A COMPARISON OF CULTURAL METHODS FOR ROUTINE DIPHTHERIA DIAGNOSIS

BY

J. D. ALLAN GRAY, M.B., B.Sc., F.R.C.P.Ed., D.P.H.

AND

DORIS M. STONE, M.D., D.P.H.

(From the Preventive Medicine Department, University of Bristol)

Current literature dealing with the bacteriological diagnosis of diphtheria has revealed a disquieting failure of methods which until recently had been accepted as all-sufficient.^{1 2} The recent epidemic of diphtheria in Bristol, associated with the presence of the gravis, mitis, and intermediate types of the organism, has supplied an opportunity of testing and comparing various methods in the hope of finding one giving reliable results with speed and reasonable ease in technique. The methods investigated have involved the use of (a) Loeffler slopes, (b) Loeffler slopes modified by the addition of glycerin, and (c) blood-tellurite media inoculated either directly from the swabs or from the growths on the Loeffler slopes. The hope of finding one all-sufficing method has not been realized, but the evidence suggests that the combined use of a Loeffler slope containing glycerin and a blood-tellurite plate would result in a marked decrease in the number of otherwise "missed" cases without an unreasonable increase in the amount of routine work involved.

Two thousand swabs have been examined by the different methods in parallel experiments. They were received from general practitioners, isolation hospitals, and other institutions, and therefore included *primary swabs* sent for the diagnosis of suspected fresh cases, or for the identification of carriers, and *repeat swabs* taken with a view to determining freedom from infectiveness in known cases and carriers. The majority were obtained from the throat and nose, but a few were from the ear and eye.

Examination of the First Thousand Swabs

A suspension of the material on the swab in three drops of sterile physiological saline solution was used to make a direct film and to inoculate two Loeffler slopes, one modified by the addition of glycerin. The direct films were stained by Neisser's method, and the presence of organisms morphologically identical with diphtheria bacilli was immediately reported to the clinician. The two slopes were left at room temperature until 5 p.m., and then incubated at 37° C. until 9 o'clock the next morning. Incubation for longer than these sixteen hours frequently allowed of the overgrowth of diphtheria bacilli by other organisms. Neisser-stained films made from the slopes were then examined and the positive results were reported.

Subinoculations were made from "likely" areas on each of the slopes on to plates of strong blood-tellurite medium. Sixteen inoculations could be made on each of the 10 cm. plates. The latter were immediately incubated at 37° C. for twenty-four hours, and subinoculations from colonies resembling those of diphtheria bacilli were made on both weak blood-tellurite plates and glycerin Loeffler slopes. After twenty-four hours' incubation at 37° C. the macroscopic appearances of the growths on the weak blood-tellurite medium were noted. At the same time films made from the glycerin Loeffler slopes and stained with Neisser's method were examined for the presence of diphtheria bacilli. Films were not made directly from the blood-tellurite medium because diphtheria bacilli grown thereon frequently fail to show metachromatic granules.^{3 4} Where divergences were found in the results obtained by the various methods isolation of the strain concerned was attempted on glycerin-agar plates with the purpose of determining the types.

Examination of the Second Thousand Swabs

Each swab was inoculated on a glycerin Loeffler slope, and immediately thereafter on a strong blood-tellurite plate. Films made from the slopes after sixteen hours' incubation at 37° C. were examined for the presence of *C. diphtheriae*. The plates after incubation at the same temperature were examined at intervals of twenty-four and forty-eight hours, and "likely" growths were subinoculated on glycerin Loeffler slopes. Films were made after incubation overnight and examined as before.

Culture Media Used

1. *Loeffler Blood Serum Containing Glycerin*.—The broth with which the ox serum was mixed was that devised by Cole and Onslow,⁵ modified by the addition of 1 per cent. glucose and 5 per cent. glycerin. In its preparation an amount of 0.5 per cent. sodium chloride in distilled water was added to the mixture of laitproto, sodium carbonate, pancreatic extract, and hydrochloric acid, sufficient to reduce the amino-acid content to approximately 40, expressed in terms of cubic centimetres of N/10 alkali per 100 c.cm. of medium.⁶

After inspissation of the mixture of serum and broth, approximately 1 c.cm. of the glucose-glycerin laitproto broth was added to each tube, and the whole was sterilized by steaming. The additional free glycerin broth has for many years been used in Bristol to keep the medium moist during storage, an advantage when few swabs are being sent to the laboratory. The free broth also encourages the growth of certain (particularly gravis) strains of *C. diphtheriae* which grow atypically on ordinary Loeffler slopes.

2. *Loeffler blood serum without glycerin* was identical with 1, except that the broth mixed with the serum and added to the slope did not contain glycerin.

3. *Strong Blood-tellurite Medium*.³—Rabbit blood was used and, as suggested by O'Meara and Parish,¹ 0.5 per cent. glucose was added to the agar to increase the whitening of the final medium subjacent to colonies of glucose-fermenting organisms such as *C. diphtheriae*. It was found essential to use a meat extract (ox muscle) for the preparation of the nutrient glucose agar, because the use of synthetic preparations such as lab-lemco greatly reduced the selectivity of the final medium for *C. diphtheriae*. A similar loss of selectivity was produced by storage of the blood-tellurite mixture for more than a fortnight before its incorporation with the glucose agar. Other workers, however, have given three weeks as the limit of storage.^{1 3} Haemolysis did not appear to produce unfavourable effects.

4. *Weak blood-tellurite medium* was identical with 3, except that the 2 per cent. potassium tellurite was diluted with seven parts of saline solution, so that its final concentration was approximately 0.02 instead of 0.17 per cent. On this medium the gravis strains of *C. diphtheriae* produced large, granular, grey, "daisy head" colonies; the mitis type convex, shiny, black colonies; and the intermediate type fine, raised, granular, black colonies. The differences, however, were insufficient by themselves to identify the various types, and not so clear as those obtained by Anderson and his co-workers,⁷ using a medium containing twice the amount of tellurite.

5. *Glycerin Agar*.—Cole and Onslow's broth containing 5 per cent. glycerin was solidified with 2 per cent. agar.

6. *Carbohydrate Media*.—These included 0.5 per cent. solutions in peptone water of starch, glycogen, dextrin, glucose, maltose, galactose, and saccharose, used for testing the fermentative reactions of the strains of *C. diphtheriae* isolated.

7. *Laitproto broth*, as prepared for 1, but without the addition of glucose and glycerin, was used for typing purposes.

Results with the First Thousand Swabs

Thirty-three of the direct films showed the presence of organisms morphologically indistinguishable from *C. diphtheriae*. Four of these swabs—all from throats—were negative by all the cultural methods. Two of these patients, moreover, did not exhibit clinical evidence of infection. Speed is desirable, admittedly, in the identification of persons harbouring diphtheria bacilli, to permit early isolation and early administration of antitoxin, but the above findings emphasize the need for caution in the interpretation of positive results in direct films, even when made from throat swabs.

Cultural Examination

The table summarizes the results of microscopical examinations of the growths on the serum slopes and blood-tellurite plates. The discrepancies between the results are in part due to unavoidable technical fallacies. For instance, many of the swabs, especially those from carriers and convalescents, contained only a very small number of diphtheria bacilli. Therefore in inoculating the serum slopes from the saline suspensions, the diphtheria bacilli may have been so unequally distributed that nearly all, and in extreme instances all, were transferred to one slope, leaving little or none for the other. Further, scantily positive serum slopes were at times overgrown with other organisms, so that the inoculum transferred to the blood-tellurite plates may not have contained diphtheria bacilli.

TABLE I.—Results of Cultural Methods with First 1,000 Swabs

Glycerin Loeffler	Non-glycerin Loeffler	Blood-tellurite from Glycerin Loeffler	Blood-tellurite from Non-glycerin Loeffler	Total Number of Diphtheria Positive	Gravis	Intermediate	Mitis	Not Typed	Saccharose Fermentation	Total
+	+	+	+	61	18	2	41	0	2	63
+	+	+	-	5	1	0	4	0	0	5
+	+	-	+	2	0	0	1	1	0	2
+	+	-	-	30	4	0	9	17	1	31
+	-	+	+	7	0	0	7	0	0	7
+	-	-	+	0	0	0	0	0	0	0
+	-	+	-	8	0	0	4	4	1	9
+	-	-	-	18	1	0	4	13	1	19
-	+	+	+	5	1	0	4	0	0	5
-	+	+	-	0	0	0	0	0	0	0
-	+	-	+	5	0	0	5	0	1	6
-	+	-	-	17	1	0	4	12	0	17
-	-	+	+	16	3	2	11	0	6	21
-	-	+	-	9	1	1	6	1	2	11
-	-	-	+	4	1	0	1	2	1	5
Totals ...	...	...	...	187	31	5	101	50	15	202

Certain saccharose-fermenting diphtheroids having morphological and staining characteristics identical with *C. diphtheriae* favoured the reporting of false positive results (see Table). This conclusion is supported by the absence of clinical evidence of infection in the individuals concerned. On the blood-tellurite medium the cultural appearances of some of these saccharose-fermenting diphtheroids were not unlike those of diphtheria bacilli of the gravis type. They gave a greyish matt growth and, when the organism fermented glucose as well as saccharose, a whitening of the subjacent medium. Fortunately they could readily be isolated from the blood-tellurite plates and tested for the fermentation of saccharose.

From 202 of the thousand swabs organisms microscopically indistinguishable from *C. diphtheriae* were isolated. In only 187 of these, however, were the organisms found to be *C. diphtheriae*, the remaining fifteen diphtheroids all fermenting saccharose. The following were the numbers of positive results obtained by the use of one medium alone or a combination of media.

Glycerin serum slopes only ...	131
Non-glycerin (Loeffler) serum slopes only ...	125
Glycerin and non-glycerin slopes together ...	158
Glycerin serum slopes and blood-tellurite plates inoculated from them ...	161
Non-glycerin serum slopes and blood-tellurite plates inoculated from them ...	152

The figures show clearly that the use of either type of serum alone permits the missing of a seriously large number of positive results. For instance, when glycerin serum slopes were used the additional use of the blood-tellurite plates gave a further thirty positive results; the corresponding figure for the non-glycerin serum slopes was twenty-seven. Of these thirty, five were gravis, three intermediate, twenty-one mitis, and one untyped. Of the twenty-seven, four were gravis, two intermediate, nineteen mitis, and two untyped. Other workers have recorded that the strains of diphtheria bacilli which fail to produce typical metachromatic granules on primary growth on Loeffler slopes, and which may therefore remain undiscovered unless blood-tellurite media is used, are invariably of the gravis type.^{1 2 7} Their results are not, however, strictly comparable with this experiment on account of the addition to the surfaces of the Loeffler slope of the glucose broth allowing the demonstration of the gravis strains. The failure in certain cases of the mitis and intermediate strains to develop on the Loeffler slopes may, as suggested before, be due to uneven distribution of the bacilli.

It is obvious that the greater the number of methods used the greater will be the number of true positive results, but the use of so many methods is impracticable in routine on account of the time involved and the amount of media used. The next best substitute appears to be the combined use of glycerin serum slopes and blood-tellurite plates inoculated from them. This combination gave a slightly higher number of true positive results than either of the other combinations or the sole use of the serum slopes. It was thought possible that inoculation of the blood-tellurite plates directly from the swabs might give a better percentage of positive results than their inoculation from the growths on the serum slopes. The experiments with the second thousand swabs were undertaken with a view to assessing the value of such a procedure.

The incorporation of the glycerin in the serum slopes gave a slightly higher proportion of positive results and so justified its use. Further, the number of additional positives obtained by the subsequent use of the blood-tellurite plates was also slightly greater than in the corresponding media without glycerin.

It is interesting to note that, although 187 of the first thousand swabs were shown by one or more of the media used to harbour *C. diphtheriae*, in only sixty-one cases did all four media yield positive results. This suggests marked paucity of the *C. diphtheriae* in relation to the other organisms present.

Results with the Second Thousand Swabs

Microscopical evidence of the presence of organisms morphologically identical with *C. diphtheriae* was found in 100 swabs. The smaller proportion of positive results compared with the first thousand swabs was due to a large number of the second thousand having been taken during the post-epidemic period. Table II shows the results obtained by the use of the different media.

TABLE II.—Results of Cultural Methods with Second 1,000 Swabs

Glycerin Serum	Blood-tellurite Medium	Number of Positives	Gravis	Mitis	Intermediate	Untyped
+	+	54	4	20	0	30
+	-	21	0	7	0	14
-	+	25	12	8	3	2
Totals ...	...	100	16	35	3	46

It will be observed that had the serum slopes alone been used the number of positive results would have been seventy-five, whereas with the blood-tellurite medium alone the number would have been seventy-nine. The advantage of the blood-tellurite medium is probably greater than the figures suggest. The routine procedure adopted involved the inoculation of the serum slopes before the blood-tellurite medium. With heavily infected swabs this was of little significance, but when the *C. diphtheriae* were scanty the chances were in favour of their growing out on the serum slopes rather than on the tellurite. In addition, the presence of saccharose-fermenting diphtheroids among the untyped strains of the serum-positive-blood-tellurite-negative group made the total of that group fictitiously high.

It will be noted that no fewer than twelve of the twenty-three typed strains in the serum-negative-blood-tellurite-positive group were of the gravis type. This may be compared with the results of Parish,² who found in his experiments that the strains missed by the sole use of serum were invariably of the gravis type.

Discussion

The difficulties met with in the bacteriological diagnosis of diphtheria and described in this paper serve to emphasize what has been stressed for a long time—namely, that it is the duty of the clinician in cases of suspected diphtheria to avoid delay in specific treatment and isolation. At best, bacteriological methods must be regarded as an adjuvant to, and not as a substitute for, clinical diagnosis. They have, however, a great use in the control of carriers and the "clearing" of convalescents. Primary examinations of direct films are useful in aiding the clinician in doubtful cases, but a dogmatic report, either positive or negative, should not be given from primary examination alone.

Serum slopes containing 5 per cent. glycerin give a slightly higher percentage of positive results than Loeffler serum without it. The main advantage of the glycerin, however, is that it retards the drying of the medium when kept in stock. The addition of about 2 c.cm. of glucose broth to the surface of the slope is recommended in the belief that certain gravis strains of *C. diphtheriae* which would otherwise fail to grow may thereby be induced to multiply.

Both types of serum used in this investigation—that is, with and without glycerin as well as the blood-tellurite medium—have been found on occasion to give "false positive" results. These have been due to the presence of saccharose-fermenting diphtheroids which, when grown on the slopes, have presented microscopical appearances identical with those of *C. diphtheriae*, and on blood-tellurite plates have given greyish-black growths, sometimes with subjacent whitening.

Such "false positives" amount probably to between 1 and 2 per cent. of the total number of swabs examined, and the fact that they can and do occur is disquieting. They are a potential danger, inasmuch as their presence in certain communities may conceivably cause a person neither suffering from diphtheria nor carrying diphtheria bacilli to be removed to an infectious diseases hospital, where he may be exposed to various infections. The application of the saccharose fermentation test should therefore be a routine measure in those cases in which the clinical findings are opposed to the bacteriological. This would obviate the need for virulence tests in many of the "scanty positive" cases.

The blood-tellurite medium is valuable for growing strains of *C. diphtheriae* which fail to grow on the serum slopes. Inoculation directly from the swabs and not from the growths on the serum slopes is recommended, since this gives better results and saves time. Even then the

time taken for characteristic colonies to develop detracts from the usefulness of the medium in the diagnosis of primary cases. When, however, speed is not of such moment, as in the determination of freedom from infection in convalescents and carriers, its use in conjunction with serum slopes is of very considerable value. It is also most helpful in the isolation of *C. diphtheriae* for virulence and typing tests. Its substitution in place of the serum slope cannot, however, be recommended for routine work, owing to the time which it requires and to the fact that it has not consistently grown all the strains which grew on the serum slopes.

In the course of the investigation it has been repeatedly noticed that diphtheria bacilli grow luxuriantly in the presence of *Staphylococcus aureus*. It appears that any relationship existing between the two organisms may be a true symbiotic effect, or, as suggested by Laybourn,⁸ the result of acid formation in the medium by the staphylococcus.

Summary

Two thousand swabs have been examined by various cultural methods, with results which justify the following conclusions.

A provisional diagnosis alone should be made on films from blood serum slopes.

Inoculation of the blood-tellurite medium should, where feasible, be part of the routine examination of all diphtheria swabs, in order to confirm or repudiate the results obtained by the use of the blood serum, to reduce the number of otherwise missed cases, and to allow of easy isolation for the performance of the saccharose fermentation and virulence tests.

The procedure suggested for routine purposes is as follows:

1. In all urgent cases examination of direct smears with immediate reporting to the clinician of positive results as "suspicious."

2. Examination of films from serum slopes (containing glycerin and free glucose broth) with reporting of all results, the clinician realizing that this report is provisional and may be reversed in the light of later results any time up to forty-eight hours.

3. A saccharose fermentation test followed, if negative, by a virulence test in all positive swabs from apparently healthy individuals.

4. Inoculation of (strong) blood-tellurite medium directly from the swab and, after twenty-four to forty-eight hours' incubation, the transference of likely growths to serum slopes (containing glycerin and free glucose broth). After incubation examination of films made from the slopes and the reporting of additional positive results to the clinician when required. These additional positive results must, of course, be controlled as above by saccharose fermentation and virulence tests.

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