THE ISOLATION OF TYPHOID BACILLI FROM FAECES BY MEANS OF BRILLIANT GREEN IN FLUID MEDIUM[1].

By C. H. BROWNING, W. GILMOUR and T. J. MACKIE.

(From the Pathological Laboratories of the University and Western Infirmary, Glasgow.)

Review of the principal methods of isolating typhoid bacilli from faeces. The methods hitherto suggested for the cultivation of typhoid bacilli from faeces have not proved entirely satisfactory though they have been of service. The constitution of media intended to aid the isolation of *B. typhosus* has been arranged according to two principles, (*a*) the use of substances which enable colonies of typhoid bacilli to be distinguished by their naked-eye appearance, and (*b*) the introduction into the medium of some substance which is intended to favour specially the growth of *B. typhosus* as compared with the other organisms which are present. Many of the media in common use fulfil both of these conditions to a greater or less extent. Thus the exclusion of Gram-positive organisms is secured by the use of crystal-violet, as in the medium of Conradi-Drigalski. This dye has very little inhibitory action on bacilli of the colon-typhoid group, although it is one of the most powerful antiseptics known so far as Gram-positive organisms are concerned. The exclusion of cocci from cultures of faeces, however, does not afford great aid in the isolation of *B. typhosus*, since the chief difficulty lies in the presence of large numbers of bacilli of the coli-group. The further addition to a solid medium of a sugar which is fermented with the production of acid by coliform bacilli, but not by *B. typhosus* and, at the same time, the

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incorporation of an indicator, is accordingly the device most commonly employed, *e.g.* in the media of MacConkey, Conradi-Drigalski and Endo. Such media have two serious disadvantages: firstly, almost every viable organism belonging to the typhoid-coli group produces a colony; hence, if typhoid bacilli are present only in scanty numbers in the faeces, it will be necessary to make the inoculation with a very considerable quantity of the material in order to include *B. typhosus* at all. Further, in order to obtain discrete colonies a large surface of medium is required. In fact, the larger the amount of faeces examined and consequently the greater the surface of medium employed, the greater will be the percentage of positive results. Obviously such a procedure may take much time to carry out. The second disadvantage of such media is that while some typical *B. coli* can be recognised with certainty there are many organisms which produce colonies more or less similar to *B. typhosus*; so that the lengthy process of examining many individual colonies may have to be resorted to.

Malachite green (derivative of tetramethyl-diamido-triphenyl-methane) and brilliant green (the corresponding tetra-ethyl compound) are bodies which possess the property of inhibiting *B. coli* to a greater extent than *B. typhosus*. These substances are both mentioned by Conradi and Drigalski (1902); but this selective action of malachite green appears to have been first of all made practical use of by Löffler; it has been employed in this country by Savage in his extensive investigations on infections with Gaertner's bacillus. Lentz and Tietz employed malachite green in a concentration of 1 : 6000 in agar as a means of obtaining elective growth of *B. typhosus* from faeces. According to their procedure, if typhoid colonies are not readily found in a plate of 10 cm. diameter inoculated with 0·1–0·2 c.cm. of a one in three suspension of faeces after 20 hours incubation at 37° C., then an emulsion of the whole growth on the plate should be made in 2 c.cm. of fluid and a loopful of this emulsion should be used to inoculate a plate of Conradi-Drigalski medium. According to Gaehtgens and Brückner this is the most efficient method so far devised for the isolation of typhoid bacilli; but they remark that a further improvement in methods of isolating *B. typhosus* is desirable. Conradi (1908) introduced later a solid agar medium containing brilliant green and picric acid, which has been further modified by Fawcus. The fact that these dyes exert a selective bactericidal action on *B. coli* as compared with *B. typhosus* has not, however, been generally accepted (Kathe and Blasius, 1909). Löffler drew attention to the fact that it was possible to inhibit the growth of

B. coli in a mixture with *B. typhosus* when inoculated into fluid media containing malachite green, so that a pure culture of typhoid bacilli was obtained. Lentz and Tietz recognised that the employment of a fluid medium would present marked advantages for the isolation of *B. typhosus* from faeces; but their attempts to employ malachite green in this way resulted in failure, as the typhoid bacilli were overgrown by other organisms.

Observations on the action of brilliant green on organisms of the typhoid-coli group. In the course of systematic investigations on the antiseptic action of benzol-derivatives[1] we found that a marked difference existed between the action of dyes which are members of the diamidotriphenylmethane group and those of the triamidotriphenylmethane series. Both groups exert a powerful action on Gram-positive organisms, *e.g.* staphylococci, anthrax bacilli (the bactericidal action of gentian violet has recently been brought into prominence by Churchman and Michael). But the diamidotriphenylmethane group (malachite and brilliant green) are also fairly actively bactericidal toward the typhoid-coli group, whereas these organisms are comparatively insusceptible to the triamidotriphenylmethane dyes (hexamethyl and hexaethyl violet). On comparing malachite and brilliant green it was found that the latter was much the more active in its inhibitory effect on the coli-group.

The examination of 54 strains of *B. coli* of the types commonly found in faeces, which had been isolated from faeces, urine, appendix abscesses, etc., and comprising B. No. 71 (MacConkey), *B. coli communis* (Escherich), *B. neapolitanus*, *B. schafferi*, *B. vesiculosus*, Grünthal's bacillus, *B. coscoroba*, B. No. 106 (MacConkey)[2], showed that these organisms were without exception more susceptible to the bactericidal action of brilliant green than were any of the 21 strains of *B. typhosus* which we have investigated. *B. coli anaerogenes* (6 strains), and certain of the "paracolon" bacilli (bacilli which do not ferment lactose and do not form indole, but which differ from the paratyphoid group either in being non-motile or in not fermenting dulcite and which probably do not possess specific pathogenic properties), also dysentery bacilli and *B. faecalis alcaligenes*, all belong to the susceptible group. More resistant than the above mentioned types, but less resistant than *B. typhosus*, is B. No. 74 (MacConkey). *B. proteus* (13 strains) is on the average

[1] We are indebted to Prof. Ehrlich and to Messrs Bayer, Cassella, and Lucius-Brüning for their kindness in placing specimens of dyes at our disposal.

[2] The classification of these organisms has been made according to MacConkey's criteria.

somewhat less resistant than *B. typhosus*, although some strains are of practically equal resistance. To the group of organisms which are more resistant to brilliant green than *B. typhosus*, belong the paratyphoid bacilli, Gaertner's bacillus and certain members of the coli-group which occur only in scanty numbers in faeces. The resistant coli-types include inosite-fermenters, *e.g. B. lactis aerogenes*, B. No. 67 (MacConkey) and also some non indole-forming types of *B. coli* (certain of which fermented lactose only after mutation, as described by Penfold).

The fluid medium containing brilliant green employed for isolating B. typhosus and B. paratyphosus.

The results following the inoculation of a medium which contains substances actively inhibitory to bacterial growth depend to a very great extent on the number of organisms which are introduced, as Krumwiede and Pratt have also noted. A small number of organisms may be entirely killed off, whereas growth may follow the introduction of a large number. This holds good both in the case of solid and of fluid media; accordingly we have concluded that it is useless to expect that a standard medium suitable for all cases can be devised. A further consideration of importance is that in making inoculations with faeces a considerable amount of organic material is introduced into the medium and there is no convenient method of standardising this element in different cases. For this reason also it is impossible to set up any single medium of fixed constitution. Accordingly we have proceeded on the principle of adding *varying amounts* of brilliant green to a series of tubes of peptone water. Each tube is then inoculated with a large loopful of faeces. If the faeces are not naturally fluid, then several volumes of sterile 0·85 per cent. NaCl solution are added and an emulsion is made. After the brilliant green peptone water cultures have been incubated for 20–24 hours at 37° C. subcultures are made from each tube on plates containing some medium with an indicator, which are then examined for *B. typhosus* after they have been incubated for 18–24 hours at 37° C.

Method. The practical details of the method are as follows:— peptone-water is prepared in the usual fashion; 20 grams of Witte's (Rostock) peptone and 5 grams of NaCl are added to 1000 c.cm. of distilled water; the mixture is steamed in a Koch's steriliser for $\frac{3}{4}$ of an hour and filtered through paper: 5 c.cm. are then distributed in 6″ × 5/8″ test-tubes which are plugged with cotton-wool and sterilised

at 120° C. for 15 minutes in the autoclave (the medium reacts faintly alkaline to litmus). The stock-solution of brilliant green (Bayer's Brilliant Green Extra Cryst.) consists of 1 per cent. of the dye in distilled water; this is freshly made up every 2–3 weeks. Immediately before use a 1:10,000 dilution of the dye is prepared by adding 0·1 c.cm. of the stock-solution to 9·9 c.cm. of distilled water. Of this dilution the following amounts are added to successive tubes of the peptone-water, viz. 0·04, 0·08, 0·12, 0·16, 0·22, 0·3 c.cm. A loopful of faeces (up to 0·4 cm. diameter where the specimen is very fluid) is then at once added to each tube and the contents are well mixed. After 20–24 hours incubation at 37° C. a loopful of material is taken from each tube and successive strokes are made on plates of MacConkey's medium (two 10 cm. plates in all are quite sufficient to accommodate 3 strokes from each dilution). The plates are then incubated as usual and are examined for typhoid bacilli.

Using this method, we have isolated *B. typhosus* from 11 cases which were clinically typhoid fever and paratyphoid bacilli from other two. In order to obtain an estimate of the number of typhoid bacilli present as shown by ordinary methods, a plate of MacConkey's medium 8 cm. in diameter was at the same time inoculated directly from the faeces in each case by making successive strokes. In seven of the 13 cases it was impossible to detect the presence of typhoid or paratyphoid bacilli in the direct plates; in five of these *B. typhosus* was recovered in the brilliant green cultures and in the other two paratyphoid bacilli. In the remaining six cases typhoid bacilli were observed in the direct cultures. It is to be noted, however, that in every instance in which typhoid bacilli were obtained by direct culture they were also demonstrated in the brilliant green cultures. It is interesting to observe that frequently a particular concentration of brilliant green yielded a practically pure growth of *B. typhosus* where only scanty colonies were found in the plate inoculated directly with faeces[1]. The advantage of making a series of cultures in medium containing different quantities of the dye is shown by the fact that there appears often to be an optimum concentration of dye favourable to the growth of the typhoid bacilli. This point is well illustrated by the following example:

[1] *B. typhosus* was identified in every instance by its culture reactions which were tested shortly after isolation (presence of motility in culture after six hours at 37° C.; fermentation of glucose and mannite without gas production, negative result with lactose, dulcite, saccharose; no indole formation). In every instance the agglutination-test carried out subsequently with a powerful anti-typhoid serum confirmed the diagnosis based on the culture reactions.

Case A. M. Subcultures on MacConkey's medium from 24-hour brilliant green peptone water cultures of faeces.

Amount of brilliant green (1:10,000) present in the peptone water cultures (5 c.cm.)	Resulting growth
0·03 c.cm.	mainly red colonies
0·07 ,,	pure *B. typhosus*
0·11 ,,	mainly *B. typhosus* with an admixture of *B. proteus*
0·16 ,,	scanty colonies of *B. typhosus*, *B. proteus* and red colonies
0·22 ,,	ditto

(A subculture made after the 0·22 c.cm. brilliant green culture had been incubated for 36 hours at 37° C. yielded a pure culture of *B. typhosus*.)

The optimum concentration of brilliant green varies from case to case; thus contrast the following example with that quoted above:

Case O. R. Subcultures on MacConkey's medium from 24-hour brilliant green peptone water cultures of faeces.

Amount of brilliant green (1:10,000) present in the peptone water cultures (5 c.cm.)	Resulting growth
0·04 c.cm.	almost all red colonies
0·08 ,,	equal numbers of *B. typhosus* and red colonies
0·12 ,,	mainly *B. typhosus*
0·16 ,,	*B. typhosus* in pure culture
0·2 ,,	ditto
0·25 ,,	ditto

It is to be noted that we endeavoured in every instance to obtain the faeces as fresh as possible. This should always be secured when examining for typhoid bacilli, as faeces on standing will become free of typhoid bacilli even when these have been introduced artificially.

The isolation of *B. typhosus* from actual cases of disease is, of course, the true test of the efficacy of a method. At the same time it was of interest to ascertain how small a number of typhoid bacilli could be recovered from an artificial mixture with faeces. Thus, a fresh specimen of normal faeces was emulsified with two volumes of 0·85 NaCl solution and strained through wire gauze in order to secure a homogeneous emulsion. A loopful (diameter of the loop 0·4 cm.) of the emulsion was introduced into a series of 5 c.cm. peptone water tubes containing brilliant green (1 : 10,000), viz. 0·04, 0·09, 0·14, 0·19, 0·24, 0·3 c.cm.; at the same time a measured amount of a dilution of a 24-hour bouillon culture of a strain of *B. typhosus* of average resistance to brilliant green was

introduced. A similar amount of the dilution of the *B. typhosus* culture was plated on agar and yielded two colonies. The number of viable organisms present in the amount of faeces employed in each tube was 2800, as ascertained by plating on agar. Subcultures made from the brilliant green tubes after incubation at 37° C. for 24 hours gave the following results:

0·14 c.cm. brilliant green	numerous red colonies (? mixture of *B. typhosus* with the *B. coli*)
0·19 ,, ,, ,,	*B. typhosus* and red colonies in equal numbers
0·24 ,, ,, ,,	ditto
0·3 ,, ,, ,,	abundant colonies of *B. typhosus* and two red colonies

Thus, a practically pure culture of *B. typhosus* was obtained from a mixture of two typhoid bacilli along with 2800 other viable organisms. A control series of brilliant green cultures of the faeces, without the addition of *B. typhosus*, yielded nothing resembling typhoid bacilli. A direct inoculation of the faeces on MacConkey's medium gave a dense growth of *B. coli*. In a series of experiments in which a larger number of typhoid bacilli (10) were added to the same quantity of normal faeces, we failed to recover a typhoid growth by plating directly on MacConkey's medium, although practically pure growths of *B. typhosus* were obtained by means of brilliant green.

The number of cases which we have investigated is comparatively small; but the successful nature of our results has led us to publish this note in the hope that the method may commend itself to those who have greater opportunities for investigating cases of typhoid fever and who, therefore, are in need of a reliable procedure for the isolation of typhoid bacilli from faeces. We may also remark that this method enables one to isolate certain types of atypical *B. coli* which are occasionally present in small numbers in faeces, in virtue of their resistance to brilliant green. Such organisms, however, have not been found to interfere with the isolation of *B. typhosus* by overgrowing it.

Different samples of the same dye are known to vary somewhat owing to the fact that most commercial dye-stuffs contain a certain quantity of indifferent impurity; accordingly, we would suggest that before proceeding to employ a given specimen of dye, its action should be tested with a sample of faeces known to contain typhoid bacilli. Under all circumstances, where it is desired to isolate *B. typhosus* from faeces by this method, a series of five or six tubes containing different amounts of brilliant green should be inoculated. If larger quantities of

faeces are to be used for inoculation or if coliform bacilli are present in very great numbers, then correspondingly large amounts of peptone water and of dye will require to be employed.

Summary.

(1) Brilliant green exerts an inhibitory effect on the growth of bacilli of the coli group commonly occurring in faeces, which is in general more marked than its action on *B. typhosus* and paratyphoid bacilli.

(2) By taking advantage of this property of brilliant green a method has been devised for isolating *B. typhosus* from faeces. The procedure adopted is the inoculation of a series of tubes of peptone-water medium containing varying amounts of brilliant green, incubating for 20–24 hours, and then the inoculation on a suitable solid medium from each tube.

(3) The reason for employing a series of concentrations of brilliant green is that the optimum concentration for the growth and isolation of *B. typhosus* varies from case to case, depending probably both on the proportion of typhoid bacilli present and on the number and character of the accompanying bacteria as well as on the organic faecal material.

(4) The method is very easily and rapidly carried out.

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