THE ISOLATION OF BACILLUS TYPHOSUS FROM EXCRETA.

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The importance of having a rapid and easy method of recovering typhoid bacilli from excreta has become of great moment, since the existence of carrier cases has been clearly recognised, and the part they play in the spread of disease realised.

During the past two years I have been investigating the different methods at our disposal, in the hope of finding one that would be easy, rapid, and sure in its results. A short résumé of the most important methods in vogue, and the conclusions drawn from working with them, may be of assistance to others engaged in the same work.

The usual routine in examining stools for B. typhosus is to make a dilution of the stool, and spread part of this on some solid medium. It is in the composition of this solid medium that the various methods differ. The text-books on the subject give a large variety of processes without pointing to any one as being especially useful. The majority contain a sugar—generally lactose—and an indicator to distinguish those organisms which ferment the sugar from those which do not.

The media which have been in use for some time may be divided into two groups.

Group 1.—These media contain no inhibitory reagents, and thus allow all organisms in the stool to grow freely; a large surface is spread so as to obtain isolated colonies. The most important of these media are litmus lactose agar and litmus glucose agar, to which nutrose, which is supposed to favour the growth of B. typhosus, is sometimes added.

Group 2.—These media are designed with the object of preventing the growth of adventitious organisms, and thus giving the typhoid bacilli a better chance of showing themselves. Some of the media contain reagents which inhibit only the growth of saprophytic non-intestinal organisms, and have no effect on either coli or typhoid bacilli. The Conradi and Drigalski medium, Endo's medium, and bile salt lactose neutral red agar are well-known examples of this class. Phenolated gelatine need not be
considered, as it is never used now. Other media of this group attempt to go even further, and to inhibit the growth of *B. coli* without affecting *B. typhosus*. Of these we have media containing caffeine, as in Gaehrtgens' modification of Endo's medium, and malachite green, as first introduced by Loeffler.

In examining stools from a case of enteric fever it is generally comparatively easy to isolate *B. typhosus* during the second or third week of the disease, when it is usually found in large numbers, being often the most numerous organism present. In such a case the media of Group 1 are sufficiently reliable for use, but a large surface of the medium must be stroked, and as there are several organisms which, growing on these media, give the typical transparent colony supposed to be distinctive of *B. typhosus*, a large amount of "fishing" of colonies is sometimes required before one arrives at the right one.

It is when the typhoid bacilli are less numerous in the stools that the advantage of the media in Group 2 should become apparent, but unfortunately experience shows that they are only a little superior to the others, and are likely to succeed only when the typhoid bacilli are slightly less numerous than in the first case. Bile salt lactose agar is to be preferred to Conradi and Drigalski's agar, as it limits the growth to purely intestinal organisms, and gives a much sharper differentiation of typhoid from coli colonies. The latter medium is troublesome to make, and the recognition of colonies on it is often difficult. On both these media coli organisms grow luxuriantly, and crowd out the typhoid bacilli, if at all sparse in numbers.

The remaining media of Group 2 are Endo's agar, containing caffeine, and Loeffler's agar, containing malachite green. These two substances have also been combined in one medium. They are supposed to inhibit the growth of *B. coli*, and not to affect the growth of *B. typhosus*. A long series of experiments with these reagents has forced me to the conclusion that they are quite useless for the detection of sparse typhoid bacilli in stools. The reason is, that while they undoubtedly have an inhibitory effect on *B. coli*, yet they inhibit just as much, if not more, the growth of *B. typhosus*. Thus, if a stool contain a few typhoid bacilli, these will be prevented from growing at all, long before the infinitely more numerous coli organisms are sufficiently inhibited to give the typhoid bacilli an opportunity to appear on the plate. Caffeine is certainly detrimental to the growth of *B. typhosus*, and inhibits it rather more than it does *B. coli*. Malachite green is a
little better, as it is more detrimental to some members of the coli group than it is to *B. typhosus*. Thus, whereas *B. coli* of Escherich and *B. acidi lactici* may be unable to grow on a malachite green medium, on which typhoid bacilli will grow, other members of the group, such as *B. lactis aerogenes* and other cane-sugar fermenters, grow in abundance, and overwhelm any typhoid bacilli that may be present. For similar reasons a combination of caffeine and malachite green is worse than useless. Malachite green also forms more or less of a precipitate when added to a medium containing peptone, a fact which may account for the varying results of different observers. Malachite green media contain no indicator to distinguish between the coli and typhoid colonies, and this is a great disadvantage, as without one it is well-nigh impossible to pick out a typhoid colony either by naked-eye or microscopic appearances.

Of all the above media I have obtained the best results with bile salt neutral red lactose agar and the original Endo medium without the caffeine, but even these media can be trusted to detect the typhoid bacilli only when they exist in large numbers in the stools.

Three recently-published methods may be mentioned more fully:—

(1) In November, 1907, Klein (*Lancet*, vol. ii., 1907, p. 1519) recommended that typhoid materials should be incubated in a fluid medium containing 0.06 per cent. bile salt and malachite green in a strength of 1 in 1,500 to 1 in 1,600. After twenty-four hours incubation portions of this were plated on Conradi and Drigalski medium, when almost pure cultures of *B. typhosus* were obtained. I have tried this method, and have found it successful only when, as stated above, the cane-sugar fermenters of the coli group are absent, and this is rarely the case with stools. I have also found that the amounts of typhoid bacilli Klein added to his experimental materials were easily demonstrable on solid media without preliminary incubation in this way.

(2) A method recently adopted by the French Army Medical Service, described by Braun (*Archives de Médecine et de Pharmacie Militaire*, September, 1908), is a modification of Endo's medium. It contains no caffeine, and is made as follows:—

A 4 per cent. nutrient agar is made neutral to litmus paper, and to every litre are added—

- 10 cc. of a 10 per cent. solution of soda,
- 10 grammes of lactose,
- 5 cc. of a 10 per cent. alcoholic solution of fuchsin,
- 25 cc. of a 10 per cent. solution of sulphite of soda.
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The resulting medium is pale pink, couleur marmelade de pomme, which is then poured into large Drigalski plates 19 cm. in diameter. It is an easy medium to work with, and gives sharp differentiation between the typhoid and coli colonies, but, as many organisms grow well on it, the typhoid bacilli are apt to be overgrown, unless a large surface is spread with the material. Nevertheless I have found it give good results when fair numbers of typhoid bacilli are present in the stool.

(3) In July last Conradi (Muenchener medizinische Wochen­schrift, July 21st, 1908) introduced a new medium, which marks a distinct advance on any hitherto devised. It is made as follows:

To 900 cc. of water are added—
30 grammes of agar,
20 grammes of Liebig's extract,
100 cc. of a 10 per cent. solution of peptone (Witte).

The reaction of the agar is brought to + 30 with normal phosphoric acid. Then to each litre are added 10 c.c. of a 1 in 1,000 watery solution of brilliant green crystals (pure, Grubler, & Co. (Hochst)), and 10 c.c. of a 1 per cent. watery solution of picric acid. Immediately after mixing the clear green agar is poured into large shallow dishes. Conradi affirms that this medium prevents altogether the growth of B. coli, but has no inhibitory effect on B. typhosus or paratyphoid organisms, and by its use these can easily be isolated, even when present in small numbers.

In connection with this it is interesting to note the results of Kybe-Burchardi (Hygienische Rundschau, November, 1908), who made quantitative estimations of various organisms when sown on this medium, as compared with the Conradi-Drigalski medium.

I. Growth of B. typhosus streaked on Conradi medium, 70-1 per cent. recovered.
II. " B. coli " Conradi 0-2 " "
III. " B. coli " Drigalski 73-2 " "
IV. " " " Conradi 0-2 " "

The estimates were done with pure cultures of the organisms.

I have tried this medium, and find that the brilliant green and picric acid undoubtedly have a marked inhibitory effect on coli organisms, though they by no means entirely prevent them from growing. At the same time they appear to have little, if any, inhibitory effect on B. typhosus. Other organisms, however, grow equally well on the medium, such as proteus, pyocyaneus, and fluorescens liquefaciens.

Of all the media hitherto devised, Conradi's is undoubtedly the
most useful and best suited for the isolation of *B. typhosus* from stools. On the other hand, it has the distinct disadvantage of being without an indicator to distinguish clearly between the various colonies which grow on it. Conradi affirms that the typhoid colonies can readily be distinguished by naked-eye and microscopic appearances, and that in this way they can even be distinguished from paratyphoid colonies. This may be possible to a finely practised eye, but to the ordinary observer typhoid, coli, proteus, &c., colonies appear to grow exactly alike, at any rate in the early stages, and one is reduced to the expedient of fishing every colony, until one finds one that is agglutinated by a typhoid serum. This entails the expenditure of much time which might be saved by the use of an indicator to distinguish the acid-forming colonies which are so numerous in faces, and which crop up on every plate even of this medium.

Recognising the value of this discovery of Conradi, I have ventured to modify the medium both in composition and reaction. I have combined the brilliant green and picric acid with lactose and bile salt, the result being a medium which has the same inhibitory effect on rival organisms as Conradi’s medium, and which at the same time shows up plainly the acid-forming colonies. The bile salt acts as the indicator by reason of its being precipitated by the acid formed from the lactose by organisms fermenting this substance, and it also assists the inhibitory action on non-intestinal bacteria. At the same time I found it necessary to increase the strength of brilliant green and picric acid owing to the “enrichment” effect of bile salt on coli organisms. The acid reaction of the medium I have also reduced to half that of Conradi’s medium. It is not possible to have a reaction of +30 without precipitating the bile salt. Such a high acidity as +30 may be useful in inhibiting the growth of some organisms, and vigorous typhoid bacilli may grow well on it, but if they have become at all enfeebled, typhoid bacilli are extremely susceptible to an increased acidity, and I find that a less acid medium is more favourable to their growth.

The medium is made as follows:—

To 900 cc. of tap water add—

5 grammes sodium taurocholate (commercial from ox bile),
30 " agar (powder),
20 " peptone (Witte),
5 " common salt.

Dissolve in steamer for three hours. Clear with white of egg,
filter through wadding, and bring to a reaction of + 15 with normal lactic acid or normal soda, as required. Dissolve 10 grammes of lactose in 100 cc. of distilled water, and add to the melted agar. Mix well, and filter through Charadin paper. To each 100 cc. of the clear bile salt lactose agar add 2 cc. of a 1 in 1,000 watery solution of brilliant green (extra pure, G. Grubler & Co.) and 2 cc. of a 1 per cent. watery solution of picric acid. Thus the finished agar contains bile salt 0.5 per cent.; brilliant green, 1-50,000; and picric acid, 1-5,000. The resulting clear bright green agar is poured without further heating into large Drigalski plates of 17-20 cm. in diameter. After solidification the plates upside down are placed to dry in the incubator, with the covers off, the edge of the dish resting on the edge of the cover, and left for two or three hours, when they are ready for use. If preferred, the covers may be left off altogether until the agar has solidified, without fear of contamination, as no ordinary air organisms will grow on them; the drying process can be done much more quickly in this way.

The medium should not be kept in flasks ready made, as it deteriorates, and re-melting the agar to pour fresh plates disintegrates the brilliant green. It is convenient to make the bile salt lactose agar in bulk, and distribute it into flasks, each containing 150 cc. When required for use, one of these is melted, and 3 cc. of each of the solutions of the dyes added and well mixed. This amount is sufficient to make three of the largest plates.

The plates are then inoculated with the material to be examined. A portion of the stool is made into an emulsion with sterile normal salt solution in a test-tube, and left standing for fifteen minutes or so. The amount of dilution depends on the consistency of the original stool. If it is very fluid, it may be used without dilution. A large loopful from the surface of the emulsion or fluid stool is placed in the centre of one of the plates, and with a glass spreader is gently but thoroughly rubbed over the whole surface of the agar. The plates are then incubated at 37° C., upside down. In cases where the number of typhoid bacilli, if present, is expected to be small, as in the case of carriers, several plates should be made from the same stool, using a fresh loopful of the material to each plate, though as a rule in favourable cases it will be found that one plate is ample to demonstrate their presence.

After twenty-four hours' incubation the typhoid colonies have attained a diameter of about 1 mm. They are round, quite transparent, and highly refracting. Where the medium is thick they
appear by transmitted light to be pale green; where it is thin they are as clear as drops of water. At this stage the colonies of the coli group have a dark green opaque spot in the centre, although the edges may be transparent. If they are strong acid-formers, the colony is surrounded by a haze in the medium, due to precipitated bile salt. Very few of these strong acid-formers appear to grow on this medium, or it may be that their acid-forming properties are inhibited. Other organisms of the coli group do not form enough acid to cause a haze round the colony, but they still form enough to render the colony opaque, and give it a dark green centre, and they are thus easily distinguished from the transparent typhoid colonies. Another type of colony which is usually present is one which appears as a thick opaque white growth without any green centre, but it is not likely to lead to confusion.

After forty-eight hours the typhoid colonies are still more easily distinguished from those of other bacilli, and it is best to wait until this period before judging a plate. They have now attained a diameter of about 2 mm. They are circular, but sometimes have an irregular margin. They are still quite transparent, without a trace of opacity in their structure, but now they have a somewhat green centre, very slightly darker than the surrounding medium. This appearance is really due to the medium at the periphery of the colony becoming a rather yellower shade, and not to any darkening of the colony itself, which still remains transparent.

At this period a coli colony, in fact almost every other colony on the plate, has an opaque dark green centre easily distinguishable from the transparent green of the typhoid colony, and the growth is thick and opaque, though the edge may be transparent. At this stage some very small transparent colonies may be seen, in appearance like a typhoid colony after twenty-four hours' incubation. These consist of B. coli and such bacilli which have been inhibited in their growth, and are now commencing to appear. They are distinguished by their small size, and before they have reached the size of a typhoid colony they will have obtained an opaque green centre like the others of their type.

Paratyphoid colonies, colonies of the food-poisoning group, and dysentery bacilli are indistinguishable in their growth from those of B. typhosus.

This description of the appearance of the colonies only applies to those that are discrete and have a clear space of medium around them, and not to those which occur in a mass of colonies closely huddled together. It is never possible to judge of the nature of
colonies in a crowded plate, as their growth becomes stunted, and they may show an entirely different appearance from that which they present when they have sufficient space to grow out. After seventy-two hours the appearances of the colonies are much the same as after forty-eight hours, but the plate may have become somewhat fogged by the acid-forming colonies. Later than this it becomes difficult to pick out colonies of *B. typhosus* with any certainty.

After forty-eight hours' growth on the plate typical colonies are fished, and tested in a hanging drop for agglutination with a typhoid serum. Those which react to the serum are sub-cultured into the usual media for the determination of their morphological and cultural characters, and their specific reactions are thoroughly worked out.