_SurveillanceVaccinePreventable_04_Diphtheria_R2.pdf?ua=1

1.6 Innovations in diphtheria diagnosis and analysis

The large epidemic of diphtheria in Russia and the NIS during the 1990s stimulated interest in developing improved diagnostic tests and increasing the competency of laboratory personnel for identifying *C. diphtheriae* and performing toxigenicity tests.

1.6.1 Tests for detection of diphtheria toxin

Modifications in procedures for the Elek test (**Chapter 4** and **Appendix 7**) improved the reproducibility of results, decreased the volume of reagents required, and decreased the time required for results from 48 hours to 16-24 hours (Engler *et al.* 1997).

Subsequently, a quantitative antigen-capture enzyme immunoassay (EIA) test for diphtheria toxin was developed, which offers rapid, sensitive, and specific alternatives to the Elek test for toxigenicity testing (Engler *et al.* 1997). Previously, the use of a qualitative immunochromatographic strip (ICS) test for diphtheria toxin was also available (Engler *et al.* 2002, Engler and Efstratiou 2000). This was prepared by the

USAID agency PATH in response to the huge outbreaks within the Former Soviet Union and was not developed commercially.

The EIA uses an equine polyclonal antibody to capture and an alkaline phosphatase-conjugated monoclonal anti-fragment A antibody to detect the diphtheria toxin. The limit of sensitivity is 0.1 ng of diphtheria toxin/ml, and results available within 3 hours of colony selection agree uniformly with Elek tests.

The ICS test also uses an equine polyclonal antibody to capture but substituted colloidal gold-labelled monoclonal anti-fragment A antibody to detect the diphtheria toxin. The limit of sensitivity for the ICS test is 0.5 ng of diphtheria toxin/ml, and results are available within 10 minutes. Furthermore, the ICS test was used to compare 850 throat swabs that were inoculated directly into broth for 16 hours and conventional culture methods; the concordance for detecting diphtheria toxin by the two methods was 99%, and the sensitivity and specificity of the ICS test for detecting diphtheria toxin were 98% and 99%, respectively. The ICS test has significant advantages over the EIA with respect to ease of test performance, the stability of reagents, and having the ability to detect diphtheria toxin production within 16 hours from the initial collection of a throat swab from a patient with suspected diphtheria.

The WHO and stakeholders are developing a Target Product Profile (TPP) for the rapid diagnosis of diphtheria toxin to improve outbreak management. The key objective is the detection of toxin production from *C. diphtheriae*, and other potentially toxigenic corynebacteria species such as *C. ulcerans* and *C. pseudotuberculosis* in a clinical setting and for the identification of patients in need of diphtheria antitoxin (DAT). A rapid point of care is envisaged that would address all these issues, for example the ICS test format, which has previously shown promising results (personal communication A. Efstratiou).

1.6.2 Molecular typing and gene sequencing

Molecular typing is still only performed in a few selected laboratories. The use of standardised molecular epidemiological tools is essential in monitoring the spread of epidemic *C. diphtheriae* strains and to differentiate between epidemic, endemic and imported cases.

Previous molecular typing methods applied to *C. diphtheriae* included restriction fragment length polymorphisms (RFLPs) using DNA and various probes, targeting the toxin gene and insertion elements. Due to the Russia/NIS epidemic, the ELWGD made a concerted effort to establish a standard genotyping method for rapid tracking of strains, namely, ribotyping. Since then, several molecular subtyping methods have been developed, such as pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphisms, and all were evaluated at the WHO Collaborating Centre in London and successfully applied to epidemiological investigations of diphtheria (De Zoysa & Efstratiou 2000; De Zoysa *et al.* 1995; De Zoysa & Efstratiou 1999; Popovic *et al.* 2000; De Zoysa *et al.* 2008). However, due to the significant progress in molecular technologies, all these methods have now been superseded by methods incorporating molecular sequencing, such as MLST (Maiden 2006; Maiden *et al.* 1998) and NGS.

In practice, the application of molecular subtyping methods and the continuous monitoring of clonal spread have a strong impact on public health control measures. Both during and after the 1990s epidemic, it was possible to distinguish between endemic, and epidemic strains and imported cases and no subsequent dissemination was reported following imported cases (Mokrousov *et al.* 2009). In Germany, the UK and France, molecular typing is also used to identify possible sources of *C. ulcerans* infections and to investigate suspected transmission from pet animals to humans (Bonmarin *et al.* 2009; De Zoysa *et al.* 2005; Lartigue *et al.* 2005; Boschert *et al.*, 2014).

CHAPTER 2 Procedures for collection, storage transportation of clinical samples and revival of isolates

The importance of speed coupled with accuracy is essential when performing the procedures outlined in this manual. It is recommended that the sample/isolate is considered as toxigenic until proven otherwise.

The range and depth of the investigation are dependent on the capacities at the disposal of the laboratory, i.e. the availability of reagents, the experience of laboratory staff and financial resources (Efstratiou *et al.* 2000).

2.1 Criteria for screening suspected specimens of *Corynebacterium* species

Due to the relatively low prevalence of diphtheria, screening of throat swabs is not routinely performed in many countries. Therefore, it is important to examine specimens for *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* if there are any specific risks reported. The definition of a suspected case of diphtheria leading to the collection of throat and nose swab according to the WHO Surveillance Standards for diphtheria are as listed below:

Clinical criteria

- Pharyngitis, nasopharyngitis, tonsillitis or laryngitis

  AND

- Adherent pseudomembrane of the pharynx, tonsils, larynx and/or nose

Epidemiological criteria

- Travel to an endemic or epidemic area within the last 10 days* or immigrants/refugees from these areas
- Recent contact with someone who has travelled overseas to an endemic area
- Recent contact with a diphtheria case in the absence of travel
- Recent consumption of raw dairy products (*C. ulcerans/C. pseudotuberculosis*)
- Recent contact with farms, farm animals or domestic animals (*C. ulcerans/ C. pseudotuberculosis*)
- Work in a clinical microbiology laboratory, or similar, where *Corynebacterium spp.* are handled

*Travel or contact with travellers in the past 10 days is most likely to be relevant for the risk of diphtheria.

In addition, swabs from chronic non-healing ulcers or skin lesions should be collected if the patient fulfils any of the following risk factors:

- Recent travel (especially to tropical regions)
- Recent contact with someone who has recently travelled to an endemic area or is an immigrant/refugee from these areas
- Recent contact with farms, farm animals or domestic animals (*C. ulcerans/C. pseudotuberculosis*)
- Work in a clinical microbiology laboratory, or similar, where *Corynebacterium spp.* are handled
- Non-healing chronic ulcer/skin infection without any of the risk factors listed above (Bernard *et al.* 2019).

It is recommended that screening of all contacts and carriers should also be done two weeks after cessation of antimicrobial therapy.

2.2 Specimen collection, storage and transport from suspected cases of respiratory or cutaneous diphtheria, and contacts

Specimen collection procedures usually induce coughing, spluttering, sneezing and watering eyes; health workers collecting specimens should be appropriately protected and follow national guidelines as described by Efstratiou and George, 1999. Droplet precautions are necessary, including a surgical mask and eye protection (**Appendix A1.1**). In addition, health workers collecting the swabs should ensure that they are vaccinated according to the recommended schedule published by WHO, and that their booster vaccines against diphtheria are up to date.

2.2.1 Collection of samples for laboratory examination

The successful isolation of *C. diphtheriae* strains depends initially on the correct collection of swabs and their subsequent rapid transfer to the laboratory. As diphtheria is most commonly an upper respiratory tract infection, specimens from the oropharynx, nasopharynx or ear should be collected (**Appendix A1.2–A1.4**). If a pseudomembrane is present, a swab from beneath the membrane should be collected (**Appendix A1.6**) as well as a piece of tissue (if possible). If cutaneous diphtheria is suspected, which is often indistinguishable from any other pyoderma, especially in parts of the world where diphtheria is endemic, swabs should be collected from any wounds or cutaneous lesions (**Appendix A1.5**). Ideally, specimens should be collected at the onset of symptoms and before antimicrobial or antitoxin therapy. All samples must be transported to the laboratory immediately after collection or kept at 4-8°C if transport delays are expected. Post-mortem specimens from the upper respiratory tract and vital organs may be examined in cases where an autopsy is required to determine if diphtheria was the cause of death.

Ideally, two Dacron or flocked applicator swabs (see the following link for choosing the best swab: https://blog.puritanmedproducts.com/medical-swabs-how-to-choose) should be collected from each suspected case and placed in a routine semi-solid

transport medium, such as Amies (Amies 1967), immediately after collection and sent to the laboratory (**Appendix 2**). Dry swabs should ideally be placed into silica gel sachets (Sinclair *et al.* 1972), particularly for swabs from endemic areas where transportation may be difficult or delayed. The swabs should be labelled accordingly with a unique identifier and the source of the specimen.

The clinician must inform the laboratory of any presumptive diagnosis of diphtheria. A guide on the data to collect and report a presumptive clinically diagnosed case has been published by WHO. (See the following link for details: https://www.who.int/immunization/monitoring_surveillance/burden/vpd/WHO_SurveillanceVaccinePreventable_04_Diphtheria_BW_R2.pdf?ua=1). Several reports (from both Europe and Australia) highlight the importance of isolating *C. diphtheriae* from blood culture and normally sterile sites from patients presenting with endocarditis (Romney *et al.* 2006; Schnell *et al.* 2010; Tiley *et al.* 1993).

2.2.2 Transport, preservation, storage and revival of cultures

If transport to the laboratory cannot be immediate (within 2-8 hours of collection), samples should be stored at 4-8°C. If Amies is not available, other commercially available transport media may be used, for example, Stuart's transport medium https://assets.fishersci.com/TFS-Assets/LSG/manuals/IFU64620.pdf. If transit times are to exceed more than one week, then silica gel packs are advisable.

Once an isolate has presumptively been identified as positive for *C. diphtheriae, C. ulcerans or C. pseudotuberculosis*, it is important to preserve the sample as a pure culture. This isolate may be sent to a reference laboratory or preserved in the original laboratory for future testing.

Traditional ways to store isolates include:

1) Short-term (up to 7 days): placed on an agar slant, incubated at 35-37°C overnight and stored in a refrigerator at 4°C

2) Long-term: cryopreservation/freezing at –20°C to –80°C in glycerol broth (**Appendix A3.1.1**); in skimmed milk tryptone glycerol glucose medium – STGG, (**Appendix A3.1.2**); or in tubes containing cryobeads, such as Microbank™ beads (ProLab Diagnostics, Richmond Hill, Ontario, Canada) (**Appendix A3.1.2.2**)

Isolates for storage should be grown in pure culture for no more than 24 hours on a trypticase soy or blood agar medium (**Appendix A4**). Media containing tellurite or antibiotics must not be used for this purpose. The storage vial should be labelled with the isolate reference number and date to link it with the patient information in the future.

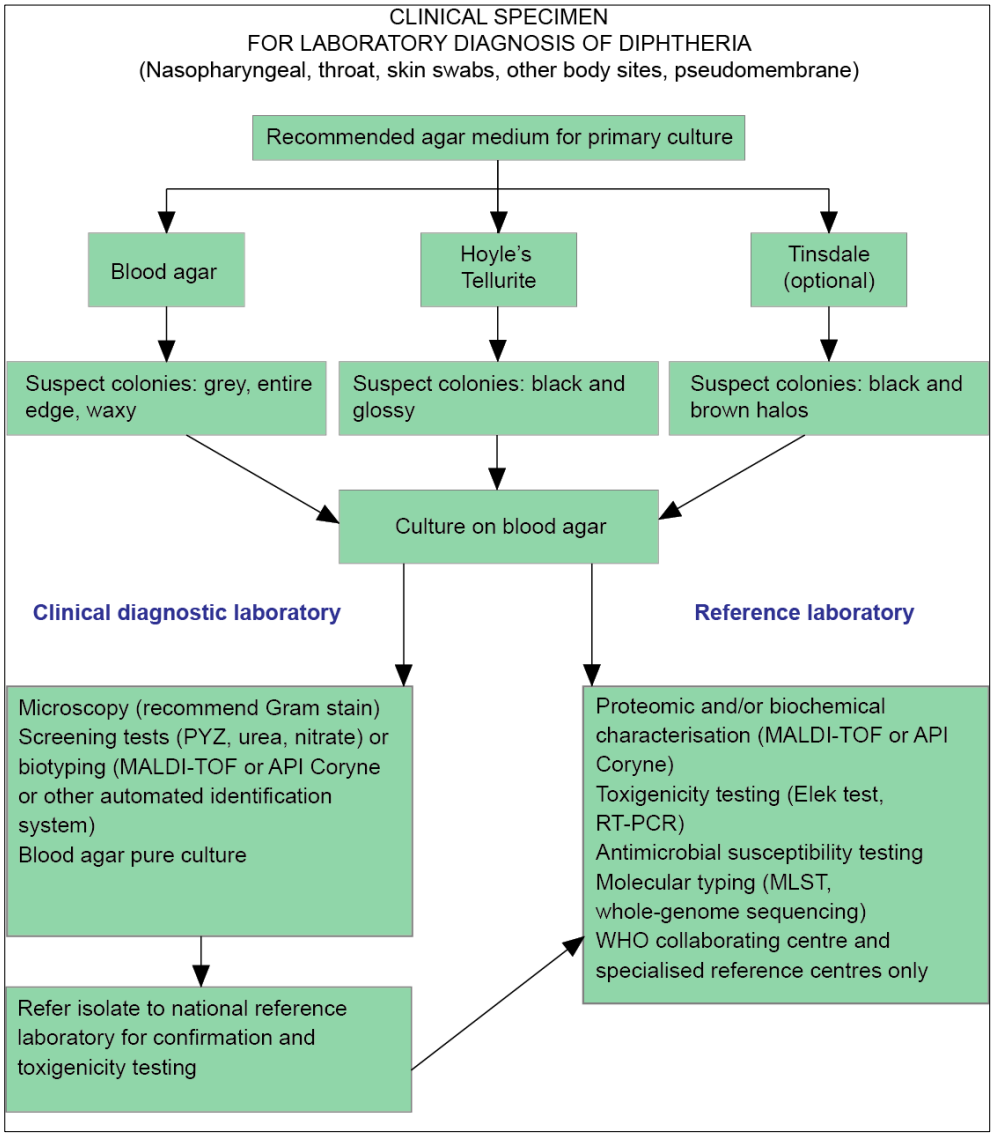
For strain revival from frozen STGG or cryobeads (**Appendix A3.2-A3.3**), it is necessary to work in a biosafety cabinet according to the laboratories safety protocol. Vials of strains should not be completely thawed and should be returned to the freezer as soon after subculture as possible.

CHAPTER 3 Procedures to isolate and biotype *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*

3.1 Laboratory procedures for primary isolation of potentially toxigenic corynebacteria

Specimens collected must be inoculated onto the correct primary culture media without delay, as the swabs may contain only a small number of corynebacteria. Also, delays may allow the natural flora from the collection site to obscure the culture by overgrowth. For this reason, Loeffler's serum medium is not ideal for primary isolation. The flow chart in **Figure 1** outlines the recommended order of procedures for the laboratory diagnosis of diphtheria and related infections.

Figure 1. Recommended procedures and order to follow for the laboratory diagnosis of diphtheria and related infections.



3.2 Primary culture and isolation

If the swab received in the laboratory has not been placed in transport medium, then it should be moistened with a few drops of sterile nutrient broth for a few minutes before culturing. If in transport media, then plate directly onto the agar plates.

The minimal culture media required for the isolation of *C. diphtheriae* and other potentially toxigenic corynebacteria are blood agar and a blood agar medium containing tellurite (Hoyle & Leeds 1941) (**Appendix A4.3**).

Swabs are firstly rubbed over a quarter of the blood agar plate surface and then the Hoyle's tellurite plate. Using sterile loops, streak the sample over each individual plate. Incubate the blood agar and the Hoyle's tellurite plates at 37°C aerobically. Examine all plates after 18-24 hours of incubation and re-incubate a further 24 hours.

Clauberg medium is also a selective culture medium containing potassium tellurite; however, some strains of staphylococci, streptococci and *Candida spp*. may grow on this medium but can be differentiated macroscopically and microscopically. Hoyle's medium is recommended (**Appendix A4.3**).

3.3 Criteria for recognising suspect colonies that require further evaluation

Primary plates must be examined after 18-48 hours of incubation, to subculture and confirm suspicious colonies as rapidly as possible (see **Figures 2, 3 and Table 1**). It is also advisable to examine colony morphology with a hand lens in reflected light. If there is no visible growth on blood agar, then further swabs should be requested immediately, as it is likely that the swab(s) have not been collected properly. Although rarely isolated, the biovar *intermedius* will take between 48 and 72 hours to grow; this is the slow-growing biovar within the *C. diphtheriae* species.

The blood agar plate is useful in the detection of β-haemolytic streptococci, *Arcanobacterium haemolyticum* and *Staphylococcus aureus*, which may often be present. In addition, some strains of *C. diphtheriae* are sensitive to potassium tellurite and will, therefore, be inhibited on tellurite medium. It is important to examine the blood agar plate carefully for any suspicious colonies of *C. diphtheriae*. Note that some strains of *S. aureus*, enterococci and other organisms may grow as black colonies on Hoyle's agar.

Figure 2. Classic colony morphology of *C. diphtheriae* and *C. ulcerans* on blood agar medium; *C. diphtheriae* (A and C) and *C. ulcerans* (B) . Images courtesy of A. Efstratiou.

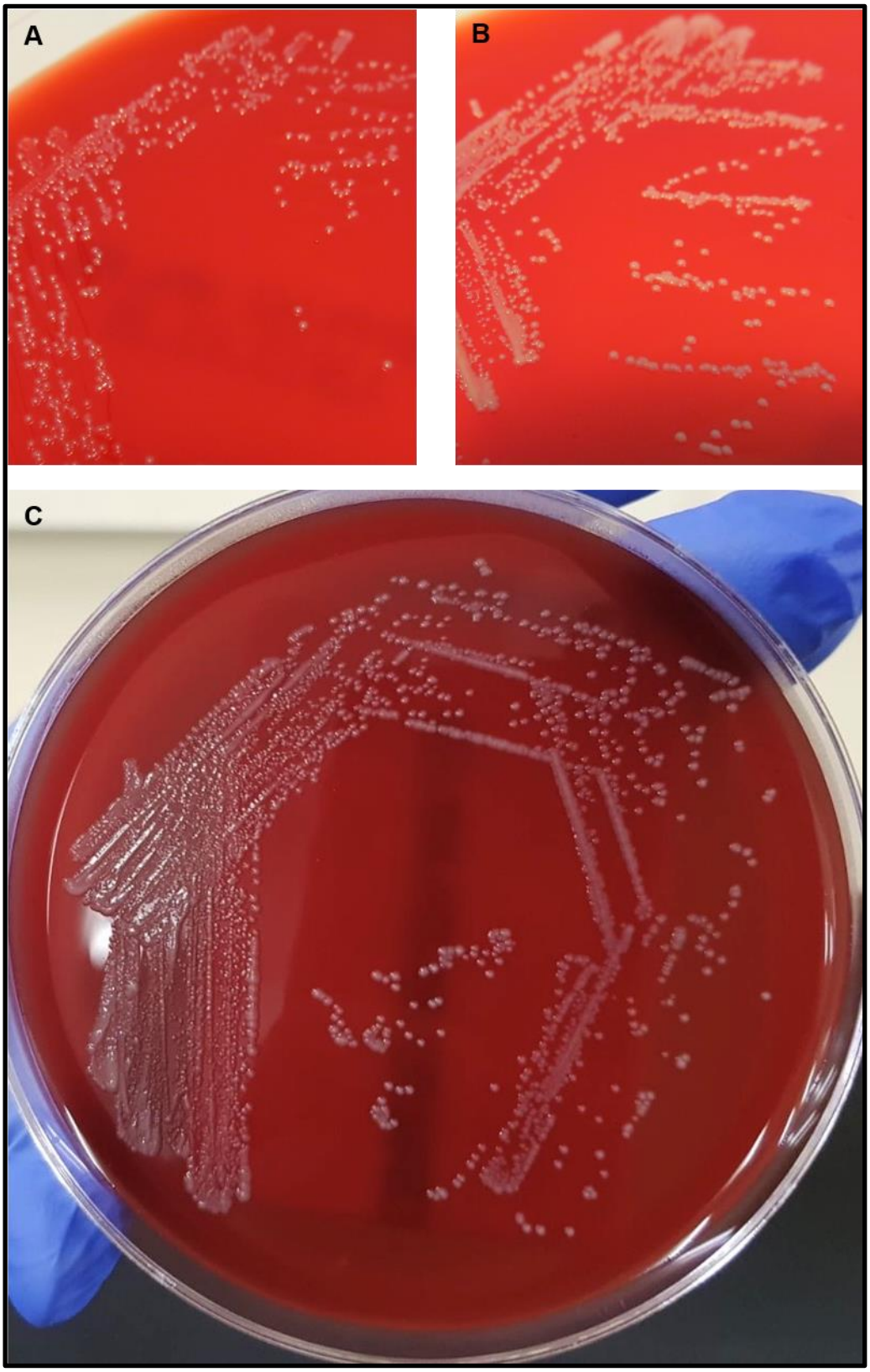
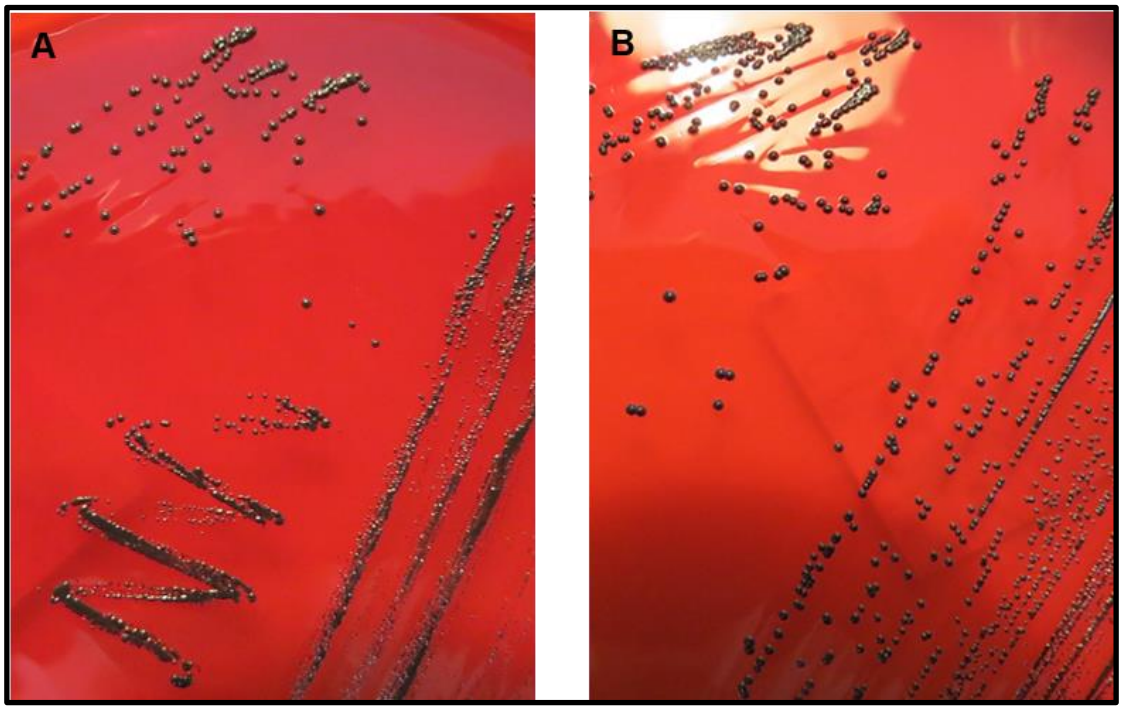


Figure 3. Morphology of *C. diphtheriae* (A) and *C. ulcerans* (B) on Hoyle's tellurite medium. Images courtesy of A. Efstratiou.



All procedures should be performed in a microbiological biosafety cabinet as toxin-producing strains of *C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis* can cause severe and sometimes fatal disease. There have been reports of laboratory-acquired infections. If available, the use of sterile disposable loops is recommended for the spreading of sample material onto culture media.

3.4 Presumptive identification and screening of potentially toxigenic and non-toxigenic *Corynebacterium* species

Tests, such as pyrazinamidase (PYZ), urea, nitrate and/or cystinase production on Tinsdale or Pizu medium (optional) are useful for the presumptive identification of potentially toxigenic corynebacteria. Tinsdale medium can also be used as part of the primary screening media directly from clinical specimens. However, this medium is very selective, and the plates have a limited shelf life (maximum 14 days) but should be used if resources are available.

3.4.1 Cystinase test (Tinsdale)

Tinsdale medium is recommended for the presumptive identification of potentially toxigenic corynebacteria as it detects cystinase enzyme (Colman *et al.* 1992). If adequately batch tested, with strong and weak enzyme-producing strains, the medium is useful for confirming suspicious colonies found on tellurite media. Only *C.*

diphtheriae, *C. ulcerans* and *C. pseudotuberculosis* will produce the characteristic black colonies surrounded by a brown halo after overnight incubation (**Appendix 5, Figure A3**). Pizu medium for cystinase detection is also used in many countries of the NIS (Feldman *et al.* 1989).

It is advisable to regularly perform quality control using culture reference strains from stock to ensure recognition of colonial morphologies and thus ensure that all tests, media and stains are working optimally (**Appendix 4 Table A1**). Type strains from international culture collections are recommended.

Table 1. Differences in the morphological appearances of typical colonies of *Corynebacterium* spp. on primary media (after 24 hours of incubation aerobically at 35-37°C).

Strain	Hoyle's Tellurite agar	Blood agar
C. diphtheriae biovar gravis	dull, grey/black, opaque colonies, 1.5-2.0 mm in diameter, matt surface, friable, tending to break into small segments when touched with a straight wire	non-haemolytic
C. diphtheriae biovar mitis	grey/black, opaque colonies, 1.5-2.0 mm in diameter, entire edge and glossy smooth surface; size variation is common	colonies may exhibit a small zone of β-haemolysis
C. diphtheriae biovar intermedius*	small, grey/black, shiny surface, discrete, translucent colonies, 0.5-1.0 mm in diameter	colonies exhibit a small zone of β-haemolysis
C. diphtheriae biovar belfanti	grey/black, opaque colonies, 1.5-2.0 mm in diameter, entire edge and glossy smooth surface; size variation is common	colonies may exhibit a small zone of β-haemolysis
C. ulcerans	grey/black, very dry opaque colonies	colonies may exhibit a small zone of β-haemolysis
C. pseudotuberculosis	grey/black, very dry opaque colonies	colonies exhibit a small zone of β-haemolysis

* *C. diphtheriae* biovar intermedius can be lipophilic (based on growth in broth enriched with Tween 80).

3.5 Microscopic examination and staining procedures for suspect colonies/cultures

The use of Albert's stain as a primary staining method is still generally undertaken in some countries as an early presumptive identification indicator for corynebacteria, as metachromatic granules are not specific to *C. diphtheriae.* Metachromatic granules can also be stained with Ponder's or Neisser's stain.

Although there are several useful stains (see **Appendix 6** and **Table A2**), confirmation of Gram-positive, club-shaped rods should be supported by the growth of corynebacteria.

Suspicious colonies from tellurite, blood agar plates or Loeffler's slopes should be prepared for staining.

The common microscopic characteristics of pathogenic corynebacteria are:

- Small Gram-positive bacilli (some strains of *C. diphtheriae* tend to over decolourise and may appear Gram variable)
- Straight or slightly club-shaped rods which are highly pleomorphic
- Cells may occur singly or in pairs, often in a "V" formation resembling Chinese letters
- Non-motile
- Non-sporing
- Non-acid fast

Metachromatic granules formed in the polar regions when grown from enrichment media such as Loffler's medium and are visible when stained with methylene blue.

The diagnosis of diphtheria must not be solely based upon direct microscopy of a smear as both false positive and false negatives may occur.

3.5.1 Gram stain

Isolates from primary culture could potentially be identified by colonial appearance, Gram stain and other preliminary screening tests. With enough experience in these methods, the *Corynebacterium* species could be presumptively identified within four hours. However, in some countries the Loeffler methylene blue stain (Sigma-Aldrich) is preferred to the Gram stain, as not all bacilli appear as Gram-positive; Gram-negative appearance could prompt the bacteriologist in excluding corynebacteria (personal communication, I. Mazurova). Other staining procedures, such as Albert's stain for metachromatic granules, are performed in some countries (**Appendix 6**).

3.6 Biochemical identification

Species identification by the recommended simple conventional tests detect a range of carbohydrates and enzymatic reactions for phenotypic identification of corynebacteria (**Table 2**). Isolates are catalase positive.

Table 2. Conventional tests

Test	Test medium
Nitrate reduction	Nitrate broth
Urea hydrolysis	Urea slope
Catalase production	Hydrogen peroxide
Cystinase activity	Tinsdale agar
Pyrazinamidase activity	Pyrazinamide substrate broth
Carbohydrate fermentation	Glucose, sucrose, maltose, glycogen/starch

The tests for PYZ activity and cystinase production are useful screening tests to distinguish between the three potentially toxigenic species and other coryneform bacteria. If screening tests are not available, conventional biochemical methods could be employed, and media can be prepared locally if the reagents are available (see the WHO 1994 manual for the laboratory diagnosis of diphtheria: Efstratiou and Maple, WHO, 1994). Where possible, toxigenicity testing should be initiated without delay.

Furthermore, several tests/systems are available to identify bacterial pathogens, from commercially available kits and diagnostic single test tablets (API® Coryne and Rosco Diagnostica) to the more complex, automated (and expensive) tests/systems. These systems provide more accurate and rapid results and are simple to use (WHO Manual 1994, as above).

Table 3. Biochemical identification of the common pathogenic *Corynebacterium* species.

Organism	CYS	PYZ	Alkaline phosphatase	Nitrate	Urease	Acid produced from:					Gelatine liquefaction
						Glucose	Ribose	Maltose	Sucrose*	Glycogen	
C. diphtheriae **biovar gravis**	+	-	-	+	-	+	+	+	-	+	N/A
biovar mitis	+	-	-	+	-	+	+	+	-	-	N/A
biovar intermedius	+	-	-	+	-	+	+	+	-	-	N/A
biovar belfanti	+	-	-	-	-	+	+	+	-	-	N/A
C. ulcerans	+	-	+	-	+	+	+	+	-	+	+ at 25°C
C. pseudotuberculosis	+	-	+	-	+	+	+	+	-	-	- at 25°C

To differentiate further between *C. pseudotuberculosis* (resistant) and *C. ulcerans* (susceptible) the vibriostatic 0129 agent can be used (Groman *et al.* 1984; Berger *et al.* 2014). N/A = Non-applicable.

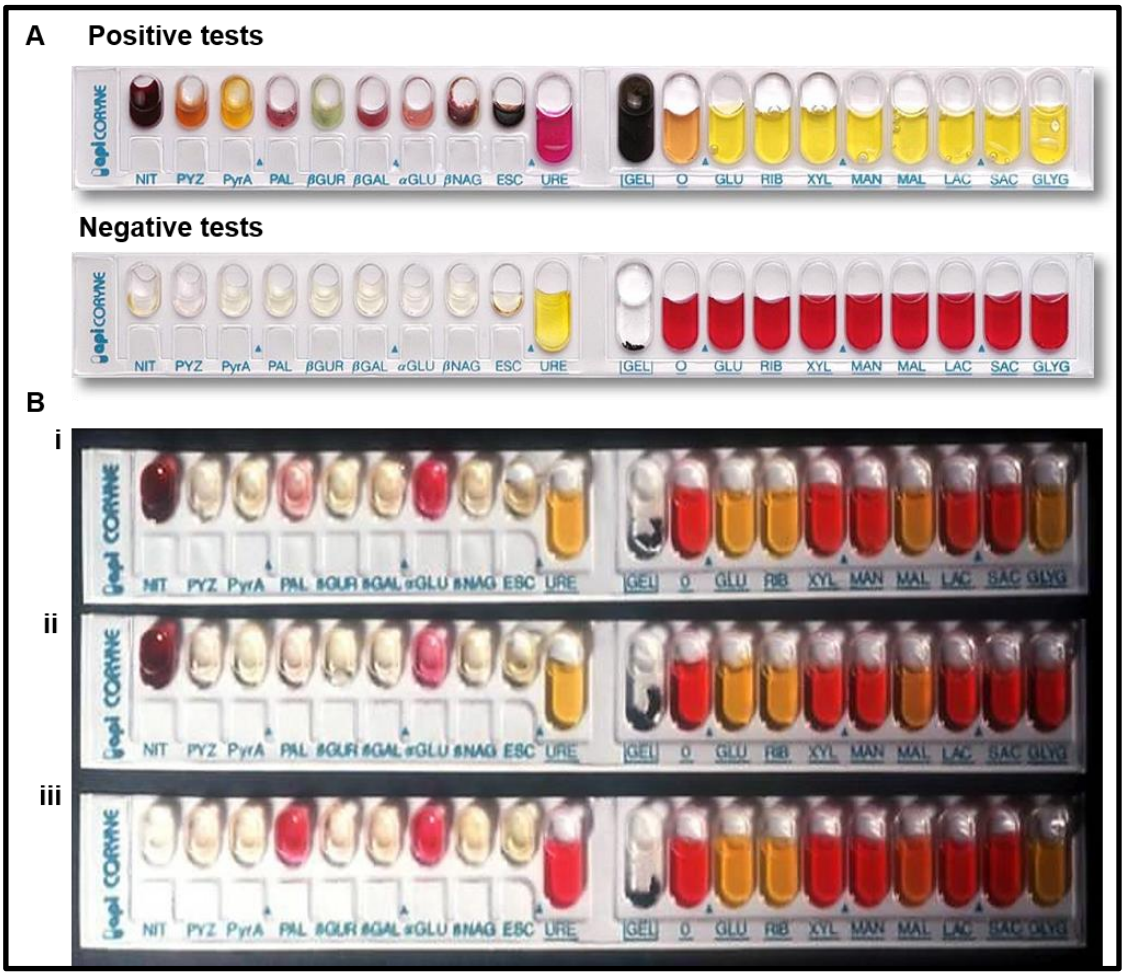
*Sucrose positive variants have been described in Brazil (de Mattos-Guaraldi & Formiga, 1998)

3.6.1 API®-Coryne test system

Always follow the manufacturer's instructions for setting up these tests (API® -Coryne, bioMèrieux, France). The kit contains 20 microtubes consisting of dehydrated substrates for detecting 11 enzymatic activities and 8 carbohydrate fermentation sugars (**Figure 4**).

Organism identification is read as a numerical analytical profile index (7-digit code) and interpreted using the available API® website which is freely available online when registering to become an APIWEB™ user (https://apiweb.biomerieux.com/login).

Figure 4. A test example of the API®-Coryne test system. (A)Top panel illustrates positive results and no colour changes in the bottom reveals negative results. (B) API® **-** Coryne test on (i) *C. diphtheriae* biovar gravis, (ii) *C. diphtheriae* biovar mitis and (iii) *C. ulcerans.* Published with permission of bioMèrieux, France.



3.6.1.1 Control strains specifically for the API® Coryne system

The following three strains should be tested to quality control positive and negative reactivity for most of the API®-Coryne tests. (No. 2 and 3 are recommended for laboratories with stricter quality control requirements and are included because of the requirements from testing regulators in some countries).

1. *Corynebacterium renale* ATCC 19412
2. *Cellulosimicrobium cellulans* ATCC 27402
3. *Microbacterium testaceum* * ATCC 15829

3.6.1.2 Quality control for the API®-Coryne system

The multitest strips and reagents are systematically quality controlled at various stages of their manufacture. Streamlined quality control may be used to confirm the acceptable performance of the API® Coryne system after shipping/storage. This methodology may be performed by following the instructions above for testing and meeting the criteria stated in CLSI M50-A Quality Control for Commercial Microbial Identification Systems:

(https://clsi.org/standards/products/microbiology/documents/m50/).

For the quality control of API® Coryne test system, refer to the package insert for the recommended controls and instructions. See the following link for details: https://www.mediray.co.nz/media/15787/om_biomerieux_test-kits_ot-20900_package_insert-20900.pdf

3.7 Minimal laboratory criteria for reporting a specimen as culture positive

Depending on the resources of the laboratory, the minimum time taken from the selection of colonies to selective media and determining toxigenicity is usually within 24-48 hours. The most widely used test for detecting toxigenicity is the Elek test (see **Chapter 4**), and results should be apparent within 24 hours. Therefore, in conjunction with a rapid test system based on, for example, PCR, confirmatory results should be available within 24 hours.

Once suspicious colonies have been confirmed as coryneforms by Gram stain, they are subcultured onto non-inhibitory blood media for the screening tests, biotyping and toxigenicity testing (usually by reference laboratories) and if necessary, microscopic morphology by Loeffler's methylene blue or Ponder's/Albert's stain.

The minimal laboratory criteria required to presumptively confirm an isolate as *C. diphtheriae, C. ulcerans* or *C. pseudotuberculosis* are as follows:

- catalase positive
- urea negative for *C. diphtheriae*, positive for *C. ulcerans* and *C. pseudotuberculosis*
- nitrate positive (except biovar belfanti, *C. ulcerans* and *C. pseudotuberculosis*)
- pyrazinamidase negative
- cystinase positive
- positive fermentation for glucose, ribose and maltose, biovar gravis and *C. ulcerans* are also glycogen positive

3.8 Laboratory data reporting

Upon isolation of a toxigenic strain of *C. diphtheriae*, *C. ulcerans* or *C. pseudotuberculosis* from a human, the following personnel must be informed immediately:

- the clinician responsible for the case
- the local public health physician
- the local consultant for the control of communicable diseases
- the national communicable disease surveillance unit

The case should be subsequently officially notified to the appropriate department/public health authorities according to the national notification system for diphtheria, and the appropriate public health actions executed.

CHAPTER 4 Phenotypic detection of toxigenicity: Elek test

4.1 Recognition and significance of non-toxigenic *C. diphtheriae*

Since mass immunisation and the decline of toxigenic isolates, non-toxigenic *C. diphtheriae* has been detected as a cause of both severe throat infections and invasive disease for over half a century in various countries. Recent invasive cases due to non-toxigenic *C. diphtheriae* have been reported in impoverished populations in Canada, injection drug users in Switzerland, Aborigines in Australia and homeless alcoholics in France (Funke *et al.* 1997; Gubler *et al.* 1998; Hogg *et al.* 1996; Romney *et al.* 2006, Lowe *et al.* 2011). Recently, cutaneous infections due to non-toxigenic *C. diphtheriae* have been found in African asylum seekers in Switzerland and Germany (Meinel *et al.* 2016). In the US, increased submissions to the CDC for confirmation and Elek testing have been observed since 2014 for domestic non-toxigenic *C. diphtheriae* isolates (**Appendix 7**) (personal communication, P Cassiday). Although most of these isolates are from wounds, isolates from respiratory sites and blood have also increased. This increase is likely due to hospital and public health laboratories using MALDI-TOF to rapidly identify isolates (**Chapter 5** and **Appendix 8**) found in mixed cultures. No such increase has been observed in the US for non-toxigenic *C. ulcerans* or *C. pseudotuberculosis*.

Since the advent of PCR (**Chapter 6 and Appendix 9**), some non-toxigenic strains have been shown to harbour the toxin gene, *tox*, without expressing diphtheria toxin (non-toxigenic, toxin gene bearing; NTTB). These NTTB strains have been reported in Russia, Lithuania, Canada and France (Bonmarin *et al.* 2009; Melnikov *et al.* 2000; Wagner *et al.* 2011). Toxigenic isolates bearing bacteriophages, which carry *tox*, can convert non-toxigenic, avirulent *C. diphtheriae* strains into toxigenic, highly virulent strains. In addition, a study of the *tox* gene in NTTB strains has revealed either deletion of one nucleotide, resulting in an open reading frame shift and formation of a stop codon, or the presence of an insertion sequence element, thus hypothesising that phage-conversion or DNA recombination could reactivate *tox* expression (Volozhantsev *et al.* 2004). Although not included in European, US and WHO case definitions, countries should record the incidence of non-toxigenic corynebacteria, in particular NTTBs, within their diphtheria surveillance frameworks.

4.2 Methodology to detect the diphtheria toxin: Elek test

Once an organism is biochemically identified as a possible *C. diphtheriae* or *C. ulcerans,* the isolate must be tested for the ability to produce diphtheria toxin. There are several *in vitro* methods available, but these are dependent upon the availability of resources and experience of laboratory staff. The method most commonly used for determining toxigenicity is the Elek immunoprecipitation test, which was improved to use a superior Elek medium and has considerably increased the clarity and accuracy of the test (Colman *et al.* 1992). This was further modified during the 1990s epidemic, to produce rapid results (16-24 hours) using only a few colonies from the primary isolation plate and reduced volumes of the specialised media (Engler *et al.* 1997).

4.3 Control strains and other quality recommendations

To aid standardisation of the test, the methodology and layout of the test strains against the three Elek controls (NCTC 10648, NCTC 3984, NCTC 10356) on both the conventional and modified Elek medium are described in **Appendix 7**.

Basal medium: The improved agar base medium (Colman *et al.* 1992) is recommended as the most suitable medium for the test. The medium must be clear to visualise even weak lines of precipitation. New batches of medium must be tested before use. The recommended storage temperature for the basal medium is six months at 4°C.

Serum: Newborn bovine and calf serum are recommended for the test (provided they are diphtheria *tox*-free). Equine serum should be avoided as this may produce cross-reactions with the same host-derived antitoxin (predominantly equine). However, the combination of the modified basal medium with the addition of newborn bovine serum produces optimal results. Each batch of serum should be checked and can be distributed into 3 ml amounts in sterile screw cap bottles and stored at –20°C. Sera stored in this way can remain stable for up to one year.

Antitoxin: Antitoxin is available from only a few sources worldwide, mostly from India. WHO has recently conducted an evaluation of some of the diphtheria antitoxin serums (DATS) available and some GMP inspection to manufacturers, however, there is no prequalification programme for these types of products. As a reference, WHO has supplied DAT in response to recent outbreaks in Bangladesh and Yemen from Premium Serums and Haffkine Bio-Pharmaceutical Corporation. See the link below from the UNICEF Supply Division for DATs.

https://www.unicef.org/supply/media/551/file/Diphtheria%20antitoxin%20(DAT)%20serum%20market%20update%20.pdf

Therefore, it is recommended that the supplier is contacted before stocks in the laboratory are low. To limit the effect of nonspecific precipitin lines, it is also important to test each antitoxin batch with Elek medium and newborn bovine serum, which has already passed a quality check. The recommended concentration is 500 IU/ml for incorporation into the antitoxin strips for the Elek test. The antitoxin is normally stored at 4°C.

CHAPTER 5 Automated identification systems

Automated identification systems have greatly increased the speed and accuracy of identifying *Corynebacterium* species, and allow for rapid and reliable initiation of effective therapy and public health response, including protective measures for health care workers and contacts. Here, several of the systems are described:

- MALDI-TOF MS
- VITEK® 2
- BD Phoenix™ System
- MicroSeq® Microbial Identification System

For all systems, manufacturer's instructions should be strictly adhered to for sample preparation, test performance and results/score interpretation. Quality control and maintenance of instruments must be followed as stipulated in user manuals.

Different MALDI-TOF MS systems for microbial identification have been developed, including:

- MALDI-TOF Biotyper® (Bruker Daltonik GmbH, Bremen, Germany)
- VITEK® MS (bioMèrieux, Marcy l'Etoile, France)
- Andromas™ system (Andromas SAS, Paris, France)

These identification systems differ mainly in the sample preparation procedures, the species coverage of the reference databases and the identification algorithm of the software (**Appendix 8**). Different sample preparation methods have been described, such as direct colony transfer, direct colony transfer-formic acid treatment and ethanol-formic acid tube extraction (Zasada *et al.* 2018).

5.1 MALDI-TOF MS

MALDI-TOF MS is a new technology for species identification based on the protein composition of microbial cells, which replaces the conventional phenotypic methods. Due to the ability to rapidly speciate a wide range of bacteria and fungi and their cost-effectiveness (excluding the cost of the MALDI-TOF instrument), it is increasingly becoming a routinely used laboratory tool for species identification (Clark *et al.* 2013).

5.1.1 MALDI-TOF Biotyper® and VITEK® MS

Among the available instruments, the two MS-based systems more frequently used are the MALDI-TOF Biotyper® and the VITEK® MS. The method allows rapid and reliable identification of clinically relevant and potentially toxigenic corynebacteria providing that a quality-controlled database of reference spectra is available (Konrad *et al.* 2010)

However, all the above methods only confirm the bacterial species, and not diphtheria toxin production. There are only limited studies evaluating the use of this tool for the identification of *Corynebacterium* species, and these studies have been done mostly using the MALDI-TOF Biotyper® system (**Appendix 8**) rather than VITEK® MS (Bao

et al. 2017, Billard-Pomares *et al.* 2017). MALDI-TOF cannot distinguish *C. diphtheriae sensu stricto* from *C. belfanti* (Dazas *et al.* 2018) or as *C. diphtheriae* subsp. *lausannense* (Taginin *et al.* 2018).

Both systems can successfully identify the species level of *Corynebacterium* species from clinical isolates but individually have their disadvantages. The MALDI-TOF Biotyper® cannot reliably differentiate between *Corynebacterium* species that cause urinary tract infections and erythrasma, but the VITEK® MS can. Whereas VITEK® MS cannot discriminate between *C. amycolatum* and *C. xerosis*, which can be clearly distinguished by the MALDI-TOF Biotyper®, both systems are problematic when identifying *C. afermentans* (Alibi *et al.*, 2017; Navas *et al.*, 2014). This should be considered when using these technologies alone for microorganism identification in a public health laboratory.

These systems cannot differentiate between biovars of *C. diphtheriae*. Although the systems have some limitations, they still can be used in clinical laboratories to detect clinically significant *Corynebacterium* species allowing for rapid and appropriate treatment for the infection. Continuous update of databases will further increase the usefulness of these rapid systems in identifying these species but in some cases not to biovar level.

5.1.2 Strain relatedness using the MALDI-TOF MS assay

The MALDI-TOF MS assay can be used as a tool for determining strain relatedness. This is particularly useful for the analysis of bacterial strains during outbreaks as it is a quick, non-laborious technique. This technique identifies bacterial isolates based upon unique protein profiles (Clark *et al.* 2013). A protein spectrum of a bacterial isolate is compared to those of reference strains on the database to identify the isolate and to determine strain relatedness. The mass protein peaks of each spectrum are compared, and a dendrogram is constructed.

5.1.3 Measuring and interpreting MALDI-TOF MS results

The manufacturers recommend a spectrum cut off score for the MALDI-TOF Biotyper®. Automated measurement and analysis of the raw spectral data are performed on a Microflex LT mass spectrometer (Bruker Daltonics) with a standard pattern-matching algorithm (BioTyper 2.0 Software). The most popular MALDI-TOF system for identifying *Corynebacterium* spp. use scores of ≥2.0 for the species level identification and ≥1.7 for the genus level identification. Scores below 1.7 are considered unreliable (Schulthess *et al.* 2014).

Resulting log (score) values:

- Above 2.0 for reliable identification on species level
- Between 1.7 and 2.0 for genus level
- Below 1.7 cannot be rated as valid according to the manufacturers' instructions.

5.2 VITEK® 2

The VITEK® 2 (bioMèrieux) is an automated microbiology system using growth-based technology. The system uses colorimetric reagent cards (ANC) that are inoculated, incubated and interpreted automatically. The ANC card helps to identify anaerobic bacteria and *Corynebacterium* species by using 64 wells with dehydrated media containing chromogenic substrates. The system library includes only eight *Corynebacterium* species. The generated laboratory report includes information on species identification level and may contain recommended supplementary tests to differentiate poorly discriminating isolates. Multicentre evaluations of the VITEK® 2 ANC card showed 95.1% correct identification, 4.9% low discrimination, 4.6% incorrect identification and 0.3% unidentified isolates with very good performance for corynebacteria, with only one strain of *C. urealyticum* misidentified from 51 *Corynebacterium* species isolates tested (Navas *et al.* 2014).

5.3 BD Phoenix™ system

The BD Phoenix™ system (Becton, Dickinson and Company, NJ, USA) is a fully automated system for the rapid identification and antimicrobial susceptibility testing (AST) of Gram-positive and Gram-negative bacteria. Similar to VITEK 2, the Phoenix system also uses colorimetric and fluorometric reactions and contains panels of dried biochemical substrates. The identification results are available within 3 hours, and most of the minimum inhibitory concentration (MIC) results are available within 6–10 hours. The identification database of the BD Phoenix™ system contains 15 *Corynebacterium* species.

5.4 MicroSEQ® microbial identification system

The MicroSEQ® microbial identification system (Applied Biosystems) is a genotypic identification system based on comparative rDNA sequencing of the 16S region. The system identifies bacteria in <24 hours and offers the option of routine bacterial identification using the first 527 bp of the rDNA, or higher resolution identification based on the full 1500 bp region. The MicroSEQ® library contains 50 *Corynebacterium* species.

Some of the automated identification systems currently include corynebacteria in their databases and, therefore, are not suitable for identifying this group of bacteria, for example, MicroScan Walk-Away® systems (Beckman Coulter, Brea, CA, USA) and FilmArray® (bioMèrieux, France). Ensure that the system used is able to identify corynebacteria

CHAPTER 6 Molecular methods confirming the presence of toxigenic *C. diphtheriae, C. ulcerans, C. pseudotuberculosis*

6.1 PCR for detecting diphtheria toxin gene

Since the early 1990s, conventional PCR has been used to detect the diphtheria toxin gene (*tox*), particularly the biologically active (Fragment A) portion (Pallen *et al.* 1994). In addition, the design of primers with specificity for different regions of this gene have been developed successfully and described in detail in the literature (Efstratiou *et al.* 1998; Hauser *et al.* 1993). Moreover, protocols have been developed for PCR detection of *tox* directly from clinical material (Nakao & Popovic 1997).

The methods described in **Appendix 9** are based on previously published primers and conditions (Pallen *et al.* 1994), while Hauser *et al.* or Sing *et al.* can be followed for the detection of both fragment A and B. (Hauser *et al.* 1993; Sing *et al.* 2011). These protocols can be used on simple boiled cell preparations or extracted DNA lysates followed by agarose gel electrophoresis to detect the amplicons.

With the development of real-time PCR, protocols have been created that allows a faster detection of *tox* than conventional PCR without further need for gel electrophoresis and UV detection steps. Moreover, this method negates the need for toxic ethidium bromide staining. Once DNA is extracted from suitable specimens or isolates and reaction mixtures are completed, PCR results are available within 60 – 90 minutes. The first real-time PCR for the detection of *tox* was published in 2002 (Mothershed *et al.,* 2002). In addition, real-time PCR showed increased sensitivity for detecting *tox* over previously described protocols for conventional PCRs.

The emergence of toxigenic *C. ulcerans* strains in patients with diphtheria-like illness prompted Sing *et al.* to sequence *tox* from *C. diphtheriae* and *C. ulcerans,* revealing differences between the *tox* sequences of these two species (Sing *et al.* 2003). As a result, the Mothershed *tox* real-time PCR did not reliably detect *tox* from some *C. ulcerans* strains (Cassiday *et al.* 2008), leading others to design PCR primers as well as hybridization probes from regions of the *tox* gene which were shown to be conserved at the sequence level from both *C. diphtheriae* and *C. ulcerans* (Sing *et al.* 2011; Badell *et al.* 2019; Schuhegger *et al.* 2008) (**Appendix 9**). De Zoysa *et al.* developed and validated a quadruplex real-time PCR for corynebacteria that has been used at PHE since 2014 (De Zoysa *et al.,* 2016). This assay improves real-time PCR by including an RNA polymerase β-subunit-encoding gene (*rpo*B) to specifically target *C. diphtheriae*, a second *rpo*B target for *C. ulcerans* and *C. pseudotuberculosis*, and a third target that identifies fragment A of *tox* from any of the three species. This method also includes an internal process control and has been optimised for the Qiagen Rotor-Gene Q platform (Qiagen GmbH, Germany) (**Appendix A9.3.3** and **Table A9**). Other multiplex assays have also been described for the identification and molecular discrimination of toxigenic and non-toxigenic *C. diphtheriae* and *C. ulcerans* using *tox*, *rpo*B and *dtx*R as gene targets (Mancini *et al.* 2012; Badell *et al.* 2019; Pimenta *et al.* 2008). More recently a triplex assay was described by Williams *et al.* (2020) as an effective diphtheria diagnostic tool that can rapidly screen isolates and clinical specimens for the three potentially toxigenic species.

6.2 PCR in the context of an outbreak investigation

Due to the difficulty in obtaining the specialised media and reagents required for the Elek test, PCR for the *tox* gene is a rapid diagnostic alternative but must be used in conjunction with a phenotypic test for toxin expression (see also WHO Surveillance Standards).

One way to conserve reagents needed for Elek testing is to use PCR to 'triage' strains, and test only *tox* PCR positive isolates by the Elek test to confirm diphtheria toxin production. However, it must be emphasised that although PCR detection of *tox* from a clinical specimen provides supportive evidence for diagnosing diphtheria, some isolates detected during a diphtheria outbreak in Russia and Ukraine were non-toxigenic toxin gene bearing NTTBs (Melnikov *et al.* 2000). Such isolates are non-toxigenic by Elek or other phenotypic testing. In addition, since *C*. *ulcerans* and *C. pseudotuberculosis* are also capable of harbouring *tox*, PCR detection of *tox* alone will not identify which organism is present. In contrast, a negative *tox* PCR result is useful for rapidly excluding toxigenicity and preventing unnecessary control measures. Until more is known about the biological, clinical and epidemiological significance of NTTB strains, a patient should be regarded as a probable diphtheria case if the PCR result is *tox* positive, but the organism has not been isolated, a histopathologic diagnosis has not been made, and there is no epidemiological link to a laboratory-confirmed case.

CHAPTER 7 Molecular typing and gene sequencing

7.1 Molecular typing of *Corynebacterium* species

The epidemiological typing of the pathogenic bacteria will help in better understanding the pathogen transmission dynamics during an outbreak situation. MLST is advantageous over other methods like ribotyping and PFGE in terms of simplicity and portability. MLST investigates genetic diversity by analysing the nucleotide variation (SNP) within the seven or more housekeeping genes, thereby providing efficient and high-resolution data suitable for epidemiological and surveillance studies. MLST enables the analysis of sequence types and clonal complexes of the organism and helps in the understanding of a specific clone that is widely spreading in the region or during the outbreak. Further whole-genome sequencing (WGS) can be useful to extend the knowledge on the molecular epidemiology of diphtheria and to predict the evolutionary relationships among the strains and to infer the global relatedness of the pathogen.

7.1.1 Multilocus sequence typing

The *C.diphtheriae* MLST scheme was developed by Bolt *et al.* (2010). The method uses the nucleotide sequence information from the internal fragments of the following seven housekeeping genes to define the sequence type (ST) for each isolate:

- ATP synthase alpha chain (*atpA*)
- DNA polymerase III alpha subunit (*dnaE*)
- Chaperone protein (*dnaK*)
- Elongation factor G (*fusA*)
- 2-isopropylmalate synthase (*leuA*)
- 2-oxoglutarate dehydrogenase E1 and E2 components (*odhA*)
- DNA-directed RNA polymerase beta chain (*rpoB*)

The details of the MLST scheme for *C. diphtheriae* are available in the PubMLST database (https://pubmlst.org/organisms/corynebacterium-diphtheriae/) and **Appendix 10**. Briefly, genomic DNA is extracted, each allele is amplified by PCR using the primers described in **Appendix Table A15**, and the resulting amplicons are visualised and checked for purity via electrophoresis on an agarose gel. The PCR-amplified DNA fragments of seven housekeeping genes are purified, and the DNA fragments on each strand sequenced by ABI 3500 Genetic Analyser (Applied Biosystems, USA) using the sequencing primers of the ABI PRISM® BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA). The allele profiles, sequence types (ST) and clonal complexes are assigned from the PubMLST database (https://pubmlst.org/bigsdb?db=pubmlst_cdiphtheriae_seqdef). Alleles and STs that have not been previously described should be submitted to the PubMLST database and assigned new allele numbers and STs. Furthermore, goeBURST analysis can be done for detailed population structure analysis (http://www.phyloviz.net/).

7.1.1.1 Multilocus sequence typing of *C. ulcerans*

As toxigenic *C. ulcerans* has gained greater importance as a diphtheria-causing pathogen (König *et al.,* 2014), a separate MLST scheme for *C. ulcerans* has been proposed by König and colleagues. Primers for *atpA*, *dnaA*, *fusA*, *odhA* and *rpoB* are identical to *C. diphtheriae,* (Bolt *et al.*, 2010). The primers used for *dnaK* and *leuA* were adapted to *C. ulcerans* according to the genome of *C. ulcerans* 809 (König *et al.,* 2014). Locus amplification and sequencing for MLST analysis are done based on the published scheme for *C. diphtheriae* with minor modifications. Each PCR is carried out in a 50 µl total volume using HotStarTaq® Master Mix kit (Qiagen).

7.1.2 goeBURST analysis

The cluster analysis of the isolates can be performed using PHYLOViZ 1.1 software (Francisco *et al.* 2012), freely available at http://www.phyloviz.net. The methods provide reproducible and comparable results needed for a global scale bacterial population analysis, in addition to their usefulness for local epidemiological surveys. The software is available as a desktop JAVA application and also as an online application. The tool allows the analysis of sequence-based typing methods that generate allelic profiles and their associated epidemiological data. The results can be displayed as an annotated graph overlaying the query results of any other epidemiological data available. PHYLOViZ uses the goeBURST algorithm, a modification of eBURST algorithm published earlier by Feil *et al.,* 2004. A complete tutorial for PHYLOViZ and a description of its features is available at http://www.phyloviz.net/wiki/tutorial.

7.1.3 Locus variant analysis

In recent years, the use of nucleotide sequence variation at multiple housekeeping loci has become increasingly popular for strain characterisation, as it has advantages for inferring levels of relatedness between strains and the reconstruction of evolutionary events.

In terms of MLST, descendants of the founding genotype will initially remain unchanged in allelic profile, but over time variants in which one of the seven alleles has changed (by point mutation or recombination) will arise. These genotypes, which have allelic profiles that differ from that of the founder at only one of the seven MLST loci, are called single-locus variants (SLVs). Eventually, SLVs will diversify further to produce variants that differ at two of the seven loci (double-locus variants [DLVs]), at three of the loci (triple-locus variants [TLVs]), and so on (Feil *et al.,* 2004). Examples of these variants are shown in **Table 4**.

Table 4. Example of single, double and triple-locus variant analysis with 7 MLST loci of *C. diphtheriae*

Strain	*atpA*	*dnaE*	*dnaK*	*fusA*	*leuA*	*odhA*	*rpoB*	ST	
1	2	10	3	1	7	3	2	ST301	
2	2	10	3	1	3	3	2	ST574	SLV of ST301
3	4	10	3	1	7	3	13	ST469	DLV of ST301
4	2	4	4	1	3	3	5	ST5	TLV of ST301

* The colour represents the variants in the allelic profile of the given sequence types (ST).

There are increasing reports of novel STs in diphtheria endemic regions. However, SNP analysis revealed that these novel STs are SLV or DLV of the existing STs, which indicates that this analysis method could help us to understand the evolution of new clones and spread of the existing clones.

7.2 Gene sequencing for identifying *Corynebacterium* species

To date, the genus *Corynebacterium* consists of >115 species isolated from human clinical and veterinary specimens, environmental samples, saline soil or the surface of smear-ripened cheese (https://lpsn.dsmz.de/genus/corynebacterium accessed 6 May 2020). Among this highly diverse group, both virulent pathogens and harmless commensals are found. Therefore, in clinical diagnosis, a reliable and fast method for determining accurate species is crucial. Sequencing of ribosomal genes (16S or 23S) provides a good tool for determining most of these species (Grimont, 1986).

However, users must be aware that some *Corynebacterium spp*. cannot be resolved by this method alone, as they are separated by <0.8% identity when compared with almost complete 16S rRNA gene sequences. These include: *C. ulcerans* and *C. pseudotuberculosis; C. afermentans, C. coyleae, C. mucifaciens* and *C. ureicelerivorans; C. aurimucosum, C. minutissimum*, and *C. singulare*; *C. sundsvallense* and *C. thomssenii; C. propinquum* and *C. pseudodiphtheriticum* (<2%); *C. xerosis, C. freneyi*, and *C. hansenii; C. macginleyi* and *C. accolens*. Resolution among these species can be done by sequencing the *rpoB* gene. Unfortunately, the 16S rRNA genes of corynebacteria show very little polymorphism; therefore, sequencing of the complete 16S gene (about 1500 bp) is necessary (Khamis *et al.* 2005). Khamis and colleagues found that a 432-452 bp fragment of *rpoB* showed sufficient discriminatory power to differentiate among *Corynebacterium spp*., with the caveat that not all species have *rpoB* sequences in public domain websites (Khamis *et al.* 2004 & 2005).

7.3 Novel advances in genomics and proteomics

The application of next-generation DNA sequencing (NGS) technologies has provided detailed insights into the genomics of corynebacteria and a greater understanding of how and why epidemic clones emerge or disappear and contribute towards preventing and managing these devastating infections.

With the triumph and the increasing affordability of mass applicable sequencing, sequencing-based methods are being explored as fast and cheap alternative typing methods and have shown considerable portability, reproducibility and discrimination. Furthermore, several genomics of *Corynebacterium spp.* are now available, which can be explored and evaluated regarding new and potentially more discriminative targets (Barh *et al.* 2011; Trost *et al.* 2010; Chorlton *et al.* 2020). This approach is being used in some national centres. Recently, NGS has been applied to both outbreaks of *C. diphtheriae* (du Plessis *et al.* 2017) and *C. ulcerans* (Meinel *et al.* 2015).

NGS is increasingly being used to explore outbreaks and transmission dynamics for these organisms (Dangel *et al.* 2019). Genomic sequencing provides a unique opportunity to explore the evolutionary drift of these organisms and should elucidate the diversity of bacteriophage insertion and associated virulence factors. *In silico* analysis has already revealed several unbiased novel targets, which have the potential to demonstrate adequate variation for a sequencing-based scheme. Genomic data of *C. diphtheriae* strains demonstrated that most of these targets were suitable for further evaluation, exhibiting between 2 and 16 variants. Therefore, sequencing-based typing methods have the advantage of not only being highly reproducible but can also be used to explore the evolutionary relationships underpinning the epidemiology.

7.3.1 Whole-genome sequencing

Generally, variation between bacterial genomes of the same species occurs for various reasons, including point mutations, homologous recombination and differences in genome content. Point mutations comprise single nucleotide polymorphisms (SNPs) and single nucleotide insertions or deletions that can vary widely depending on the species (Schurch *et al.* 2018). SNPs are the most common and simplest form of DNA variation and are an important driver of bacterial evolution and expansion.

SNPs are the most common type of variation to occur. This is the variation of a single nucleotide (adenine, cytosine, guanine or thymine) in a genetic sequence and may result in subtle changes within the genome. Their accumulation results in the majority of diversity amongst genomes (Gouy & Gautier, 1982). Due to the degeneracy in the amino acid code, the majority of SNPs are synonymous (“silent”) mutations and do not result in a change in the functionality of the gene expression. However, non-synonymous mutations lead to a change in the amino acid and hence potentially alter the gene or protein expression. The ratio of the number of non-synonymous nucleotide changes per non-synonymous site (d*N*) and the number of synonymous changes per synonymous site (d*S*) is often used to determine the rate of evolution in, or between, organisms. Therefore, SNPs can be used as a stable signal for disseminating a particular strain. This use is extended to population genetics for estimating genetic variation, identification of relatedness or parentage, measuring population structure and changes in population size over time (Morin *et al.* 2004).

Recently, whole genome sequencing has allowed for the development of a typing scheme known as core genome MLST (cgMLST) and is currently being used in several outbreak investigations hospitals to decipher information on the relatedness of the isolates through SNP based phylogeny. The method demonstrates good typing ability by extending the traditional MLST concept to the entire genome. This provides additional higher resolution information on genetic diversity of the species and highlights that cgMLST can probably become the gold standard for strain subtyping in epidemiological investigations (Venditti *et al.* 2018). Besides, pangenome analysis showed better discrimination within the strains compared to the separate analysis of core or accessory genome of the species due to the often-changing nature of the accessory genome. Pangenome represents all genes, whether constant or variable that are found in members of a species. Several studies emphasize the utility of WGS in understanding the evolution and pathogenicity of different *C. diphtheriae* strains (Sangal & Hoskisson, 2016).

7.3.2 Transcriptomics

The genomic approach identifies the DNA sequence of a certain organism, though this knowledge alone does not define the gene function to external stimuli. Genes are not active all the time and are expressed when necessary to act in cellular biological processes. The set of genes that are expressed in a cell under a certain physiological condition or stage of development at a specific time is called the transcriptome. Transcriptome studies aim to analyse the collection of all transcripts and provide information on the regulation of genes and may be used to infer the functions of uncharacterised genes. One of the applications of this approach is to provide information about the host defence response to the survival and proliferation of bacterial pathogens. Diverse application of transcriptomics includes microarray and RNA sequencing (Lowe *et al.* 2017).

Although the *C. diphtheriae* genome was sequenced more than a decade ago, not much is known about its transcriptome. RNA sequencing is considered an ideal tool for the analysis of complete transcriptomes and is applied in the exploration of expression profile, and characterisation of differentially expressed genes. Thus, it represents an important tool to uncover the mechanisms of virulence and pathogenicity in microorganisms.

RNA sequencing of *C. diphtheriae* investigated the alteration of the transcription profile between a wild type strain and a ΔdtxR mutant, and also detected the operon structures from the transcriptome data of the wild-type strain. Approximately 15% of the genome was differentially transcribed and findings suggest that dtxR may also play a role in other regulatory functions, in addition to regulating iron and diphtheria toxin metabolism (Wittchen *et al.,* 2018).

CHAPTER 8 Procedures for serological testing to assess individual and population immunity/susceptibility to diphtheria

Immunity against diphtheria is antibody-mediated, and as diphtheria morbidity is almost entirely due to diphtheria toxin, protection against disease is dependent on antibodies against the toxin. Since serum antibody titre against pathogens, including toxins known to rise during infection, measuring serum antibody titre is sometimes useful for laboratory diagnosis of diphtheria but is not a recommended criterion for confirmation.

8.1 Procedures for assaying diphtheria antitoxin

The earliest methods for measuring serum antitoxin levels were originally developed by Behring, Ehrlich and Roux (1892-1895) and used guinea pigs as a sensitive detection system for the titration of diphtheria toxin and determining the neutralizing capacity of serum antitoxin. The method in guinea pigs is still indicated in the European Pharmacopoeia to determine the level of antitoxic globulins in horse or other mammals' immune sera for immunotherapy. Alternative tests using cultured Vero cells (*in vitro* TNT) have been developed as reliable alternatives to the *in vivo* TNT (Dular 1993; Kriz *et al.* 1974; Melville-Smith & Balfour 1988; Miyamura *et al.* 1974a; Miyamura *et al.* 1974b). Vero cells have been identified as a suitable model for the specific detection of functional diphtheria antibodies both in human and animal sera (Aggerbeck & Heron 1991; Miyamura *et al.* 1974b; Gupta *et al.*1994).

Diphtheria toxin-sensitive cell line, such as Vero cells (**Appendix 12**), are grown on multi-well tissue culture plates and incubated with a mixture of fixed pre-determined concentration of diphtheria toxin and a graded concentration of test serum and reference diphtheria antitoxin of known neutralizing activity in International Units (IU). The endpoint is taken as the lowest concentration of test and reference antitoxin, which is able to protect Vero cells from cytotoxic effect of diphtheria toxin, determined by adding a chemical dye that can differentiate visually between live and dead cells. Concentration of test serum sample is calculated relative to a reference standard and expressed in IU/ml. Each test must include positive and negative controls to be valid (Begg & WHO, 1994).

Because *in vitro* TNT is relatively time-consuming and requires specialized tissue culture facilities, it is not well suited for individual or population immunity screening, particularly in clinical laboratories. Instead, several immunoassay methods have been developed for the routine measurement of diphtheria antitoxin levels in human sera (Kristiansen *et al.* 1997). These include ELISA and multiplex immunoassay (MIA), which are described below and modified ELISA methods, such as double-antigen enzyme-linked immunosorbent assay (DAE) (Aggerbeck & Heron 1991), dDA-DELFIA (Aggerbeck *et al.* 1996; Bonin *et al.* 1999) and ToBI (Hendriksen *et al.* 1989). There are several commercial assay kits available for diphtheria serology, mostly based on a direct ELISA format and these are frequently included in external quality assessment schemes for diphtheria antibody testing (Di Giovine *et al.* 2010; Von Hunolstein *et al.* 2014).

Measuring serum antibody titre also gives critical information for estimating population immunity to diphtheria. Widely adopted criteria for the immune status of an individual have been established and described in the WHO position paper (2017) (see **Table 5**).

However, as the immunoassay methods are surrogate models for *in vivo* and *in vitro* TNT and validation studies are often limited, assigning protective levels for diphtheria antitoxin in the population using these methods could be problematic. Validation using well-defined standardized reagents is essential for assuring accuracy and reproducibility of assays. Immunoassays for diphtheria should be appropriately validated and standardized with titres reported in IU. The use of an international standard preparation or an appropriately calibrated secondary reference preparation is required for determining diphtheria antibody levels and is important to compare data obtained from different clinical trials and population immunity studies. An International Standard for Diphtheria Antitoxin, Human (NIBSC product code 10/262) was established by the WHO in 2012 and is suitable for calibrating diphtheria immunoassays. The standard was shown to be commutable with human serum samples in commonly used immunoassays (Stickings *et al.* 2013).

Table 5. Antitoxin levels and immunity to diphtheria: interpretation guidelines refer to antibody levels determined using a functional assay.

Antitoxin level	Interpretation
<0.01 IU/ml	Individual is susceptible
0.01 IU/ml	Lowest level of circulating antitoxin giving some degree of protection
0.01 – 0.09 IU/ml	Level of circulating antitoxin giving some degree of protection
0.1 IU/ml	A protective level of circulating antitoxin
≥0.1 IU/ml	A level of circulating antitoxin giving long-term protection

8.2 ELISA assays

ELISA and EIA are biochemical techniques used in immunology for determining serum antibody concentrations. In contrast to the *in vivo* or *in vitro* TNT, ELISA can be used specifically to measure IgM or IgG antibodies and will detect total antibody levels (i.e. not only the functional antibodies that neutralize the diphtheria toxin). ELISA has been developed as an in-house assay, but also some available commercial kits can be used.

8.2.1 Performance characteristics

ELISA offers significant advantages in terms of cost, speed, ease of use and adaptability to automation. In addition, the amount of serum sample required for the test is low since samples are typically diluted prior to testing. Nevertheless, there are potential disadvantages since it measures total binding (i.e. functional and some non-functional) antibodies and therefore does not always correlate well with the *in vitro* TNT (Melville-Smith & Balfour 1988; Walory *et al.* 2000), particularly at lower antibody titres. Previous studies showed that the correlation between ELISA and *in vitro* TNT expressed as Pearson's correlation coefficient was about R=0.81, but for sera with an

antibody titre below 0.1 IU/ml the correlation coefficient was around R=0.5 (Walory *et al.* 2000). The use of different protocols, reagents or commercial kits impact on the performance characteristics of this method (von Hunolstein *et al.* 2014). The precision of the method for sera within the range (0.1-1.0 IU/ml) as measured by the coefficient of variation is about 10%. This imprecision increases considerably when antibody levels are outside this range (Walory *et al.* 2000). The diagnostic accuracy of ELISA tests in comparison to *in vitro* TNT, including the commercial kits, is limited because some of them work with a breakpoint titre of 0.1 IU/ml, without discriminating between equivocal (weakly protective) and negative sera (no protective sera). Sensitivity and specificity vary widely between different ELISA kits or in-house methods, and many authors reported the occurrence of false positive as well as false negative *(*Skogen *et al.* 1999; Walory *et al.* 2000). Antibody levels below 0.1 IU/ml in the *in vitro* TNT, showed 2–20 times higher antibody values in ELISA (Walory *et al.* 2000; von Hunolstein *et al.* 2014). This is likely to be because the ELISA system appears to detect low levels of specific IgG that is unable to neutralise diphtheria toxin in the TNT. ELISA could be used for screening purposes, but samples with antibody levels below 0.1 IU/ml should ideally be re-determined by *in vitro* TNT to ascertain if the sample is likely to provide weak or no protection. Moreover, setting a grey zone could be good practice, ideally between 0.1-0.15 IU/ml, and re-determine these sera by *in vitro* TNT (Budd *et al.* 2004).

8.2.2 Bead-based multiplex assay or multiplex immunoassay

The Luminex technology using fluorescent distinct microspheres as a carrier for different antigens enables the detection of multiple analytes in one single serum sample. Several studies have demonstrated the ability to effectively multiplex a range of assays, including antibody detection and quantification of vaccination samples (Lal *et al.* 2005; Pickering *et al.* 2007; Caboré *et al.* 2016). This technology has been used to develop a rapid and reproducible assay for the simultaneous determination of serum antibodies against three different antigens of *B. pertussis* (pertussis toxin, filamentous haemaglutinin and pertactin) and diphtheria and tetanus toxins (van Gageldonk *et al.* 2008; Caboré *et al.* 2016; Sonobe *et al.* 2007). It uses far less serum than what would be used if measurements for different analytes were made independently. In this pentaplex immunoassay, purified antigens are coupled to activated carboxylated microspheres by using a two-step carbodiimide reaction. For diphtheria, the performance of the MIA was shown to improve when diphtheria toxoid, rather than toxin was used as the antigen (van Gageldonk *et al.* 2011).

The use of a multiplex assay offers significant advantages compared to conventional techniques in terms of speed, and economy of sample and antigen used.

CHAPTER 9 Procedures for antimicrobial susceptibility testing of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*

9.1 Purpose of antimicrobial susceptibility testing

The management and treatment of any suspected diphtheria case is the prompt administration of diphtheria antitoxin, which neutralises any unbound toxin. The antitoxin should be administered before laboratory confirmation. Appropriate antibiotics, such as penicillin or erythromycin will aid in speeding up the successful eradication of the organisms from the respiratory tract, thus decreasing the toxin burden in the patient, as well as preventing and/or limiting further spread of the organism to contacts. In countries where contact tracing is undertaken, contacts of a definitive case are investigated by public health services and prophylactically given a macrolide (mostly erythromycin) or other efficacious antibiotics to limit the spread of infection (Perkins *et al.* 2010).

We highly recommend that AST be carried out on:

- **all** *C. diphtheriae* isolates (irrespective of toxin production)
- **all** *C. diphtheriae* isolates (cases and carriers)
- **all** clinically significant strains of *C. ulcerans* and *C. pseudotuberculosis*, as these can also on occasion cause serious disease and even death, among at risk patient populations, such as the impoverished, homeless, alcoholics or injecting drug users (Gruner *et al.* 1994; Harnisch *et al.* 1989; Lowe *et al.* 2011; Pedersen *et al.* 1977; Romney *et al.* 2006).
- **clinically relevant strains** of *C. jeikeium, C. amycolatum* or other species isolated from body sites, many of which can be multidrug-resistant.

9.2 Review of common treatment choices and *in vitro* susceptibility of *C. diphtheriae*

Despite infections caused by potentially toxigenic corynebacteria having been described for many years, the actual numbers of publications describing AST data, specifically for *C. diphtheriae*, and particularly in the last decade, are sparse. Penicillin and erythromycin have historically always been recommended for use in treatment of suspected/cases of diphtheria.

In 1971, *C. diphtheriae* strains were found to be susceptible to commonly used drug classes tested, particularly those used for acute care treatment, namely penicillin and erythromycin (McLaughlin *et al.* 1971). However, in one recent study of 195 *C. diphtheriae* isolates, nearly 17% of strains were non-susceptible to erythromycin, and a significant number showed intermediate or resistant MICs to one or more cephalosporins as well as to other drug classes. Subsequently, susceptibility to all drug classes tested has been described by investigators from different countries (von

Hunolstein *et al.* 2003; Zamiri & McEntegart 1972). Intermediate MIC ranges have been observed for ciprofloxacin and cefotaxime (Zasada *et al.* 2010), ceftriaxone (Bernard *et al.* 2015); and first-generation cephalosporins (Patey *et al.* 1995). Reports of resistance have been described for tetracyclines (Funke *et al.* 1999; Kneen *et al.* 1998), erythromycin (Kneen *et al.* 1998) and to combinations of erythromycin and tetracycline, tetracycline and choramphenicol, and one strain to three antibiotics , erythromycin, tetracycline and chloramphenicol (Kneen *et al.* 1998). In Canada, strains have been observed with intermediate MIC ranges to ciprofloxacin alone, or resistant to tetracycline and trimethoprim/sulfamethoxozole together or alone (Bernard and Funke, 2015). A strain recovered from a cutaneous infection in a Canadian male who had previously travelled to India was found to be resistant to four antibiotics (chloramphenicol, erythromycin, clindamycin and trimethoprim-sulfamethoxazole) (Mina *et al.* 2011). Resistance to erythromycin and clindamycin/lincomycin, first described in Canada in 1973 (Jellard & Lipinski 1973) is increasingly being observed (Bernard and Funke, 2015; Kneen *et al.* 1998; Patey *et al.* 1995), maybe inducible (Coyle *et al.* 1979) and has been associated with the presence of a plasmid-borne *erm*X gene (Roberts 2008).

A descriptive study undertaken during outbreaks in Indonesia evaluated the first-line antibiotic susceptibility patterns of *C. diphtheriae* isolates (Husada *et al.* 2019). Sensitivity by E-test to five antibiotics (penicillin, oxacillin, erythromycin, azithromycin and clarithromycin) was undertaken using CLSI (2015) standards. Their susceptibility to erythromycin was considerably higher than that to penicillin, and their overall recommendation was that there should be a regular update of antibiotic selection to the national guidelines.

Increasing multidrug-resistant corynebacteria are challenges in many countries (Mina *et al.* 2011; Pereira *et al.* 2008), such as increases in clindamycin resistance detected in *C. ulcerans* in Germany (data from the German Consiliary Laboratory on Diphtheria, personal communication, Berger and Sing). Therefore, AST is highly recommended, particularly if clinically, macrolides are to be used *in lieu* of penicillin.

9.3 Methods for antimicrobial susceptibility testing of *Corynebacterium* species

Currently, two guidelines are available for testing and interpreting results:

- The Clinical and Laboratory Standards Institute (CLSI) M45 2015 guideline does not have data/methods for testing *Corynebacterium* species by disk diffusion methods but instead recommends broth microdilution as the gold standard method (see Weiss *et al.* 1996).
- The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has developed zone diameters for the standardized disk diffusion testing (Leclercq *et al.* 2013) and breakpoints for interpreting MICs using broth microdilution for *Corynebacterium* spp. including *C. diphtheriae*.

(http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf)

Although six clinically relevant antimicrobial classes are listed (**Table 6**), zone diameters or MICs for testing erythromycin as one of the most relevant antimicrobials is still lacking. Breakpoints for corynebacteria were developed by EUCAST for species other than *C. diphtheriae*. In an ongoing study, the preliminary results indicate that the current breakpoints for benzylpenicillin and rifampicin are not useful for *C. diphtheriae*.

Table 6. CLSI and EUCAST recommended antimicrobial agents and interpretation of results for testing *C. diphtheriae, C. ulcerans, C. pseudotuberculosis* and other *Corynebacterium* species by disk diffusion and MIC.

Antimicrobial class	Antimicrobial agent	Disc content (ug)	Zone diameter (mm) and breakpoint (EUCAST)	MIC value and breakpoint (CLSI)	MIC value and breakpoint (CLSI)
Penicillins	Penicillin	-	-	-	S ≤0.12 I=0.25-2 R ≥4
Macrolides	Erythromycin	-	-	-	S≤0.5 I=1 R ≥2
Lincosamides	Clindamycin	2	R <20 S ≥20	S≤0.5 R>0.5	S≤0.5 I=1-2 R ≥4
Ansamycins	Rifampicin	5	R <25 S ≥30	S≤0.06 R>0.5	S≤1 I=2 R ≥4
Oxazoldinones	Linezolid	10	R <25 S ≥25	S≤2 R>2	S≤2
Tetracyclines	Tetracycline	30	R <25 S ≥25	S≤2 R>2	S≤4 I=8 R ≥16

Definitions of SIR:

Susceptible (S) isolates are inhibited by the usually achievable concentrations of the antimicrobial agent and infection is expected to respond when the recommended dosage is used for the site of infection.

Intermediate (I) isolates have antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates with normal recommended doses implying clinical efficacy.

Resistant (R) isolates are not inhibited by the usually achievable concentrations of the agent and/or that demonstrate zone diameters that fall in the range where specific microbial resistance mechanisms (*e.g.*, β-lactamases) are likely and infection is not expected to respond to treatment with highest recommended doses.

CHAPTER 10 Quality management

It is strongly recommended that tests undertaken within clinical diagnostic laboratories should be assured by a comprehensive quality assurance scheme, which should be subject to third party accreditation to the internationally recognised (ISO/IEC 15189, ISO 17025, ISO9001, CAP) standard for laboratory competence.

10.1 Basis of laboratory quality assurance

Laboratory Quality Assurance (LQA) is the total process whereby the quality of laboratory results, from specimen collection to analysis of tests and reporting of results, can be guaranteed and enable clinical diagnosis and effective treatment. LQA helps define the procedures, documents and controls used to enhance the quality and integrity of the activities and the final results generated. Components of QA include good laboratory practice, internal quality control, audit, validation, internal quality assessment (IQA), accreditation, evaluation, education and external quality assessment (EQA).

Objectives of a well-organised LQA system include:

- Preventing risks
- Detecting deviations
- Correcting and preventing further errors
- Improving efficiency
- Ensuring data quality and integrity

Most European laboratories must comply with accreditation or certification standards and regulations through third party assessments (regulating authorities and accreditation bodies) for national recognition and status. The responsibility ultimately lies with the head or chief of the laboratory to establish, implement and ensure compliance with LQA, although all laboratory personnel should understand and adhere to most aspects of the LQA.

Elements that constitute a successful QA system are described below.

10.2 Staff and staffing levels

All diagnostic and reference laboratories for diphtheria should have staff who are appropriately qualified, trained and experienced to perform the tasks safely and accurately and to ensure good quality results are reported. A clear organogram should be prepared to illustrate the hierarchy and lines of responsibility and ideally should include the director or chief of the laboratory, the head of each unit if appropriate (e.g., identification and toxigenicity testing, serology, molecular typing), the quality manager, scientific and technical staff, and auxiliary and administrative support. Each post should have a job description describing their roles and responsibilities, with academic training, skills and experience required for each post.

In addition, staff who routinely handle cultures of potentially toxigenic corynebacteria, should be fully vaccinated (including booster vaccinations) according to the respective national immunisation guidelines.

Staffing levels should be adequate to perform all services of a diphtheria laboratory without compromising safety or the integrity of the results generated. There should be at least one person with a minimum of 12 months experience in the specialised techniques used and the services provided. To cover staff absence and build capacity and competency, at least one other person should work alongside the experienced person to gain experience training and understanding. In addition, new staff should receive extensive training, and all staff should be encouraged to attend both internal and external training courses, according to the needs of the staff and the laboratory. Such training and test witnessing should be documented to record the skills gained as part of the staffs' continuing personal development and education.

10.3 Space allocation

A dedicated diphtheria laboratory should have adequate space to safely perform all activities and is usually within a state or government centre or institute, which is recognised by the country's Ministry of Health, or equivalent. Therefore, there should be sufficient infrastructure and management to allow enough rooms to separate tasks into infectious from non-infectious work. Space permitting, specific area and preferably specific rooms should be designated for:

- Reagents and consumables storage
- Washing, preparation and sterilisation
- Specimen receipt and recording
- Bacteriology activities (e.g., identification, toxigenicity testing, DNA extraction)
- Serological activities (including cell culture for Vero cell assay)
- Specialised activities (e.g., unidirectional pathway for PCR preparation, PCR amplification, gel electrophoresis)
- Documentation, archiving and control
- The administrative area

Some of these can be shared within other departments of the centre or institute for cost-effectiveness, as long as it is safe to do so and does not jeopardise the health of other staff employed, or cross-contaminate PCR products/master mixes/reagents.

General characteristics that the laboratory should comply with are:

- Adequate lighting and ventilation appropriate for the activities performed. The workbench surfaces should be smooth, easy to clean and resistant to chemicals
- Safety systems to combat fire, electrical emergencies, biological and/or chemical spillages and other potential local events
- Appropriate personal protective equipment

- Sustainable hot and cold water, and electricity to cover adequate use for the duration of the activities. This should be sufficient for essential equipment such as incubators, biological safety cabinets, freezers, etc. Desirably, a standby generator is available, especially if the power supply is erratic
- Adequate space to store both supplies for immediate use and long-term storage away from active working areas
- Hand washbasins, with running water, should be provided in each laboratory room, preferably located near the door
- An autoclave within the same building as the laboratory
- Facilities for storage of outer garments and personal items, and for eating and drinking, outside the working areas
- When installing equipment, biosafety and other safety standards should be adhered to

10.4 Standard Operating Procedures

Standard Operating Procedures (SOPs) describe in detail the activities performed in the laboratory to:

- Provide uniformity, consistency and reliability in each of the activities performed in the laboratory
- Reduce systematic errors
- Provide guidance for staff new to learning the procedures

They should be written by specialised staff, reviewed by those competent in the procedure, read and acknowledged by the relevant staff and approved by the laboratory manager or director. To aid clarity, SOPs can be divided into sequentially numbered sections and subdivided as appropriate; a common format consists of:

- Title of procedure
- Code or SOP number
- The author and authoriser of the SOP
- An ‘effective from’ and ‘review’ date
- A summary or brief description of the procedure
- A list of related safety documents, including MSDS, COSHH and risk assessments and other cross-referenced SOPs
- Materials, reagents and equipment used for the procedure
- A chronological description of method, written to be understood to those with and without experience
- Expected results and the recommended interpretations
- Any quality control or assurance set up for the procedure
- An acknowledgement form indicating the SOP has been signed and understood

SOP's should be clear and concise, as this will encourage staff to read, understand and comply with procedures. Any changes to SOPs should be implemented and finalised in the same manner as a new SOP.

10.5 Documentation and equipment

This applies to the set of quality manuals, SOPs, forms, reports and record of data that serve as evidence of LQA and permit the traceability of data. The laboratory should have the necessary equipment for the accurate performance of all tests performed and monitored where required (e.g., incubator temperatures, airflow of safety cabinets).

10.6 Reference materials and reagents

The control strains of *Corynebacterium* species recommended for the phenotypic tests are those used in the UK and may be obtained from the National Collection of Type Cultures website (http://www.phe-culturecollections.org.uk/, last accessed 1 June 2021). For a reference laboratory specialising in diphtheria diagnosis, control strains are recommended to be subcultured every seven days and stored at 6-8°C.

The stock controls and other cultures of *C. diphtheriae* and other potentially toxigenic corynebacteria are maintained in 16% (v/v) glycerol broth and stored at –20°C or –70°C (**Appendix A3.1.1**). Clinical laboratory practice is to take a fresh bead from the stock and culture appropriately for use, every time one needs the strain for quality control (QC). However, this is not the case for a diphtheria reference laboratory where subculture is required at least every two weeks.

Reagents are chemical or biological materials used to perform specific assays. Laboratories should hold a reserve stock to efficiently perform the assays and guarantee provision of a good diphtheria diagnostic/typing service. It is recommended to check the quality of a reagent using the appropriate controls when a different lot number has been received.

A logbook or folder should be kept with the following details for all reference materials and reagents:

- Name and catalogue number of the reference material/reagent
- Supplier
- Lot number
- Date of analysis or QC certificate
- Expiry date
- Concentration (where applicable)
- Initials of the person responsible
- Location of storage, e.g. flammable/toxic
- Temperature monitoring of storage

10.7 Laboratory safety

Potentially toxigenic *C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis* are classified according to regulations for dangerous pathogens in each country for example in the UK it is the Advisory Committee on Dangerous Pathogens (ACDP) with a classification of Hazard Group 2. Each laboratory should have its own local safety manual which describes the essential biosafety, chemical, fire and electrical safety requirements to protect staff, the community and the environment. All staff should be familiar with the contents of the manual and should proceed accordingly. All new staff should be required to read the manual and made aware of the risks involved in working in a diphtheria laboratory before starting work. They must comply with personal protective equipment regulations for that laboratory and wear suitable protective clothing when handling these pathogens. They must be competent in the relevant SOPs, safety protocols and risk assessments. All staff that routinely handle cultures of potentially toxigenic corynebacteria should be fully vaccinated (including booster vaccinations) according to the respective national immunisation guidelines. Ideally, serum antibody levels should be checked every three years to ensure laboratory staff have adequate immunity.

10.8 Audits and accreditation

Audits are a way of independently examining the documentation and processes to assess whether they are of a certain standard and are appropriate for the service provided. Audits may be internal, performed by staff that do not have direct involvement in the processes being assessed, or external by regulating authorities or accreditation bodies for QA purposes. Audits should be viewed as a way of maintaining and/or improving the quality of the service by identifying weaknesses and undertaking corrective actions and should be regularly undertaken and recorded accordingly.

Accreditation provides documentation that the laboratory has the capability to detect, identify and promptly report potentially toxigenic corynebacteria. The process also provides a learning opportunity, a mechanism for identifying resources, training needs and a measure of progress.

There is no defined WHO mechanism for the accreditation of national diphtheria laboratories. Accreditation is usually achieved according to the criteria established within each country or region.

10.9 Training workshops and internal quality assurance/external quality assurance

Numerous workshops have been held in the European, South-East Asia, Western Pacific, Eastern Mediterranean and African regions by PHE during the last 15 years, under the auspices of WHO HQ, WHO EURO, WHO WPRO, WHO EMRO, WHO SEARO, CDC, various European programmes, ‘DIPNET’ and PHE (International Health Regulations programme). These workshops are key to maintain awareness and microbiological expertise. It is essential to maintain this level of training globally on a regular basis.

A recent diphtheria gap analysis within the European and Western Pacific Regions identified training as a key priority not only within these Regions but also globally.

https://ecdc.europa.eu/sites/portal/files/documents/Diphtheria%20Gap%20Analysis%20final%20with%20cover%20for%20web.pdf

Practical workshops and regular EQA studies are crucial and beneficial to update personnel on current practices of laboratory diagnostics of diphtheria. It is essential to maintain the level of expertise among countries globally (Both *et al.* 2014; Di Giovine *et al.* 2010; Neal *et al.* 2009).

10.10 Benefits of EQA

The EQA allows the participating laboratories to assess its own performance with specimens distributed for investigation in comparison with the expected results and compare performance with other sites. EQA can:

- Highlight issues at an early stage with inadequacies with kits and procedures
- Provide objective evidence and efficacy of testing pathways
- Monitor internal QC procedures
- Provide an educational stimulus for improvement
- Identify any staff training needs

The last EQA conducted under the auspices of the WHO Collaborating Centre and ECDC was in 2013 (Both *et al.* 2014). EQA distributions are being planned under the auspices of both WHO and ECDC in the future. It is important to maintain this high standard and to continue offering training and EQA programmes within this specialised area of microbiology globally.

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APPENDIX 1 Collecting clinical specimens

A1. Procedures for collecting samples for the laboratory diagnosis of diphtheria

Ideally, two samples should be collected from each suspected case; a nasopharyngeal and oropharyngeal swab and placed into the appropriate transport media.

A1.1 Materials required for clinical sampling

- Strong light source for illuminating the pharynx
- Dacron cotton-tipped or flocked swab
- Amies transport medium or other suitable transport medium
- Sterile tongue depressor
- Saline solution
- Skin punch or scalpel
- Eppendorf tube
- Gloves
- Surgical mask
- Goggles

A1.2 Oropharyngeal/throat swabs

1. Pharynx should be clearly visible and well illuminated.
2. Depress the tongue with a tongue depressor, swab the throat without touching the tongue, uvula or inside of the cheeks.
3. Rub vigorously over any membrane, white spots or inflamed areas; slight pressure with a rotating movement must be applied to the swab.
4. Place in a routine semi-solid transport medium or into a silica gel sachet.

A1.3 Nasopharyngeal swabs

1. Insert the swab into one nostril, beyond the anterior nares.
2. Gently introduce the swab along the floor of the nasal cavity, under the middle turbinate until the pharyngeal wall is reached, rotating swab 2-3 times. Force must not be used to overcome any obstruction.
3. Place in a routine semi-solid transport medium or into a silica gel sachet.

A1.4 Nasal swabs

1. Insert the swab into the nose through one nostril beyond the anterior nares.
2. Gently introduce the swab along the floor of the nasal cavity.
3. Place in a routine semi-solid transport medium or into a silica gel sachet.

A1.5 Cutaneous lesions

1. Lesions should be moistened with sterile normal saline and crusted material removed.
2. Press the swab firmly into the lesion.
3. Place into a routine semi-solid transport medium or into a silica gel sachet.

A1.6 Pseudomembrane

1. To be undertaken preferably by an infectious disease specialist as there is a considerable risk of severe bleeding. If a membrane is present, lift the edge of the pseudomembrane and swab beneath it.
2. Using sterile forceps gently lift the pseudomembrane where possible and aseptically remove pieces of the membrane.
3. Place the membrane into either Amies transport medium or a small volume (2 ml) of sterile broth or saline.

Figure A1. Case of pharyngeal diphtheria with classic pseudomembrane. Image courtesy of Prof Ismoedijanto Moedjito, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.

APPENDIX 2 Temporary storage and transportation of isolates

A2.1 Silica gel packets for temporary storage and transportation of swabs and bacterial isolates

Commercially available:

Desiccant activated silica gel packets 1.5 g foil bags. 3000 bags/drum: 75% white gel – 25% blue gel. Packet size = 89 mm tall x 55 mm wide. Hargo Corporation USA (+ 937 298 4008)

A2.1.1 Use of silica packets for temporary storage and transporting bacterial cultures

Silica gel packages are effective for 1-2 weeks transportation of clinical swabs and bacterial isolates.

- Will work at room temperature; will remain viable for more than 1 week
- No need for dry ice
- Less expensive to transport – low weight (2 g/sachet)

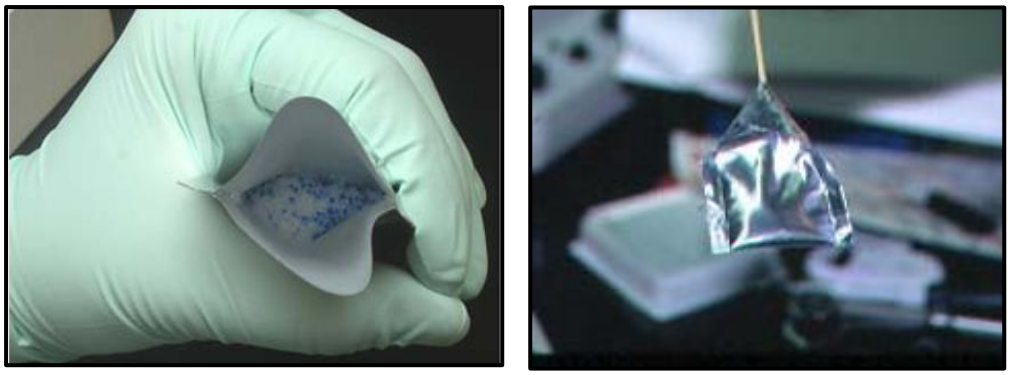
Note: Unused silica packages should be kept apart from any moisture inside the manufacture drums or in well-sealed zip bags.

A2.1.2 Procedure for storage and transporting isolates or clinical swabs:

1. Open the top of the silica package with 70% ethanol-sterilised scissors.
2. Check for the blue (25% indicator) silica gel inside the silica package:
    a. If some blue indicator silica still visible (**Figure A2**), proceed with steps 3-5.
    b. If no visible blue silica is found inside the package (only transparent or pinkish/violet), moisture is present, and the package must be discarded and not used to transport bacterial isolates or clinical swabs.

Figure A2. Silica gel packs for temporary storage and transportation. A. visible blue silica indicates the package is good to use. B. Illustration of how to fold the aluminium package with a cotton swab inside. Modified and courtesy of Centers for Diseases Control and Prevention, Atlanta, Georgia, USA.

A **B**



3. Using a sterile cotton swab roll it over all culture growth area (use care to touch only the end of the swab shaft to avoid contamination) and place the loaded swab into the pack or place the clinical swab directly into the silica gel pack.
4. Fold the two sides of the aluminium package similar to the way a lollypop is packaged.
5. Tape the sides and the swab in place to avoid leakage moisture. Clearly mark the package with patient details

A2.1.3 Procedure for retrieving isolates transported in silica packets:

1. Work over large paper towels if available, as sometimes the silica particles fall off the swab.
2. Carefully remove the tape and open the foil packet taking care not to contaminate the lower end of the swab.
3. Roll the swab over the appropriate agar plate.
4. Carefully break off (or cut with a sterile scissors) the tip of the swab into a test-tube or bottle containing an appropriate liquid broth medium (ensure the swab is far enough into the tube that it will not flip out of the tube)
5. Incubate the plate and tube at 37°C overnight. In most cases, the plate will have growth, and broth will not need to be plated.
6. In rare instances where there is no growth on the plate or for a potential strain of *C. diphtheriae* biovar intermedius, inoculate some of the overnight broth culture onto a fresh plate and re-incubate for at least another 48h.

APPENDIX 3 Long-term storage of strains

A3.1 Storage of strains

Ideally, strains of the isolate should be pure and their identification confirmed before cryo-storage. Subculture into a storage medium should not be performed from a selective medium. Take care when performing this procedure as aerosols are created. Work in a biosafety cabinet.

1. Using a sterile cotton swab, gently scrape most of the growth off a pure blood plate (avoid the dense area), and place in a 1 ml vial of storage broth, and emulsify in the broth
2. Label the tube with reference number and date of storage.
3. Freeze at –25°C or below.

A3.1.1 Glycerol broth 16% (v/v) for frozen storage of isolates.

Base	Oxoid Nutrient Broth No.2 (CM0067)	25 g
	Glycerol (warmed before use)	168 g
	Distilled or deionised water	1 L
Method	1. Mix gently to dissolve	
	2. Check pH and note (Should be pH 7.0)	
	3. Autoclave at 121°C, 10 lb for 15 minutes. Allow to cool to 50°C	
Aseptically add	25 ml defibrinated horse blood per litre	
Dispense	Into 1 ml sterile plastic screw-capped cryovials and refrigerate until use	

1. Label the Columbia blood agar plates before removing the vials from the freezer.
2. Remove the vials of strains from the freezer into a suitable container/rack and transfer them to a biosafety cabinet. Allow to stand for ~10 minutes.
3. Using a sterile disposable loop, gently scrape out some of the frozen culture from the frozen broth and subculture onto a Columbia blood agar plate.
4. Return the frozen broth to the freezer immediately after use allowing only partial thawing.

A3.1.2 Skimmed milk tryptone glucose glycerol (STGG) medium

A3.1.2.1 Preparation of STGG medium

Add the following to 100 ml distilled water

2 g skim milk powder

3 g TSB

0.5 g glucose

10 ml glycerol

Mix to dissolve all the ingredients

Dispense 1 ml into 1.5 ml screw cap vials

Loosen the screw caps and autoclave at 121°C for 10 minutes

Tighten the caps after autoclaving and store at –20°C until use.

Quality control – Sterility check. Inoculate a blood and chocolate agar plate with 200 µl of either of the storage media and incubate at 37°C for up to 48 hours to ensure total sterility. No growth of any organism is expected.

A3.1.2.2 Cryobeads

Commercially available Cryobeads, such as Microbank beads (ProLab Diagnostics, Ontario, Canada) are useful as they cause less freeze-thaw damage to the organism and have the advantage to easily select a few beads to send (frozen) to another laboratory while maintaining the original culture.

A3.2 Reviving strains from frozen glycerol blood broth

To revive an isolate preserved in broth, work in an aseptically biosafety cabinet, allow the frozen tube to thaw for a few minutes (avoid thawing the tube completely), then aseptically remove a loopful of broth and streak onto the first quadrant of the plate. Return the tube to the freezer or keep in a cold rack or on dry ice until returning it to the freezer to prevent freeze-thaw damage to the organism. Continue streaking the plate as normal. Incubate the plate at 35-37°C for 24 hours.

A3.3 Reviving isolates preserved on cryobeads

To revive an isolate preserved on cryobeads, work in an aseptically biosafety cabinet, use a sterile loop or needle to aseptically remove one bead from the tube and place it onto an agar plate. There is no need to thaw the tube of beads entirely, only enough to remove one bead. Return the tube to the freezer or keep in a cold rack or on dry ice until returning it to the freezer to prevent freeze-thaw damage to the organism. Streak the bead in the first quadrant of the plate and then continue streaking the plate as normal. The bead may remain on the plate or discarded as biohazardous waste. Incubate the plate at 35-37°C for 24 hours.

A3.4 Loeffler's serum slopes

Formula	Nutrient broth	100 ml
	Normal horse serum	300 ml
	Glucose	2.0 g

Method (makes approximately 120 bijoux bottles)

1. Add glucose to sterile nutrient broth and shake to dissolve
2. Place in steamer for 5 minutes

Cool To 56°C

Aseptically add
1. Sterile horse serum into a sterile bottle
2. The cooled nutrient broth + glucose
3. Mix thoroughly

Aliquot
1. 3.0 ml amounts into sterile bijoux bottles
2. Place angled in the inspissator and sterilise by heating for 60 minutes at 75-80°C on two consecutive days.
3. Store at 4°C

DO NOT AUTOCLAVE THIS MEDIUM. YOU MUST USE AN INSPISSATOR. Serum-containing media cannot tolerate higher temperatures and are rendered sterile by heating at 75-80ºC for 60 minutes on two consecutive days. This process of sterilisation is called inspissation.

APPENDIX 4 Preparation of bacteriological media

A4.1 Blood agar plates – 5% to 10% horse or sheep blood agar plate

Base	Oxoid Blood agar (BA) base No.2 (CM271)	40 g
	Distilled or deionised water	2 L
Method	1. Boil to dissolve agar	
	2. Check pH and note	
	3. Autoclave at 121°C, 15 lb for 15 minutes	
	4. Final pH is 7.4±0.2	
Cool	To 40°C	
Aseptically add	50 ml defibrinated horse blood (or sheep blood) per L (Oxoid SR0050C)	
Pour	25 ml volumes in triple vent Petri dishes	
Label	BA + date of preparation, store at 2-8°C until use	

A4.2 Columbia blood agar

Base	Columbia (COL) agar base (Oxoid CM0331)	39 g
	Distilled water	1 L
Method	1. Boil to dissolve agar	
	2. Check pH and note	
	3. Autoclave at 121°C, 15 lb for 15 minutes	
	4. Or melt down bottles from stock	
Cool	To 40°C	
Aseptically add	50 ml defibrinated horse (or sheep) blood per L	

Pour	25 ml volumes into triple vent Petri dishes	
Label	COL + Date, store at 2-8°C until use	

A4.3 Tellurite-containing blood agar plate (Hoyle's Tellurite)

Base	Oxoid Hoyles medium base (CM0083)	40 g
	Distilled water	1 L
Method	1. Boil to dissolve agar	
	2. Check pH and note	
	3. Distribute in flasks if the intention is to keep as a stock item	
	4. Autoclave at 121°C, 15 lb for 20 minutes or 10 lb for 20 minutes if the volume is less than 500 ml	
Cool	To 50°C	
Aseptically add	1. 50 ml lysed defibrinated horse (or sheep) blood* (use the oldest blood) or purchase laked blood from for example, Oxoid (100 ml: SR0048C)	
	2. 3 ml (105 mg/ml)	
Pour	25 ml in triple vent Petri dishes	

Base could be kept as a stock item; 500 ml in 20 oz bottles. To reheat, autoclave for 10 mins/15 lb.

*Freeze and thaw unopened sterile horse or sheep blood for five consecutive days until the blood cells have completely lysed.

A4.4 Tinsdale medium

Base	Difco™ Tinsdale agar base (Difco 278610)	18 g
	Distilled water	400 ml (4 x 100 ml volumes of base)

Method	1. Mix dry reagent in purified water
	2. Heat while stirring and boil for 1 minute to dissolve
	3. Dispense 100 ml amounts into appropriate flasks
	4. Autoclave at 121°C for 15 minutes
Cool	To 50°C
	Difco™ Tinsdale supplement (234210), reconstituted to 15 ml with sterile distilled water
Aseptically add	15 ml supplement to each 100 ml base
	Mix well ensuring no bubbles form
	Final pH of medium is 7.4±0.2
Pour	Five plates (i.e. 20 ml per plate)

A4.5 PIZU medium for detecting cystinase

In some NIS countries, an in-house modified Pizu method is used to determine the production of cystinase (Feldman *et al.* 1989).

A4.5.1 Preparation of medium

Base	Mueller Hinton agar	1.7 g
	L-cystine	0.03 g
	Sodium bicarbonate	0.1 g
	Sodium thiosulphate	0.15 g
	Lead acetate	0.1 g
	Horse serum	10 ml
	Distilled water	90 ml

Method	1. Mix Mueller Hinton agar with 90 ml distilled water
	2. Heat until dissolved
	3. In a tube containing 2 ml distilled water, add 0.1 g sodium bicarbonate and heat in a boiling water bath
	4. Dissolve 0.03 g L-cystine in the sodium bicarbonate solution.

Sterilise	10% solution of lead acetate and 10% solution of sodium thiosulphate by heating in a boiling water bath for approx. 30 minutes
Cool	To 38-39°C
Aseptically add	In the following order add: 10 ml horse serum 1 ml 10% lead acetate solution 1.5 ml 10% sodium thiosulphate solution Mix well with no bubbles
Distribute	Into sterile tubes to produce stabs of up to 3 cm.

Recipe courtesy of Dr Siva Gabrielian, (registered medium No. 1877 A2, 15.12.2006)

A4.5.3 Control strains

It is advisable to regularly culture reference strains from stock to ensure recognition of colonial morphologies and that all media are working optimally (**Table A1**). Type strains from international culture collections are recommended.

Table A1. Quality control strains for media and expected results

NCTC 10356 *C. diphtheriae* biovar belfanti non-toxigenic	Elek negative control CYS positive control
NCTC 10648 *C. diphtheriae* biovar gravis toxigenic	Elek positive control
NCTC 3984 *C. diphtheriae* biovar gravis toxigenic or **ATCC 19409**	Elek positive control
NCTC 764 *C. striatum Klebsiella pneumoniae* ATCC 700603 can also be used as the CYS negative control for Tinsdale medium.	CYS negative control
NCTC 12077 *C. ulcerans*	GLY positive control PYZ negative control Urea positive control NIT negative control
NCTC 12078 *C. xerosis*	GLY negative control PYZ positive control Urea negative control NIT positive control

GLY, glycogen; CYS, cystinase; PYZ, pyrazinamidase; NIT, nitrate

APPENDIX 5 Screening and identification tests

A5.1 Tinsdale

Recommended controls:

Positive = non-toxigenic *C. diphtheriae* biovar belfanti, NCTC 10356

Negative = *C. striatum,* NCTC 764

1. Demarcate a small section on a Tinsdale agar plate for the positive and negative controls
2. Inoculate with the above controls or other appropriate known strains, stabbing the strain into the agar as well as inoculating the surface
3. Inoculate the remainder of the plate with the test strain and stab it into the agar as for the control
4. Incubate plate overnight at 37°C

A5.1.1 Reading test

The cystinase plate may be read on the open bench.

1. Examine the plates after overnight incubation, looking for the presence of black colonies surrounded by a brown halo or a brown halo around the stab area.

2. Reactions: - Positive: brown halo around black colonies or stab area (**Figure A3** and **A4**)

   - Negative: absence of brown halo; some coryneform bacteria may produce black colonies only

3. Interpretation: - Positive: pathogenic corynebacteria

   - *C. diphtheriae*

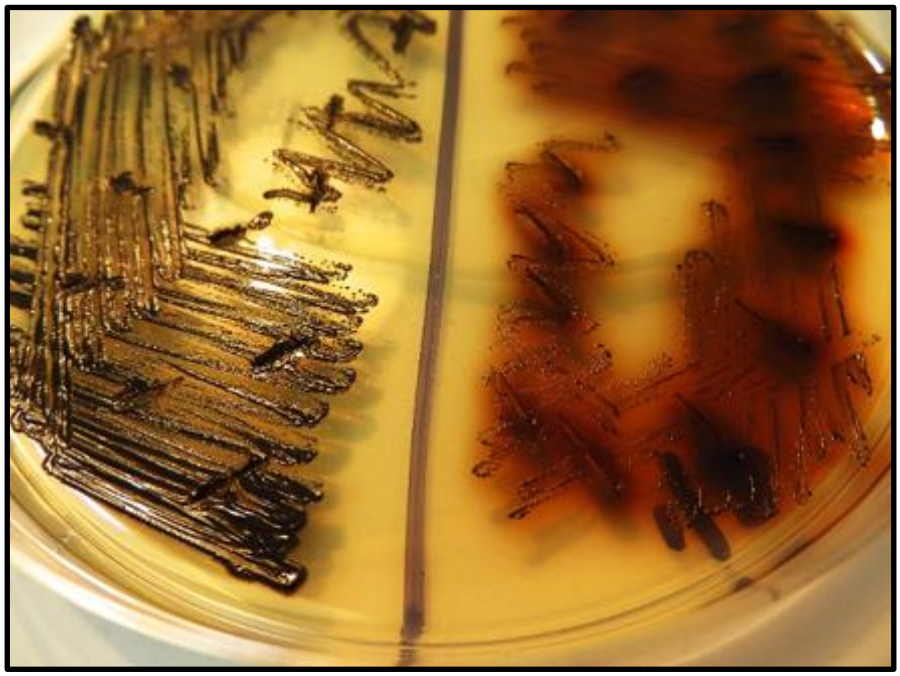
   - *C. pseudotuberculosis*

   - *C. ulcerans*

   - Negative: other non-pathogenic corynebacteria

Figure A3. Cystinase test – Tinsdale medium. A. Left: Other corynebacteria form black colonies (and no brown halo production), Right: *C.diphtheriae, ulcerans* or *pseudotuberculosis* (brown halo production) on Tinsdale medium. **B.** Tinsdale medium showing colonies of cystinase positive corynebacteria as black colonies with a brown halo. Image courtesy of A. Efstratiou.

A



B

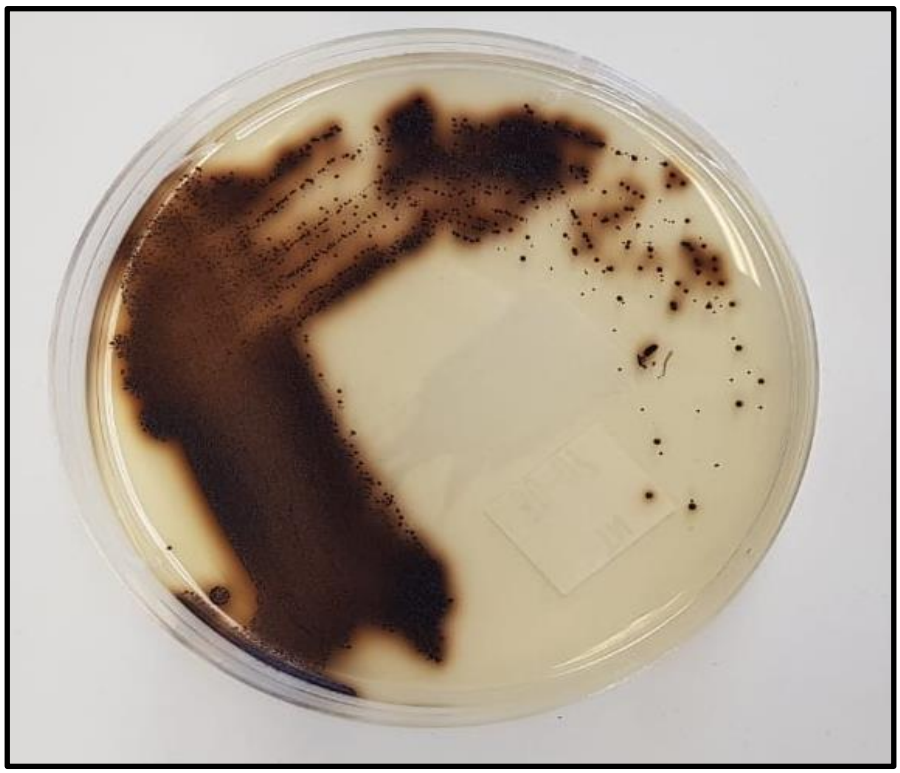
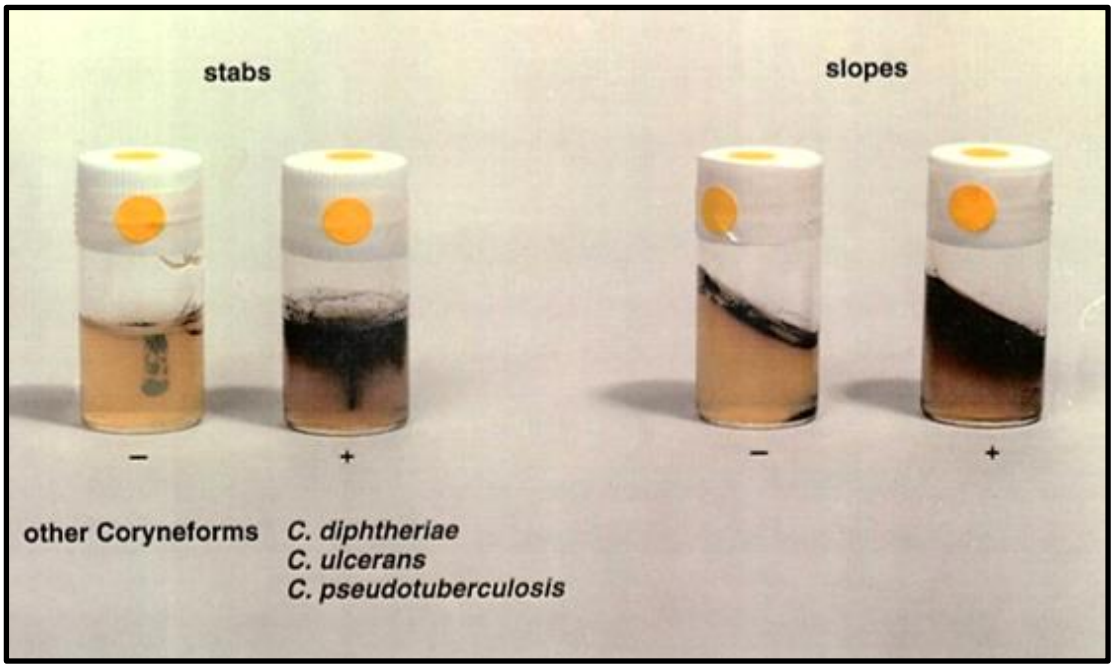


Figure A4. Cystinase test as agar slope culture: Tinsdale or Pizu medium. Potentially toxigenic species will produce a diffused, black colouration as shown. Image courtesy of A. Efstratiou.



A5.2 Pyrazinamidase test

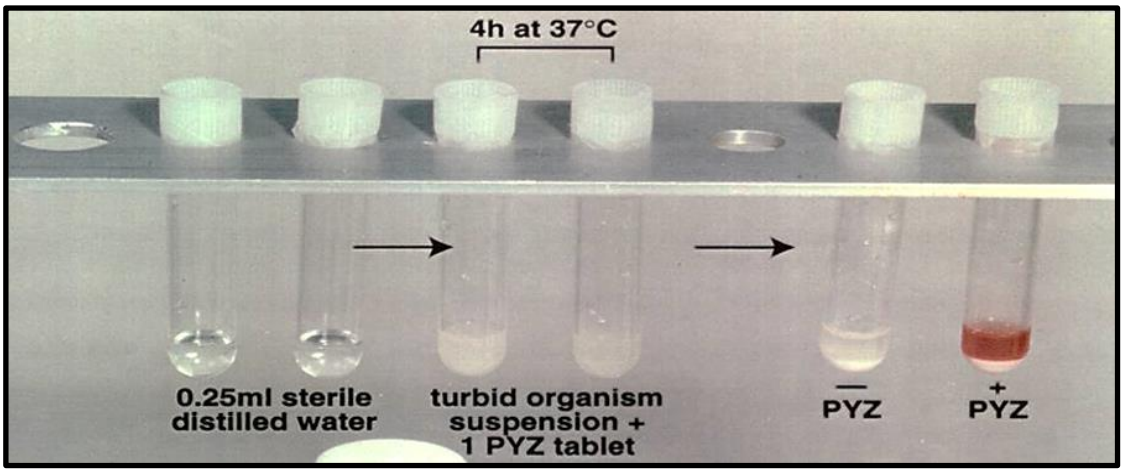
A rapid test is available commercially that can differentiate pathogenic corynebacteria (*C.diphtheriae, C.pseudotuberculosis* and *C.ulcerans*) from the other species of corynebacteria. There are however, other non-toxigenic species that can also be PYZ negative for example, *C.macginleyi*, *C.resistens* . However, the test itself is simple, rapid (4 hours) and cost-effective. The most well-known manufacturer for these reagents is Rosco Diagnostica A/S, Denmark and these are distributed globally from various suppliers. **Please also refer to the manufacturer's instructions.**

Reagents	Pyrazinamidase Diagnostic tablets (UK supplier BioConnections; CK4537)
	Pyrazinamidase Reagent (UK supplier BioConnections; CK9801)
	OR
	API® PYZ reagent (bioMérieux; 70590)
Recommended controls:	Positive = *C. xerosis,* NCTC 12078
	Negative = *C. ulcerans,* NCTC 12077

If commercial tests are difficult to procure in some countries, then the test could be developed using specific chemical reagents as described by Efstratiou and Maple (1994).

Limitations: The positive reaction may fade. If needed, it can be restored by adding another drop of reagent. The test should be read after 5-10 minutes and then discarded. In addition, there are a few other species of corynebacteria that may be negative but are rarely encountered in upper respiratory tract specimens.

Figure A5. Pyrazinamidase (PYZ) test. Image courtesy of A. Efstratiou.



APPENDIX 6 Staining methods for laboratory identification of *C. diphtheriae*

Staining is the primary test that can be performed directly on the samples to study specific features such as the morphology (size, shape), structural details and composition. Below are the different staining methods for *C. diphtheriae* with specific usage.

Table A2. Stain preparation and staining methods for laboratory identification of *C. diphtheriae*

Stain	Ingredients	Preparation	Method	Result	Usage
Gram stain	**1. Crystal violet** Crystal violet 1 g 5% Sodium bicarbonate 1 ml Distilled water 99 ml **2. Gram's Iodine** Iodine crystals 2 g sodium Hydroxide 10 ml Distilled water 90 ml **3. Acetone/alcohol** **4. Safranin (counterstain)** Safranin 0.34 g Absolute alcohol 10 ml Distilled water 90 ml	• Add 1 g of crystal violet and the sodium bicarbonate into a mortar • Grind using a pestle to get a good paste • Then add water and mix well • Filter through a filter paper • Add NaOH to the iodine crystals • Grind using a mortar to get a good paste • Add distilled water and mix well • Filter through a filter paper • Grind the dye in alcohol and then add water • Filter through a filter paper	1. Prepare a heat-fixed smear of the suspected *C. diphtheriae* culture 2. Flood slide with crystal violet and allow to stand for 1 minute 3. Gently rinse with tap water 4. Cover with iodine solution for 1 minute 5. Rinse with tap water 6. Decolourise by adding acetone/alcohol solution for 5 – 10 seconds. Take care not to over decolourise 7. Counterstain with Safranin for 45 seconds 8. Gently rinse with tap water and blot dry 9. Examine under oil immersion objective	*C. diphtheriae* is weakly Gram-positive; occasionally, it may be entirely Gram-negative, or Gram variable.	Used to differentiate Gram-positive and Gram-negative bacteria and yeast and yeast-like organism

Stain	Ingredients	Preparation	Method	Result	Usage
Ponder's stain	Toluidine blue 0.02 g Glacial acetic acid 1.0 ml Alcohol (95%) 2.0 ml Distilled water 100.0 ml	• Grind toluidine blue in alcohol • Mix with water and then add glacial acetic acid • Filter through a filter paper	1. Prepare and fix by heat slide preparations of the suspected *C. diphtheriae*, from Loeffler serum medium 2. Cover slide with Ponder's stain for 8 minutes 3. Do not wash 4. Air dry	Metachromatic granules stain purple, and the body appears light blue	To stain metachromatic granules
Neisser's stain	**Solution 1, part A** Methylene blue 0.1 g Ethanol (95%) 5 ml Glacial acetic acid 5 ml Distilled water 100 ml **Solution 1, part B** Crystal violet 0.33 g Ethanol (95%) 3.3 ml Distilled water 100 ml **Solution 2** Counterstain of choice, e.g. eosin, chrysoidine or Bismarck brown	• Dissolve the dye in the water and add the acid and ethanol • Dissolve the dye in the ethanol-water mixture • To use, mix 2 parts of Part A to 1 of Part B, (e.g., 20 ml Part A and 10 ml Part B) • Prepare fresh monthly	1. Prepare thin smears on the microscope slides and thoroughly air dry. Do not heat-fix. 2. Cover slide with Solution 1 for 30 seconds 3. Rinse with water 4. Counterstain with Solution 2 for 1 minute 5. Rinse well with water; blot dry	Yellow-brown is positive Blue-violet is negative	To stain metachromatic granules

All staining reagents should be kept in well-closed stoppered bottles and protected from direct sunlight and stored below 30°C. Commercially prepared staining reagents are more widely available; however, it is important for QC purposes to record batch numbers and the dates used. Positive and negative control slides using known or reference strains should be used every time the staining procedure is performed, except for Gram staining (which is used more frequently), where control slides may be done with each new batch used.

APPENDIX 7 Elek toxigenicity test

A7.1 Elek agar medium materials and preparation

Solution A (500 ml)

Material:

1. BD Bacto™ Proteose Peptone No. 2 (212120) 20 g
2. Deionised or distilled water 500 ml
3. Maltose 3.0 g
4. 10N NaOH 3.25 ml
5. Lactic acid 0.7 ml
6. 1N HCl
7. Whatman filter paper No. 12
8. pH meter
9. Magnetic stirrer with a hotplate
10. Magnet

Preparation:

1. Dissolve peptone in water.
2. Add 3.25 ml NaOH (40% w/v = 10N solution)
3. Mix and heat to boiling in a steamer or hotplate
4. Let it cool
5. Preferably filter through Whatman® glass fibre filter (Grade GF/F) or Whatman® filter paper no. 12 to remove precipitated phosphates
6. Add 0.7 ml lactic acid solution (AnalaR NORMAPUR®, 88.0-92.0%)
7. Add 3.0 g maltose
8. Mix the solution thoroughly
9. Adjust pH to 7.8 with 5N or 1N HCl using a pH metre

Solution B – Elek basal medium (500 ml)

Material:

1. Sodium chloride 5.0 g
2. 'Lab M' agar (Neogen, code MC2) 10.0 g

   Or

   Bacto™ Agar (Becton Dickinson 214010) 10.0 g
3. Deionised or distilled water 500.0 ml

Preparation:

1. Mix the sodium chloride and the agar in the distilled water
2. Autoclave then steam to dissolve
3. Cool to 50°C and adjust pH to 7.8±0.2 with 1N NaOH (AnalaR)

A7.2 Preparation of Elek agar medium

Material:

1. Solution A
2. Solution B
3. Water bath
4. McCartney bottles

Procedure:

1. Warm up solution A to 50°C *
2. Mix with solution B (Elek basal agar medium)
3. Distribute in 15 ml volumes in McCartney bottles (GW162)
4. Autoclave at115°C (10 psi) for 10 minutes
5. Store in the refrigerator at 4°C until use

A7.3 Preparation of Elek agar medium plates

Material:

1. Elek basal agar medium 15 ml (McCartney bottle)
2. Newborn bovine serum** 3 ml aliquot
3. 90 mm sterile Petri dish
4. 50 mm single vent sterile Petri dish

Procedure:

1. Melt 15 ml Elek agar medium by letting the flask stand in a 50°C water bath
2. Add 3 ml sterile newborn bovine serum** to the heated base and mix gently
3. For conventional Elek plates, pour about 18 ml*** of the mixture into 90 mm sterile Petri dish, using aseptic technique
4. For modified Elek plates, aliquot 3 ml volume into 50 mm single vent sterile Petri dish. One bottle of Elek medium (15 ml) is sufficient to prepare 4-5 plates (volume should not be less than 3 ml for each plate)

5. Allow to cool down on the bench and label with the date of preparation
6. Store at 2-8°C for up to 1 week or 2 weeks if plates are cling-filmed.

*Peptone must be from a batch with known properties

**It is advisable to use newborn bovine serum but if difficult to procure then use either sheep or horse serum that is 'diphtheria antibody free'

***Calculate the total volume to be prepared by estimating a total of 18 ml media per plate

A7.4 Preparation of antitoxin strips and discs

Material:

1. Diphtheria antitoxin, usually supplied in vials reconstituted at 1000 IU/ml; should be stored at 4°C
2. Mast Bacteruritest Dipstrips (BTRI; Mast Diagnostics Ltd, UK) Mast Discs (BDO638W; Mast Diagnostics Ltd, UK)

   OR

   Whatman® No. 1 or No.3 filter paper is also suitable for preparing the strips
3. Petri dish
4. Forceps

Procedure:

1. Open the vial at the neck using ampoule protector and aseptically transfer the contents to a sterile tube
2. Dilute antitoxin to 500 IU/ml with sterile distilled water. The diluted antitoxin is stable for 6 months if stored at 4°C
3. Pour diluted antitoxin into a sterile Petri dish
4. Using sterile forceps dip the sterile paper strips/discs into the diluted antitoxin solution
5. Drain excess antitoxin and place in sterile Petri dishes, no more than 2 layers high
6. Place Petri dish at 37°C until strips/discs are completely dry
7. Transfer strips/discs into a suitable sterile container, using sterile forceps
8. Label container with the date of preparation and expiry date
9. Store in sterile capped containers at 2-8°C. They should remain stable for a minimum of 6 months; however, this should be carefully controlled by testing the strips at regular intervals (i.e. monthly)

A7.5 Setting up the Elek toxigenicity test

Material:

1. Elek agar media plates
2. Antitoxin strips or disks
3. Culture isolate

Procedure:

1. Label a prepared plate as per template in **Figures A6** and **A7**. Ensure there is no moisture on the surface. Two unknown strains can be tested on one plate.
2. In a biosafety cabinet and wearing gloves, inoculate the plate with the test strains and the control strains, as illustrated in **Figures A6** and **A7**.
3. Using sterile forceps, place an antitoxin strip (500 units/ml) on the 90 mm plate or a disc on the 50 mm plate, as per template in **Figures A6** and **A7**. Ensure that the single line of the organism does not touch the disc/strip.
4. Incubate plate(s) aerobically at 35-37°C for 24 and 48 hours.

A7.5.1. Recommended controls:

Positive = toxigenic *C. diphtheriae* biovar gravis, NCTC 10648

Weak positive = toxigenic *C. diphtheriae* biovar gravis, NCTC 3984/ATCC 19409

Negative = non-toxigenic *C. diphtheriae* biovar belfanti, NCTC 10356

A7.6 Reading test

Carefully examine the plates after 16-24 hours of incubation and again after 48 hours, using a suitable light source (transmitted light plus hand lens). Look for precipitin lines of identity between the test strains and the strong and weak positive control strains. The negative control strain must not demonstrate any precipitin lines.

Do <u>not</u> re-incubate for longer than 48 hours as nonspecific precipitin lines may develop.

As both non-toxigenic and toxigenic colony variants may be present from a single throat culture, two individual colonies and a 'sweep' of five to six colonies together should be examined for toxigenicity of a culture.

A7.7 Interpretation of the Elek test

Classic precipitin lines forming an ‘arc’ with the positive controls denote a toxin-producing strain. White lines of precipitation commencing about 10 mm from the filter paper strip and occurring at an angle of about 45° to the line of growth are interpreted as positive toxigenic strains. If the test strain shows similar lines to the toxin positive control, then it should be regarded as being toxigenic. Non-toxigenic strains will not show these lines. Secondary lines of precipitation due to soluble antigens other than diphtheria toxin can be produced by both toxigenic and non-toxigenic strains.

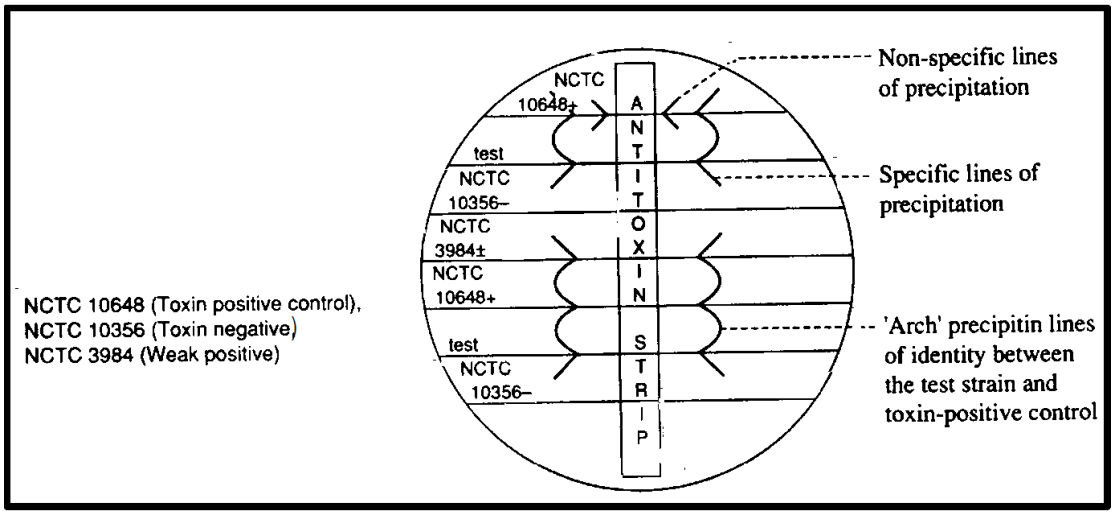
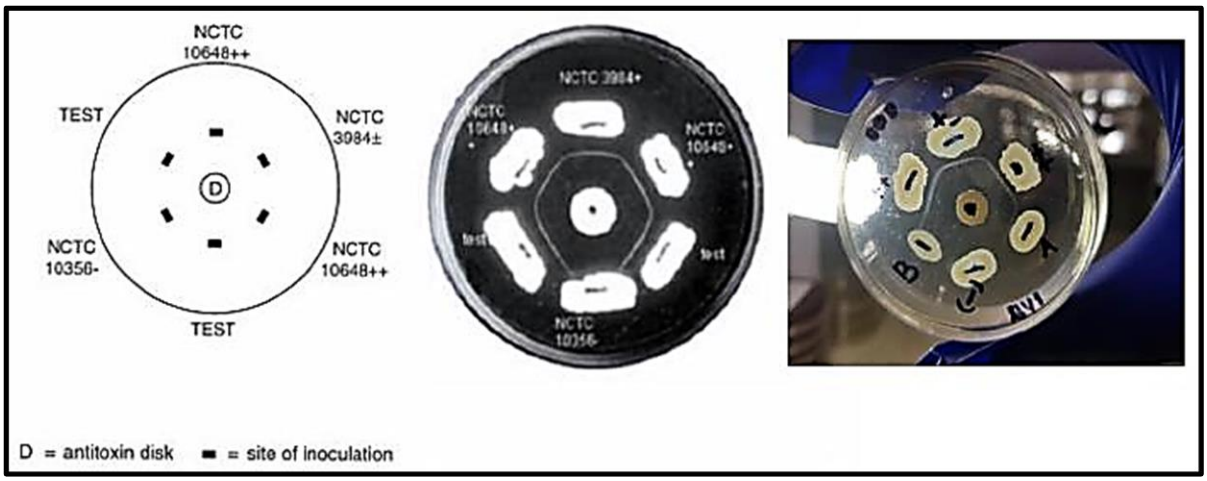


Figure A6. Conventional Elek test for toxigenicity testing. Diagrammatic representation and interpretation. Images courtesy of A. Efstratiou.

Figure A7. Modified Elek test.



APPENDIX 8 Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry (MS) as a tool for rapid identification of *Corynebacterium* species

This protocol uses a Microflex LT instrument (Bruker Daltonics GmbH, Germany) for the acquisition of mass spectra within a range of 2 to 20 kDA according to the instructions of the manufacturer. Software used includes Biotyper 2.0 database and FlexControl software (version 3.0) (Bruker Daltonics).

A8.1 Matrix solution composition

Material:

Solution A

Acetonitrile (AN), HPLC grade	500 µl
Aqua dest, HPLC grade	475 µl
Tri-fluor-acetic-acid (TFA), HPLC grade	25 µl

Solution B

Saturated α-cyano-4-hydroxycinnamic acid (HCCA)*

matrix solution (Bruker Daltonics)

Procedure:

1. Add 250 µl of Solution A to 250 µl of Solution B (HCCA) until all matrix crystals are dissolved.
2. Matrix solution can be stored for two weeks in the dark at room temperature according to the manufacturers' instructions.

A8.2 Sample preparation (protein extraction protocol)

The protocol below should be used only if the reading is below 2.0

Material:

Fresh bacterial culture	1 – 5 colonies
Aqua dest	300 µl
Ethanol, HPLC grade	900 µl
Formic acid (70%), HPLC grade	50 µl
Acetonitrile (AN), HPLC grade	50 µl
Target plate (Bruker Daltonics)	
Matrix solution	1 µl

*Saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker Daltonics)

Procedure:

1. Suspend 1 to 5 single colonies (up to 5-10 mg) of a fresh bacterial culture in 300 µl aqua dest and 900 µl Ethanol and mix thoroughly
2. Centrifuge at maximum speed for 2 minutes
3. Decant supernatant, centrifuge again and remove residual fluid
4. Add 50 µl of 70% formic acid to the pellet and mix by vortexing, add 50 µl AN and mix carefully
5. Centrifuge at maximum speed for 2 minutes
6. Transfer 1 µl of supernatant onto the target plate and leave to dry at room temperature
7. Overlay the spot with 1 µl of matrix solution and dry at room temperature again.

A8.3 Measurements and interpretation of MALDI-TOF MS results

Automated measurement and analysis of the raw spectral data are performed on a Microflex LT mass spectrometer (Bruker Daltonics) with a standard pattern matching algorithm (BioTyper 2.0 Software).

Resulting log (score) values:

- Above 2.0 for reliable identification on species level
- Between 1.7 and 2.0 for genus level
- Below 1.7 cannot be rated as valid according to the manufacturers' instructions

A8.4 Quality control systems

MALDI-TOF MS results can be impaired by technical problems. The calibration control proposed by Bruker should be used before each run (Croxatto *et al.* 2012).

Internal QC (machine calibration)

- Laboratories must perform internal QC before using MALDI-TOF MS for identification
- Internal QC consists of an automatic instrument calibration using a manufacturer-specified calibration standard
- Depending on the system, calibrators include a manufactured extract of *Escherichia coli* (*E. coli*) or a specific *E. coli* calibration strain

- Generally, Bruker system uses reference strain *Escherichia coli* K-12 (genotype GM48) as a standard for calibration and as a reference for QC
- Laboratories should ensure that they follow manufacturers' specifications for preparing, using and storing calibrators
- Laboratories must perform calibration before every run and should document calibration results

External QC (positive and negative controls for each run)

- Laboratories should perform external QC using appropriate positive and negative controls
- Bruker system uses *Staphylococcus aureus* as a positive control and matrix as negative controls in each run
- For positive controls, laboratories should test well-characterized strains using the same methodology they use for patient isolates. The negative control consists of reagents spotted directly on the target plate or slide
- Laboratories that work platforms with reusable targets test a blank negative control to ensure adequate cleaning of the target
- Results of QC testing should be documented and periodically reviewed to assess the instrument performance and the testing consistency among users

A8.5 Maintenance of the MALDI-TOF system

- Results can be impaired by poor cleaning of the microplate between runs which is a problem encountered only by Bruker users. This can be avoided by using disposable microplates which are now available for Bruker users
- The presence of dust on plastic joints can lead to functional disturbances. This can be reduced by placing the machine in a quiet area without drafts /dust free
- Maintenance should ideally be done before the "dirtiness rate" reaches 80%, i.e. about four times a year if three to five microplates are tested per day
- Maintenance frequency should be increased if the apparatus is heavily used or located in a crowded/dusty area

A8.6 Discrepancies/Troubleshooting

- Any discrepancies in the results can be resolved by performing additional methods like sequencing of 16S rRNA or *rpoB* gene
- Further, MALDI-TOF MS cannot reliably discriminate between closely related species. For instance, the system cannot differentiate *C. pseudodiphtheriticum*

and *C. propinquum*. In addition, *C. ulcerans* and *C. pseudotuberculosis* may also have close scores (>2.0) to each other and may not be clearly differentiated (Suwantarat *et al.* 2016; Vila *et al.* 2012). In such cases, biochemical tests like urease can be used to differentiate these species along with MALDI-TOF MS identification. Similarly, lipophilic *Corynebacterium* species like *Corynebacterium urealyticum* tend to have lower scores of identification by MALDI-TOF MS. This could be due to the slow growth characteristics with tiny colony appearance, which makes it difficult to smear colonies onto the MS plate. Increased incubation time can help to get more visible colonies and thus increase the identification scores

- Other possibilities for the failure to identify organisms in the database include identification of organism with thick-cell walls, mucoid properties of the colonies, and pigmentation

Note:

- Fresh isolates should be used whenever possible
- 10^6 bacteria per well is necessary to consistently obtain a spectrum (to score above 2)
- Reagents used should be checked routinely for the expiry/QC compliance
- Reference databases need to be updated continuously to account for new species or taxonomic revisions

Figure A8. The process of MALDI-TOF MS (Clark *et al.*, 2013).

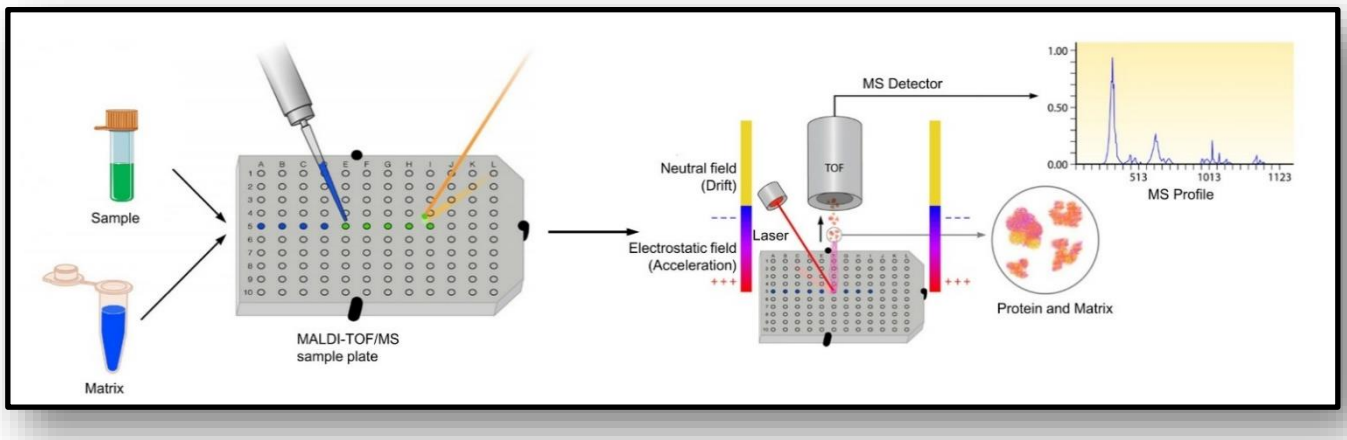
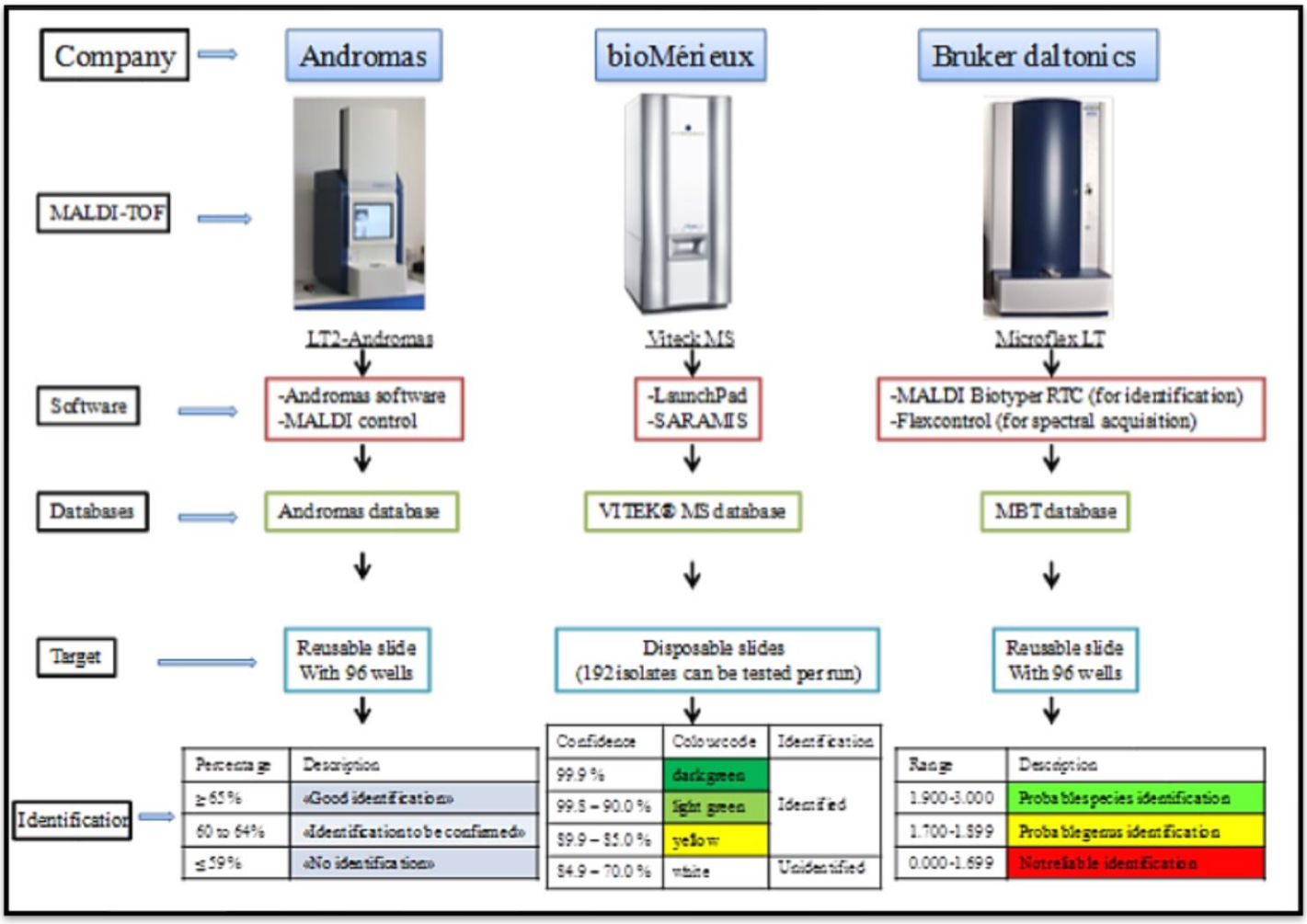


Figure A9. Three different MALDI-TOF MS systems for microbial identification. (Lo *et al.* 2017)



References

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Vila J, *et al.* 2012 Identification of clinically relevant Corynebacterium spp., Arcanobacterium haemolyticum, and Rhodococcus equi by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Journal of clinical microbiology. 50(5):1745-7.

Suwantarat N, *et al.* 2015 Practical utility and accuracy of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of Corynebacterium species and other medically relevant coryneform-like bacteria. American journal of clinical pathology. 145(1):22-8.

Croxatto A, Prod'hom G, and Greub G. 2012 Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS microbiology reviews. 36(2):380-407.

Lo CI, *et al.*, 2017 Value of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry in clinical microbiology and infectious diseases in Africa and tropical areas. Afr J Microbiol Res. 11:1360-70.

APPENDIX 9 Conventional and qPCR

The sample preparation must be performed in a class 1-biosafety cabinet or a dedicated clean area in the laboratory.

A9.1 Sample preparation from bacterial cultures (de Zoysa *et al.* 2016) – crude extraction/boiling

1. Gloves must be worn for this procedure.
2. Distribute 0.5 ml distilled water (PCR grade water) to sterile microtubes with safety locks
3. Transfer a 1 µl loopful of each test and the two control organisms freshly cultured on blood agar, to sterile microtubes containing the 0.5 ml of water.
4. Place the tubes containing the suspensions in a dry heating block pre-set at 100°C (or alternative, i.e. boiling water bath with tube holder) and heat the tubes for 15 minutes (ideally in a biosafety cabinet)
5. Centrifuge for 1 minute at 10,000 g in a microcentrifuge
6. Transfer the supernatant with the template DNA into a clean tube or directly into the tube containing the master mix (see below).

Control organisms: NCTC 10356 (Tox negative *C. diphtheriae*) and NCTC 10648 (Tox positive *C. diphtheriae*). It is not necessary to use the phenotypic weak toxin producer NCTC 3984.

A9.1.1 Alterative sample preparation for bacterial cultures and clinical specimens (using extraction kit)

Bacterial DNA can also be prepared from either a swab or cultured bacteria (Hauser *et al.*, 1993; Sing *et al.*, 2011), using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany according to the manufacturer's instructions, and as described below) or any other extraction kit or platform that has been fully validated. .

Throat swabs collected from patients:

1. Cut or break off the swab tip and suspend in 200 µl of tissue lysis buffer and 40 µl of proteinase K solution (20 mg/ml)
2. Incubate at 55°C for at least 30 minutes
3. After complete disintegration of the swab piece, which can be examined visually, add 200 µl of binding buffer
4. Incubate further at 70°C for 10 minutes
5. Add 100 µl of isopropanol and transfer the mixture to the High Pure spin column

Cultured bacteria:

1. Suspend single colonies in 200 µl of phosphate buffered saline (PBS) buffer and 15 µl of a lysozyme solution (10 mg/ml in Tris-HCl, pH 8.0)
2. Incubate at 37°C for 10 minutes
3. Add 200 µl of binding buffer and 40 µl of a proteinase K solution (20 mg/ml)
4. Incubate further at 70°C for 10 minutes
5. Add 100 µl of isopropanol and transfer the mixture to the High Pure spin column
6. Following the centrifugation and wash steps, elute the bacterial DNA with 200 µl of elution buffer
7. A 2 µl aliquot (for cultured bacteria) or a 5 µl aliquot (for processed throat swabs) is used for the PCR.

A9.2 PCR mixture preparation: conventional and modified sample preparation

The two PCR mixes described in the following section of the manual are;

1. PCR mix for the conventional assay (**Table A4**)
2. PCR mix for the modified conventional assay (**Table A6**)

The modified version is based on a ready-to-use mixture containing buffer, $MgCl_2$, dNTPs and Taq polymerase in a Master Mix. In the conventional PCR protocol, all the components are separated.

PCR mixture preparations MUST BE PERFORMED in a PCR cabinet with UV decontamination (option) or dedicated clean area in the laboratory or in a separate lab (clean room free of DNA and/or cultures). The cabinet should ideally not be used for bacterial samples or other potentially contaminated substances.

Each run should contain the test sample plus positive and negative controls. For example:

- Test strain (in duplicate)
- 1 positive control (positive sample, toxin gene positive)
- 1 negative control (negative sample, toxin gene negative)
- 1 extraction negative control (PCR grade water control from the extraction)
- 1 PCR negative control (PCR grade water added instead of template DNA)

Table A3. Primer sequences.

Primer name	Primer sequence	Primer direction
Primer 1	5'-ATC-CAC-TTT-TAG-TGC-GAG-AAC-CTT-GGT-CA-3'	Forward
Primer 2	5'-GAA-AAC-TTT-TCT-TCG-TAC-CAC-GGG-ACT-AA-3'	Reverse

A9.2.1 Conventional PCR mix preparation

1. Prepare the PCR reaction mixture in a microtube by adding the reagents according to **Table A4**. Prepare the mixture depending upon the number of reactions, for example, if n=10 samples then prepare a mix of reagents (excluding DNA template) for n+1 (11) and aliquot 23 µl to each tube or plate well.

Table A4. Conventional PCR mix

Conventional PCR mix reagents	Quantity x1 (µl)	Final concentration
10 x Reaction buffer	2.5 µl	1x
$MgCl_2$ (50 mM)	0.75 µl	1.5 mM
Nucleotides: (10 mM each of dATP, dCTP, dGTP and dTTP)	0.5 µl	200 µM each dNTP
Taq polymerase (5 units/µl)	0.5 µl	1.25 units
Primer 1 (15 pmol/µl)	1 µl	0.6 pmol/µl
Primer 2 (15 pmol/µl)	1 µl	0.6 pmol/µl
Water (PCR grade)	16.75 µl	-
Total volume:	**23 µl**	
Add 2 µl of DNA template in each tube (final volume = 25 µl)		

2. Vortex the PCR reaction mixture.

3. Label the required number of sterile PCR grade microtubes appropriately (i.e. test strain, positive, weak positive, negative control strains; extraction negative control [no template control], PCR negative control [water control]).

4. To each PCR tube add:

48 µl of PCR mix,

1 µl of DNA template and

1 µl of PCR grade water

Total PCR volume = 50 µl

5. Vortex.
6. Centrifuge the mixture for a few seconds in a microcentrifuge to settle fluid and ensure there are no bubbles
7. Place all tubes in a thermal cycler and start the PCR programme with the parameters as described in **Table A5**.
8. The PCR products can be run on a conventional agarose gel and stained in ethidium bromide as described in **A9.2.3** or they can be run on a pre-cast agarose gel containing ethidium bromide (E-gel Invitrogen) see **A9.2.4**. There are also several alternative dyes that are not carcinogenic that can be used (e.g. 'SYBBR Safe').

Table A5. Conventional PCR amplification conditions.

Number of cycles	Working temperatures (in °C)	PCR stage
1 cycle	96°C for 2 minutes	Denaturation
35 cycles	94°C for 15 seconds 50°C for 15 seconds 72°C for 30 seconds	Denaturation Annealing Extension
1 cycle	72°C for 10 minutes	Extension

A9.2.2 Modified conventional PCR mix preparation

This is an example of a simplified PCR mix, using the HotStarTaq Mastermix (Qiagen). This Taq DNA polymerase requires a 15-minute denaturation step at the start of the PCR.

1. Prepare the PCR reaction mixture in a microtube by adding the reagents according to **Table A6**.

Table A6. Modified conventional PCR mix

Modified conventional PCR mix reagents	Quantity x1 (µl)	Final concentration
HotStarTaq® Master Mix 2x	12.5 µl	1x
Primer 1 (15 pmol/µl)	1 µl	0.6 pmol/µl
Primer 2 (15 pmol/µl)	1 µl	0.6 pmol/µl
Water (PCR grade)	8.5 µl	-
Total volume:	**23 µl**	
Add 2 µl of DNA template in each tube		

2. Vortex the PCR reaction mixture
3. Label sterile PCR grade microtubes appropriately (i.e. test, control strains, extraction negative control [no template control], PCR negative control [water control])
4. To each tube add:

18 µl of PCR mix

2 µl of DNA template/water control

Total PCR volume = 20 µl

5. Vortex
6. Centrifuge the mixture for a few seconds in a microcentrifuge and ensure there are no bubbles.
7. Place all tubes in a thermal cycler and start the PCR programme with the parameters as described in **Table A7**.
8. PCR products can be run on a conventional agarose gel and stained in ethidium bromide (see **A9.2.3**) or they can be run on a pre-cast agarose gel containing ethidium bromide (E-gel Invitrogen) (see **A9.2.4**). There are also several alternative dyes that are not carcinogenic that can be used (e.g. 'SYBBR Safe').

Table A7. Modified conventional PCR amplification conditions.

Number of cycles	Conditions	PCR stage
1 cycle	96°C for 15 minutes	Denaturation
35 cycles	94°C for 15 seconds 50°C for 15 seconds 72°C for 30 seconds	Denaturation Annealing Extension
1 cycle	72°C for 10 minutes	Extension

A9.2.3 Analysis of PCR products: conventional gel electrophoresis

PCR products can be run on a conventional agarose gel and stained in ethidium bromide or they can be run on a pre-cast agarose gel containing ethidium bromide (E-gel, Invitrogen). See ***safety information box***.

Safety information box:

ETHIDIUM BROMIDE IS CARCINOGENIC

IT MUST THEREFORE BE DISCARDED WITH CARE VIA THE LOCALLY AGREED TOXIC WASTE DISCARD SYSTEM. ALWAYS WEAR GLOVES WHEN HANDLING ETHIDIUM BROMIDE OR STAINED GELS

Preparation of 3% agarose gel:

1. Weigh 3 g of agarose and add to 100 ml of 1x Tris-Borate EDTA (TBE) buffer in a conical flask. The TBE is prepared as a 5x stock solution as described in **Table A8**.

   Precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the 5x stock solution in glass bottles at room temperature and discard any batches that develop a precipitate.

2. Bring to boil in the steamer, but do not allow to burn. Set aside to cool to hand-hot temperature (about 50°C).
3. Prepare the gel tray by wiping with 70% alcohol and sealing with masking tape. Ensure gel tray is on a flat surface.
3. Pour gel into a gel tray and allow to set.
4. Place the gel tray in the electrophoresis tank containing TBE buffer pH 8.2.

Wear gloves when handling E-gel and wear a UV visor when using the UV transilluminator.

Table A8. TBE 5x buffer pH 8.0

Chemical	Amount
Tris base	54 g
EDTA 0.5 M (pH 8.0)	20 ml
Boric acid 0.9 M	27.5 g
Add distilled H_20 to bring up to 1000 ml	

A9.2.3.1 Electrophoresis of the PCR products

1. Mix 10 µl of 1x gel loading buffer (e.g. BlueJuice) with 5 µl of PCR product.
2. Load 15 µl of samples into the wells. Add a suitable DNA size standard (e.g. 100 bp Ladder) into one or more wells, as appropriate.
3. The samples are run at 150V for approximately an hour.
4. Once finished running, transfer the gel into a specially selected container with ethidium bromide.
5. The gel is stained with ethidium bromide for 30 minutes and viewed on a UV transilluminator. Alternatively, ethidium bromide could be pre-added to the gel during preparation.
6. A positive reaction for the fragment A portion of the gene is represented by a single band of 246 bp (**Figure A10**).

Wear gloves when handling ethidium bromide and wear a UV visor when using the UV transilluminator.

Ethidium bromide staining**:

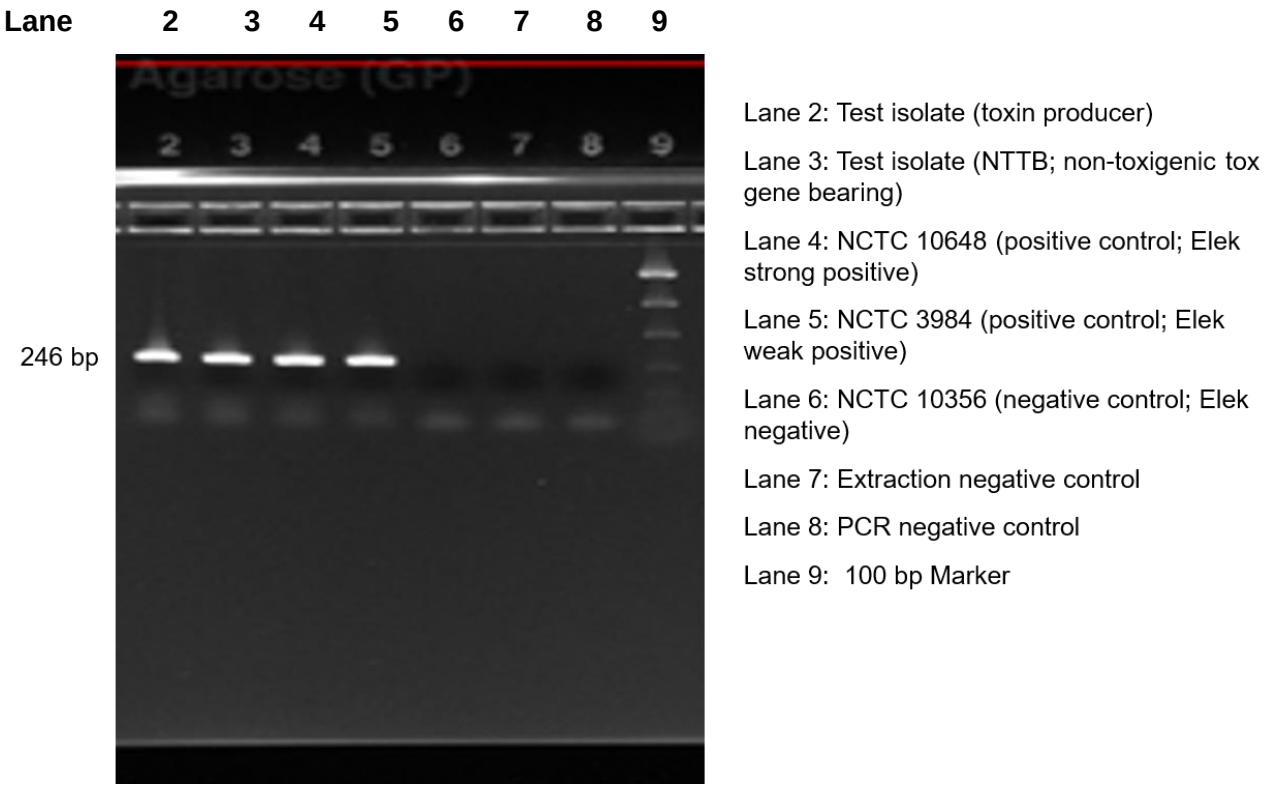
- Stock solution: 10 mg/ml in distilled water.
- Working solution: stock solution diluted to 0.5-1 µg/ml.
- Stain for 30 minutes.

A9.2.4 Using pre-cast gels (Invitrogen E-Gels) for running PCR products

Wear gloves and plug E-Gel PowerBase to the electricity socket.

1. Open the package containing the gel and carefully take the gel out and insert the gel cassette into the Gel PowerBase. Remove the comb carefully.
2. Mix 10 µl gel loading buffer (e,g. BlueJuice) with 5 µl PCR product and load into the appropriate well. Load 10 µl DNA size standard (e.g. E-gel standard) into one or more wells, as appropriate.
3. Run the gel for 15 minutes, and visualise the gel on a UV transilluminator

Figure A10. PCR for the detection of diphtheria toxin gene (246 bp). Courtesy of A. Efstratiou.



A9.3 qPCR for detection of tox gene and Corynebacterium species (DeZoysa *et al.* 2016)

A9.3.1 Purpose and rationale of *Corynebacterium* species qPCR assay

This assay simultaneously detects potentially toxigenic and non-toxigenic *C. diphtheriae*, *C. ulcerans/C. pseudotuberculosis* in DNA extracts from cultures. The assay targets specific regions of the *C. diphtheriae* and *C. ulcerans rpo*B genes and the presence of the toxin gene is determined by targeting the 'A portion' (the active portion) of the diphtheria toxin gene. The *C. ulcerans rpoB* gene PCR also detects *C. pseudotuberculosis* strains. The green fluorescent protein (gfp) gene from *Aequorea victoria* cloned into a plasmid is used as an internal process control (IPC) in order to detect PCR inhibition. This assay specifically validated for bacterial isolates has been published by De Zoysa *et al.* (2016).

Expression of diphtheria toxin by toxin gene bearing strains must always be confirmed by the Elek test.

A9.3.2 Type of sample

This version of the test has only been validated on DNA extracts from submitted cultures.

A9.3.3 Type of RT-PCR assay: dual labelled hybridisation probes

The assay is a quadruplex assay, optimised on the Qiagen Rotor-Gene Q platform, using the channels as shown below (**Table A9**). The Rotor-Gene platform does not require the use of a passive reference (e.g. ROX), and choice of Texas Red as a dye below is not compatible with mastermixes that contain ROX as a passive reference.

Table A9. Qiagen Rotor-Gene Q channels and probes

Channel	Excitation (nm)	Detection (nm)	Dye/Quencher combination	Probe target
Blue	450-490	510-530	FAM/BHQ-1	***C. ulcerans* and *C. pseudotuberculosis* (*rpoB* gene)**
Green	515-535	560-580	HEX/BHQ-1	***C. diphtheriae* (*rpoB* gene)**
Yellow	560-590	640-650	Texas Red/BHQ-2	**Diphtheria toxin gene (*tox*)**
Red	620-650	675-690	Cy5/BHQ-2	**IPC (*gfp* gene)**

Note: You can substitute the IPC (gfp) target with the 16S target as described by Badell *et al.* 2019.

A9.3.4 Materials

- Sterile microfuge tubes
- Gilson pipettes: P1000, P200, P20, P10 (or equivalent)
- Sterile filtered tips for above
- PCR grade Tris-EDTA (TE) buffer pH 8.0
- Primers and probes (see **Table A10**)
- Positive controls DNA (extracted from toxigenic *C. diphtheriae* [NCTC 10648] and non-toxigenic *C. ulcerans* [NCTC 12077]).
- IPC: The IPC DNA described here comprises the pGFP plasmid, which contains the *gfp* gene (from *Aequorea victoria*) cloned into a bacterial plasmid. 10 µl aliquots of a 500 copies/µl stock are prepared and stored -20°C or below. 90µl of PCR grade water is added to one aliquot on the day of use for a 50 copies/µl working solution. An alternative commercial IPC control can be used instead.
- Nuclease-free water.

- 0.2 ml or 0.1 ml – depending on the real-time platform and real-time PCR tubes.
- 0.5 ml/1.5 ml amber sterile tubes (to store primers aliquoted properly).
- PCR grade Tris-HCl buffer pH 8.0.

Primers choice and management

Primers must be synthesized to a purity stated by the manufacturer as suitable for real-time PCR (e.g. HPLC-purified or Eurofins "PCR primer"). On receipt, resuspend in TE (if necessary) to a concentration of 100 µM (100 pmol/µL). Use immediately to make the 20x primer/probe mix (see **Table A10**) or store at -20°C or below until required (see manufacturer's requirement). Primers and probes must be stored separate from any bacterial DNA (to avoid contamination). Probes are light sensitive and should be handled in dark/amber microfuge tubes at all times.

Table A10. Primers and probes for multiplex qPCR

Target gene	Oligo name	Sequence	Fragment
C. diphtheriae rpoB	dip_rpobF	CGTTCGCAAAGATTACGGAACCA	97bp
	dip_rpobR	CACTCAGGCGTACCAATCAAC	
	C. dip HP	**HEX-AGGTTCCGGGGCTTCTCGATATTCA-BHQ1**	
C. ulcerans rpoB	ulc_rpobF	TTCGCATGGCTCATTGGCAC	98bp
	ulc_rpobR	TCCAGGATGTCTTCCAGTCC	
	CulcHP	**FAM-CCAGCAGGAGGAGCTGGGTGAA-BHQ1**	
tox	toxAF	CTTTTCTTCGTACCACGGGACTAA	117bp
	toxAR	CTATAAAACCCTTTCCAATCATCGTC	
	diptoxHP	**Texas Red - AAGGTATACAAAAGCCAAAATCTGGTACACAAGG-BHQ2**	
gfp	gfp_FP	CCTGTCCTTTTACCAGACAACCA	77bp
	gfp_RP	GGTCTCTCTTTTCGTTGGGATCT	
	gfp_HP	**Cy5-TACCTGTCCACACAATCTGCCCTTTCG-BHQ2**	

Note: These probe dye labels have been chosen for the Rotor-Gene Q platform. They may need to be changed if the assay is adapted to run on another platform.

A9.3.5 DNA extraction

1. Prepare DNA extracts of bacterial isolates as described in **Section A9.1**.
2. Also extract DNA from positive control strains NCTC 10648 (toxigenic *C. diphtheriae*) and NCTC 12077 (non-toxigenic *C. ulcerans*). (This can be done ahead of time and the extracted DNA stored frozen.)
3. Include the IPC as extraction sample. Take out an aliquot of IPC plasmid stock and dilute it 1/10 before use by adding 90 µl TE 1x pH 8.0 to the tube and mixing it.
4. Optional: prepare purified DNA stocks of positive control strains: If you are performing a quantitative qPCR using commercial standards, follow the manufacturer's instructions. To prepare your own standard curve controls, follow the instructions below:
    a. Extract DNA from positive control strains NCTC 10648 (toxigenic *C. diphtheriae*) and NCTC 12077 (non-toxigenic *C. ulcerans*) using a commercial kit (e.g. as described in **Section A9.1.1**)
    b. Quantify the DNA by using a suitable method (e.g. Qubit Fluorometer).
    c. Prepare serial dilutions of the DNA (e.g. 1000, 100 and 10 genome copies/µl) in 10 mM Tris-HCl pH 8.0.
    d. Prepare aliquots for single-use (5 µl); store at –20°C.

A9.3.6 Preparation of a 20x primer/probe mix

1. Prepare the primer/probe mix in advance:
    a. Mix the 100 µM (100 pmol/µl) stocks of primers (forward and reverse) and probes as described in **Table A11**.
    b. Label the mixture as "Dip4plex", indicating the final volume on the tube
    c. Before using each new batch of primer/probe mixture to test samples, perform a QC run using the positive control samples plus ≥1 negative control (non-template control, NTC)
    d. The "Dip4plex" tube must be stored in a clean laboratory in a freezer at -20°C

Table A11. Preparation of 20x primer/probe mix (Dip4plex mix)

Reagent		1 ml mix	1.5 ml mix	2 ml mix	Final conc. in Dip4plex mix
Primer/probe [100 pmol/µl stock]	dip rpob-F	50 µl	75 µl	100 µl	5 µM
	dip rpob-R	50 µl	75 µl	100 µl	5 µM
	C. dip HP	20 µl	30 µl	40 µl	2 µM
	ulc rpob-F	50 µl	75 µl	100 µl	5 µM
	ulc rpob-R	50 µl	75 µl	100 µl	5 µM
	C-ulc HP	20 µl	30 µl	40 µl	2 µM
	toxA-F	50 µl	75 µl	100 µl	5 µM

	toxA-R	50 µl	75 µl	100 µl	5 µM
	Diptox HP	20 µl	30 µl	40 µl	2 µM
	gfp-FP	50 µl	75 µl	100 µl	5 µM
	gfp-RP	50 µl	75 µl	100 µl	5 µM
	gfp HP	20 µl	30 µl	40 µl	2 µM
Buffer	TE 1x pH 8.0	520 µl	780 µl	1040 µl	
Final volume		**1000 µl**	**1500 µl**		

2. In the PCR clean room or clean designated area, prepare the q-PCR reaction mix in a 1.5 ml tube as described in **Table A12**.

Table A12. RT-PCR reaction mix

Reagent	qPCR mix x1 (µl)	Final concentration
PCR grade H_2O	2 µl	-
Dip4plex 20x	1 µl	1x
pGFP [50 copies/µl]	2 µl	5 copies/µl
Rotor-Gene Multiplex PCR Mix (2x)	10 µl	1x
Dispense 15 µl in each tube		
Add 5 µl of DNA template		

3. Gently vortex the 1.5 ml centrifuge tube and spin to settle fluid before dispensing 15 µl of the reaction mix into 200 µl or 100 µl qPCR tubes.
4. In another cabinet, add to each tube 5 µl of DNA template previously extracted.
5. Set up the real-time machine, running the programme shown in **Table A13**.

Table A13. Cycling conditions

Cycles	Temperature	Step
1 cycle	95°C for 5 minutes	PCR activation step
45 cycles	95°C for 10 seconds	Denaturation
	60°C for 20 seconds	Annealing/extension

A9.3.7 Data analysis

1. After the qPCR run has completed, analyse the results to determine the Ct values (the cycle at which the fluorescence crosses the threshold line). For the Rotor-Gene, a threshold value of 0.05 is recommended. The optimal threshold value will have to be determined separately for a different PCR platform.
2. Check the threshold to avoid a false positive result, especially when the background fluorescence of the negative control samples rises slightly. If this happens, you may raise the threshold above 0.05 to prevent false Ct values.

A9.3.8 Interpretation of results

1. Check that the positive control samples have produced the expected results (see **Table A14**). If they have not, there may be a problem with the PCR run. If you use the same material for the positive controls in every run (e.g. purified DNA), check that the Ct values for the standards lie within the minimum and maximum (mean ± 2 standard deviations) expected for each target (you will need to define this using data from ~20 runs). If they don't, this could be indicative of probe degradation and loss of sensitivity. Record this information for quality purposes.
2. Interpret the PCR results for the test samples according to **Table A14**.
3. If the PCR result indicates a toxin gene bearing *C. diphtheriae,* or a toxin gene bearing *C. ulcerans*/*C. pseudotuberculosis* the result needs to be confirmed by phenotypic Elek test.
4. If the result is *Inhibitory* or *Equivocal*, consider whether to repeat the PCR (and possibly the DNA extraction).

Table A14. Interpreting results from the multiplex qPCR

C. diphtheriae	*C. ulcerans/C. pseudotuberculosis*	Toxin gene	IPC	Result
+	-	+	+	Toxigenic *C. diphtheriae*
+	-	-	+	Non-toxigenic *C. diphtheriae*
-	+	+	+	Toxigenic *C. ulcerans*
-	+	-	+	Non-toxigenic *C. ulcerans*
-	-	-	+	Negative for *C. diphtheriae*/ *C. ulcerans*
-	-	-	-	Inhibitory PCR
+	-	-	-	Equivocal PCR (needs to be repeat)
-	-	+	+	Equivocal PCR (needs to be repeat)

Once corynebacteria have been identified using the qPCR, further tests are used to confirm identification and determine the expression of toxigenicity by the isolate. These are usually performed in National Reference Laboratories or by referral to the WHO Collaborating Centre for Diphtheria in the UK.

A9.4 Modifications to the real-time multiplex qPCR

The method by de Zoysa *et al.,* (2016) described above has been further developed by Badell *et al.* (2019) by replacing the *gfp* IPC with broad range primers and a probe directed at the 16S rRNA gene. This acts as both an extraction control and an IPC (to detect inhibition). This modified method was validated by the authors for use on both bacterial cultures and clinical specimens. The method of de Zoysa *et al.* has also been successfully run on the Roche LightCycler 480 platform (Badell *et al.* 2019) and the Applied Biosystems 7500/7500Fast platform (unpublished communications). In the case of the 7500/7500Fast platform, the extension time needs to be increased from 20 to 30 seconds. More recently, a triplex assay has been described by Williams *et al.* (2020) for use with both cultures and clinical specimens.

References:

Badell E, *et al.*, 2019 Improved quadruplex real-time PCR assay for the diagnosis of diphtheria. J Med Microbiol. 68: 1455-1465

De Zoysa A, *et al.*, 2016 Development, validation and implementation of a quadruplex real-time PCR assay for identification of potentially toxigenic corynebacteria. J Med Microbiol. 65(12):1521-1527.

Hauser, D., *et al.*, 1993 Polymerase chain reaction assay for diagnosis of potentially toxinogenic *Corynebacterium diphtheriae* strains: correlation with ADP-ribosylation activity assay. J.Clin.Microbiol. 31: 2720-2723.

Sing, A., *et al.*, 2011 Rapid detection and molecular differentiation of toxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains by LightCycler PCR. J.Clin.Microbiol. 49: 2485-2489.

APPENDIX 10 Multilocus sequence typing

MLST of *C. diphtheriae* and *C. ulcerans* uses the nucleotide sequence information from the internal fragments of the below described seven housekeeping genes to define the sequence type (ST) for each isolate.

A10.1 Multilocus Sequence Typing for *C. diphtheriae*

Table A15. MLST primers for PCR

Gene	Direction	Primer sequence	Amplicon size (bp)
atpA	Fwd	gcgattgcgaactacacc	1029
	Rev	Ctcgaggaatacctracc	
dnaE	Fwd	tgcgtcatctgattgaaa	858
	Rev	cggtccaataagacacca	
dnaK	Fwd	acttgggtggcggtactt	696
	Rev	tggtgaacgtctcggaac	
fusA	Fwd	taccgcgagaagctcgtt	683
	Rev	gaaggttgggtcctcttc	
leuA	Fwd	cgtgcacttctacaactc	865
	Rev	accgtgatcggtcttcat	
odhA	Fwd	cggcaaggaaascatgac	505
	Rev	gttgtcgccraacatctg	
rpoB	Fwd	aagcgcaagatccaggac	845
	Rev	tcgaactcgtcgtcatcc	

Table A16. MLST primers for sequencing

Gene	Direction	Primer sequence	Allele size (bp)
atpA	Fwd	agaaggcgacgaagtmaagc	378
	Rev	crgaatcagaagctggwgca	
dnaE	Fwd	gtgcgacaagctggtgtg	354
	Rev	ggcttwcggccattyttg	
dnaK	Fwd	agatggctatgcagcgtct	345
	Rev	gatgagcttggtcatcacg	
fusA	Fwd	cgtaagctgaccgttaactc	360
	Rev	ccatggactcraggatga	
leuA	Fwd	ccyatcatcatcaayctgcc	384
	Rev	cagctggttgcagtaytc	
odhA	Fwd	tbcaagatcgcatygarrc	381
	Rev	twggctcgatgtgkccttc	
rpoB	Fwd	cgwatgaacatyggbcaggt	342
	Rev	tccatytcrccraarcgctg	

A10.1.1 PCR reaction preparation (to be done for each allele)

For 1 PCR with a final volume of 25 µl. To minimise pipetting errors, make up multiple reactions 'supermix', then aliquot in batches before adding the chromosomal DNA.

Table A17. PCR reaction preparation for MLST for *C. diphtheriae*

For allele – *dnaK, fusA, lenA, odhA, rpoB*	
Multiplex Master Mix (Qiagen)	10 µl
Q solution	5 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
Water	5 µl
Template DNA	3 µl
Final volume	**25 µl**

Table A18. PCR reaction preparation for MLST for *C. diphtheriae*

For allele – *atpA, dnaE*	
Multiplex Master Mix (Qiagen)	10 µl
Q solution	5 µl
25 M $MgCl_2$	2 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
Water	3 µl
Template DNA	3 µl
Final volume	**25 µl**

Table A19. PCR thermal cycling conditions

Cycling conditions	**Temperature**	**Time per cycle**
1 cycle	95°C	1 minute
35 cycles	96°C 58°C 72°C	1 minute 1 minute 2 minutes
1 cycle	72°C	5 minutes

Table A20. Sequencing PCR reaction preparation

Reaction Component	Volume
RR mix	0.5 µl
Sequencing buffer	1.75 µl
Forward primer (2 pmol/µl)	0.5 µl
Reverse primer (2 pmol/µl)	0.5 µl
Water	6.25 µl
Template DNA	1 µl
Final volume	**10 µl**

Table A21. PCR cycling conditions

Cycling conditions	Temperature	Time per cycle
1 cycle	96°C	1 minute
25 cycles	96°C 50°C 60°C	10 seconds 5 seconds 4 minutes

A10.2 Multilocus Sequence Typing (MLST) of *C. ulcerans*

A separate MLST scheme for the *C. ulcerans* has been proposed by König C and colleagues, as toxigenic *C. ulcerans* is gaining greater importance as a diphtheria-causing pathogen (König *et al.*, 2014). Primers for *atpA*, *dnaA*, *fusA*, *odhA* and *rpoB* are identical as *C. diphtheriae* from the reference Bolt *et al.*, 2010. The primer used for *dnaK* and *leuA* was adapted to *C. ulcerans* according to the genome of *C. ulcerans* 809 (König *et al.*, 2014). Locus amplification and sequencing for MLST analysis are done based on the published scheme for *C. diphtheriae* with minor modifications. Each PCR was carried out in a 50 µl total volume using HotStarTaq Master Mix (Qiagen) (**Table A6**).

Table A22. MLST primers for PCR for *C. ulcerans*

Gene	Direction	Primer sequence	Amplicon size (bp)
atpA	Fwd	gcgattgcgaactacacc	1029
	Rev	ctcgaggaatacctracc	
dnaE	Fwd	tgcgtcatctgattgaaa	858
	Rev	cggtccaataagacacca	
dnaK	Fwd	acttgggtggcggaacct	687
	Rev	tggtaaaggtctcagaa	
fusA	Fwd	taccgcgagaagctcgtt	683
	Rev	gaaggttgggtcctcttc	
leuA	Fwd	cgttcacttctacaattc	864
	Rev	gccgtggtcagttttcat	
odhA	Fwd	cggcaaggaaascatgac	505
	Rev	gttgtcgccraacatctg	
rpoB	Fwd	aagcgcaagatccaggac	845
	Rev	tcgaactcgtcgtcatcc	

Table A23. MLST primers for sequencing for *C. ulcerans*

Gene	Direction	Primer sequence	Amplicon size (bp)
atpA	Fwd	gcgattgcgaactacacc	378
	Rev	ctcgaggaatacctracc	
dnaE	Fwd	tgcgtcatctgattgaaa	354
	Rev	cggtccaataagacacca	
dnaK	Fwd	acttgggtggcggaacct	345
	Rev	tggtaaaggtctcagaa	
fusA	Fwd	taccgcgagaagctcgtt	360
	Rev	gaaggttgggtcctcttc	
leuA	Fwd	cgttcacttctacaattc	384
	Rev	gccgtggtcagttttcat	
odhA	Fwd	cggcaaggaaascatgac	381
	Rev	gttgtcgccraacatctg	
rpoB	Fwd	aagcgcaagatccaggac	342
	Rev	tcgaactcgtcgtcatcc	

Table A24. PCR cycling conditions: (*atpK*, *dnaE*, *dnaK*, *fusA*, *odhA* ***and*** *rpoB)*

Cycling conditions	Temperature	Time per cycle
1 cycle	95°C	15 minutes
35 cycles	94°C 58°C 72°C	1 minute 1 minute 2 minutes
1 cycle	72°C	5 minutes

Table A25. PCR cycling conditions: (*leuA*)

Cycling conditions	Temperature	Time per cycle
1 cycle	95°C	15 minutes
10 cycles	94°C 60-50°C 72°C	1 minute 1 minute (minus 1°C per cycle) 2 minutes
25 cycles	94°C 60-50°C 72°C	1 minute 1 minute 2 minutes
1 cycle	72°C	5 minutes

References:

Bolt, F., *et al.* 2010 Multilocus sequence typing identifies evidence for recombination and two distinct lineages of *Corynebacterium diphtheriae*. J.Clin.Microbiol. 48: 4177-4185.

König, C., *et al.*, 2014 Multilocus Sequence Typing of *Corynebacterium ulcerans* provides evidence for zoonotic transmission and for increased prevalence of certain sequence types among toxigenic strains. J Clin Microbiol. 52:4318-4324

APPENDIX 11 ELISA assays

The general steps of the indirect solid-phase ELISA for the quantification of serum specific anti-diphtheria toxin antibodies are universal, whether the assay is performed using a commercial kit or an in-house assay:

1. Coating step: a diphtheria toxoid of 1/100 known concentration is used to coat the surface of microtiter plate wells at 37°C for 1 hour or at 4°C overnight. For safety and practical reasons, the toxoid is always preferred to toxin as coating antigen. However, different toxoid preparations might affect the results. In the case of commercial kits, the plate is usually already pre-coated with the antigen.
2. The plate is then washed with a wash buffer to eliminate excess of coating antigen. The washing procedure is a critical step and requires special attention. An improperly washed plate will give inaccurate results, with poor precision and high backgrounds and low sensitivity.
3. Blocking step: a concentrated solution of non-interacting protein, such as bovine serum albumin (BSA) is added to all plate wells as blocking agent. Temperature and incubation time might be similar to that of the coating step.
4. The plate is then washed with a wash buffer to eliminate the excess of blocking solution
5. The serum samples of unknown antibody concentration, usually diluted 1/100 into a common dilution buffer, are then added to the wells. A calibration curve is prepared with a reference diphtheria antitoxin human serum, calibrated in IU. Positive and negative controls have to be present on each plate. Generally, the plate is incubated at 37°C for 1 hour or at 4°C overnight.
6. The plate is then washed again, and a peroxidase-labelled anti-human IgM/IgG conjugate as detection antibody is applied to all plate wells and incubated according to instructions.
7. After, the plate is washed, in order to remove excess unbound enzyme-antibody conjugate; the peroxidase substrate is then added. This will be converted by the enzyme to elicit a chromogenic signal. The reaction is usually carried out in the dark and at room temperature.
8. The enzymatic reaction is then stopped and the optical density of each well measured on a microplate reader at a wavelength specific for the reaction product.
9. The results are quantified comparing the chromogenic signal of the serum sample with the reference standard serum. The titres are expressed in IU/ml. To be valid, a calibration curve and a positive and negative control must be included in each plate and must be in the range established during validation of the method or on the QC certificate.

The details of the reagents used in the commercial ELISA are not always provided by the manufacturer. The differences in reagents generate different performance characteristics of the kits.

APPENDIX 12 Diphtheria antitoxin assay in Vero cell (*in vitro* toxin neutralisation)

The method described here involves staining for cell viability with the yellow tetrazolium salt (MTT), adapted from NIBSC method (see below link).

12.1 Materials

12.1.1 Critical reagents and standards

- Diphtheria antitoxin: 1st WHO International Standard for diphtheria antitoxin, equine (DI). or the 1st WHO International Standard for Diphtheria Antitoxin Human (10/262) are available from NIBSC (https://www.nibsc.org/science_and_research/bacteriology/diphtheria.aspx)
- In-house standards or a panel of positive control human serum samples of different level of defined activities in IU (e.g. 0.1, 0.01, 0.001 IU/ml) may be included to monitor assay performance.
- Diphtheria toxin: A purified preparation of diphtheria toxin of defined activity (minimum cytopathic dose) and stability should be used.
- Vero cells: Vero cells are available on request from Chief, Biologicals, WHO, Geneva, Switzerland. Cells may also be obtained from culture collections (ATCC, EDQM) or other sources provided that their robustness and sensitivity to diphtheria toxin is known.

12.1.2 Equipment

- Laminar airflow cabinet
- Incubator (+36 to +38°C)
- Flat bottomed sterile 96 well tissue culture plates
- Multichannel manual or electronic pipettes 50-200 µl
- Sterile tips for pipettes
- Sterile serological pipettes (5-25 ml) and electronic pipette controller
- Haemocytometer (cell counting chamber) with Neubauer rulings or Burker counting chamber for cell counts
- Microscope
- Tissue culture flasks, 75 cm^2 (or 150 cm^2)
- Polyester pressure-sensitive film or microtiter sealing tapes
- pH indicator paper (optional if metabolic activity used as an end-point)

12.1.3 Buffers and reagents

- Minimal Essential Medium, MEM (commercially available from suitable suppliers)
- Foetal or newborn calf serum (must be confirmed free from diphtheria antitoxin)
- Antibiotic solution containing penicillin (10,000 IU/ml) and streptomycin (10 mg/ml)

- 200 mM L-glutamine solution
- 10% D (+)-Glucose solution
- 1M HEPES buffer
- Hanks' balanced salt solution (HBSS) or PBS
- 0.25% trypsin/EDTA solution
- Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT
- Sodium lauryl sulphate, SDS
- *N,*N-dimethylformamide, DMF

12.1.4 Complete culture medium for Vero cells

Supplement MEM with:

- calf serum (final concentration 5-10% v/v)
- L-glutamine (2 mM)
- D-glucose (0.1% w/v)
- HEPES (0.01 M)
- penicillin (100 U/ml)
- streptomycin (100 µg/ml)

Other preparations of cell culture medium may also be suitable for use.

- Trypan blue (0.4%) solution

Note: Medium and all the solutions have to be sterile.

If MTT dye is used for staining viable cells, extraction buffer is prepared with solution of sodium lauryl sulphate (SDS, 10% w/v) in dimethylformamide (DMF, 50% v/v, pH to 4.7). Alternatively, other staining reagents may be used (e.g. crystal violet staining solution) prepared with 5 g of crystal violet dissolved in 100 ml of 37% formaldehyde, 200 ml abs. ethanol, 1665 ml distilled water, 35 ml 2M Tris base and 10 g calcium chloride.

12.2 Procedures

All procedures are performed aseptically using the laminar airflow cabinet.

12.2.1 Culture, harvesting and counting of Vero cells

Established cultures of Vero cells can be maintained in 75 cm^2 tissue culture flasks in complete medium. Depending on the split ratio following passage and the percentage of serum in the medium, a confluent monolayer of cells is obtained after 4-6 days. The following procedure is suitable for routine passage and harvesting of Vero cell cultures. The cells are handled aseptically in the laminar airflow cabinet. Note that one T-75 tissue culture flask of Vero cells at 80-90% confluence will provide enough cells for 3 x 96-well tissue culture plates (at a cell density of 4 x 10^5 cells/ml).

1. Remove the supernatant from a flask containing a confluent monolayer of Vero cells using a sterile pipette.
2. Add 1 ml of sterile HBSS (or PBS) solution to the flask rinse the cells and then remove using a sterile pipette.
3. Add 1 ml of sterile trypsin-EDTA solution to the flask and place in a 37°C incubator until the cells are detached from the flask (2-5 minutes). The trypsin/EDTA solution should be pre-warmed to 37°C to speed up the trypsinisation process.
4. Add approximately 5 ml of complete medium to the flask to randomize the trypsin and resuspend the cell suspension using a sterile pipette to obtain a suspension of single cells for counting (gently mix the cell suspension within the sterile serological pipette to disperse cell clusters).
5. Prepare a 1 in 5 dilution of the cell suspension in 0.4% trypan blue solution and complete medium (e.g. 100 µl cell suspension + 100 µl 0.4% trypan blue solution + 300 µl complete medium). Depending on the total number of cells present a lower or higher dilution may be required. As a guide, the cell suspension should be diluted such that the total number of cells counted exceeds 100 (minimum required for statistical significance).
6. Prepare the haemocytometer by placing the coverslip over the mirrored counting surface. It may be necessary to moisten the edges of the chamber (this can be done by breathing on the glass) such that Newton's rings (rainbow-like interference patterns) appear indicating that the coverslip is in the correct position to allow accurate cell counting (the depth of the counting chamber is 0.1 mm).
7. Using a pipette, introduce a small sample (approximately 10 µl) of the diluted cell suspension into the counting chamber such that the mirrored surface is just covered. The chamber fills by capillary action. Fill both sides of the chamber to allow for counting in duplicate.
8. The entire grid on a standard haemocytometer is comprised of nine large squares (bounded by 3 lines), each of which has a surface area of 1 mm^2. The total volume of each large square is 1 x 10^{-4} cm^3 (0.0001 ml).
9. Count the number of cells in one large square and calculate the cell concentration as follows: *cells/ml = total cell count in one large square x* 10^4.
10. For example, if 150 cells are counted in one large square (1mm^2), the concentration of the cell suspension = 150 x 10^4 cells/ml. If fewer than 100 cells are counted in 1 large square it may be necessary to count multiple large squares (for example, the 4 corner squares plus the centre square) and divide the total cell count by the total number of large squares used for counting.
11. For the Vero cell assay, prepare a cell suspension containing approximately 4 x 10^5 cells/ml in complete medium. Note that the cell suspension should be prepared immediately before use and after all dilutions and neutralization steps have been performed.
12. To maintain the culture of Vero cells, seed approximately 1 x 10^6 cells into a new 75 cm^2 tissue culture flask and add 10 – 15 ml of complete medium prior to incubation at 37°C.

12.2.2 Determination of the test dose of diphtheria toxin

The protocol described here is performed using the Lcd/1000 level of toxin defined by the lowest concentration of toxin (in Lf/ml) which when mixed with 0.001 IU/ml of antitoxin is capable of causing cytotoxic effects on Vero cells after 6 days of culture. At this toxin dose level, the sensitivity of the assay is approximately 0.002 IU/ml for the DI equine antitoxin. The sensitivity of Vero cells to diphtheria toxin may vary when different batches of cells and/or serum are used. As a result, the Lcd/1000 should be determined by each individual laboratory or whenever one of these variables is changed. These parameters should also be confirmed for every new lot of diphtheria toxin and reference antitoxin used. The test dose of toxin is determined by titration of a stable, purified diphtheria toxin against a suitable reference antitoxin as follows:

1. In a sterile 96-well tissue culture plate, fill all the wells of columns 2–11 with 50 µl of complete medium using a multichannel micropipette.
2. Dilute the diphtheria toxin in complete medium to give a starting concentration of approximately 0.02 Lf/ml.
3. Add 100 µl of the diluted diphtheria toxin solution to each well in column 1 using a micropipette.
4. Prepare serial twofold dilutions in 50 µl volumes starting at column 1 through to column 11 using a multichannel micropipette. Discard 50 µl from column 11.
5. Prepare a dilution of the reference diphtheria antitoxin in complete medium to give a diphtheria antitoxin concentration of 0.001 IU/ml.
6. Add 50 µl of the diluted diphtheria antitoxin preparation to all wells in columns 1-11.
7. Add 100 µl of complete medium to 4 “cell control” wells in column 12.
8. Add 50 µl of complete medium and 50 µl of diluted diphtheria antitoxin to 4 “antitoxin control” wells in column 12.
9. Allow the plate to stand at room temperature for 1 hour to allow toxin neutralization to occur.
10. Prepare a suspension of Vero cells in complete medium containing approximately 4 x 10^5 cells/ml (as described previously).
11. Add 50 µl of the cell suspension to all wells of the microplate. The total volume in all wells is 150 µl.
12. Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air. Note that the use of pressure film to seal plates is an important step for methods based on colour changes in the culture medium to determine assay end-points.
13. Incubate for 6 days at 37°C in 5% CO_2 incubator.
14. Perform staining of Vero cells (see example below) or follow an alternative detection method.

The Lcd/1000 dose of diphtheria toxin is defined as the lowest concentration of toxin causing more than 50% cytotoxicity in Vero cells in the presence of 0.001 IU/ml diphtheria antitoxin.

Note that the minimum cytotoxic dose (MCD) of diphtheria toxin can be determined using the same procedure but without the addition of diphtheria antitoxin. To determine the MCD, 50 µl of complete medium should be added in step 6 instead of the diluted diphtheria antitoxin. The antitoxin control wells in column 12 should contain complete medium only and become blank control wells. Because this titration is performed without diphtheria antitoxin, it may be necessary to use a lower starting concentration of diphtheria toxin to determine the MCD.

12.2.3 Staining of Vero cells

The MTT is reduced in metabolically active, viable cells to form insoluble purple formazan crystals which are then randomised by the addition of detergent or solvent:

1. After 6 days of incubation at 37°C, remove the plate sealer and check the wells for microbial contamination.
2. Prepare a solution of MTT in PBS (5 mg/ml). Sterilise by passing through a 0.2 µM syringe filter. Add 10 µl of the sterile MTT solution to each well of the microplate using a multichannel micropipette.
3. Return the microplate to the 37°C incubator for 4 h to allow metabolism of the MTT by viable cells and formation of the blue formazan product.
4. Carefully remove the medium from all wells using a multichannel micropipette (set to >160 µl).
5. Add 100 µl of extraction buffer to all wells and return the microplate to the 37°C incubator and leave overnight to allow extraction and solubilisation of the formazan product.
6. Alternatively, monolayers of Vero cells can be stained for 5 minutes with crystal violet solution dissolved in formalin-ethanol. After staining the solution is discarded and wells rinsed with hot running tap water.
7. The plates are examined visually and the absorbance could be measured at 550-570 nm on a microplate reader. Once staining is complete, the colour is extremely stable at room temperature.

The presence of a dark blue colour indicates viable cells in both methods. A light blue colour indicates partial toxicity, while the absence of colour indicates complete toxicity and cell death.

12.2.4 Determination of potency of the antitoxin

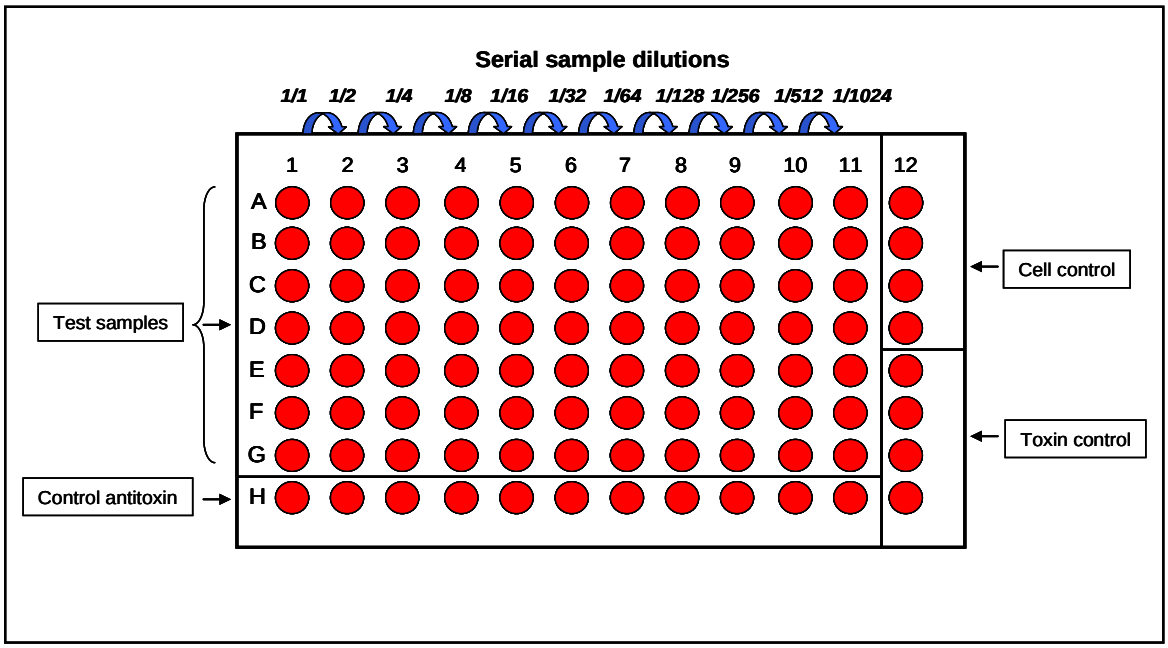
Serial twofold dilutions of test and reference serum sample are prepared in complete medium in a 96-well tissue culture microplate. After the addition of the test dose of diphtheria toxin (defined in Lcd/1000 and previously determined), the mixtures are incubated at room temperature for 1 h to allow toxin randomisation to occur. Vero cells are then added, and the plates incubated for 6 days. After 6 days of culture, the MTT assay or cell staining is performed to determine assay end-points. Reference antitoxin is included on each plate to calculate activity in IU/ml. As an internal control, antitoxin samples of known defined low/high activity may be titrated within each assay.

1. Fill all the wells of columns 2–11 with 50 µl of complete medium using a multichannel micropipette.
2. Fill the first four wells in column 12 (12A–12D) with 100 µl of complete medium using a multichannel micropipette (“cell control”).
3. Fill the last four wells in column 12 (12E–12H) with 50 µl of complete medium using a multichannel micropipette (“toxin control”).
4. Add 100 µl of each test serum sample into the appropriate well in column 1 (sera from all animals should be randomised across the plates).
5. Prepare a suitable dilution of the reference antitoxin in complete medium (for assays performed at the Lcd/1000 dose level, a starting concentration of 0.064 IU/ml should be suitable). Add 100 µl of the diluted reference antitoxin to the appropriate well in column one in every plate.
6. Make a twofold dilution series in 50 µl volumes starting at column 1 through to column 11 using a multichannel micropipette. Discard 50 µl from column 11 to randomised volumes.
7. Prepare a dilution of the test toxin in complete medium as determined previously. Test dose of toxin is defined as Lcd/1000.
8. Add 50 µl of the diluted diphtheria toxin solution to all wells in columns 1-11 using a multichannel micropipette. Add 50 µl of the diluted diphtheria toxin solution to the last four wells in column 12 (12E – 12H, toxin control).
9. Mix antitoxin with toxin by gently shaking and cover the plate with a lid.
10. Incubate at room temperature (20–25°C) for one hour to allow toxin neutralization to occur.
11. Meanwhile, prepare a Vero cell suspension in complete medium containing approximately 4 x 10^5 cells/ml (as described previously).
12. Add 50 µl of the cell suspension to all wells of the microplate. The total volume in all wells should be 150 µl.
13. Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air. Note that the use of pressure film to seal plates is an important step for methods based on colour changes in the culture medium to determine assay endpoints.
14. Incubate for 6 days at 37°C in 5% CO_2 incubator.
15. Perform the MTT assay or cell staining as described previously.

12.3 Calculation of results

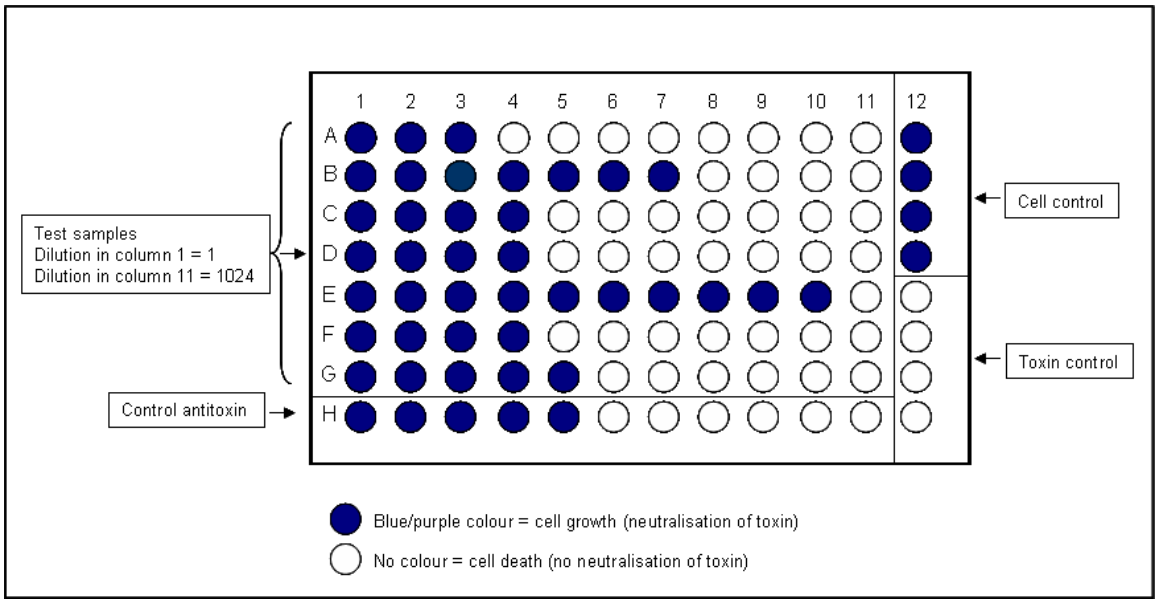
The endpoint of each test and reference serum sample is defined as the last well showing neutralization of toxin which in turn can be defined as an OD value greater than the 50% control OD value (if OD recorded). The endpoint is recorded as a score based on the dilution of the serum sample at the endpoint.

Figure A11. Example arrangement of microtiter plate layout for titration of sera using the Vero cells where only one reference sample is included.



The plate format can be modified to include additional serum controls. The position of the reference (control) antitoxin should be randomised when multiple plates are used.

Figure A12. Example of the Vero cell assay following the MTT extraction or staining



The end-point scores for individual test and reference serum samples should then be converted to titres in IU/ml by comparison with the endpoint of the reference antitoxin on each plate. The antibody concentration of each serum under test can be calculated by multiplying the dilution ratio (sample titre endpoint or score/standard titre end-point or score) with the calibrated concentration in IU of a reference standard.

Note that any dilution of the serum samples prior to titration in the assay must be taken into account to obtain the final end-point titre in IU/ml.

12.4 Validity of the test

- The test is not valid if no toxicity is observed in the wells containing Vero cells and diphtheria toxin ("toxin control").
- The test is not valid if the wells containing Vero cells alone (in complete medium, "cell control") do not show positive cell growth with a confluent monolayer of cells after 6 days of culture. As a guide, the OD 570 nm after MTT staining should be >1.
- The end-point for the reference antitoxin should be 0.002 IU/ml for assays performed at the Lcd/1000 dose level.
- The results of the negative control serum must be below the limit of detection and calculated value for the two positive control sera (if used) are within the agreed limits so that the difference between the titres of the lowest and highest standard dilution is not more that 4-fold.

APPENDIX 13 Antimicrobial Susceptibility Testing

A13.1 Materials required for disk and gradient MIC strips (E-test) antimicrobial susceptibility testing

- Normal sterile saline medium (1.5 ml quantities)
- Mueller Hinton blood agar with 5% sheep blood
- Antimicrobial disks and E-test strips
- Sterile cotton swabs
- 0.5 McFarland ($BaSO_4$) turbidity standard
- Sterile forceps / needle / disk dispenser
- Sliding callipers / ruler
- QC reference strains

A13.2 AST methods

A13.2.1 Disk diffusion by Kirby-Bauer method

- Direct colony suspension is recommended for routine AST of *Corynebacterium spp.* The accuracy and reproducibility of AST are dependent on maintaining a consistent standard set of quality procedures.
- Antimicrobial agents for testing are available as commercial disks of standard size and strength.

A13.2.2 Preparation of bacterial inoculum for disk susceptibility and MIC

- The inoculum is prepared by the direct colony suspension method from the primary culture
- Using a sterile needle/loop, touch eight 8 – 10 well-isolated colonies of the same morphological type.
- Inoculate into 1.5 ml of sterile saline
- Compare the prepared suspension to a 0.5 McFarland standard using a turbidity metre or then visually in adequate light against a card with a white background and contrasting black lines. This results in a suspension containing approximately 1 – 2 x 10^8 CFU/ml. If required, adjust the turbidity using normal saline.

A13.2.3 Inoculation of test plate

- Inoculate the Mueller Hinton blood agar plates within 15 minutes of preparation of suspension so that the density does not change.
- Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level.
- Inoculate the dried surface of a Mueller Hinton agar plate by streaking the swab over the entire sterile agar surface. Repeat procedure by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum.
- The lid may be left slightly ajar for 3-5 minutes to allow excess surface moisture to be absorbed before applying the antimicrobial disks.
- Using sterile forceps/needle, apply antimicrobial disks onto the surface of the inoculated agar plate within 15 minutes of inoculation of culture.
- Make sure that the discs are no closer than 24 mm from centre to centre. Ordinarily, no more than six disks should be placed on a 100 mm plate.
- Incubate the plates in an inverted position in an incubator set to 35±2°C within 15 minutes, in ambient air.

A13.2.4 Reading and interpretation of results

- Examine plates after 16 to 20 hours of incubation.
- If there is insufficient growth, re-incubate immediately for a further 24 hours – this could indicate that the initial bacterial inoculum was not 0.5 McFarland, and the test should be repeated.
- Measure the diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk. The point of abrupt diminution of growth, which in most cases corresponds with the point of complete inhibition of growth, is the zone edge. Zones of inhibition are uniformly circular.
- Zones are measured to the nearest whole millimetre, using sliding callipers or a ruler, which is held on the back of the inverted Petri plate, with reflected light.
- Interpret results using the appropriate guidelines.

A13.2.5 Minimum inhibitory concentration testing using E-Test

- MIC test strips directly quantify antimicrobial susceptibility in terms of discrete MIC values of *Corynebacterium* species including *C. diphtheriae*. The accuracy and reproducibility of testing are dependent on maintaining a consistent standard set of quality procedures.
- Although not the gold standard, determination of MICs by the antibiotic gradient method (e.g. E-test) are preferable for sporadic isolates of potentially toxigenic corynebacteria. E-test is a well-established method for antimicrobial resistance testing in microbiology laboratories globally and comprises a predefined gradient of antibiotic concentrations on a plastic strip. It is a useful method when the

numbers of isolates do not justify the use of broth microdilution methods. The recommended media for E-test is Mueller Hinton agar with 5% sheep blood or Iso-Sensitest agar with 5% defibrinated horse blood.

The preparation of bacterial inoculum is as described in A13.2.2

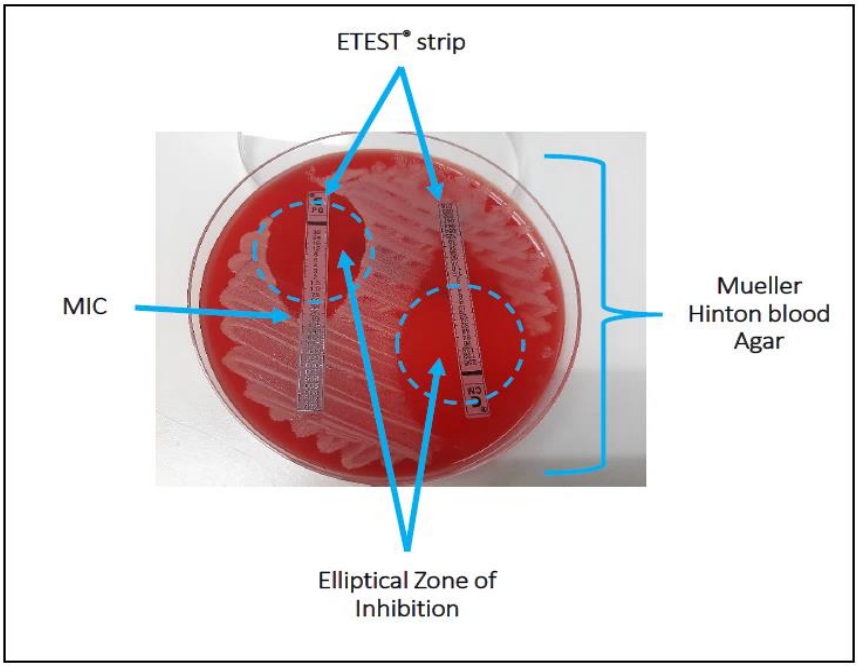
A13.2.6 Inoculation of test plates

- Inoculate the Mueller Hinton blood agar plates within 15 minutes of preparation of suspension so that the density does not change.
- Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level.
- Inoculate the dried surface of a Mueller Hinton agar plate by streaking the swab over the entire sterile agar surface. Repeat procedure by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum.
- The lid may be left slightly ajar for 3-5 minutes to allow excess surface moisture to be absorbed before applying the MIC test strips.
- Using sterile forceps, apply the strips onto the inoculated agar plate within 15 minutes of inoculation of culture (maximum of two strips per plate).
- Ensure that the MIC scale is facing upwards and that the strip is in complete contact with the agar surface.
- Once applied, the strip cannot be moved because of the instantaneous release of antibiotic into the agar.
- Incubate the plates in an inverted position in an incubator set to 35±2°C, in ambient air, within 15 minutes of applying the strips.

A13.2.7 Reading and interpretation of results

- Examine plates after 16 to 20 hours of incubation and re-incubate a further 12 hours if insufficient growth is visible. This could indicate that the initial bacterial inoculum was not 0.5 McFarland; the test should be repeated.
- Read MIC at the point where the symmetrical inhibition ellipse intersects the MIC reading scale. If a MIC value falls between a twofold dilution, always round up to the highest value.
- Remember to read the MIC value at complete inhibition of all growth, including isolated colonies.
- If the elliptical zone of inhibition differs on either side of the strip, read the MIC at the greater value.
- Ignore any growth at the edge of the strip.
- Interpret results using the appropriate guidelines. MIC values are read directly from the scale and reported in µg/ml.

- **Figure A13. Interpretation of ETEST® gradient diffusion method to determine the MIC of antimicrobial agent tested (Published with permission of bioMèrieux, France).**



A13.3 Broth microdilution MIC test

Selection of antimicrobial agents for routine testing

- The broth microdilution MIC method previously published (Clinical Laboratory Standards Institute 2020; Clinical Laboratory Standards Institute M45-2) can be done using commercially prepared Sensititre plates (Trek Diagnostic Systems, East Grinstead, UK; Sifin, Germany), which contain antibiotics diluted out in ranges appropriate for CSLI guidelines interpretation. This method, although accurate, has some limitations, including it being time-consuming and expensive.
- Follow the manufacturer's instructions for inoculum preparation, plate inoculation and incubation and result interpretation.

A13.4 Quality control of AST

It is essential that all components involved in AST are quality controlled to ensure:

- Reproducibility of AST results
- Accuracy of the procedures
- Acceptable performance of all reagents used

- Accurate techniques and methods used by personnel performing the tests
- *S. pneumoniae* ATCC 49619 and *E. coli* ATCC 25922 are recommended

References AMR

- The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 9.0, 2019. http://www.eucast.org.
- CLSI, Clinical and Laboratory Standards Institute (2016). Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria M45, 3rd edition. Wayne: Clinical and Laboratory Standards Institute.