OBSERVATIONS ON THE GROWTH OF MENINGOCOCCI
IN VITRO IN RELATION TO VIRULENCE.¹

A REPORT TO THE MEDICAL RESEARCH COUNCIL ON WORK CARRIED OUT
AT THE UNIVERSITY OF CAMBRIDGE PATHOLOGICAL LABORATORY
AND FIELD LABORATORIES.

By E. G. D. MURRAY AND R. AYRTON.

(Continued from page 444, Vol. XLIV.)

VI.—THE RELATION OF MEDIUM TO VIABILITY AND
MAINTENANCE OF VIRULENCE.

In the foregoing sections we have discussed the influence of the constitution of the medium upon physiological characters of the meningococcus which can be measured in terms of reproduction in vitro and adaptation to a parasitic existence. In the present section we wish to consider briefly a few scattered observations on the duration of the life and the virulence of cultures of that organism in vitro.

It is generally agreed that the life of a culture of meningococcus on the ordinarily used media is short, and it is very commonly stated that the culture may die within forty-eight hours. Various media have been considered satisfactory because stock cultures remained alive for one or two months, and it is quite likely that the strains used had been subjected to subculture before the test was performed. In our experience, before we had investigated the facts detailed in the preceding sections, Dorsett's egg medium maintained the life of meningococcal cultures better than the other media we had tried. A large proportion of cultures on egg could be recovered when they were a year old and only an occasional one died in less than six months, but absolute certainty only prevailed with monthly subculture. In any case direct subculture from an egg slope a month or more old on to agar media was often a matter of difficulty, it frequently failed to give any growth and quite commonly only a few scattered colonies resulted, even with quite heavy inoculation. Subculture from egg to egg gave better results, but, even so, the appearance of only a few scattered colonies was a far too frequent occurrence; and these scattered colonies commonly had to be subcultured daily for a few generations before vigorous growth obtained. Thus it became our usual practice, when growth of a particular strain living on egg was required on agar medium, to resort to an intermediate young subculture on egg.

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When we first observed the difference in virulence of cultures grown on particular samples of EDB/N and EDB/S media, we were examining the influence of repeated subculture on the growth of the meningococcus. Two series of subcultures were being run at the same time: one at twelve and the other at twenty-four-hourly intervals. In the twenty-four-hourly series there was a marked initial lag with each subculture, but in the twelve-hourly series growth appeared more and more rapidly with successive subculture, until, after a few generations, four hours' incubation yielded a considerable growth. In the course of thirty-six days the EDB/N medium with which we had started came to an end and we proceeded with a batch of EDB/S; these were the same batches of media on which we had noticed the difference in virulence, but we did not at first observe any marked difference in the growth as these generations were carried out on slopes, in test-tubes plugged with wool in the ordinary way. We had previously noticed that cultures which were kept at 37°C for fourteen to thirty days occasionally gave rise to scattered colonies superimposed on the old growth, so these various cultures were all kept for a month to watch for this secondary growth, without any precautions to prevent drying of the medium. Out of the 108 cultures grown on EDB/N (No. 86) (71 from the 12-hour and 37 from the 24-hour series) only 4 showed secondary growth; whereas of 37 cultures grown on EDB/S (No. 88) (25 of the 12-hour and 12 of the 24-hour series) every one gave good secondary growth.

In view of the experiments described in Section V, the outstanding feature of this observation depends upon the fact that growth yielded by EDB/S, No. 88, was virulent, whereas the same strain grown on EDB/N, No. 86, failed to kill mice. Backed by our observation on virulence in relation to these media, secondary growth immediately became a character of importance in our eyes, and caused us to think of the possibility of making a medium which would maintain a culture alive for a considerable time without loss of virulence.

At the time we thought these important differences might be due to the digest having been contaminated or not during its preparation and we resolved to test the viability of cultures on EDB/N and EDB/S media, with the result that in this case the non-sterile proved to be the better, but still fell far short of egg for this purpose, as is shown in Table IX.

<table>
<thead>
<tr>
<th>TABLE IX.</th>
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<tbody>
<tr>
<td>Medium kept at 37°C with waxed plugs</td>
<td>Subcultured from</td>
<td>Number of cultures after 9 months later, by subculture on EDB/N medium</td>
</tr>
<tr>
<td>EDB/N No. 72</td>
<td>Bacteriolysis Expt. 50, XVII</td>
<td>24</td>
</tr>
<tr>
<td>EDB/S No. 138</td>
<td>Bacteriolysis Expt. 59, XVII</td>
<td>25</td>
</tr>
<tr>
<td>Egg</td>
<td>EDB/N 72</td>
<td>18</td>
</tr>
<tr>
<td>Egg</td>
<td>EDB/S 138</td>
<td>16</td>
</tr>
</tbody>
</table>

Media 72 and 138 showed an identical increment in the "Sörensen figure" due to added digest; but there is considerable evidence that the
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centration of digest 122 used for making No. 138 and other media was not favourable for virulence, and some evidence that No. 72 was a better medium from this point of view. No very precise information on this point is available because we had not yet recognized the principle of the "virulence range."

The recognition of the "virulence range" was immediately applied in the form of our early $EDB/V$ medium and when this was used we observed that there was a rapid regrowth of the meningococcus over the area from which the original growth had been cleanly removed. This observation was followed by the experiments relating to inorganic salts in the medium (Section IV (d)) and the relation of regrowth of the culture to potassium salts was consequently noticed.

Previously we had tried a medium described by Wadsworth (1903), a weak agar jelly, containing about seventy-five per cent of serum or ascites fluid, in which the pneumococcus maintained its virulence at a constant level for several weeks (Wadsworth and Kirkbridge, 1918); some cultures of meningococcus died quite soon and others survived for a long time in this medium. But we were struck by the advantages of the weak agar jelly and the benefit of being able to dispense with waxed plugs. We therefore made $EDB/V$ medium, containing the required amount of digest and salts, but only 0.5 per cent. of agar; this medium we call "F." In order to have present the accessory growth factors required by the meningococcus, our practice is to dilute "EB" or "EH agar" with extract to which we have added the required amount of digest and salts, and, after adjusting the reaction, to distribute it in wool-plugged tubes under a layer of liquid paraffin and autoclave it.

We have not had this medium in use sufficiently long to be able to discuss its properties fully, but we may say that it promises well, for the following reasons: The meningococcus grows readily in it in primary culture from cerebro-spinal fluid and a profuse growth is obtained on subculture on to our ordinary medium ($EHD/V$), even when the culture is five months old. The minimal lethal dose of this culture was 2 milligrammes for 20 grammes of mouse when put into "F" medium and it showed no alteration in killing power in four months; after five months it killed more often than not in a 2-milligramme dose (slightly irregular) and with absolute certainty in a 4-milligramme dose for 20 grammes of mouse. This is a considerable improvement on our experience with egg medium. Furthermore we have never worked with such virulent strains as those isolated on "F" medium.

During the last three years we received seventeen cultures of freshly isolated strains of meningococcus from private friends and as the result of an appeal by the Secretary of the Medical Research Council and the Principal Medical Officer of the Ministry of Health. These were grown on various media of which we know nothing, nor do we know how often they had been subcultured before we received them, but the relation of
the minimal lethal dose of the cultures to the medium on which they were sent to us is set out in Table X and has certain points of interest.

TABLE X.

<table>
<thead>
<tr>
<th>Minimal lethal dose for 20 gm. of mouse</th>
<th>&gt; 8 mgm.</th>
<th>8 mgm.</th>
<th>4 mgm.</th>
<th>2 mgm.</th>
<th>Totals on each medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsett’s egg</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Insipissated serum</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Trypagar</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Unknown agar</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EDB/N</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Totals of each M.L.D. 8 3 1 5 17

The striking feature of these results is that no strain received on insipissated serum was virulent; but the other figures are difficult to interpret without further investigation of the problem.

We are greatly indebted to Professor H. R. Dean for his personal appeal to a wide circle of workers, asking them to collect primary cultures on our media and they have kindly consented to help us in this direction. During the last three months we have received ten suitable cultures on “F” egg and serum; each set having been inoculated direct from the same sample of cerebro-spinal fluid. Up to the present all the cultures on “F,” nearly all on egg, and certain of the serum cultures have been virulent. Of these two were received from the same source as the serum cultures given in Table X.

It is yet too early to discuss these results; but at present it appears that the primary culture may be virulent even when the medium is not the most suitable, although on subculture its virulence may be lost. Of the virulent cultures shown in Table X, we know that the one we received on “Trypagar” was inoculated direct from the cerebro-spinal fluid.

TABLE XI.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Generations at 24-hourly intervals</th>
<th>Dose of living meningococci per 20 gm. of mouse (2 mice were inoculated with each dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 mgm.</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = one mouse died in under 48 hours.
(+)= one mouse died between 48 and 86 hours.
0 = one mouse definitely survived.
Blank space = dose not tried.

There is one other aspect of the maintenance of virulence in vitro which deserves consideration. It is well known that many parasitic bacteria lose their virulence if frequently subcultured at intervals of twenty-four hours
and that the meningococcus is particularly apt to behave in this way. It is especially interesting, therefore, to notice the relation of killing power to successive subculture at twenty-four-hourly intervals on \textit{EHD/V} medium and the results of two such experiments are given in Table XI.

These experiments suggest that it may yet be possible to produce a medium and a method of using it, which will allow of repeated subculture without loss of virulence. The fluctuations in the time the mice took to die gains in interest when compared with the fluctuations in yield of growth shown in Table II (Section III), for here again the rise and fall may be irregular after the first two generations. It is conceivable that this fluctuation in killing power and yield of growth may be determined by the proportion of dead cocci contained in a given mass of growth; this interpretation is particularly suggested by the fact that the mass representing one minimal lethal dose of killed cocci, when the organisms are entire, is very many times greater than that representing one minimal lethal dose of the given living meningococcus culture.

We readily admit that the observations contained in this section are most incomplete, but, at least, they indicate that much may yet be done by a thorough investigation of the influence of medium on the maintenance of viability and virulence of cultures \textit{in vitro}.

\textbf{VII.—Discussion.}

Dopter (1921, p. 416) remarks that the immunization of animals against the meningococcus is a very delicate process which presents many technical difficulties, and this observation is emphasized by his discussion of the methods advocated by eminent authorities. There appears to be no doubt that the immunization of horses, with the object of producing a potent therapeutic anti-meningococcal serum, is by no means accomplished with any degree of certainty. Flexner, Dopter, Gordon, Nicolle and others have produced unassailable evidence that a highly potent therapeutic serum can be produced occasionally, but it is quite evident that failures have been a common experience. Similar failures appeared to us to call for a close study of the characters of meningococcal antigens and the present paper deals with part of this investigation.

A study of the literature of anti-meningococcal serum reveals no convincing evidence of any character which might serve as a guide in the production of a successful serum. The virulence of the cultures used as antigens does not appear to have been investigated, and the explanation of this undoubtedly lies in the fact that it is generally admitted that the attempted titration of meningococcal virulence has resulted in failure.

In this paper we have discussed in detail some of the inherent difficulties presented by the cultivation of the meningococcus \textit{in vitro}, and, although we cannot claim to have made an entirely satisfactory medium, it will be admitted that we have established that the constitution of the medium exercises an important influence on the "virulence" of the culture.
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We have no evidence to show how important it may or may not be to use only highly virulent meningococcal cultures as antigens for the production of potent therapeutic serums. Those concerned with the production of serums commonly express as their opinion that it is desirable to use only freshly isolated strains, but judging by their behaviour towards mice, the freshly isolated strains shown in Table X of this paper are very different organisms from those we are obtaining in primary culture on our “F” medium. Thus, “freshly isolated strain” becomes a term of no exact meaning without qualifying it by describing the properties of the medium. Possibly “freshly isolated” strains are more likely to represent correctly the prevalent agglutinable types.

A few months ago we started to immunize horses in terms of the degree of virulence of cultures (titrated in mice), and it is a matter for regret that this part of our investigation had to be abandoned, through circumstances not under our control, just at a time when we appeared to have mastered some of the chief difficulties in the manipulation of meningococcal virulence.

The work of Cotoni, Truche, and Raphael (1922), although dealing with the pneumococcus, bears on this question with considerable weight. In discussing the protective power of active immunization with vaccines and the production of potent protective serums, they repeatedly emphasize that satisfactory results have been obtained only when very virulent cultures were used. They even go so far as to say (p. 82) : “It is impossible to obtain an active serum with an avirulent or slightly virulent pneumococcus,” and (p. 78) “To prepare a multivalent serum it appears to us to be an absolute necessity to use a very virulent pneumococcus.”

There is no doubt that the position of anti-meningococcal therapeutic serum still is most unsatisfactory. The identification of agglutinating types and the application of this knowledge was undoubtedly a step forward on the evidence of Gordon, Nicolle, Netter, but the production of a potent serum of any type is not a certainty. The use of virulent cultures as antigens may prove to be merely “clutching at a straw,” but whether this is the case or not can only be known when the method has been tried. In any case it is important to remember that a fundamental principle of Pasteur’s active immunization was to use successive doses of increasing virulence, and that we can make no claim to greater success to-day than he achieved.

The evidence we have brought forward is a step towards making it possible to test whether the virulence of a meningococcal culture bears any relation to its antigenic capacity. But for the present we must content ourselves with agreeing with Nicolle and Césari (1924, p. 76) that this information is most desirable.

We suggest that the experiments described in this paper show that the general question of culture media needs further investigation. There are certainly components in media which influence to a profound degree the
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physiological state of the micro-organisms grown on them. Since writing this paper we have read the interesting work of Felton and Dougherty (1924), who show that the virulence of a strain of pneumococcus can be enormously increased by repeated subculture in milk at intervals of two to eight hours (p. 141), and that although similar subculture in meat extract or ordinary broth results only in lowering the virulence, an increased amount of “peptone” will allow of it being maintained (p. 164). This work raises the hope that with further experiment it may be possible to maintain, or even increase, meningococcal virulence by suitable cultural methods.

It still remains an open question whether the cultures we are accustomed to use may be considered to be normal healthy organisms and representative of their kind. That is to say, we do not know whether the physiological state of the organisms in our cultures in vitro is identical with that of those actively causing disease in their natural host. Up to the present we have not been able to detect a difference between naturally virulent primary cultures and those in which the virulence has been raised by the method described by Murray (1924, p. 194), but in both cases we are dealing with cultures.

In this respect it is interesting to note that we have seen, on several occasions, a definite early purulent meningitis in mice that have been inoculated intraperitoneally with cultures grown on our medium standardized by virulence tests. Microscopically the scanty pus was quite typical of the disease and the meningococcus was recovered in culture. One of our most striking instances of meningitis in a mouse resulted from a culture whose virulence had been raised by the in vitro method. But since we have not made a systematic investigation of the point, no definite conclusion can be drawn.

The special advisory committee upon bacteriological studies of cerebrospinal fever during the epidemic 1915 (Medical Research Committee, 1916, p. 20), in discussing culture media for the growth of the meningococcus, paraphrase Gordon (1916), as follows:

“The requirements of a good routine medium for the purpose have been stated as follows:

(1) The meningococcus must grow on it readily and with certainty.
(2) It must be easily and cheaply made and must not involve ingredients now difficult to procure in this country.
(3) It must be of such a nature that it can be stored and sent out in bulk from a central laboratory.
(4) It should preferably be transparent.
(5) The viability of the meningococcus on it should be as prolonged as possible.”

Our $EDB/V$ or $EHD/V$ medium fulfils all these requirements quite as well and certain of them better than any of the many media we have tried. Furthermore, the evidence we have brought forward in this paper
allows us to add another very important requirement: That the medium
must allow the meningococcus culture grown on it to develop and main-
tain the physiological characters contributing to virulence. We might
also add requirements relating to the physical characters of the growth,
particularly percentage of adventitious moisture.

We are only too well aware of the tentative nature of many of our
observations and that a fuller investigation of many points would add to
the value of our paper; but work of this nature could easily be prolonged
for an indefinite time and still remain incomplete. It will be admitted,
perhaps, that we have at least recognized a problem requiring solution and
taken a step in a direction from which useful results may be forthcoming.

In conclusion, we wish to thank those who have kindly sent us cultures
and all who have promised to do so should they get cases. We are par-
ticularly indebted to Professor H. R. Dean and Dr. Duncan Forbes, who
have kindly made personal appeals for cultures to be sent to us on our own
media.

Finally, we wish to thank J. Bain and E. Pleasance for their pains-
taking and willing assistance, which has contributed so largely to the
success of the work.

VIII.—Conclusions.

(1) That it is extremely difficult to make any two batches of a given
medium sufficiently alike to obtain identical cultural results with the
meningococcus.

(2) That this is largely due to our insufficient knowledge of
(a) the raw materials required, and
(b) the relative concentrations of the ingredients necessary to afford
the optimal conditions required by the organism to develop
their natural physiological state essential to a successful
parasitic existence.

(3) That the yield and physical characters of the growth are insufficient
criteria whereby to judge a given medium as good or bad, since the killing
power of a culture appears to be to a certain degree independent of these.
That the present state of our knowledge requires that several characters be
examined simultaneously in judging a medium for the meningococcus; such as :

(a) The alacrity with which growth takes place, and
(b) The yield of growth,
(c) The physical characters of the growth,
(d) The viability of the culture,
(e) The virulence, and
(f) The maintenance of virulence with age and subculture.

(4) With the kind of medium considered in this paper, a good deal of
truth is expressed by saying :

(a) That the virulence of the culture is chiefly affected by substances
conducted by the digest;
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(b) That the added inorganic salts, and possibly the physical state of the agar, contribute largely to the physical characters of the growth; although other factors are also concerned;

(c) That the yield and viability of the culture is determined by all the factors being correctly balanced.

(Elimination of by-products has not been discussed.)

(5) That there is an optimal range of concentration for tryptic digest of heart muscle, over which virulent cultures of the meningococcus are obtained.

(6) That media made with due consideration of the "virulence range" of the digest used are favourable to the viability and maintenance of virulence of the culture.

IX.—REFERENCES.


COTONI, TRUCHE et RAPHAEL (1922). "Pneumocoques et affections pneumocoociques" (Monographies de l'Institut Pasteur), Paris.


