

# The Expanded Programme on Immunization in the European Region of WHO

## Diphtheria

### Manual for the Laboratory Diagnosis of Diphtheria



Copenhagen 1994

1254

## **TARGET 5**

### **REDUCING COMMUNICABLE DISEASE**

By the year 2000, there should be no indigenous cases of poliomyelitis, diphtheria, neonatal tetanus, measles, mumps and congenital rubella in the Region and there should be a sustained and continuing reduction in the incidence and adverse consequences of other communicable diseases, notably HIV infection.

### **Keywords**

**DIPHTHERIA – Laboratory Diagnosis  
EUROPE  
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## Diphtheria

### Laboratory Diagnosis of Diphtheria

by  
Dr Androulla Efstratiou  
Co-ordinator for the European Laboratory  
Working Group on Diphtheria  
Head, Diphtheria Reference Laboratory  
Respiratory and Systemic Infection Laboratory  
Central Public Health Laboratory  
61 Colindale Avenue  
London NW9 5HT  
United Kingdom

Fax: +44 81 205 6528  
Tel: +44 81 200 4400

and

Dr P.A. Christopher Maple  
Clinical Scientist  
Diphtheria Reference Laboratory



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# BACKGROUND

In 1990, the report of a WHO meeting recommended that a group be set up to produce two manuals, for laboratory workers, clinicians and epidemiologists to help eliminate diphtheria. One part would deal with epidemiological surveillance and control of diphtheria; the other would outline procedures for the routine isolation and identification of *Corynebacterium diphtheriae*. Further, the recommendations of the 1990 meeting were that a network of collaborating centres should be established within Europe to improve the exchange of information about diphtheria. This was reiterated at the emergency meeting convened by WHO in St Petersburg, Russia (July 1993) to discuss the alarming situation in Russia, the Ukraine and surrounding areas. An important recommendation from this meeting was to form a group of participating scientists in order to develop guidelines and outline future study needs and directions for laboratories. A 'European Laboratory Working Group on Diphtheria' consisting of selected representatives from Diphtheria Reference Centres was therefore formed (see section 1.5). The aims of the Group are to strengthen laboratory collaboration and support, particularly to those in greater need and to increase current knowledge and develop new technology relating to the laboratory diagnosis and epidemiological characterisation of *C.diphtheriae* on a global basis.

This manual on laboratory diagnostic procedures replaces the previous WHO "Guidelines for the laboratory Diagnosis of diphtheria" prepared by Mr R Brooks of Swansea Public Health Laboratory (UK, 1981). It is aimed for global – as opposed to solely European Region – use and will hopefully fulfil the needs of laboratories with both minimal and maximal resources.



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# WHO MANUAL FOR LABORATORY DIAGNOSIS OF DIPHTHERIA

## 1. INTRODUCTION

### 1.1 AIMS AND OBJECTIVES OF THE LABORATORY MANUAL

The aims and objectives of this manual are to describe microbiological procedures for the identification and toxigenicity testing of *Corynebacterium diphtheriae*; and how the laboratory may assist the clinician in the diagnosis, treatment and prevention of diphtheria. Serological procedures for assessing immunity and aspects of epidemiological typing of *C. diphtheriae* will also be addressed. The manual is intended for wide use in many different countries, therefore, methodologies which may not be applicable in developing countries will be included with the aim of promoting and developing laboratory technologies within the international network of diphtheria reference centres and beyond.

### 1.2 ROLE OF THE LABORATORY IN THE DIAGNOSIS OF DIPHTHERIA

In many advanced cases a clinical diagnosis of diphtheria would normally precede the microbiological diagnosis. However, the first indication of the likelihood of the disease is often given by the microbiology laboratory reporting the presence of the causative organism *C. diphtheriae* in routine throat and other swabs taken from the respiratory tract. Early and accurate diagnosis is of the utmost importance. Clinical diagnosis, particularly in countries where the disease is uncommon is not made easily and may be confused with other infections such as tonsillitis and streptococcal sore 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, 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 from further investigation therefore avoiding unnecessary treatment or control measures such as isolation.

### 1.3 ROLE OF THE LABORATORY IN SEROLOGICAL TESTING FOR POPULATION AND INDIVIDUAL IMMUNITY/SUSCEPTIBILITY TO DIPHTHERIA

Since the early 1980s, diphtheria has begun to increase globally, particularly within the Eastern European region. Several factors have contributed to the rise and continuation of these epidemics which include, low immunisation coverage rates in some areas, lack of immunity amongst adults and the general unavailability of vaccines in some countries. In view of the evidence from these current epidemics, it is apparent that adults are a high risk group for the disease.

Population immunity studies using tissue culture toxin neutralisation, ELISA or passive haemagglutination have been performed in some European countries (Galazka 1993). There is no agreed methodology for serological testing but it is hoped that in the future, standard techniques will be established for serological testing of diphtheria immunity. This manual addresses the tests currently used by many centres where facilities are available.

## 1.4 ROLE OF REFERENCE LABORATORIES IN CONFIRMATORY AND SPECIALISED TESTING

As a result of the St Petersburg meeting a 'European Laboratory Working Group on Diphtheria' was established which consists of selected representatives from different diphtheria reference centres (as listed in section 1.5). The aims of this group are to develop guidelines and outline future study needs and directions for themselves and other laboratories. Therefore, the objectives of a reference centre are to strengthen laboratory collaboration and support, particularly to those in greater need and to increase current knowledge and develop new technology relating to the laboratory diagnosis and epidemiological surveillance of *C.diphtheriae*. A network of designated reference laboratories has therefore been established. Another important role for reference laboratories is teaching and training of scientists, particularly from developing countries, in the procedures necessary for the correct laboratory diagnosis of diphtheria. In some countries, laboratory workshops are held to encourage and update personnel in these procedures. In the UK, two such workshops have so far been held for UK laboratory workers.

## 1.5 LISTING OF WHO DESIGNATED AND OTHER DIPHTHERIA REFERENCE CENTRES (FROM WHICH FURTHER ADVICE AND GUIDANCE MAY BE SOUGHT)

The following laboratories and named participants are within the formal European Laboratory Working Group on Diphtheria:

Dr C Andronescu  
WHO Collaborating Centre for Reference and Research on Diphtheria  
Institutul Cantacuzino  
103 Spl Independentei, CP 1-325 Bucharest, Romania  
TEL: 401 312 3800/2159  
FAX: 401 638 2720

Dr A Efstratiou: European Group Co-ordinator  
Streptococcus and Diphtheria Reference Units  
Respiratory and Systemic Infection Laboratory  
Central Public Health Laboratory  
Colindale Avenue, London NW9 5HT, United Kingdom  
TEL: 81 200 4400 Ext. 4270  
FAX: 81 205 6528

Professor P Grimont: Database Co-ordinator  
Unite des Enterobacteries, Institut Pasteur  
28 Rue du Dr Roux, 75724 Paris Cedex 15, France  
TEL: 33 1 45 68 8340  
FAX: 33 1 45 68 8837

Dr T Glushkevich  
Central Sanitary Epidemiological Station  
Varoslavskaya Str 41, 25207 Kiev, Ukraine  
TEL: 7044 416 3817  
FAX: 7044 417 3775

Dr M Kiredjian  
Laboratoire des Identifications Bacteriennes  
Institut Pasteur  
28 Rue du Dr Roux, 75724 Paris Cedex 15, France  
TEL: 33 1 45 68 8336  
FAX: 33 1 45 68 8953

Dr I Mazurova  
Diphtheria Laboratory  
Gabrichevsky Institute of Epidemiology and Microbiology  
Admiral Makarov Str 10, Moscow A-212, Russia 125212  
TEL: 7 095 452 1816  
FAX: 7 095 452 1830

Dr T Popovic / Dr M Reeves: 'Special Link' to European Group  
Division of Bacterial and Mycotic Diseases  
National Center for Infectious Diseases  
Centers for Disease Control  
Bldg 1, Room 4065, MS/C09, Atlanta, Georgia 30333, USA  
TEL: 404 639 3331 / 1380  
FAX: 404 639 3926 / 3296

Dr Y Rikushin/Dr G Tseneva  
Pasteur Institute  
14 Mira Str, 197101 St Petersburg, Russia  
TEL: 7 812 232 2136  
FAX: 7 812 232 9217

Professor W Thilo  
Robert Koch Institut  
Bundesinstitut für Infektionskrankheiten  
und nicht übertragbare Krankheiten  
Nationales Referenzzentrum  
Für Diphtherie und Tetanus  
Postfach 34/13161 Berlin, Germany  
TEL: 49 30 4547 3060  
FAX: 49 30 4547 3006

Dr J Vuopio-Varkila  
Nosocomial and Diphtheria Laboratories  
KTL National Public Health Institute  
Mannerheimintie 166, SF-00300 Helsinki, Finland  
TEL: 358 0474 4240  
FAX: 358 0474 4238

## 2. PROCEDURES FOR THE ISOLATION, IDENTIFICATION, TOXIGENICITY TESTING AND REPORTING OF *C. DIPHTHERIAE*

### 2.1 INTRODUCTION

The importance of speed coupled with accuracy is essential when performing these procedures. However, the range of investigations is dependent upon the availability of reagents, experience of laboratory staff and of course financial resources. The flowchart in Figure 1 outlines the currently recommended procedures for the laboratory diagnosis of diphtheria.

### 2.2 COLLECTION AND TRANSPORT OF SPECIMENS FOR LABORATORY EXAMINATION

The successful isolation of *C.diphtheriae* depends initially on the collection of swabs and their subsequent transfer to the laboratory. As diphtheria is most commonly an upper respiratory tract infection, ear, throat, nasopharyngeal and nasal swabs should be taken. If present, membranous material should be examined also. The only other common form is cutaneous diphtheria which is often indistinguishable from any other pyoderma, especially in parts of the world where diphtheria is endemic. Swabs should be taken from any wounds or skin lesions in patients with suspected diphtheria. Post-mortem specimens from the upper respiratory tract and vital organs may be examined in extreme cases where autopsy is required to confirm the cause of death as diphtheria.

After collection the swabs must be sent to the laboratory immediately as rapid inoculation of special culture media is important. At the same time the clinician should inform the laboratory of any presumptive diagnosis of Diphtheria. Recent blood culture isolations of *C.diphtheriae* from patients with endocarditis from both Europe and Australia highlights the importance of correct isolation and screening procedures for these organisms from normally sterile sites. (Lortholary *et al.* 1993, Tiley *et al.* 1993).

#### 2.2.1 Materials required for sampling

- Strong light source for illuminating the pharynx
- Sterile cotton wool swab in a sterile container
- Sterile tongue depressor
- Sterile saline for skin lesions

#### 2.2.2 Procedures for collection of samples (Appendix 2.2.2)

#### 2.2.3 Materials required for transport

If specimens cannot be transported to the laboratory immediately, the use of a transport medium such as that described by Amies (1976) should be considered (Appendix 2.2.3). According to Brooks (1981) if transit times are to exceed 24 hours the use of a silica-gel transport medium should be considered (Sinclair *et al.* 1972, Facklam *et al.* 1978).

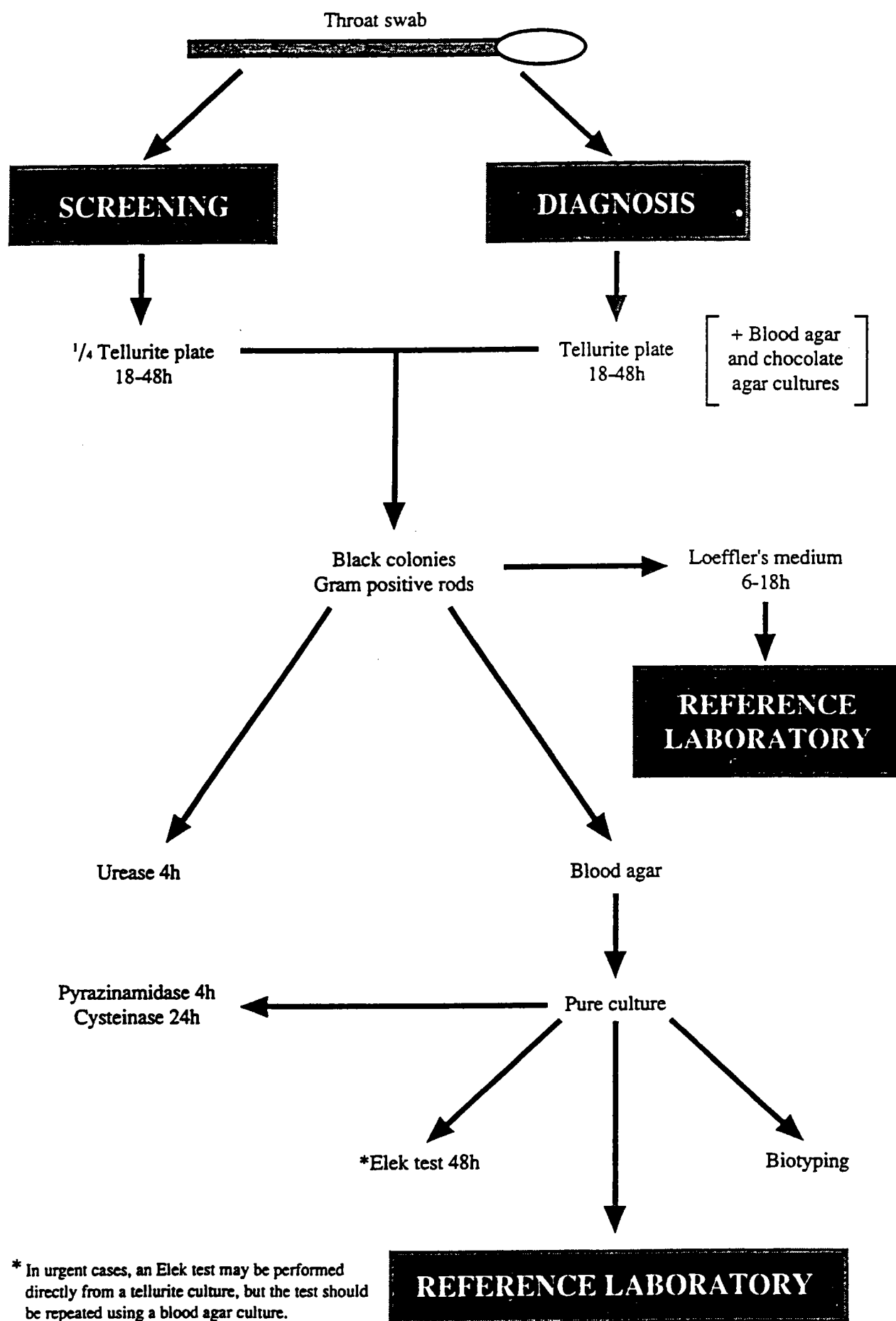


Figure 1. Laboratory Diagnosis of Diphtheria

#### 2.2.4 Minimum clinical and epidemiological data required to accompany specimens

For surveillance and monitoring purposes it is important that the laboratory ensures that it receives the following information for each specimen;

Patient details:	Name, age, sex Hospital where admitted Physician caring for patient
Laboratory details:	Source of specimen(s) Date(s) collected
Clinical details:	Symptoms Onset date Treatment – antibiotics antitoxin
Epidemiological Information:	Case, contact or carrier Immunisation history Travel history Contact list

### 2.3 LABORATORY PROCEDURES FOR PRIMARY ISOLATION

Specimens must be inoculated onto culture media without delay. The diagnosis of diphtheria based upon direct microscopy of a smear is not to be advised as both false positive and false negatives may occur. For the examination of swabs taken from carriers, contacts and convalescent patients, the use of selective culture media is important as these swabs may only contain small numbers of *C.diphtheriae* which may be obscured on other media such as Loeffler's by overgrowth with other bacteria. For this reason, Loeffler's serum medium is not recommended for primary isolation.

#### 2.3.1 Culture media (Appendix 2.3.1)

The minimal culture media required along with useful and rapid screening tests for the isolation of *C.diphtheriae* are:

- Columbia blood agar plate
- Tellurite blood agar plate
- Tinsdale medium (screening test)
- Pyrazinamide (for pyrazinamidase screening test)
- Loeffler serum slope

Primary culture onto blood agar and a selective tellurite medium such as Hoyle's is essential (Hoyle 1941). However, any infusion blood agar base to which potassium tellurite and blood are added produces good results.

In recent years, Tinsdale's medium for the detection of the enzyme cysteinase has been recommended for the rapid isolation of *C.diphtheriae* (Colman *et al.* 1992). If adequately batch tested, with strong and weak enzyme producing strains, the medium is very useful for the confirmation of suspicious colonies found on generally accepted tellurite media, as only *C.diphtheriae*, *C.ulcerans* and *C.pseudotuberculosis* will produce the characteristic black colonies surrounded by a brown halo after overnight incubation.

Another very useful test for the presumptive identification of *C.diphtheriae* is the pyrazinamidase test. These tests along with others are described in Appendix 2.4.3.

2.3.2 Inoculation and incubation of culture media

Unless otherwise indicated all incubations are performed aerobically at 37°C for 18–24h. If available, the use of disposable loops is to be recommended for inoculation of culture media. However, this is not always possible. Therefore, it is preferable to have several wire loops available which avoids the need to wait for the loop to cool after heating and the danger of using a hot loop. Bunsen burners are used to sterilise the loops.

2.3.3 Procedures for obtaining pure cultures

The procedures for obtaining pure cultures are as follows:- In brief, if the swab is received in the laboratory without the use of any transport medium, then it should be moistened with a few drops of sterile nutrient broth. The swab is then rubbed over a quarter of the surface of firstly, the columbia blood agar or blood agar plate and then the tellurite plate (area 1 in Figure 2). Using sterile loops inoculate each plate as described in Figure 2. The plates are incubated as previously described. For economy, once experience has been gained by laboratory staff, a half plate for each swab may be used. If a half plate technique is used each half must be clearly labelled and special care taken to ensure that each sample is plated in the correct place.

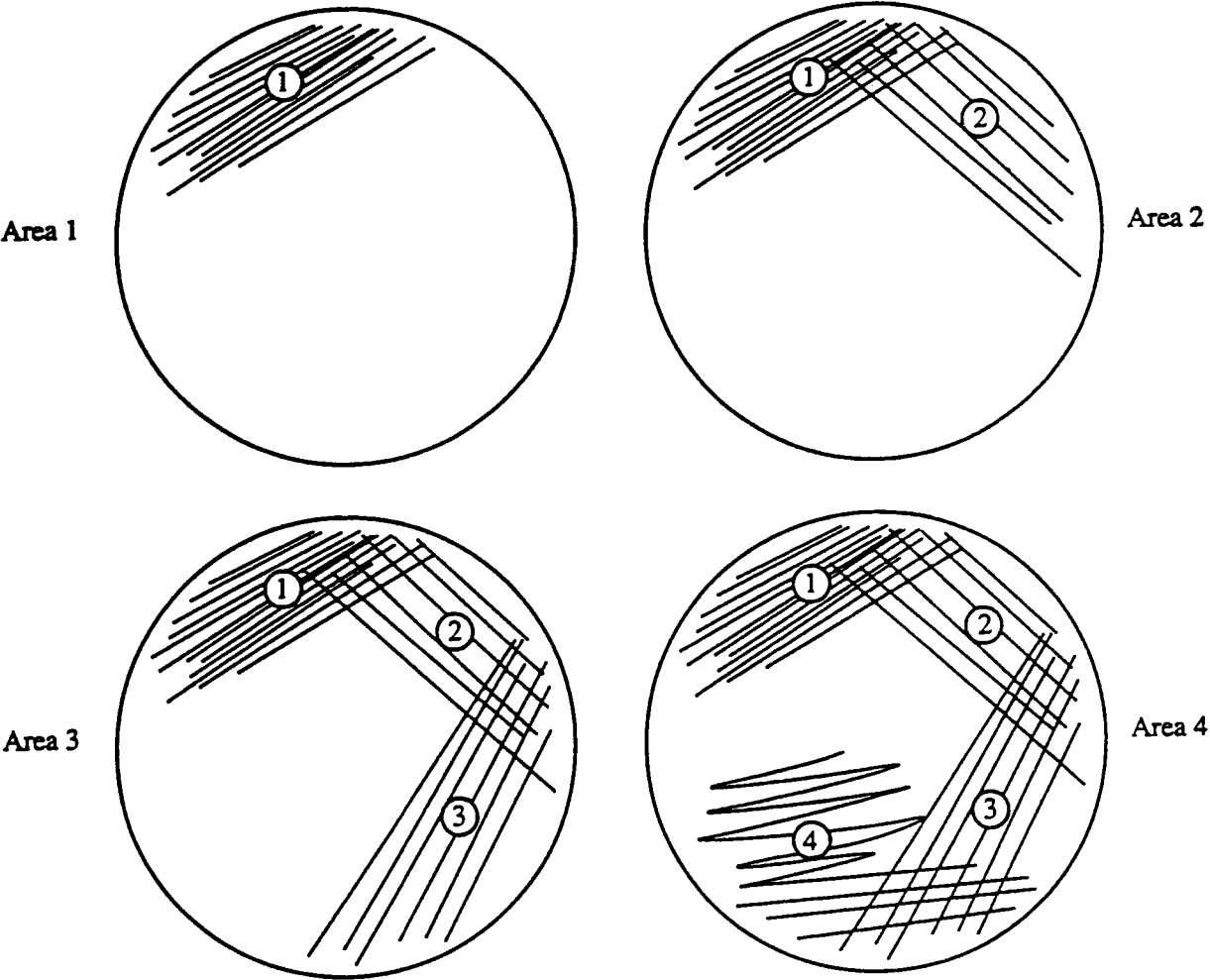


Figure 2. Primary plating of swabs for the isolation of *C. diphtheriae*



### 2.3.4 Criteria for recognizing suspect colonies that require further evaluation

Primary plates must be examined after 18–24 hours incubation, so that any suspicious colonies can be subcultured as rapidly as possible. It is also advisable to examine colony morphology with a hand lens in reflected light. If there is no visible growth on both blood agar and tellurite plates, then further swabs should be requested immediately, as it is likely that the swab(s) have not been collected properly.

The blood agar plate is useful in that it is used for the detection of pyogenic streptococci such as Lancefield groups A, C or G 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 therefore, to examine the blood agar plate carefully for suspect colonies of *C.diphtheriae* which should be subcultured to blood agar for further (Sections 2.4 and 2.5). It is advisable to replate stock cultures of *C.diphtheriae* regularly to ensure recognition of colonial morphologies. Type strains from international culture collections are recommended.

## 2.4 LABORATORY PROCEDURES FOR SCREENING TESTS TO ALLOW RAPID CONFIRMATION OF PUTATIVE *C. DIPHTHERIAE*

### 2.4.1 Colonial morphology on conventional media (Appendix 2.3.1)

Differences in the morphological appearances of typical colonies of *C.diphtheriae* on tellurite blood agar (after 48h incubation aerobically) may be described as follows:

- |   |   |
|---|---|
| <b><i>C.diphtheriae</i> var <i>gravis</i>:</b>      | Dull, dry, grey, opaque colonies, 1.5–2mm in diameter with a matt surface. Colonies are friable, they tend to 'break' into small segments when touched with a straight wire. They can also be 'pushed' across the surface of the medium without breaking. Non-haemolytic usually. |
| <b><i>C.diphtheriae</i> var <i>mitis</i>:</b>       | Grey, opaque colonies, 1.5–2mm in diameter with an entire edge and smooth surface. Variation in size is a common characteristic. They tend to exhibit a small zone of beta haemolysis on blood agar.  |
| <b><i>C.diphtheriae</i> var <i>intermedius</i>:</b> | Small, grey, discrete, translucent, colonies, 0.5–1mm in diameter.  |

### 2.4.2 Staining Procedures (Appendix 2.4.2).

Films of suspicious colonies from tellurite or blood agar plates should be prepared. The common microscopic characteristics of pathogenic corynebacteria are listed below:

- They are usually Gram positive but some strains of *C.diphtheriae* tend to over decolourise and appear Gram variable
- Straight or slightly curved rods with tapered ends, pleomorphic
- Non-motile
- Non-sporing
- Non-acid fast
- Stain unevenly with the formation of metachromatic granules

2.4.3 Screening tests: Pyrazinamidase, Cysteinase (Appendix 2.4.3).

There are predominantly three biotypes of *C.diphtheriae* described as *gravis*, *mitis* and *intermedius*. The biotype *intermedius* has not been seen in Europe for many years and, most of infections are caused by the other two biotypes.

Useful screening tests for the presumptive identification of these biotypes, and of *C.ulcerans* and *C.pseudotuberculosis*, are the pyrazinamidase and cysteinase tests (Appendix 2.4.3). Our finding however, that two species of *C.pseudodiphtheriticum* did not produce the enzyme, indicates that it should not be the only screening test used. A combination of the cysteinase and pyrazinamidase tests should be adequate (Figure 3).

Minimal laboratory information required to report a specimen as negative for *C.diphtheriae*

Once suspicious colonies have been confirmed as coryneforms by Gram stain, they are subcultured onto non-inhibitory media; blood agar for screening tests, biotyping and toxigenicity testing and Loeffler's medium for demonstrating characteristic microscopic morphology by Albert's stain or Loeffler's methylene blue.

The tests for pyrazinamidase (PYZ) activity and cysteinase production are useful screening tests to distinguish between the three potentially toxigenic species and other coryneforms (see Section 2.4.3). If screening tests are not available, conventional biochemical methods should be employed (Section 2.5.1 and Table 1). Where possible toxigenicity testing should be initiated without delay. Most biotypes of *C.diphtheriae* tend to be:-

- catalase positive
- urea negative
- nitrate positive (except '*belfanti*')
- pyrazinamidase negative
- cysteinase positive and ferment glucose, maltose, starch (*gravis* only).

TABLE 1 BIOCHEMICAL IDENTIFICATION OF PATHOGENIC CORYNEBACTERIA

ORGANISM	CYS	PYZ	GLUCOSE	MALTOSE	SUCROSE	STARCH	NITRATE	UREA
<i>C.diphtheriae</i>								
var <i>gravis</i>	+	-	+	+	-	+	+	-
var <i>mitis</i>	+	-	+	+	-	-	+	-
var <i>intermedius</i>	+	-	+	+	-	-	+	-
var <i>belfanti</i>	+	-	+	+	-	-	-	-
<i>C.ulcerans</i>	+	-	+	+	-	+	+	+
<i>C.pseudotuberculosis</i>	+	-	+	+	-	+	+	+
<i>C.pseudodiphtheriticum</i>	-	+	-	-	-	-	+	+
<i>C.xerosis</i>	-	+	+	+	+	-	+	-

CYS: Cysteinase production on Tinsdale  
Positive, characteristic black colonies surrounded by a brown halo after overnight incubation

PYZ: Pyrazinamidase activity, 4h or 24h tube test  
Orange colouration after reaction with development reagent denotes a positive

## 2.5 LABORATORY PROCEDURES FOR DEFINITIVE CONFIRMATION OF TOXIGENIC *C.DIPHThERIAE*

A procedure for the identification of *C.diphtheriae* is outlined in Figure 1. This includes biochemical identification and determination of toxigenicity status of the isolate.

### 2.5.1 Biochemical testing and biotyping

Materials required and methodologies: (Appendix 2.5.1) Although of little help to the management of the patient, biochemical testing is essential for biotyping purposes, for contact tracing and for epidemiological studies. The simple conventional tests to be recommended are predominantly, the reduction of nitrate, hydrolysis of urea, catalase production, cysteinase activity, pyrazinamidase activity and fermentation tests for glucose, sucrose, maltose and starch (Table 1). However, commercial kits such as the API CORYNE (bioMérieux) and individual test systems (Rosco Diagnostica) are available.

#### Conventional Tests

1. Reduction of nitrate
  - nitrate broth
2. Hydrolysis of urea
  - Christensen's urea slope
3. Sugar fermentation tests (Hiss Serum Water Sugars)
  - Glucose
  - Sucrose
  - Maltose
  - Starch
4. Catalase production

#### Commercial Tests

Rosco Diagnostica have developed a range of diagnostic tablets containing chromogenic and modified substrates which are able to detect preformed enzymes and are useful in the rapid identification of bacteria. Tests currently available which are useful for the rapid identification of *C.diphtheriae* are: PYZ test, urea hydrolysis, nitrate reduction and the fermentation of glucose, sucrose and maltose (starch is not available). These tests are either 4 or 24 hour incubations at 37°C (Appendix 2.5.1).

There is also a 24 hour kit (API CORYNE system) incorporating 20 tests for the identification of corynebacteria (Appendix 2.5.1).

### 2.5.2 Toxin testing, with the *in vitro* conventional Elek test

The most important test for the microbiological diagnosis of diphtheria is the detection of toxin-producing strains. Not all *C.diphtheriae* isolates are able to produce toxin. The clinical and epidemiological significance of non-toxigenic *C.diphtheriae* are different. It is therefore of utmost importance to obtain an accurate result as soon as possible, in order to confirm the diagnosis of diphtheria and to contain possible spread of the disease by identifying contacts who may be carriers. There are several *in vitro* and *in vivo* methods available but these are dependent upon

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 has recently been improved (Colman *et al.* 1992). The improved test uses a superior Elek medium which has considerably increased the clarity and accuracy of the test. Iron limitation is an essential factor in the expression of the gene for diphtheria toxin, even amongst weakly toxigenic strains. The layout of the test strains against the three Elek controls (NCTC 10648, NCTC 3984, NCTC 10356) is described in Figure 4. The methodology for the test is described in Appendix 2.5.2.

### Interpretation of the test (Figure 5)

The Elek test should be examined at 24 and 48 h. Classic precipitin lines forming an 'arc' with the positive controls denotes a toxin producing strain. Non-specific precipitin lines are apparent if the test is incubated longer than 48h. It is usual to examine the Elek plate for white lines of precipitation commencing about 10mm from the filter paper strip and occurring at an angle of about 45° to the line of growth. These are shown by the positive toxigenic control strains. If the test strain shows similar lines 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.

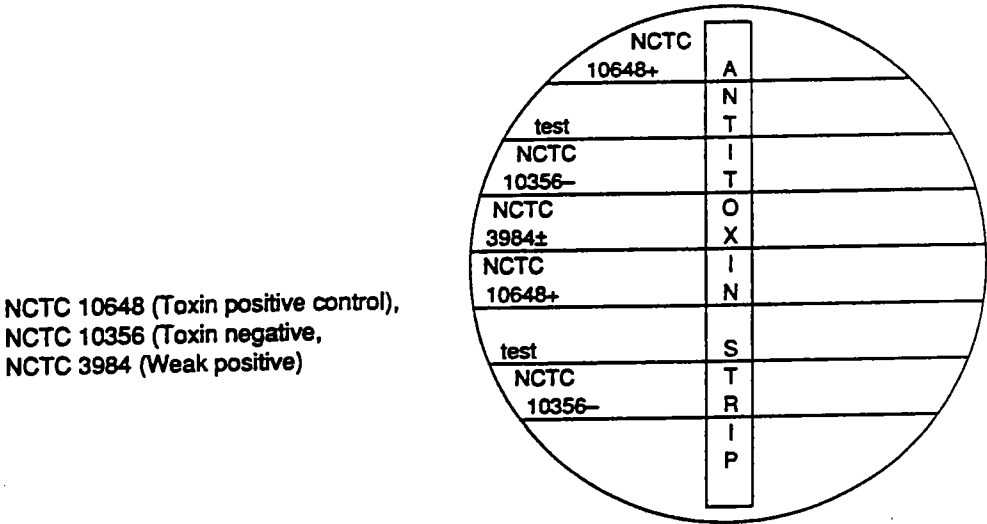


Figure 4. Diagrammatic representation of the Elek Plate

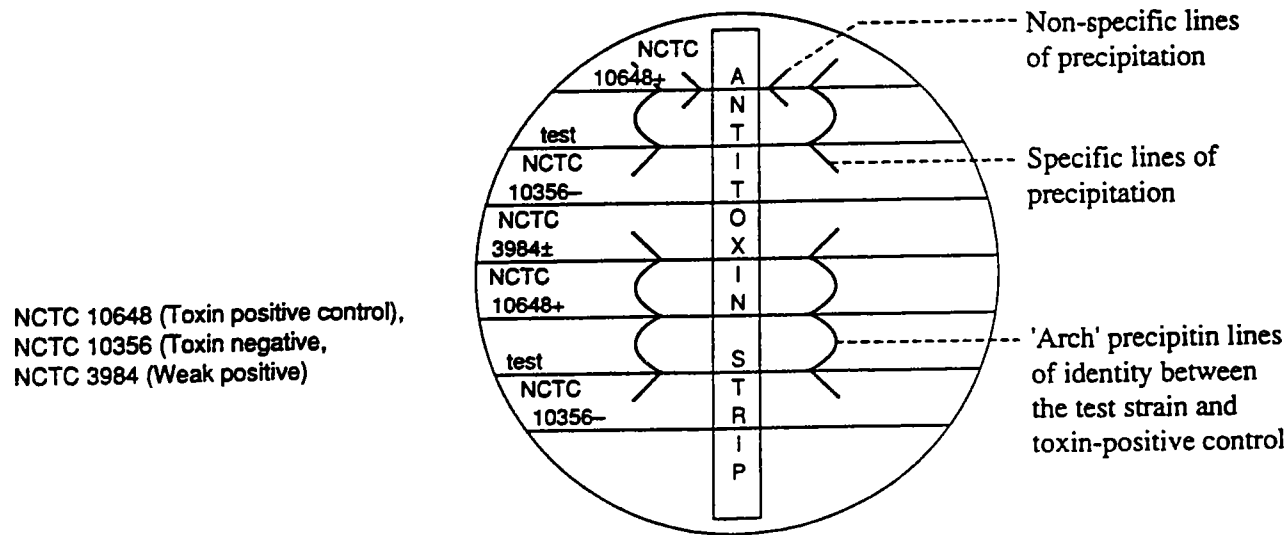


Figure 5. Interpretation of the Elek Plate

## Notes on Elek Test

1. The modified agar base medium is recommended as the most suitable medium for the test. The medium must be clear so as 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 4°C.
2. Newborn bovine serum is recommended for the test, but several other sera have been used. Rabbit, calf and adult bovine serum produce reasonable results. 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 3ml amounts in sterile screw cap bottles and stored at -20°C. Sera stored in this way can remain stable for up to one year.
3. The antitoxin recommended is available from the Swiss Serum and Vaccine Institute, Berne, Switzerland with the recommended concentration being 500 units for incorporation into the antitoxin strips for the Elek test. It is advisable to obtain the antitoxin from the same manufacturer as some batches of antitoxin from different manufacturers cause excessive precipitin lines of non-specificity. The antitoxin is normally stored at 4°C.
4. The control strains of *C.diphtheriae* recommended for the test are those used in the UK and may be obtained from the Curator at National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT.

The stock control and other cultures of *C.diphtheriae* are maintained in 16% (v/v) glycerol broth and stored at -20°C or -70°C (Appendix 2.5.1).

## Other immunoassays for detection of diphtheria toxin

Other methods that have been employed for the detection of diphtheria toxin from bacterial cultures are the verocell immunoassay, western blotting, dot blotting and a capture ELISA assay. However, these tests, although sensitive and specific, are tedious and time consuming and are not recommended for routine use.

### 2.5.3 *In vivo* Toxin testing

The most reliable test for detecting toxin production is the subcutaneous test for virulence in guinea pigs. The virulence test involves injection of live Loeffler-slope grown cultures into non-protected and protected (previously injected with 200 units of diphtheria antitoxin, immediately prior to the inoculation of the live culture) guinea pigs. (Appendix 2.5.3). This is a test that is not to be recommended for inexperienced laboratory personnel.

#### Interpretation and notes on the subcutaneous test for virulence (Appendix 2.5.3)

If an isolate of *C.diphtheriae* is weakly toxigenic the test may take 7–10 days before characteristic features develop (Appendix 2.5.3), although, most results are apparent within 48–72h.

### 2.5.4 Genotypic tests based on the Polymerase Chain Reaction (PCR) for detection of the Diphtheria Toxin Gene

The use of PCR has recently been introduced to detect the diphtheria toxin gene, particularly the biologically active (Fragment A) portion (Pallen *et al.* 1994), which usually produces an amplicon of 248bp. Primers specific for different regions of the gene have been described and used successfully (Lucchini *et al.* 1992, Hauser *et al.* 1993).

For diagnostic purposes, primers specific for the A portion are used to amplify a toxin gene fragment from simpleboiled cell preparations. The PCR method is extremely simple and rapid: a result may be obtained in 5–6 hours from the selection of colonies to the final result (Figure 6 and Appendix 2.5.4).

PCR is a powerful tool with several advantages over the traditional methods in that it works on mixed cultures, cultures from inhibitory media and does not require difficult to standardise biological reagents.

There are also disadvantages using this system, thus it is advisable to use PCR only as an adjunct to conventional methods such as the Elek test. A disadvantage with PCR is that some *C.diphtheriae* isolates have been found to carry the toxin gene, but are unable to express the protein biologically. Such isolates are relatively rare worldwide (a small number have been identified in the mid-USA, Canada and Trinidad (Efstratiou *et al.* 1993). It is thus unlikely that these isolates will compromise the utility of the PCR assay except perhaps in certain defined localities.

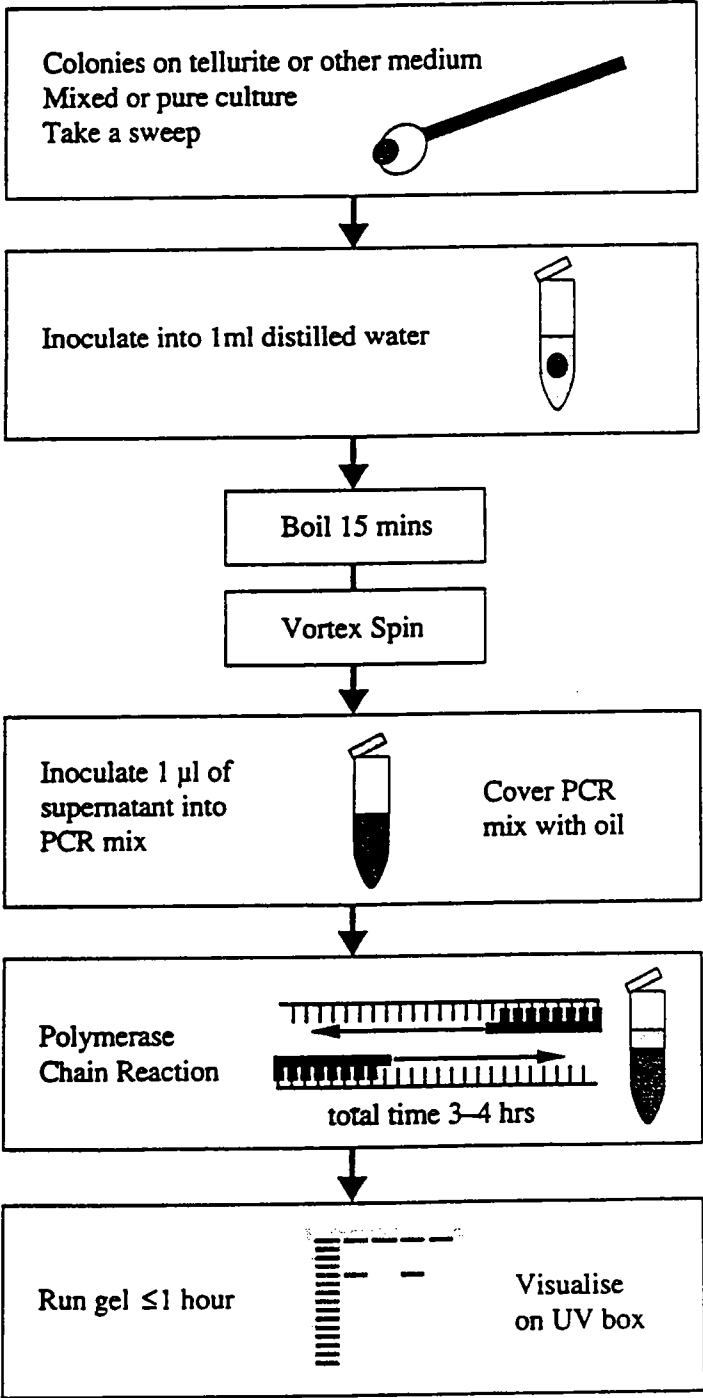


Figure 6. Protocol for the detection of the *tox* gene by PCR

### 2.5.5 Minimal laboratory information required to report a specimen as positive for toxigenic *C. diphtheriae*

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

### 2.5.6 Recognition and significance of non-toxigenic *C. diphtheriae*

Non-toxigenic *C.diphtheriae var gravis* have emerged recently as a pathogen within the UK among persons with severe throat infections. (Efstratiou *et al.* 1993). A recent report drew attention to its isolation from patients attending a genito-urinary medicine clinic at a London hospital (Wilson *et al.* 1992). All patients, the majority of whom were homosexual men, presented with pharyngitis. Molecular typing confirmed the clonality of these isolates. Other 'clones' have also been identified within the UK. The number of infections caused by non-toxigenic *C.diphtheriae var gravis* has significantly increased and this observation has also been noted in Australia where several cases of endocarditis caused by this non-toxigenic biotype have been described (Efstratiou *et al.* 1993, Tiley *et al.* 1993). Reports of similar cases have also been documented in Europe. The apparent increase of this biotype is of concern in view of its association with serious invasive disease (Lortholary *et al.* 1993). The global incidence of infections caused by non-toxigenic biotypes is unknown but it would be worthwhile for countries to record the incidence within their surveillance framework for diphtheria. The isolation of a non-toxigenic *C.diphtheriae* from the throat culture of a patient who has severe or mild pharyngitis without the presence of a membrane is not defined as a case of diphtheria. A reliable case reporting system should be available in every country. Infections caused by non-toxigenic *mitis* are generally uncommon in Europe, however, they do occur and are invariably associated either with cutaneous infections or pharyngitis.

## 2.6 LABORATORY RESPONSIBILITY FOR REPORTING SAMPLES POSITIVE FOR TOXIGENIC *C. DIPHTHERIAE*

### 2.6.1 Who needs to know and when

Upon isolation of a toxigenic strain of *C.diphtheriae*, 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.

### 3. PROCEDURES FOR SEROLOGICAL TESTING FOR POPULATION AND INDIVIDUAL IMMUNITY/SUSCEPTIBILITY TO DIPHTHERIA

#### 3.1 LABORATORY PROCEDURES FOR ASSAYING DIPHTHERIA ANTITOXIN IN PATIENT SERUM SAMPLES

The earliest methods for measuring serum antitoxin levels were developed by Behring, Ehrlich and Roux (1892–1895) and all of them used the guinea pig as a sensitive detection system for titrating toxin neutralisation by serum antitoxin. This model has been retained to the present day and national and internationally agreed methodologies have been published (British Pharmacopoeia, 1993; European Pharmacopoeia, 1971). These bioassays compare the dose of antitoxin necessary to protect guinea pigs or rabbits against the erythrogenic effect (the degree of redness and inflammation at the inoculation site) of a fixed dose of diphtheria toxin to the dose of a standard preparation of diphtheria antitoxin which gives the same protective effect.

*In vivo* toxin neutralisation testing using guinea pigs or rabbits is regarded as the gold standard test for determining serum antitoxin levels. Despite the modifications in technique established by Romer (1909) and Jensen (1933) for the testing of human serum, *in vivo* testing is still elaborate, time-consuming, and expensive in its requirement of animal biomass and test serum. Fortunately, alternative tests using cultured animal cells have been developed as reliable alternatives to *in vivo* testing. In 1957, Placido Sousa and Evans reported that monkey cell cultures were as susceptible as the skin of the guinea pig to the action of diphtheria toxin and that the cytopathic effect of the toxin could be neutralised by specific antitoxin. Since this report a considerable amount of evidence has been amassed (Kriz *et al.*, 1974; Miyamura *et al.*, 1974a; Kjeldsen *et al.*, 1988; Melville-Smith & Balfour, 1988) showing tissue culture neutralisation to be as reliable as *in vivo* neutralisation for titrating the antitoxic potency of sera. In our laboratory, we use a modified version (Miyamura *et al.*, 1974b; Aggerbeck & Heron, 1991; Hansen *et al.*, 1989) of the monkey cell assay – the vero cell assay for determining the diphtheria antitoxin content of human serum. Full details of methodologies in current use are available from the Diphtheria Reference Laboratory, London (Full address in Section 1.5).

ELISA (Camargo *et al.*, Schou *et al.* 1987; Ramsay *et al.*, 1991) and passive haemagglutination (Ajello *et al.*, 1991; Galazka & Kardymowicz, 1989; Kameyama *et al.*, 1989) tests have also been developed and used for measuring serum antitoxin levels. The attraction of both these methods is their rapidity and convenience, however there have been a number of reports suggesting that they may be less reliable than tissue culture neutralisation assays. Passive haemagglutination assays have been found to lack sensitivity for sera containing <0.1 IU/ml antitoxin (Kriz *et al.*, 1978; Cellesi *et al.*, 1989) with an ensuing risk of false-negative interpretations. Similarly, ELISA tests have been shown to have a poor correlation with tissue culture and *in vivo* neutralisation tests for sera containing <0.1 IU/ml antitoxin (Melville-Smith & Balfour, 1988; Cutts & Begg, 1992; Cohen *et al.*, 1991) with a significant risk of false positive interpretations of immunity. It is considered that the ELISA false-positive reactions are due to the binding of non-neutralising antibodies (Padovan *et al.*, 1991; Sesardic & Corbel, 1992). ELISA tests have been used for preliminary screening of sera followed by retesting of sera with antitoxin titres of <0.1 IU/ml by tissue culture/*in vivo* neutralisation assays (Melville-Smith & Balfour, 1988). Our laboratory has not adopted this approach because it has been reported that even for sera with antitoxin concentrations above 0.1 IU/ml ELISA may not be a reliable predictor of immunity (Sesardic & Corbel, 1992).



### Antitoxin levels and immunity to diphtheria

Schick testing and observations of circulating antitoxin levels in individuals developing diphtheria (Parish & Wright, 1938; Ipsen, 1946; Topley & Wilson, 1938; Andrewes *et al.*, 1923; Bjorkholm *et al.*, 1986) have resulted in the following widely adopted interpretations of measured antitoxin levels (Mofredj & Guerin, 1993; Christenson *et al.*, 1989; Bannister & Corbel, 1991; Mark *et al.*, 1989).

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	levels of antitoxin giving SOME degree of protection
0.1 IU/ml	a PROTECTIVE level of circulating antitoxin
≥1.0 IU/ml	a level of antitoxin giving LONG-TERM protection

## 4. ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *C. DIPHTHERIAE*

### 4.1 INTRODUCTION

Penicillin and erythromycin appear to be the agents of choice (Farizo *et al.*, 1993; Björkholm *et al.* 1987; Harnisch *et al.* 1989) for

- eradicating colonisation of *C. diphtheriae* in clinical cases of diphtheria thereby limiting toxin production and reducing the likelihood of transmission
- eradicating *C. diphtheriae* colonisation in carriers
- use as post-exposure prophylaxis for preventing colonisation of *C. diphtheriae* in contacts of clinical cases or carriers

#### 4.2.1 In vitro susceptibility of *C. diphtheriae* to $\beta$ -lactams and macrolide/lincosamide antibiotics

*C. diphtheriae* strains, even those isolated after failed courses of penicillin treatment, are invariably susceptible to penicillin (Long, 1947; Public Health Laboratory Service 1948; Jackson *et al.* 1950; Zamiri and McEntegart, 1972). A wide range of values for penicillin susceptibility have been reported in the literature, however, a number of authors have correctly pointed out that the divergence of results is probably due to differences in methodology rather than differences between strains (Long, 1947; Public Health Laboratory Service, 1948; Jackson, 1950). A number of *in vitro* studies (Zamiri and McEntegart, 1972, Gordon *et al.* 1971) have shown penicillin to be more active against *C. diphtheriae* than ampicillin. Cephalosporins such as cephalothin and cephalexin are less active against *C. diphtheriae* than penicillin (Gordon *et al.* 1971) although cephaloridine has been shown to be as active as penicillin (Zamiri and McEntegart, 1972). One report (Public Health Laboratory Service, 1948) has suggested that strains of the *gravis* biotype are more resistant to penicillin than the *intermedius* and *mitis* biotypes. However, this observation has not been reproduced in other studies (Jackson, 1950, Zamiri and McEntegart, 1972).

*C. diphtheriae* is usually susceptible to erythromycin (Zamiri and McEntegart, 1972; Gordon *et al.* 1971) which is marginally more active *in vitro* than penicillin. Clindamycin has also been shown to be more active than penicillin *in vitro* (Zamiri & McEntegart, 1972). Newer macrolides which only require one or two doses daily, such as dirithromycin, clarithromycin and roxithromycin are less active ( $MIC_{50}$  0.03 – 0.06 mg/L) than erythromycin ( $MIC_{50}$  0.008 mg/L) (Bauernfeind, 1993). Macrolide resistance in *C. diphtheriae* can occur (Jellard and Lipinski, 1973; Coyle *et al.* 1979). For the period 1972 to 1983, erythromycin resistance was found in 105 (1.9%) of 5,672 strains of Canadian origin (Dixon, 1984). Erythromycin resistance in *C. diphtheriae* appears to be of the inducible (MLS) type (Coyle *et al.* 1979) and to be plasmid mediated (Schiller *et al.* 1980).

#### 4.2.2 In vitro susceptibility of *C. diphtheriae* to other antibiotics

Rifampicin has been found to be highly active against *C. diphtheriae* and, on the basis of this, it has been proposed and used for the eradication of *C. diphtheriae* colonisation (Gordon *et al.* 1971; McLaughlin *et al.* 1971). We would advise caution with this approach as we have recently detected rifampicin resistance in a small number of international isolates referred to our Laboratory (Maple *et al.* 1994). In general, we have found *C. diphtheriae* to be sensitive to chloramphenicol, gentamicin, streptomycin and tetracycline as have other workers (Gordon *et al.* 1971; Jackson *et al.* 1950). Tetracycline resistance has been reported in *C. diphtheriae* (Rockhill *et al.* 1982).

### 4.3 METHODS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

It is not realistic to recommend a single standard methodology for general antimicrobial susceptibility testing to be used in all countries (World Health Organisation, 1977). However, we are of the opinion that a small group of reference laboratories working closely together should strive to agree upon as many basic points of susceptibility testing methodology as possible. Standard methods could include types of growth media, inoculum size, choice of breakpoints and use of the same control strains. Full details of methodologies currently used at the Diphtheria Reference Laboratory, London (see address – Section 1.5) are available upon request.

We must not be complacent in our present knowledge that many strains of *C. diphtheriae* are susceptible to a wide range of antibiotics. Emergence of resistance to penicillins or further dissemination of erythromycin resistance could cause major problems. When testing for penicillin resistance, we should consider looking for  $\beta$ -lactamase production and resistance due to low-affinity penicillin-binding proteins. Screening for plasmid content of strains and research into the potential for the spread of plasmid/transposon-mediated resistance should be considered by reference laboratories if further resistance problems arise.

## 5. SPECIALISED TESTING UNDERTAKEN BY REFERENCE LABORATORIES

This section will describe the schemes available for the epidemiological typing of *C.diphtheriae*. These methods are undertaken by reference laboratories within the European Laboratory Working Group on Diphtheria.

### 5.1 CONVENTIONAL TYPING METHODS

There have been several schemes described based on traditional methods such as serotyping (Hewitt 1947), phage typing and bacteriocin typing (Gibson and Colman 1973). The preferred method is phage typing which is currently being performed by the WHO Reference Centre in Bucharest, Romania.

#### 5.1.1 Phage Typing

The Romanian phage typing schemes were originally published by Saragea and Maximescu in 1969. This system is widely used as the epidemiological typing tool for *C.diphtheriae* in the WHO Reference Centre in Bucharest. The original phage typing set consists of 22 phages whereby the three biotypes of *C.diphtheriae* may be subdivided into 21 phage types which correlate with biotype and toxigenicity status. The Diphtheria Laboratory in Bucharest recommends phage typing as a suitable method for the characterisation of *C.diphtheriae* in epidemiological situations. Further advice relating to technique is available from the Diphtheria Reference Centre in Bucharest.

### 5.2 MOLECULAR TYPING METHODS FOR *C.DIPHTHERIAE*

The application of molecular typing methods for *C.diphtheriae* has been rewarding and is becoming increasingly important. Pappenheimer and Murphy (1984) first demonstrated the resolving power of molecular methods when they analyzed *C.diphtheriae* isolates using DNA restriction patterns and hybridisation patterns with different DNA probes targeting different areas of the toxin gene.

The following techniques are now available for molecular typing of *C.diphtheriae* within the Reference Centres listed. Further information relating to methodologies may be obtained from those centres (in brackets).

#### 5.2.1 Restriction Fragment Length Polymorphisms of rRNA Genes or Ribotyping (UK, FINLAND, FRANCE, RUSSIA and USA)

RFLPs of rRNA genes is a useful way of discriminating between closely related isolates. Ribosomal RNA gene sequences are highly conserved and are present as multiple copies in the bacterial genome of all bacteria. The technique relies upon the electrophoresis of chromosomal DNA restriction enzyme digests, Southern blotting (transfer of the DNA fingerprints to a nylon membrane) followed by hybridisation with a non-radioactive (biotinylated) cDNA transcript of RNA (Figure 7).

So far, results from various centres indicate geographical heterogeneity between isolates and homogeneity between clusters/outbreaks. Computerised database storage of the molecular patterns generated could aid in the confirmation and identification of apparent clones in different parts of the world. This is currently being addressed by the European laboratory Working Group on Diphtheria. Ribotyping is an effective and discriminatory typing method and is of immense value in exploring epidemiologically related collections of *C.diphtheriae* isolates.

### 5.2.2 DNA Amplification Fingerprinting or RAPD (UK, FRANCE and RUSSIA)

DNA amplification fingerprinting using PCR has become a key procedure in molecular biology. This technique has recently been applied to *C.diphtheriae* and shows promise. In brief, target DNA, in this case boiled cell lysates of bacteria, are enzymatically amplified as directed by a pair of highly specific oligonucleotide primers, each approximately 10 bases long. Each primer amplifies discrete and limited portions of a genome producing a set of amplification products. When separated by electrophoresis in agarose gels and stained, each spectrum of products resolves into a banding pattern or fingerprint (Welsh *et al.* 1990).

This typing method does have potential, it is rapid, simple, does not use cloned probes and is independent of prior DNA sequence information. However, much work is required to address the important questions of reproducibility and discrimination. Some Diphtheria Centres are currently exploring the feasibility of this method.

### 5.2.3 Pulsed Field Gel Electrophoresis (FRANCE, UK and USA)

This technique has not yet been documented for *C.diphtheriae* but is currently being evaluated by laboratories in France, the UK and USA for epidemic and sporadic isolates of *C.diphtheriae*. In brief, restriction enzymes that have 'rare cutting' sites within the bacterial chromosome are used to digest chromosomal DNA preparations in agarose blocks. The resulting large molecular weight fragment is separated by pulsed field gel electrophoresis (PFGE) and the bands visualised by ethidium bromide staining. All PFGE systems rely upon the phenomenon of DNA reorientation for fragment separation by subjecting the molecules to at least two alternating electric fields. These systems do, however, vary in their ability to produce clear resolution of bands in straight lanes, speed of separation and the range of molecular weights that can be separated.

### 5.2.4 Multilocus Enzyme Electrophoresis (USA, FINLAND, and FRANCE)

Multilocus enzyme electrophoresis (MEE) has been used fairly extensively in large scale studies to estimate the genetic diversity and structure in natural populations of a variety of bacterial species (Selander *et al.* 1986). Using MEE, bacteria are differentiated by analysis of the electrophoretic mobility of a range of soluble basic metabolic enzymes extracted from the organisms. MEE detects changes in the electrophoretic mobilities of metabolic enzymes and identifies allelic variations at the chromosomal loci encoding the enzymes. Within a bacterial population, several variant forms of individual enzymes (allozymes) exist, each encoded by different alleles at a specific genetic locus. Single amino acid changes in the enzymic proteins encoded by different genes or alleles are sufficient to affect their electrophoretic mobility and allow their differentiation. The discrimination of the technique can be enhanced by analysis of multiple enzymes, and a panel of 10–15 enzymes is used commonly for MEE. Combined data for the range of enzymes tested is then used to assign an electrophoretic type (ET) to a particular isolate or strain which identifies the organism and allows its comparison with others.

MEE is used extensively within centres in the USA, France and now Finland for the characterisation of epidemic *C.diphtheriae*. In epidemics where specific diseases arise, comparison of clones causing the outbreak with others of the same species allows the appearance of new genetically distinct clones to be recognised. In addition to its use for epidemiological typing, MEE has also been used for taxonomic studies of bacteria.

### 5.2.5 SDS Polyacrylamide Gel Electrophoresis (UK)

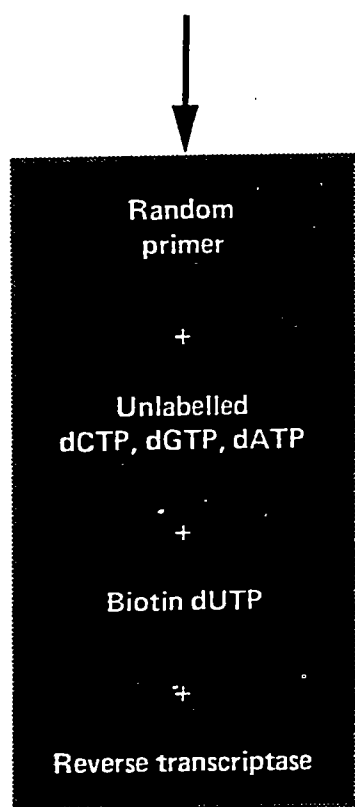
Polyacrylamide gel electrophoresis in the presence of SDS is the technique used to generate protein, polypeptide or peptide profiles from microorganisms. SDS PAGE protein profiles have been used as a successful typing tool

for *C.diphtheriae* (Hallas 1988). In various studies marked differences have been observed amongst isolates from one species of *C.diphtheriae*. The method provides a stable typing system in comparison to some conventional methods, but molecular typing methods appear to be more discriminatory (Efstratiou *et al.* 1993).

Synthesis of biotin labelled  
DNA probe (cDNA)  
transcript of total ribosomal  
RNA from *C. diphtheriae*  
NCTC 11397

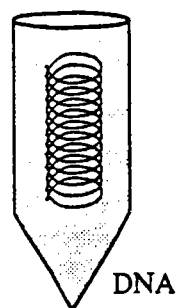
*C. diphtheriae* rRNA in water  
+ heat

RNA linear

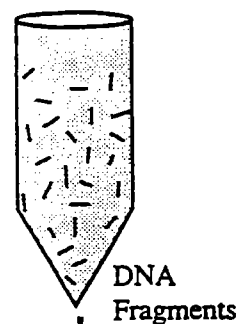


cDNA — biotin labelled  
(ribosomal probe)

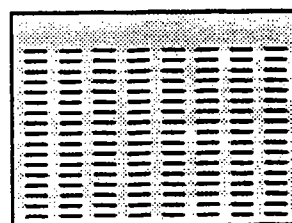
Extraction of chromosomal DNA  
(G.E.S. method)



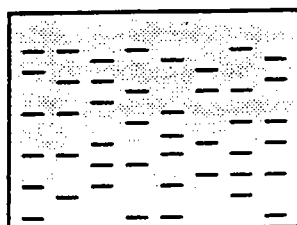
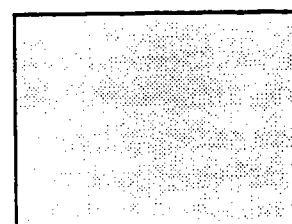
Cut with  
restriction  
enzymes  
( Eco R1  
BstE II )



Agarose gel  
electrophoresis



Southern blotting  
of DNA fragments



Hybridization and gene detection

Figure 7. Ribotyping method for *Corynebacterium diphtheriae*

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# APPENDIX

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## 2.2.2 COLLECTION OF SPECIMENS FOR *C.DIPHTHERIAE*

### THROAT SWABS

---

1. Pharynx should be clearly visible and well illuminated.
  2. Depress the tongue with an applicator and swab the throat without touching the tongue 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. If any membrane is present, lift the edge and swab beneath it to reach the deeply located organisms.
- 

### NASOPHARYNGEAL SPECIMENS

---

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, under the middle turbinate until the pharyngeal wall is reached. Force must not be used to overcome any obstruction.
- 

### SKIN DIPHTHERIA AND OTHER LESIONS

---

1. Lesions should be cleansed with sterile normal saline and crusted material removed.
  2. Press the swab firmly into the lesion.
-

### 2.2.3 AMIES TRANSPORT MEDIUM (MODIFIED STUART'S)

Distilled or deionised water	1 litre
Agar	4.0 g

Heat until dissolved by boiling, and add while hot but not boiling, the following ingredients:

NaCl	3.0 g
KCl	0.2 g
Na <sub>2</sub> PO <sub>4</sub> anhydrous	1.15g
KH <sub>2</sub> PO <sub>4</sub>	0.2 g
Sodium thioglycollate	1.0 g
CaCl <sub>2</sub> 1% (w/v) solution freshly prepared	10.0 ml
MgCl <sub>2</sub> .6H <sub>2</sub> O – 1% solution	10.0 ml

1. Stir until dissolved and add 10 g charcoal (pharmaceutical neutral grade).
2. Mix thoroughly and distribute in 1/4 oz (7 ml) bijoux bottles, filling them almost to the brim. Apply screw cap and screw down tightly.
3. Autoclave at 121°C for 15 minutes. Invert the bottles during cooling to distribute the charcoal evenly, making sure that the caps are tightened securely. Final pH, 7.2. Store in the dark in a cool place.

### 2.3.1 BLOOD AGAR

<b>Formula</b>	Oxoid agar No.2	40g
	Distilled Water	2 litre
<b>Method</b>	Boil to dissolve agar	
<b>pH</b>	Check and note	
<b>Autoclave/Media Preparator</b>	15 minutes 15lb 121°C	
	Or melt down bottles from stock	
<b>Cool</b>	To 40°C	
<b>Aseptically add</b>	50ml defibrinated horse blood per litre	
<b>Pour</b>	15ml on 12ml peptone water agar bases in triple vent plates	
<b>Label</b>	BA + Date	

#### NB: BULK BATCHES OF BASE

This agar is usually soft when melted down and extra agar is added to compensate.

### 2.3.1 COLUMBIA BLOOD AGAR

<b>Formula</b>	Oxoid No. 1 agar	39g
	Distilled water	1 litre
<b>Method</b>	Boil to dissolve agar	
<b>pH</b>	Check and note	
<b>Autoclave/Media Preparator</b>	15 minutes 15lb 121°C	
	Or melt down bottles from stock	
<b>Cool</b>	To 40°C	
<b>Aseptically add</b>	50ml defibrinated Horse Blood per litre	
<b>Pour</b>	15ml on 12ml peptone water bases in triple vent plates	
<b>Label</b>	COL + Date	

#### NB: BULK BATCHES OF BASE

This agar is usually soft when melted down and extra agar is added to compensate.

### 2.3.1 COLONIAL BIOTYPES OF C.DIPHTHERIAE ON TELLURITE MEDIUM

- GRAVIS:** 1–2 mm diameter, circular, convex, grey colonies with an entire edge, matt surface, friable.
- MITIS:** 1–2 mm diameter, circular, convex, grey colonies with an entire edge, smooth surface soft.
- INTERMEDIUS:** 0.5–1 mm diameter, circular flat, greyish-black colonies with an entire edge delicate and discreet in appearance.

### HOYLES TELLURITE

<b>Base</b>	Oxoid Hoyles Medium Base	40 g
	Distilled water	1000 ml
Heat to dissolve agar		
<b>pH</b>	Check and note	
<b>Distribute</b>	In flasks	
<b>Autoclave</b>	121°C for 20 minutes or 10lb/20 minutes if volumes less than 500ml	
<b>Cool</b>	To 50°C	
<b>Aseptically add</b>	50ml lysed horse blood 16ml 2% Potassium Tellurite Use the oldest blood	
<b>Pour</b>	25ml in triple vent plates	

Base should be kept as a stock item; 500ml in 20oz bottles. Autoclave 10mins/15lb.

### 2.3.1 LOEFFLERS SERUM SLOPES

(For the cultivation of *Corynebacterium diphtheriae*)

---

Formula	Nutrient Broth	250.0 ml
	Normal Horse Serum	750.0 ml
	Glucose	5.0 g

---

**Method** (400ml; approximately 120 bijoux bottles)

1. To 100ml sterile Nutrient Broth add 2.0 g Glucose and shake to dissolve. Place in steamer for 5 minutes. Remove and cool to 56°C.
2. Aseptically transfer 300ml sterile Horse Serum into a sterile bottle and then the cooled Nutrient Broth + Glucose. Mix thoroughly.
3. Aseptically fill 3.0 ml amounts into sterile bijoux bottles.
4. Slope in the inspissator and sterilise by heating for 60 minutes at 75–80°C on two consecutive days.

Store at 4°C.

---

### 2.3.1 PEPTONE WATER

---

Formula	Lab M Peptone No.1	10.0 g
	Sodium Chloride	5.0 g
	Distilled water	1.0 litre

---

**Method** 5 litres

1. In 5 litres distilled water dissolve

Peptone	50.0 g
Sodium Chloride	25.0 g
  2. Check and note reaction.
  3. Fill 2–5ml amounts or as required.  
4oz and 20oz bottles. Label PW + Batch number
  4. Sterilise at 10lb for 10 minutes, 20oz at 10 minutes 15lb 121°C.
-

### 2.3.1 PEPTONE WATER AGAR BASE

Formula	Lab M Peptone No.1	10.0 g
	Sodium Chloride	5.0 g
	Japanese Agar	15.0 g
	Distilled Water	1.0 litre
Method	Boil to dissolve agar.	
pH	Check and note.	
Autoclave/Media preparator	15 minutes 15lb 121°C.	
Cool	To 55°C.	
Dispense	12ml in triple vent plates.	
Storage	Pack in baskets lined with cellophane wrap. Following overnight incubation, store in cold room.	



## 2.4.2 ALBERT'S STAIN

This stain is used to demonstrate metachromatic granules in bacteria, eg. *Corynebacterium diphtheriae*.

---

1.	Albert's Stain	- 5 min
2.	Rinse in tap water	
3.	Jensen's iodine	- 1 min
4.	Wash, blot and dry	

---

Result	Metachromatic granules	- black
	Protoplasm and other organisms	- green

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Albert's Stain	Toluidine blue	0.15g
	Malachite green	0.2 g
	Glacial acetic acid	1.0 ml
	Alcohol (95%)	2.0 ml
	Distilled water	100.0 ml
Dissolve dyes in alcohol and then add other reagents		
Allow to stand for one day and filter.		

---

Iodine Solution (Jensen's)	Iodine crystals	1.0 g
	Potassium iodide	2.0 g
	Distilled water	100.0 ml
Dissolve iodine in strong Potassium iodide (2 g + 2 ml water), then dilute.		

---

## 2.4.2 GRAM STAIN

The method gives reliable results, particularly eliminating false results due to over or under decolourising.

---

Solutions required	1. Ammonium oxalate – crystal violet	
	Crystal violet	20 g
	Ethanol (95%) or methylated spirits (64 OP, 95%)	200 ml
	Filter before use	
	Ammonium oxalate 1% aqueous solution	800 ml
	2. Lugol's Iodine solution	
	Iodine	10 g
	Potassium iodide	20 g
	Distilled or deionized water	1000 ml
	3. Gram's iodine	
	Iodine	10 g
	Potassium iodide	6 g
	Ethanol (absolute) or	
	Methylated spirit (74 OP, absolute)	90 ml
	Distilled or deionized water	10 ml
	4. Iodine – acetone	
	Lugol's Iodine	35 ml
	Acetone	965 ml
	5. Counter-stain of choice e.g., Safranin, neutral red or Bismarck brown.	

---

Method	1. Prepare and fix by heat slide preparations of the suspected <i>C.diphtheriae</i>
	2. Cover slide with solution 1 and allow to act for 30 seconds
	3. Pour off and wash freely with iodine solution 2
	Cover with fresh iodine solution and allow to act to 30 seconds
	4. Pour off iodine solution and wash freely with iodine-acetone solution 4
	Cover with fresh iodine-acetone and allow to act for 30 seconds
	5. Wash thoroughly with water
	6. Counterstain according to choice
	7. Wash with water, blot and dry

---

Generally, *C.diphtheriae* is weakly Gram positive, so that occasionally it may be entirely Gram negative, or Gram variable. Diphtheroids on the other hand strongly retain the stain and are invariably Gram positive.

## 2.4.2 LOEFFLER METHYLENE BLUE STAIN

Saturated Solution	Methylene blue – 1% in ethanol (95%)
Staining Solution	<p>Potassium hydroxide KOH aqueous solution 1%(w/v)</p> <p>Saturated solution of methylene blue in distilled deionized water 30%</p> <p>Add the KOH solution to the water, mix well. Follow with the saturated methylene blue, mixing well.</p> <p>Staining is much better if the reagent is allowed to ripen before use. This process can be hastened by only half filling the stock reagent bottle and replacing the stopper or screw cap with a light cotton wool plug so that aeration can take place. The process of ripening may take several months so it is recommended that large batches of the staining solution are prepared and that fresh batches are prepared well in advance of current batches being used up.</p> <p>Loeffler's methylene blue stain is excellent for staining the corynebacteria where beading, barring and metachromatic granules may be readily demonstrated.</p>
Method	<ol style="list-style-type: none"><li>1. Prepare and fix by heat slide preparations of the suspected <i>C.diphtheriae</i></li><li>2. Cover slide with stain and leave for 1–3 minutes</li><li>3. Rinse with water</li><li>4. Blot and dry</li></ol> <p>Typical <i>C.diphtheriae</i> cell morphology is usually well demonstrated on Loeffler serum medium.</p>

## 2.4.3 SCREENING FOR *CORYNEBACTERIUM DIPHTHERIAE* USING THE CYSTEINASE TEST

### SETTING UP TEST

---

1. Demarcate a small section on a Tinsdale agar plate as a positive control area and inoculate heavily with a known *C.diphtheriae* stain. Inoculate the remainder of the plate with the test strain.
  2. Incubate plate overnight.
- 

### READING TEST

---

– test 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.
  2. Reactions:
    - Positive: black colonies with brown halo
    - Negative: absence of brown halo; some coryneforms may produce black colonies
  3. Interpretation:
    - Positive: pathogenic corynebacteria
      - *C.diphtheriae*
      - *C.pseudotuberculosis*
      - *C.ulcerans*
    - Negative: other corynebacteria
- 

### TINSDALE MEDIUM FOR THE DETECTION OF CYSTEINASE

---

1. Formula

Tinsdale Base	200 ml
Difco Tinsdale Supplement	15 ml
– 1 vial + 15 ml sterile distilled water	
  2. Method
    - melt base and cool to 56°C
    - add Difco Tinsdale Supplement
    - mix well with no bubbles
    - pour 10 plates (ie 20 ml per plate)
- 

NOTE: A 'strip test' system for detection of cysteinase is used within the Ukraine (Pers. Comm. T. Glushkevich, Kiev).

### 2.4.3 PYRAZINAMIDASE TEST FOR CORYNEBACTERIA COLINDALE METHOD

This test is performed using growth from a blood agar plate culture of the test organism. It is advisable to carry it out in a Class I safety exhaust cabinet.

---

#### Reagents

1. Pyrazinamide Solution
  - Sigma Ref p 7136
  - 2mg/ml in distilled water
  - sterilise by filtration through a 0.45 $\mu$  membrane filter
  - dispense into sterile bijoux
  - store at –20°C

**CAUTION: Hazardous in powder form. Avoid skin contact. Do not inhale power.**

2. PYZ Reagent: ferrous ammonium sulphate
  - 20% w/v in sterile distilled water
  - store at –20°C

---

#### Control Strains

1. Positive control: NCTC 12078, *C.xerosis*  
Negative control: NCTC 12077, *C.ulcerans*

---

#### Method

1. Distribute 0.25 ml pyrazinamide solution into three sterile capped tubes.
2. Prepare a turbid suspension (equivalent to McFarland's No 8) of the test strain in one tube. Prepare positive and negative control suspensions in the other two tubes.
3. Incubate for at 37°C for 4 hours or overnight.
4. After incubation, add 1 drop of PYZ reagent to each suspension.

---

#### Interpretation

- Positive = Other *Corynebacteria*  
Negative = *Corynebacterium diphtheriae*, *C.ulcerans*, *C.pseudotuberculosis*
-

### 2.4.3 SCREENING FOR *CORYNEBACTERIUM DIPHTHERIAE* USING COMMERCIALY AVAILABLE PYRAZINAMIDASE

A new rapid test is available commercially that can differentiate pathogenic corynebacteria (*diphtheriae*, *pseudotuberculosis* and *ulcerans*) from the other species of corynebacteria. It is simple, rapid (3–4 hours) and cost effective.

A full identification scheme is also available from the manufacturer A/S Rosco, offering identification of 26 corynebacteria species in 4 hours.

#### SETTING UP TEST

---

1. Transfer 0.25 ml of sterile distilled water to sterile 3 x 1/2 tubes (prepare 1 tube for each test strain and 2 additional for control strains).
  2. In a Class I Safety Exhaust Cabinet prepare a milky suspension (at least McFarland No.8) of the test strain in the 0.25 ml sterile distilled water. Prepare positive and negative control strains in the same way.
  3. Using pre-flamed forceps, add 1 Rosco\* Diagnostic Tablet (598–21) to each tube.
  4. Incubate the tubes at 37°C for 4 hours or 18–24 hours.
- 

#### READING TEST

---

- test may be read on the open bench
1. Add 1 drop of ferrous ammonium sulphate (5% w/v in distilled water, freshly prepared or stored at –20°C).
  2. Reactions:
    - Positive: red/orange
    - Negative: colourless/pale yellow
  3. Interpretation:
    - Negative: pathogenic corynebacteria
      - *C.diphtheriae*
      - *C.pseudotuberculosis*
      - *C.ulcerans*
    - Positive: other corynebacteria
- 

#### European Supplier

- \* Rosco Diagnostica, Taastrupgaardsvej 30, DK–2630, Taastrup DENMARK  
TEL: 45 42 99 3377, FAX: 45 42 52 7374

### 2.5.1 CHRISTENSENS UREA MEDIUM BASE

(Christensen 1946)

Formula	Neutralised Bacteriological peptone, Oxoid	1.0 g
	Sodium chloride	5.0 g
	Potassium dihydrogen orthophosphate $\text{KH}_2\text{PO}_4$	2.0 g
	Glucose	1.0 g
	1% Phenol red solution (aqueous)	1.2 ml
	Oxoid agar No.1	10.0 g
	Distilled water	1.0 litre
<hr/>		
Method	(1 litre)	
	1. Dissolve above ingredients in 1 litre distilled water.	
	2. Heat to dissolve agar.	
	3. Adjust pH to 6.8 with approximately 5ml NaOH (Normal)	
	4. Fill 50ml into 20 oz bottle	
	5. Autoclave 10 minutes 15lb 121°C	

### 2.5.1 CHRISTENSEN'S UREA SLOPES

(Christensen 1946)

Formula	Christensen's Urea Base	1.0 litre
	40% Urea solution	50.0 ml
<hr/>		
Method	(500ml; approximately 200 slopes)	
	a. Melt 500ml urea base in the steamer.	
	b. Remove from steamer and allow to cool to 50°C.	
	c. Aseptically add 25ml 40% urea solution. Mix thoroughly.	
	d. Aseptically fill 2.5 ml amounts into sterile 5" x 1/2" tubes. Slope immediately and allow to set.	

COLOUR CODE: Mauve

Hydrolysis of urea is indicated by a deep pink colouration of the medium.

Absence of hydrolysis; colourless.

## 2.5.1 NITRATE BROTH

Formula	Nutrient Broth No.2 Oxoid CM67	25.0 g
	Potassium Nitrate	1.0 g
	Distilled water	2.0 litre
Method	a. In 2.0 litres distilled water dissolve	
	Nutrient Broth No.2	50.0 g
	Potassium Nitrate	2.0 g
	Check and note pH	
	b. Fill 3.0 ml amounts into 6 x 1/2" tubes	
	c. Sterilise by autoclaving for 10 minutes at 115°C (10lb)	

COLOUR CODE: Brown/Red

Cultures are incubated for 24–48 hours at 37°C.

The presence of nitrate is detected by immersing a nitrate detection stick or 'N-stix' into the culture.

A pink colouration denotes the presence of nitrate.



## 2.5.1 SERUM WATER CARBOHYDRATE MEDIUM

(Hiss Serum Water Sugars Robinsons Modification)

<b>Formula</b>	
<b>Base</b>	Peptone Oxoid L37 5.0 g
	Na <sub>2</sub> HPO <sub>4</sub> di Sodium Hydrogen Orthophosphate anhydrous 1.0 g
	Sterile Horse Serum 178.0 ml
	Andrades Indicator 7.8 ml
	Distilled Water 1000.0 ml
	Add appropriate sugar to final conc. 1% or starch to 0.5%
<b>Method</b>	<ul style="list-style-type: none"> <li>– Dissolve Peptone and Na<sub>2</sub>HPO<sub>4</sub> in warm distilled water and steam for 15 minutes. Filter. Cool.</li> <li>– Adjust pH to 7.4</li> <li>– Add serum, mix well and steam for 20 minutes.</li> <li>– Add indicator and adjust pH to 7.7</li> </ul>
<b>For Base Only</b>	Distribute in convenient amounts. Autoclave 10 minutes/10lb/115°
<b>For Complete Medium</b>	
<b>Either</b>	Add appropriate sugar to base (except Ethanol and Starch)
	<b>Distribute</b> In 4.5ml in bijoux
	<b>Autoclave</b> At 30 minutes 5lb 108°C
<b>or</b>	Aseptically add 10ml 10% filter sterilised sugar solution to each 90ml pre-sterilised base.
	<b>Distribute</b> 4.5ml in sterile bijoux
<b>Ethanol</b>	Aseptically add 10ml to each 90ml of pre-sterilised base. Distribute aseptically – <b>CARE DO NOT FLAME</b>
<b>Starch Method</b>	<ul style="list-style-type: none"> <li>– Serum water base (No starch) supplied</li> <li>– 0.15g soluble starch in screw-capped bottles supplied.</li> </ul>
<b>To Use</b>	<ol style="list-style-type: none"> <li>1. Add loopful of starch to 3ml of media. OR (PREFERRED METHOD)</li> <li>2. Add 5ml of sterile distilled water to 0.15g starch and bring to boil, shaking continuously.</li> </ol> <p>Boil for 5 minutes approximately (loosened cap) Cool. Shaking at intervals. Add 0.15ml to each 3ml of medium, (about 5 drops, with a sterile plugged Pasteur pipette).</p>
<b>Code</b>	Usual appropriate to sugar, none on Base, label clearly.
<b>Normal Diphtheria set</b>	Glucose, Maltose, Sucrose, Starch.
<b>Full Corynebacterium set</b>	Glucose, Maltose, Galactose, Dextrin, Sucrose, Lactose.

2.5.1 16% GLYCEROL BROTH

Formula	Oxoid Nutrient Broth No.2	6.25 g
	Glycerol (warmed before use)	42.00 g (NB g not ml)
	Distilled water	208.00 ml
Method	Mix gently to dissolve.	
pH	Check and note.	
Dispense	1ml in plastic, screw-capped vials	
Autoclave	10 minutes 10lbs 115°C	

## 2.5.1 IDENTIFICATION OF *CORYNEBACTERIUM DIPHTHERIAE* USING THE ROSCO SYSTEM

### SETTING UP TESTS

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1. In a Class I Safety Exhaust Cabinet, prepare a milky suspension (at least McFarland No.8) of the test strain in a bijoux of sterile saline.
2. Transfer 0.25 ml of suspension to each of five sterile capped tubes.
3. Using pre-flamed forceps, add 1 Rosco tablet of each of the following:
  - Nitrate Reduction Diagnostic Tablet, 437–21
  - Urease Diagnostic Tablet, 575–21
  - Glucose Tablet, 526–21
  - Maltose Tablet, 575–21
  - Sucrose Tablet, 538–21
4. Add 1 drop of inoculum to a \*Hiss's Starch Serum Water.
5. Incubate the Rosco test at 37°C for 4 hours or 18–24 hours and the Hiss's Starch Serum Water for 24 hours.

\*Starch tablets are not yet available from Rosco.

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### READING TESTS

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#### A. NITRATE

1. After incubation, add:
  - 1 drop dimethylnaphthylamine solution (Rosco 918–31)
  - 1 drop sulfanilic acid solution (Rosco 919–31)
2. Read within 2 minutes:
  - **Positive:** red/pink
  - **Negative:** colourless/light rose
3. Please note: if test is read after 4 hours incubation, it cannot be reincubated.

#### B. UREASE TEST

1. Observe for colour change:
  - **Positive:** red/purple
  - **Negative:** yellow/orange

#### C. SUGAR FERMENTATION TESTS

1. Observe Rosco tests for colour change:
    - **Positive:** yellow/yellow-orange
    - **Negative:** red/red-orange
  2. Observe Hiss's Starch for colour change and possible
    - **Positive:** pink
    - **Negative:** colourless
-

## 2.5.1 API CORYNE TEST

### SETTING UP TEST

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– this protocol is a slight modification of that described in the API instruction sheet supplied with the kit.

1. Label an API tray. Add water to the base, draining off any excess.
  2. Note the haemolytic reaction of your test strain on Columbia Blood Agar.
  3. Working in a Class I Safety Cabinet and wearing latex gloves, harvest all the growth on the Columbia plate using a sterile swab and produce a dense suspension in the suspension medium. The turbidity should be **greater than 6** when compared with the turbidity control provided.
  4. Inoculate the strip with a sterile pipette (Pastette)
    - first 11 tests: – **NIT to GEL**
      - **NIT to ESC**: 6 drops of suspension
      - **URE**: fill tube portion only
      - **GEL**: fill tube and cupule
    - last 9 tests: **0 to GLYG**
      - add 0.5 ml suspension to an ampoule of **GP Medium** and mix well
      - distribute this new suspension to the tubes only of the last 9 tests
  5. Overlay the cupules of **URE** and **0 to GLYG** with mineral oil, forming a slightly convex meniscus.
  6. Incubate a 37°C overnight.
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### READING TESTS

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– tests may be read on the open bench wearing latex gloves with the exception of the catalase test.

1. Add reagents:
    - **NIT test**: 1 drop **NIT 1** and **NIT 2**
    - **PYZ test**: 1 drop **PYZ**
    - **PyrA, PAL, B GUR, B GAL, alpha GLU, B NAG tests**: 1 drop of **ZYM A** and **ZYM B**
    - **do not read the catalase reaction at this point** (see instruction 4)
  2. Wait 10 minutes, then read the reactions referring to the reading table provided.
  3. Record the reactions on your result sheet.
  4. Place the strip in the safety cabinet, add 1 drop of 3%  $\text{H}_2\text{O}_2$  to the cupule **ESC**. Leaving the strip in the cabinet, observe the cupule for the evolution of gas bubbles.
  5. Interpretation of results:
    - compare the Profile Number obtained in the “API Coryne Analytical Profile Index”
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## 2.5.2 MATERIALS FOR THE ELEK TOXIGENICITY TEST

### BASAL MEDIUM

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A.	*Difco Proteose Peptone No.2	20 g
	De-ionised or distilled water	500 ml

Dissolve peptone.

Add NaOH (40%w/v = 10N solution) 3.25ml

Mix, heat to boiling in a steamer, cool.

Filter through Whatman glass fibre filter (Grade GF/F) to remove precipitated phosphates.

Add lactic acid (Analar), 90% soln 0.7 ml

Mix and then add maltose 3.0 g

Dissolve; adjust pH to 7.8 with 5N or 1N HCl using a pH meter

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B.	Sodium chloride	5.0 g
	'Lab M' agar (code MC2)	10.0 g
	De-ionised or distilled water	500.0 ml

Mix. Allow to stand cold and then steam to dissolve.

Cool to 50°C and adjust pH to 7.8 with 1N NaOH (Analar)

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Warm A to 50°C and then mix A and B, distribute in 15 ml volumes in McCartney bottles and autoclave at 116°C (10 psi) for 10 minutes.

\*Peptone must be from a batch with known properties

## 2.5.2 PREPARATION OF ANTITOXIN STRIPS FOR THE ELEK TEST

### MATERIALS REQUIRED

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#### DIPHTHERIA ANTITOXIN

Supplied as 10 vials each containing 5 ml of Diphtheria Antitoxin BP at 2,000 IU/ml, produced by the Swiss Serum and Vaccine Institute, Berne.

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<b>Source</b>	Swiss Serum and Vaccine Institute Berne SWITZERLAND
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<b>Mast Bacteruritest Dipstrips (Europe)</b>	Order Code: BTRI Source: Mast Diagnostics Ltd Mast House Derby Road Bootle, LIVERPOOL L20 1EA, UK
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OR Whatman No.1 or No.3 filter paper are also suitable for preparing the strips.

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<b>Method (Single Dipstrip)</b>	<ol style="list-style-type: none"><li>1. Dilute antitoxin to 500 IU/ml with sterile distilled water (5 ml antitoxin + 15 ml water). The dilute antitoxin is stable for 6 months if stored at 4°C.</li><li>2. Aseptically immerse sterile dipstrip into the diluted antitoxin.</li><li>3. Drain excess antitoxin.</li><li>4. The strip is now ready for use.</li></ol>
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<b>Method (Bulk Preparation)</b>	<ol style="list-style-type: none"><li>1. As (1) and (2) above.</li><li>2. Aseptically place the moistened dipstrips into a sterile, capped large-mouth container (e.g. a "Honey jar").</li><li>3. Freeze-dry dipstrips.</li><li>4. Store in sterile capped containers at 4°C. They should remain stable for a minimum of 6 months. It is advisable to test their stability at 6 months and regularly thereafter.</li></ol>
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