A SIMPLE TELLURITE-CHOCOLATE-AGAR MEDIUM FOR THE TYPING AND ISOLATION OF *CORYNEBACTERIUM DIPHTHERIAE*

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(With Plate IV, containing 3 Figures)

The importance, to the Public Health Service, of investigating the types of *Corynebacterium diphtheriae* occurring in a district is now well established, and would probably be more widely used if a simpler technique were available for the making of the necessary selective tellurite medium. This problem is especially important for those working for some of the smaller Sanitary Authorities, where the laboratory staffs are small and facilities for involved technical work do not exist.

In Huddersfield it became desirable to carry out an investigation into the prevailing types, but with the arrangements existing it was impossible to devote the amount of time to preparing media, which would be called for if any technique was to be adopted involving the use of the medium described by the Leeds workers (Anderson, *et al.* (1931)). Apart from this there was the difficulty of obtaining, sufficiently frequently, fresh rabbit red blood cells for the small batches of medium required. As the incidence of diphtheria in Huddersfield is now fairly low, the number of swabs to be examined is relatively small; consequently, as plate media do not keep very well, frequently made small batches became a necessity.

In view of the above considerations, when the diphtheria typing investigation was commenced in Huddersfield, it was decided to embody in it a second investigation into the various tellurite plate media available with the object of determining whether it would be possible to produce a selective medium which would comply with certain conditions.

These conditions may be summarized thus:

(1) The medium would be quickly and simply made, not calling for repeated filtration through close filters, and no great difficulty should arise with the adjustment of the hydrogen-ion concentration.

(2) The ingredients would be such that they could be conveniently stored for long periods and would, at the same time, be instantly available to produce a batch of medium at a moment's notice. Such ingredients as fresh blood should not be required.

(3) It would be possible to make, at any time, a batch of any size, large or small, to meet with immediate requirements, with equal facility.

(4) The medium would be reasonably selective for *C. diphtheriae* and would produce a good growth of characteristic colonies for each of the three types described by McLeod and his co-workers.

TYPES OF MEDIA INVESTIGATED

At the commencement of the investigation it was felt that, perhaps, an agar medium, using animal serum for enrichment, offered the best chance of securing the desired results. Ox serum is reasonably easy to obtain and it was thought that storage in the form of a serum-water base for the agar could be arranged. In order to determine whether a serum-containing medium would be capable of complying with the condition regarding the production of type-characteristic colonies, it was decided to observe the growth of a series of strains on the well-known coagulated serum medium of Conradi & Troch (1912). On this medium 110 strains were examined including representatives of each of the types but, while there was some differentiation, no reliance could be placed on colony formation as a type-distinguishing feature. In spite of these results a trial was made of various kinds of serum-water containing varying amounts of potassium tellurite and solidified with agar, but in each case the results corresponded more or less closely with those obtained using Conradi-Troch.

Following the failure to find a suitable medium using ox serum as the enriching agent, it was decided to examine the possibilities of the tellurite blood agar described by Horgan & Marshall (1932). When this medium was first used it was made with citrated horse blood as the source of red cells. This, however, was felt to be departing from the letter of the hypothesis, as the horse blood could be obtained only on certain days and, of course, could not be stored for long periods. Human blood was next used but was found, as described in the original paper, not to be wholly satisfactory. This is probably due to a high bacterial immunity to *C. diphtheriae* on the part of the individual from whom the blood was taken. In any case it was found to be extremely difficult to identify with the appropriate type the tiny colonies which *C. diphtheriae* forms on this medium. Accordingly after examining 70 strains of the various types on it the medium was discarded and a fresh start made.

An examination of the literature of *C. diphtheriae* revealed the fact that a medium had been described which conformed with the first three conditions set out above. This medium was described as a "blood agar tellurite arsenite selective medium for *C. diphtheriae*" by Prof. W. J. Wilson of Belfast in 1934. Used as a simple tellurite medium without the addition of sodium arsenite, this was found to comply with the first three of the conditions above set out. The medium, as originally described, consists simply of nutrient agar, 0·02 per cent sodium tellurite and 5 per cent of a preparation of laked ox blood. This laked blood mixture is very easy to procure and prepare, and will keep almost

indefinitely, certainly much longer than the 18 months claimed in the original paper. It is prepared as follows. A litre bottle, containing 10 g. of sodium citrate dissolved in 10 c.c. of water, is sterilized in an autoclave, is stoppered and is taken to the slaughter house. When an ox is being "stuck" a litre of the blood is caught in a sterile funnel and is directed into the bottle and vigorously shaken. To the blood 1·25 c.c. of pure formalin and 30 c.c. of methyl ether are added and the bottle is tightly corked and put away till the preparation is required for making a batch of medium.

Unfortunately no record was obtainable regarding the behaviour of the various types of *C. diphtheriae* when planted out on Prof. Wilson's medium. Consequently it was decided to make up a quantity of it and inoculate with cultures of specimens of *gravis*, *mitis* and *intermediate* types as well as with specimen mixed cultures. This showed that while on the whole there was a tendency to produce type-true colonies, this faculty was very poorly developed, and it was almost impossible to differentiate one type from another or to distinguish between certain strains of *C. diphtheriae* and such organisms as *C. hofmanii* which grew freely on the medium as well. However, it was felt that here was a starting point which offered more than anything that had been tried before, and many experiments were carried out using the "laked blood mixture" as an enriching agent for various types of special nutrient agar, at all times keeping in mind the necessity of simplicity in preparation. Finally, it was found that a simple nutrient agar containing "proteose peptone" with the *p*H adjusted to 7·6 would produce the characteristic colonies of the *gravis*, *mitis* and *intermediate* types, provided the medium was converted into a "chocolate medium" by heating at 75° C. for 10 to 15 min. The preparation of this medium is given in detail below.

Preparation of medium

1. *Broth*

(The best results are to be obtained by mixing the broth base with the plain agar just before pouring into plates.)

Lemco	20 g.
"Difco" proteose peptone	20 g.
Sodium chloride	10 g.
Distilled water	1000 c.c.

Dissolve by steaming in the usual way. Adjust reaction with *N*/1 NaOH till alkali to phenolphthalein. Heat to bring down phosphates. 30 min. at 90° C. Filter and bring to *p*H 7·6 with *N*/1 HCl. Bottle in screw-top bottles as described by McCartney (1933). (100 c.c. in an 8 oz. "medical flat" obtainable from the United Glass Bottle Company.) Stopper tightly and sterilize by autoclaving for 15 min. at 15 lb. pressure.

2. *Agar*

"Difco" "bacto" agar	30 g.
Distilled water	1000 c.c.

Dissolve the agar by boiling, bottle, placing every 100 c.c. in a 12 oz. "medical flat" (U.G.B.). Sterilize in autoclave 20 min. at 20 lb. pressure.

3. *Laked blood mixture*

Prepared as described above. This is best stored in 200 c.c. lots in 8 oz. screw-top "medical flat" bottles.

4. *Potassium tellurite* (1 per cent solution)

A bottle containing 100 c.c. of the broth is placed in a water-bath at 55° C. 10 c.c. of the laked blood mixture and 4 c.c. of the potassium tellurite 1 per cent solution are added and the whole well mixed. 100 c.c. of the agar are melted, by immersing the bottle in boiling water, and the melted agar is cooled to 55° C. The broth-blood-tellurite mixture is now added to the 100 c.c. of agar in the large bottle and mixed by inverting it gently several times. The agar mixture is now heated by gradually raising the temperature of the water-bath to 75° C., at which temperature it is maintained for 15 min. The medium is now ready for pouring into plates. These plates show a finely grained chocolate medium if the medium has been properly made.

To obtain the best results several points require emphasis.

(1) The broth should be heated above 100° C. only on one occasion. Hence the mixing of the broth to the agar just prior to pouring the plates.

(2) The blood and tellurite should be added to the broth and mixed before the broth, etc. is mixed with the agar. This ensures uniform mixing and prevents the formation of bubbles and froth.

(3) The blood-broth mixture should be added *to the agar* (which must be in a large enough bottle to accommodate the whole) and should be mixed by inverting the bottle slowly several times. This, again, prevents the formation of froth and lumps in the agar.

The technique just described gives about 210 c.c. of medium or about 12 generous plates. Actually a batch of any size may be made provided the same proportions of each ingredient are observed. The 100 c.c. amounts in the bottles have been adopted because it is so convenient to make 200 c.c. or multiples of this at a time.

GROWTH AND COLONY CHARACTERISTICS OF *C. DIPHTHERIAE* ON THE MEDIUM DESCRIBED

C. diphtheriae gravis

18–24 *hours*. Medium small, discrete, irregular colonies, with "nippled" or conical centre, somewhat crenated edge, grey in colour, darker towards the centre, size 2 to 4 mm. in diameter.

36–48 *hours*. Large, slate-grey coloured colonies, well separated specimens often reaching a size of 5 to 7 mm. in diameter, showing a central nipple, radial striations, irregular outline and a granular frosted surface, so aptly described as "daisy head".

Compared with appearances on McLeod's medium, the colonies are slightly larger in size and greyer in colour; this is probably due to the lower concentration of tellurite. (Pl. IV, fig. 1.)

C. diphtheriae mitis

18–24 *hours*. Medium small, discrete, regular colonies, spherical or lenticular in shape, colour typical, being grey at the periphery shading to black at the centre, size 1 to 3 mm. in diameter.

36–48 *hours*. Large, grey-black or black colonies varying in size up to 5 mm. in diameter. In form they may be either spherical or slightly conical, the outline is regular and sometimes slightly hazy, the surface is shiny, reflecting light freely and the colour shades from a greyish colour at the periphery to a dense black at the centre (Pl. IV, fig. 2).

C. diphtheriae intermediate

18–24 *hours*. Small metallic grey, discrete colonies, irregular in outline, edge somewhat crenated, with central papilla showing a characteristic "punched-out" appearance, size about 1 mm. in diameter.

36–48 *hours*. Small grey-black granular colonies about 1 to 3 mm. in diameter, irregular edge, whole appearance very rough with central papilla. These colonies have a peculiar "cut-out" appearance, not unlike pieces of confetti, which is very characteristic (Pl. IV, fig. 3).

Atypical strains

A number of strains approximating to *C. diphtheriae* type IV of Wright and Christison have been examined in parallel on McLeod's medium and on the medium here described. In each case exact correspondence between the two media has been found, the strains showing typical "daisy-head" colonies though they do not ferment starch but give *gravis* reactions on broth.

It will be seen from the foregoing that the medium described is eminently suitable for type identification and can be relied upon to produce typical colonies with each of the three types originally described. It has been used in the Huddersfield Public Health Laboratory as the routine medium for a typing investigation on 215 strains of *C. diphtheriae* and in every case the type characteristics have been found to correspond with the colony formation. A number of strains were planted in parallel on this medium and on McLeod's and here again an exact parallelism was found, the colonies on each being typical of that type to which they were proved, by other methods, to belong.

This is shown in Table I, only virulent strains being considered.

The technique of innoculation of the plates described in Table I was as follows: the strain was grown in pure culture for 24 hours on a modified Hiss' serum water medium and one loopful of this was emulsified in 4 c.c. of sterile normal saline. A loopful of the resulting emulsion was spread on the plate with a long platinum loop. In this way well-separated typical colonies were obtained.

All the strains were tested for fermentation reactions on modified Hiss' serum water containing the various carbohydrates (starch, glucose and saccharose) and for broth characteristics on a protose-peptone-lemco broth which has been found to be most suitable for the purpose. Virulence tests were carried out by the intradermal method.

Table I. *Comparison of standard strains grown on McLeod's medium and on the chocolate-tellurite-agar*

Strain	Colonies on McLeod's medium	Colonies on chocolate-tellurite-agar
D 43, *mitis*	Medium small black conico-lenticular	Large black conical hazy entire edge
D 65, *gravis*	Medium large black "daisy head"	Large slate-grey "daisy head" (Pl. IV, fig. 3)
E 6, *intermediate*	Small black irregular conical	Medium black crenated conical (Pl. IV, fig. 3)
D 64, *gravis*	Large black "daisy head"	Large slate-grey "daisy head"
D 67, *mitis*	Small black spherical	Large black conico-spherical (Pl. IV, fig. 2)
D 66, *gravis*	Large black "daisy head"	Large slate-grey "daisy head"
4120, *intermediate*	Small black irregular conical	Medium black irregular crenated conical
ML 10, *mitis*	Small black spherical	Medium small black spherico-conical
E 9 Atypical (Type IV)	Large black "daisy head"	Large slate-grey "daisy head"
E 3, Atypical (Type IV)	Large black "daisy head"	Large slate-grey "daisy head"

IDENTIFICATION OF *C. DIPHTHERIAE* WITH THE CHOCOLATE-TELLURITE MEDIUM

So far, the use of this medium has been described in relation to its value for the identification of types of *C. diphtheriae*, but a description of it would be incomplete without reference to its value as a "diagnostic" medium. For this purpose, then, it is necessary to consider what, if any, other organisms grow on it. Actually the only ones that are likely to cause any difficulty are the "diphtheroids" (*C. hofmanii* and *C. xerosis*) and *Micrococcus catarrhalis*.

Corynebacterium hofmanii produces two kinds of colonies, one black and the other pearly white. The difficulty arises principally with the former which may resemble *mitis* type colonies very closely at 48 hours; earlier, however, they are smaller and lack the characteristic grey colour, while in form the dewdrop appearance presented by the colonies is very characteristic. *C. xerosis* is much more like *C. hofmanii* than *C. diphtheriae* and is jet black from the first, so in a carefully observed culture should not be mistaken. *Micrococcus catarrhalis*, again, is much smaller than *Corynebacterium diphtheriae*, the colonies are more conical and are very tenacious, being extremely difficult to pick off the medium.

The practical application of this is the method of examination of routine swabs now in use in the Huddersfield Laboratory. Each swab received is inoculated on a Loeffler slope and, at the same time, on a portion of a tellurite-chocolate-agar plate. The plate and the Loeffler slope are read after 18 hours incubation, the slope in the ordinary way by staining a film by Albert's stain and placing it under the microscope, the plate by observation of the colonies with a hand lens. When examining a plate thus it is often helpful to pick off a *suspicious colony, make a film of it, stain it with Albert's stain and examine it microscopically for, unlike most tellurite media, colonies developing on this one present a fairly typical morphology of the constituent organisms.*

https://doi.org/10.1017/S0022172400035270 Published online by Cambridge University Press

Up to the present it has been found that every swab giving a positive reading on the Loeffler slope has given a corresponding positive reading on the plate. On the other hand, positive readings on the plate are often accompanied by negative findings on the Loeffler slope. In these cases the growth on the plate is usually only very scanty, perhaps only three or four colonies. This is particularly remarkable in the case of "discharge" swabs from the Fever Hospital where one would be inclined to expect the organisms to be present in small numbers.

The results of this method of examination applied to swabs are shown in Table II.

Table II. *Summary of* 1375 *swabs examined by "plate" and "slope" methods*

Swabs reading	Loeffler slope culture	Tellurite plate culture
Positive	227	293
Negative	1148	1082
Total no. of swabs examined	1375	1375

Positive on Loeffler only	0
,, tellurite and Loeffler	227
,, tellurite only	66

Therefore, had the Loeffler slope only been relied upon, 66 positive swabs would have been missed.

Further investigations along these lines are being carried out to see if it would be possible to dispense with the use of the Loeffler slope altogether from routine swab examinations.

Discussion

The need for a simply made tellurite medium has been felt since the value of classifying the types of *C. diphtheriae* became understood. Many efforts have been made with this in view, but in nearly every case something has had to be sacrificed in the interests of simplicity. It is fairly clear, however, that the medium here described is not only simple to make, but appears to do everything that the more complicated media do in the way of producing type-characteristic colonies.

The ingredients are not difficult to obtain. The broth described is used, in single strength, as the standard broth for diphtheria work in the Huddersfield Laboratory and has been found by much experimental work to be the most satisfactory for this purpose. The agar is a simple 3 per cent solution of a high quality product which is specified because it is sufficiently pure not to require clearing. The laked blood solution is included because it appears to be the simplest method of obtaining the necessary native protein enrichment, and because it supplies it in a form which, once procured, can be readily placed in store over long periods, and yet is available at any time a batch of medium is required. The question of inhibition of growth by the ether and formalin

does not seem to exist as far as *C. diphtheriae* is concerned; they are probably evaporated in the heating process. The percentage of tellurite used was found, by lengthy experiment, to be the most satisfactory. It, combined with the *p*H of the broth, appears to be quite sufficient to inhibit the growth of unwanted organisms. No advantage was found to result from increasing the tellurite, though with a lower hydrogen-ion concentration more tellurite might possibly be added with advantage. Glass in a recent paper (1937) states that the concentration should not exceed 0·06 per cent and the work done during this investigation supports this.

The technique described for the preparation of the medium might possibly be simplified by making up the broth and agar together as a nutrient agar. This method was tried during the earlier experiments but the results appeared to be less constant. This may be due to the effect of repeated heating on the proteose-peptone. The colonies were smaller and less characteristic in a nutrient agar which had been heated and cooled several times.

The question of using laked bloods and of heating blood-agar media was also very fully discussed by Glass in the paper mentioned above. This author describes experiments showing laked blood to be inferior, as an enriching agent, to blood not so treated. He also shows growth on a heated blood proteose-peptone-tellurite-agar to be inferior to such a medium not so treated. In this investigation these disadvantages were not met with. The blood used was ox blood which Glass does not mention in his experiments and no difference could be observed between the fresh and the preserved blood. As far as heating the medium to 75° C. is concerned, this appears to be a great advantage. On the heated or "chocolate" medium the colonies are not only more numerous but heating appears to be actually necessary to produce the type-characteristic colonies. It may be that this effect is due to the final loss, by evaporation, of the preserving agents, the ether and formalin, in the laked blood.

The value of this medium is enhanced by the fact that it places a selective medium always at hand for use in the examination of routine swabs. It is particularly valuable when the question of Fever Hospital "discharge" swabs with scanty numbers of organisms come under consideration. Its convenience coupled with its reliability in this work raise the question of its replacing Loeffler's serum medium as the routine medium in the Huddersfield Laboratory, and investigations with this in view are at present being undertaken.

SUMMARY

1. A simply made selective tellurite medium for the isolation of *Corynebacterium diphtheriae* and the identification of its types has been described.

2. The ingredients of this medium are easily obtained and can be stored for any length of time making the medium readily available at short notice.

3. Large or small quantities of the medium can be made with equal facility.

4. When grown on the medium, *C. diphtheriae* will produce colonies characteristic of its type, be it *gravis*, *mitis* or *intermediate*.

5. The appearance of the colonies has been described and has been shown to resemble closely, in the case of each type respectively, the appearances described by the Leeds workers (Anderson *et al.* (1931)) on their medium in the original papers.

6. The possibilities of the use of this medium for diagnostic routine swab examinations have been briefly referred to.

ACKNOWLEDGEMENT. My thanks are due to Prof. W. J. Wilson, Queen's University, Belfast, for assistance and guidance in the course of this investigation, and to Dr John M. Gibson, Medical Officer of Health, Huddersfield, for permission to publish the results herein recorded.

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EXPLANATION OF PLATE IV

Corynebacterium diphtheriae

Photomicrographs of all colonies taken at 48 hours on chocolate-tellurite-agar. Magnification × 1·5.

Fig. 1. *C. diphtheriae gravis*, strain D 65.

Fig. 2. *C. diphtheriae mitis*, strain D 67.

Fig. 3. *C. diphtheriae intermediate*, strain E 6.

(*MS. received for publication* 13. II. 1937.—Ed.)

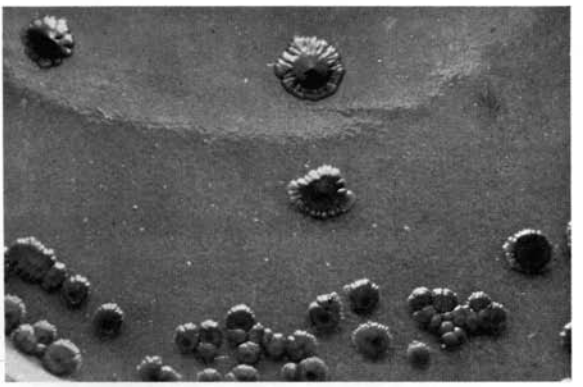

Fig. 1.

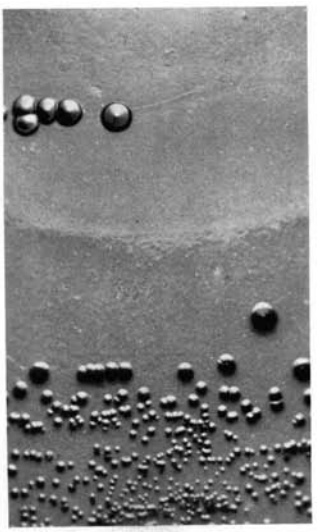

Fig. 2.

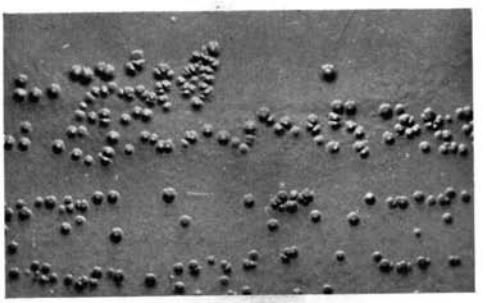

Fig. 3.

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