LABORATORY TECHNIQUES

Broadsheet No 125

July 1990

Bacteriological diagnosis of diphtheria

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Bacteriological diagnosis of diphtheria

Diphtheria is no longer diagnosed easily on clinical grounds. The rarity of the disease means that many clinicians never encounter a case, and they may fail to appreciate the clinical importance of a pharyngeal membrane, especially if it is thin. Often the first indication of the likelihood of diphtheria is usually given by the laboratory reporting the presence of the causative organism *Corynebacterium diphtheriae* in routine throat and other swabs taken from the respiratory tract. The mortality in those who have not been immunised has changed little, despite the rarity of the disease in Great Britain. The pattern of diphtheria in this country over the past 20 years or so has been of small, sporadic outbreaks often presenting with a fatal case. The ability of the laboratory to make a diagnosis both quickly and reliably, or at least to alert the clinician to the possibility of diphtheria in this situation is therefore of paramount importance.

COLLECTION AND TRANSPORT OF SPECIMENS

The successful isolation of *C diphtheriae* depends in the first instance on the proper collection of swabs and their subsequent transport to the laboratory. As diphtheria is most commonly an upper respiratory tract infection, throat and nasopharyngeal swabs need to be taken. The only other form which occurs with some frequency is skin diphtheria, often indistinguishable clinically from any other pyoderma, especially in parts of the world where diphtheria is endemic. Less common forms are diphtheria of the lip, the conjunctiva, and the vulva. As there seems to be an association between respiratory tract diphtheria and infection of other sites it is wise to take both throat and nasopharyngeal swabs at the same time.

After collection swabs should be sent to the laboratory immediately as the rapid inoculation of special culture media is most important; at the same time the clinician should inform the laboratory of any presumptive diagnosis of diphtheria. If specimens cannot be sent to the laboratory immediately then the swabs must be placed in a transport medium

such as that described by Amies.¹ *C diphtheriae* shows little reduction in viability during transport in this medium within 24 hours but subsequently there is a gradual reduction in its survival.

PROCESSING OF SPECIMENS

Specimens must be inoculated on culture media without delay; the diagnosis of diphtheria based on the microscopical examination of a direct smear is ill advised as both false positive and false negative results are likely to occur. Culture on blood agar and a selective tellurite medium is essential, but there is little need for the continued use of Loeffler's serum medium for primary isolation purposes. If after culture from a suspected case there is little or no growth on both blood agar and tellurite blood agar plates then further swabs should be requested with a reminder that the taking of swabs should be done with care.

CULTURE IN THE LABORATORY

The ideal medium should be easy to prepare, of constant composition, and should permit rapid growth of all types of *C diphtheriae* while suppressing the growth of other bacteria. Time honoured tellurite media, which have proved their usefulness and are easily prepared, are the whole blood medium attributed to Downie² and the laked blood medium of Hoyle.³ Downie's medium (containing whole sheep blood) is particularly useful and is strongly recommended. It is not necessary to prepare the base medium as originally described as almost any good infusion blood agar base or Columbia blood agar base can be used, providing that the base medium is adjusted to pH 7.8. Colonial morphology develops better on media containing sheep blood rather than horse blood. By frequent examination of cultures of stock strains of *C diphtheriae* on Downie's medium made with horse blood, including strains from recent cases of diphtheria, confidence in recognising the essential colonial features of the biotypes can soon be gained. In recent years modified Tinsdale's medium has been recommended for the rapid isolation of *C diphtheriae*,^{4,5} particularly for use by inex-

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Accepted for publication
23 January 1990

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perienced staff. The medium is difficult to prepare, however, and has a very short storage life. Commercially prepared Tinsdale's medium has also been found to be unreliable, the main problem being that the medium is often too inhibitory. Because of these deficiencies it is not recommended as a primary isolation medium, although good results can be obtained in experienced hands. If properly batch tested, with strongly and weakly halo-producing strains of *C diphtheriae*, Tinsdale's medium is very useful for checking suspicious colonies found on the more generally accepted tellurite media, as only *C diphtheriae*, *C ulcerans*, and *C ovis* (*pseudotuberculosis*) are expected to produce characteristic brownish-black haloes.

Inoculated plates are incubated in a normal atmosphere at 35–36°C and examined after overnight incubation so that subcultures of any suspicious colonies can be made as early in the day as possible. This will permit adequate growth (four to six hours) for toxigenicity and biochemical tests to be set up the day the primary plates are examined. Colonies should be examined in reflected light with a ×8 hand lens or preferably with a plate or dissection microscope. It is important to remember that sometimes one or more of the typical colonial features may be absent; that coryneform (diphtheroids) organisms may also exhibit similar colonial morphology; and that occasionally tellurite sensitive strains of *C diphtheriae* are encountered which means that blood agar plates must be examined with equal care. Culture plates should be incubated for a full 48 hours before being discarded as negative. For biotyping purposes the best colonial features are produced after 24–48 hours of incubation, but the essential skill to be learned is the early recognition of suspicious colonies, so that crucial toxigenicity tests can be put up without delay. To gain experience in recognising colonies of *C diphtheriae* on tellurite blood agar plates, stock cultures should be plated out at regular intervals and carefully examined. Type strains from national collections of type cultures are suitable for this purpose, providing that cultures showing typical colonial morphology are requested. Time spent in examining typical *C diphtheriae* colonies will be rewarded by having to put up fewer identification tests.

IDENTIFICATION OF *C DIPHTHERIAE*

The most important test is for toxigenicity and this must be done without delay. Pure cultures of suspicious colonies must be obtained for accurate identification tests. The textbook descriptions of the three colonial biotypes, *gravis*, *mitis*, and *intermedius*, are not usually apparent until 24 hours or more after incubation begins. On whole blood tellurite medium, after overnight incubation, *gravis* colonies are about 1–2 mm in diameter, circular with an entire edge, convex, grey in colour with a matt or frosted glass surface and friable consistency—occasional strains may show a faint zone of haemolysis. *Mitis* colonies are about 1–2 mm in diameter, circular with an entire edge, convex,

grey in colour with a smooth surface and soft consistency, and usually show well defined zones of haemolysis. *Intermedius* colonies are about 0.5–1 mm in diameter, circular with an entire edge, flat, greyish-black in colour with paler periphery and a smooth or finely granular surface—generally, *intermedius* colonies are much more delicate and discrete in appearance than the other two biotypes. *Intermedius* biotype *C diphtheriae* has not been seen in Great Britain for many years; most of the small number of infections have been caused by *gravis* or *mitis* biotypes.

Films are made from suspicious colonies found on tellurite or blood agar plates and are stained by Gram's method. All the corynebacterium group are Gram positive, but *C diphtheriae* tends to decolourise easily and frequently appears as either Gram variable or Gram negative; coryneform organisms (diphtheroids) mostly remain strongly Gram positive. It is advisable to control the staining procedure with known Gram positive and Gram negative organisms. *C diphtheriae* grown on blood tellurite medium and stained by Gram's method differs in morphology from the classic descriptions in textbooks obtained from cultures grown on Loeffler's serum medium or Dorset's egg medium and stained by Loeffler's methylene blue. Comparisons will need to be made with stock culture strains grown on blood tellurite medium to gain experience. A representative of each colony type which has *C diphtheriae* morphology with Gram's stain is subcultured to a Columbia blood agar plate for purity and for toxigenicity and biochemical testing, and to Dorset's egg medium for determining microscopical morphology by Loeffler's methylene blue stain and Albert's stain. The latter is particularly good for showing the presence of metachromatic granules. Generally speaking, there is little difference between the morphology of *C diphtheriae* grown on Loeffler's serum medium and Dorset's egg medium after staining with Loeffler's methylene blue, though the latter seems to produce more involution forms. Dorset's egg medium is recommended in preference to Loeffler's serum medium because it is considerably more consistent in producing the distinctive morphology of each of the three main biotypes, particularly when using culture media purchased from commercial sources.

The subcultures on Columbia blood agar are incubated at 35–36°C for four to six hours, when there should be sufficient growth for setting up toxigenicity and biochemical tests; if growth is sparse it should be reserved for the toxigenicity test. The growth on blood agar should be checked for purity by Gram stained films; the Dorset's egg medium subcultures can be left for 18–24 hours at 35–36°C after which growth is checked for distinctive *C diphtheriae* microscopical morphology by making films—stained by Loeffler's methylene blue and Albert's stain. The subcultures are further cultured sometime during the day on to tellurite blood agar and Columbia blood agar for making a more leisurely study of colonial morphology, for biotyping purposes, or for

repeating anomalous toxigenicity or biochemical tests.

For toxigenicity testing it is essential to obtain a result as soon as possible as delay may have serious consequences for the patient. Tests can be *in vivo* or *in vitro*. *In vivo* tests using guinea pigs, for example, are slow to produce results; few laboratories now have animal facilities and such tests are best carried out in reference laboratories. The *in vitro* modified Elek's immunodiffusion test is highly suitable for the clinical laboratory.⁶ Using a suitable base medium, satisfactory serum additive, and reliable diphtheria antitoxin, all previously tested and known to work satisfactorily, and with the proper use of control organisms, the result of Elek's test (figure) should be available within 24 hours and certainly by 36 hours after setting up the test. A good laboratory will have the materials constantly to hand and will carry out regular tests on control cultures to ensure that when the emergency arises an unequivocal test result can be given to the clinician. The following is an account of a method of confirmed reliability.

- 1 Melt 15 ml amounts of special agar base.⁶ Cool to 50–55°C. The use of Difco Proteose Peptone No 2, specially prepared for maximum toxin production, is strongly recommended.
- 2 Add 3 ml sterile newborn calf serum (Cat No 29–121–49 from Flow Laboratories Ltd. PO Box 17, Second Avenue Industrial Estate, Irvine KA12 8NB Scotland). Mix well and pour into 9 cm diameter clear, scratch free, Petri dishes.
- 3 Allow to set, then dry surfaces of medium—usually about one hour in the incubator at 35–36°C.

Interpretation

- 1 Strongly positive control—toxicogenic (NCTC 10648)
- 2 Test or unknown strain—toxicogenic
- 3 Negative control—non-toxicogenic (NCTC 10356)
- 4 Weakly positive control—toxicogenic (NCTC 3984)
- 5 Strongly positive control—toxicogenic
- 6 Duplicate test or unknown—toxicogenic
- 7 Weakly positive control—toxicogenic (NCTC 3984)

- 4 Draw lines on the base of the dish as follows: One line across the centre and seven lines 50 mm long and 10 mm apart at right angles to it.

- 5 Streak test and control cultures along the seven lines, using a 1 mm loop—disposable plastic loops are ideal for this purpose. Inocula are taken from a four to six hour growth on Columbia blood agar which has been inoculated by 10 hours and produces sufficient growth for the test by 16 hours.

- 6 Lay a dried diphtheria antitoxin filter paper strip along the centre line at right angles to the seven lines of test and control cultures. Incubate at 35–36°C. The strips are prepared by soaking Mast Bacteruritest strips (Code BTR 1 from Mast Diagnostics, Mast House, Derby Road, Bootle, Merseyside L201EA) in diluted refined diphtheria antitoxin followed by drying. Five hundred units is usually a satisfactory dilution but the antitoxin may need to be titrated at 250, 500, 750 and 1000 units/ml. The best concentration is found by using control strains of *C diphtheriae*, NCTC 10648—strongly toxigenic, lines appear in 12 to 18 hours; NCTC 3984, weakly toxigenic—with satisfactory media and reagents lines appear in 12 to 18 hours; NCTC 10356, non-toxicogenic, may show secondary lines after 48 hours. With less than optimal conditions NCTC 3984 may not produce lines, even at 48 hours, but if placed next to NCTC 10648 on the Elek plate, the lines of the latter will “arc” towards NCTC 3984—that is, lines of identity. Failure of NCTC 3984 to produce lines or to cause the lines of NCTC 10648 to “arc” towards it means the test is void unless, of course, NCTC 10648 and the test organisms show distinct lines within 48 hours. Lines appearing after 48 hours are usually secondary and may even join up by “arc-ing”. A diphtheria antitoxin which produces many secondary lines should not be used. A suitable antitoxin is currently available from the Swiss Serum and Vaccine Institute, Bern, Switzerland (imported by Regent Laboratories Ltd, Cunard Road, London, NW10 6PN).

It is essential to place the test (unknown) culture next to the strongly toxigenic control culture (NCTC 10684) so that any lines of precipitation will join with those of the control causing “arc-ing”—a reaction of identity.

The Elek plate is examined the next morning, again at 18 hours, 24 hours and finally at 48 hours. Lines are best seen by transmitted light against a dark background with the aid of a hand lens. Look for white lines of precipitation starting about 4–5 mm from the filter paper strip and occurring at an angle of about 45° to the line of growth. These should be shown by the positive control toxigenic strains. If the test or unknown organism shows similar lines then it should be regarded as being toxigenic. The plates are reincubated and examined later in the day and finally on the following day—that is, after 48 hours. The toxin line of the positive control strain should join the toxin line of a positive test strain to form an arch or “arc”

Corynebacteria isolated from human sources

Organism	Glucose	Maltose	Starch	Sucrose	Nitrate reduction	Urea hydrolysis
<i>C diphtheriae gravis</i>	Acid	Acid	Acid	— (Rare A)	+	—
<i>C diphtheriae mitis</i>	Acid	Acid	—	— (Rare A)	+ (Rare —)	—
<i>C diphtheriae intermedius</i>	Acid	Acid	—	—	+	—
<i>C ulcerans</i>	Acid	Acid	Acid	—	—	+ (Rare —)
<i>C xerosis</i>	Acid	Acid	—	Acid	+	—
<i>C hofmannii</i>	—	—	—	—	+	+

C ulcerans is distinguished from *C diphtheriae* biotypes by the hydrolysis of urea, fermentation (sometimes slowly) of trehalose, and the liquefaction of gelatin, usually within two to three days at 22°C.

C diphtheriae mitis strains which are nitrate negative are sometimes referred to as *C belfanti* or *C diphtheriae mitis* var *belfanti*.

A = acid.

between the two strains. If the test strain produces toxin more slowly than the positive control strain—that is, weakly toxigenic—the arch formed may be bent slightly. If the test strain does not produce toxin the toxin line will be absent and the toxin line from the positive control strain will pass straight through the line of growth of the strain. Secondary lines of precipitation due to soluble antigens other than diphtheria toxin are produced by both toxigenic and non-toxigenic strains. If positive, weakly positive, and negative control strains are included in the test there should be no difficulty in identifying the true toxin lines.

BIOCHEMICAL TESTING

Though of little help to the management of the patient, biochemical testing (table) is essential for biotyping purposes, for contact tracing, and for epidemiological studies. *C diphtheriae* is catalase and usually nitrate positive; occasional nitrate negative *mitis* biotypes are encountered. A simple nitrate reduction test has been described by Cook.⁷ Acid without gas is produced from glucose and maltose. Trehalose and sucrose are negative, but in recent years sucrose fermenting strains of the *mitis* biotype have been encountered in the United Kingdom. The *gravis* biotype also ferments starch and glycogen. None of the *C diphtheriae* biotypes hydrolyse urea. Traditionally Hiss's serum water sugars have been used for carbohydrate fermentation tests but these tend to stagnate in laboratory cupboards as they are used for little else. Andrade's peptone water sugars, which have a much longer shelf life, can be used, providing that a few drops of Hiss's serum water are added to each tube or bottle just before use. Ideally, the organisms to be tested can be suspended in 5 ml of Hiss's serum water and 3–5 drops of the suspension added to each carbohydrate tube or bottle.⁸ Newborn calf serum can be used for preparing the Hiss's serum water, but as with other animal sera the final medium needs to be sterilised by autoclaving (Appendix), which also destroys naturally occurring enzymes, which may cause non-specific carbohydrate fermentation. Starch peptone water is unstable. It is recommended that starch solution is added to the Andrade's peptone water at the time of use. This is done by preparing a 2.5% solution of soluble starch

in distilled or deionised water and sterilising in the autoclave at 115°C for 10 minutes. Add 0.6 ml of this to 3 ml of base medium (final concentration 0.4%). Not all batches of soluble starch are satisfactory and they should be pre-tested in made-up form with known starch fermenting and non-fermenting strains of corynebacteria. The 2.5% starch solution is usually stable for about three months at 4°C. It is recommended that known starch positive and starch negative controls are included with each carbohydrate fermentation test or batch of tests.

Incubate the carbohydrate fermentation tests at 35–36°C. Results can usually be read after 24 hours. Occasionally with slow growing strains it may be necessary to reincubate and read after 48 hours and 72 hours. In most cases fermentation of a particular carbohydrate by *C diphtheriae* is detected not only by the Andrade's indicator turning distinctly pink but also by the cloudy deposit of coagulation of even the small amount of serum present in the Hiss's serum water additive. All the tests to which the suspected diphtheria bacillus are subjected must be controlled. The cultures from the National Collection of Type Cultures used for Elek's immunodiffusion test are ideal for this purpose as they should always be readily available in a viable state. NCTC 10648 and NCTC 3984 are both *gravis* biotypes and therefore ferment starch and glycogen in addition to glucose and maltose and are nitrate positive. The microscopical and cultural morphology is also adequate enough to represent the *gravis* biotype. NCTC 10356 is a *mitis* biotype and is therefore starch and glycogen negative. It is also nitrate negative and suitable as a negative control organism for the reduction test; it also has very reasonable *mitis* biotype microscopic and cultural properties.

Appendix

HISS'S SERUM WATER

Peptone water, adjusted to pH 7.6–7.8, 4 parts; newborn calf serum 1 part.

Check that the pH remains unchanged, otherwise, readjust. Distribute into screw-capped bottles or tubes in suitable quantities and sterilise in the autoclave at 115°C for 10 minutes.

To be able to see bacterial growth after inoculation it is preferable to have the clearest medium possible. Batches of serum differ in their coagulability by heat, and using the above concentrations of serum may cause some batches of medium to become cloudy. It is useful to add varying concentrations of serum to the basal peptone water, such as 10%, 15%, 20%, 25%, to autoclave them as described above and to choose the highest concentration that does not cause cloudiness after heat sterilisation. 25%, to autoclave them as described above and to choose the highest concentration that does not cause cloudiness after heat sterilisation.

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