

A SIMPLE MODIFICATION OF WILSON AND BLAIR'S MEDIUM FOR THE ISOLATION OF TYPHOID AND PARATYPHOID BACILLI

(BRILLIANT GREEN, BISMUTH-AMMONIUM-CITRATE AND SODIUM
SULPHITE MEDIUM—B.B.S. MEDIUM)

By SAGA TYAGARAJA, M.A., M.D. (CANTAB.)

From the City Microbiological Laboratory, Colombo

A SELECTIVE medium for the isolation of *B. typhosus* from faeces has been a long-felt desideratum, particularly so in the tropics with the endemic and epidemic character of such infections.

Several selective media had been described from time to time, but for general routine purposes they did not prove altogether satisfactory. In 1927 Wilson & Blair evolved a medium that seemed very suitable for this purpose. Their later investigations (1931) led to a modification of the original medium which left its basic elements unaltered; this modification is known as "the new standard bismuth sulphite brilliant green medium".

Many workers have found this medium to be a definite advance in media technique for the isolation of *B. typhosus*. A further advance was recorded when Tabet (1938) discovered that by varying the concentrations of some of the ingredients the media could be used more efficiently. At the same time, it is of interest to note that Tabet found that the medium was rendered inefficient by the omission of any one of its component ingredients.

My own experiments confirm the generally recognized value of these selective media, but since they generally tend to the suppression of *B. coli* and not of *B. aerogenes*, their utility is necessarily limited when this organism is present in the faeces. In fact, in Ceylon *B. aerogenes* in varying numbers is very often present in faeces, sometimes even in pure culture. Moreover, the preparation of Wilson & Blair's medium and its various modifications is by no means easy in spite of the fact that the introduction of bismuth-ammonium-citrate scales has somewhat simplified the process. Furthermore, the media are not stable and do not keep for more than a short period.

Because of the many difficulties that have to be surmounted it seems highly desirable that investigations should be continued into the possibility of evolving a simpler and more stable medium. The evolution of such a medium, provided it shows no impairment in its selectivity towards *B. typhosus* and *B. paratyphosus* and also exerts sufficient inhibition towards *B. aerogenes*, would prove very welcome for general routine purposes and would certainly be

appreciated by smaller institutions. This investigation was accordingly carried out in the following stages:

(i) *The ingredients required.* The first problem that had to be investigated was whether all the ingredients recommended by Wilson & Blair were necessary for the preparation of such a medium. Experiments were therefore conducted in a routine manner by making several series of agar plates, some of which contained all the ingredients while in others some of the ingredients were omitted. The plates were inoculated with 24 hr. old cultures of *B. typhosus*, *B. aerogenes* and *B. coli* and the resultant growth and the amount of inhibition produced were noted at intervals of 24 hr. The process of exclusion of some of the ingredients was done in gradual stages by observing the effect produced on the growths of the organisms and choosing the optimum. The results obtained indicated that some of the ingredients could well be dispensed with. The omissions seemed to have affected the results only in so far as the pigmentation of the colonies was concerned. If suitable selectivity can be obtained, then no special stress need be attached to pigmentation, as fallacious pigmentation has been noted not infrequently. The ingredients that can be omitted were found to be glucose, ferrous sulphate and sodium phosphate.

(ii) *The optimum concentrations of the necessary ingredients.* An endeavour was next made to ascertain the optimum quantities of the ingredients necessary to standardize the medium. This was done by trying out various concentrations, using inoculations of the same cultures, and noting the growth obtained with *B. typhosus* as compared with the degree of inhibition shown towards *B. aerogenes* and *B. coli*.

The concentrations of the stock ingredients used were 1% brilliant green, 12% bismuth-ammonium-citrate scales and 40% anhydrous sodium sulphite. The solution of bismuth-ammonium-citrate scales should be prepared fresh on each occasion, but the brilliant green and the sodium sulphite may be kept for a long time provided the latter is well shaken before use, as the sulphite may not be fully dissolved.

Tables 1 and 2 give some of the characteristic results obtained by varying the concentrations. In the series of experiments in Table 1 no brilliant green was used. It will be noted that within 24 hr. growth of *B. typhosus* was present in all, but the inhibition of *B. aerogenes* and *B. coli* was most marked in No. 4. Within 48 hr. the inhibition was best in No. 4, whilst the growth of *B. typhosus* had increased.

These series of experiments show that inhibition of *B. aerogenes* and *B. coli* may be obtained by using certain balanced amounts of bismuth citrate and sodium sulphite without the use of brilliant green. The inhibition thus obtained is however not of a permanent nature for growths of these organisms appear within 48 hr.

In Table 2 it will be noted that different balanced amounts of bismuth citrate and sodium sulphite were used whilst the amount of brilliant green added was the same in the first four. The results obtained seem to be about

Table 1. Showing the effects of different concentrations of bismuth citrate and sodium sulphite

No.	Bismuth citrate c.c.	Sodium sulphite c.c.	Within 24 hr.			Within 48 hr.		
			<i>B. typhosus</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. typhosus</i>	<i>B. aerogenes</i>	<i>B. coli</i>
1	0.8	0.8	+	*	*	+	+	+
2	0.8	1.2	+	*	*	+	+	+
3	0.6	1.2	+	+	+	+	++	++
4	4.0	4.0	++	-	-	++	+	+

++ good growth; + fair growth; * slight growth; - no growth.

Table 2. Showing the effects of different concentrations of bismuth citrate and sodium sulphite with brilliant green

No.	Bismuth citrate c.c.	Sodium sulphite c.c.	Brilliant green c.c.	Within 24 hr.			Within 48 hr.		
				<i>B. typhosus</i>	<i>B. aerogenes</i>	<i>B. coli</i>	<i>B. typhosus</i>	<i>B. aerogenes</i>	<i>B. coli</i>
1	4.0	4.0	0.1	++	-	-	+++	*	-
2	6.0	6.0	0.1	+	-	-	++	*	-
3	7.0	7.0	0.1	+	-	-	++	*	-
4	8.0	8.0	0.1	+	-	-	++	*	-
5	4.0	4.0	0.2	*	-	-	+	*	-

+++ very good growth; ++ good growth; + fair growth; * slight growth; - no growth.

the same in regard to inhibition, but the growth of *B. typhosus* is best in No. 1. Apparently increase of bismuth citrate and sodium sulphite is of no advantage, the optimum amounts being 4 c.c. of each in 100 c.c. of nutrient agar.

To arrive at the optimum concentration of brilliant green, a similar series of experiments was carried out, and it was found that concentrations below 0.075 c.c. in 100 c.c. of agar were not sufficiently inhibitory. The characteristic result obtained by increasing the amount over 0.1 c.c. is given in Table 2, No. 5. Here there is some inhibition of *B. typhosus* whilst the suppression of *B. aerogenes* is not complete. After several such experiments, it was found that 0.1 c.c. of brilliant green was the optimum amount.

The following therefore seem to be the optimum amounts of the three ingredients for use in 100 c.c. of nutrient agar:

- 0.1 c.c. of 1% brilliant green,
- 4 c.c. of 12% bismuth-ammonium-citrate,
- 4 c.c. of 40% sodium sulphite.

(iii) *The choice of the nutrient broth and the optimum pH.* For the completion of the medium, there now remains the choice of broth and the adjustment of the pH. In the preparation of any specialized medium it is important to pay attention to the choice of the nutrient broth that is to be used. Various experiments were therefore made to compare the values of the nutrient broths prepared (a) with beef extract and (b) with broth freshly prepared from bullock's heart. The other constituents added to the broth were the same in both cases. The results obtained from such experiments showed that the medium

containing the heart broth was superior in respect of colour, stability and the growth factor.

This medium, when prepared according to the details given in the next section, is opaque and of a light amber colour, in fact like ordinary cloudy agar. The growth of *B. typhosus* is good and fairly characteristic, and the different batches of the medium are apparently identical. On the other hand the medium prepared with beef extract failed to give repeatedly consistent results.

As regards the choice of peptone, both Witte's and Bacto-peptone were tried out and both acted equally satisfactorily. To assess the optimum pH a series of experiments was carried out at pH's ranging from 6.9 to 8, the adjustments being made before the ingredients were added to the agar. The medium worked satisfactorily from pH 7.2 to 8; but towards the alkaline side, i.e. from 7.6 upwards, it was found to be better as regards growth, keeping properties and uniformity of results.

It was therefore decided to adjust the agar to between pH 7.8 and 8 before adding the chemical ingredients.

This simplified medium containing brilliant green, bismuth-ammonium-citrate scales and sodium sulphite may conveniently be termed the "B.B.S." medium and will be referred to as such.

METHOD OF PREPARATION

The method of preparing this B.B.S. medium is as follows.

Heart broth agar

The nutrient broth is first made by taking 500 g. of freshly minced bullock's heart, free from fat, and adding it to 1000 c.c. of water. This is left overnight in an ice chest to extract. It is then well boiled and filtered through muslin, and sufficient water is added to make up the volume to 1000 c.c. Then 10 g. of (Witte's or Bacto) peptone and 5 g. of sodium chloride are added and the fluid again boiled. It is then cooled and filtered. The broth is now adjusted to about pH 7.8 and autoclaved for 30 min. at 120° C.

This broth may be kept for stock and used when required. To every 100 c.c. of this broth 3 g. of agar (Bacto) are added and the solution autoclaved for 30 min. at 120° C. It is then cooled down to 55° C. and white of egg is added to clear. After steaming for 1 hr. in a sterilizer, it is filtered through glass wool and filter paper. The pH is then adjusted to between 7.8 and 8 and the nutrient agar sterilized for half an hour on three successive days.

The medium

The nutrient agar containing heart broth is now ready for the addition of the three ingredients. The agar is cooled down to about 60° C. and the ingredients are added in the following order. First 0.1 c.c. of 1% brilliant green is added and the agar is shaken. Then 4 c.c. of 12% freshly prepared bismuth-ammonium-citrate solution are added and the agar again shaken and lastly 4 c.c. of 40% sodium sulphite solution are added.

The sodium sulphite solution should be well shaken before using and it should be added slowly, agitating the agar all the time. The medium is now complete and requires no further sterilization.

The medium should be poured into sterile tubes and stored at ordinary room temperature.

When required one of these tubes is melted by placing it in boiling water. When the agar becomes liquid it is carefully rotated and poured on to plates, which would be ready for use in about 10 min.

Keeping properties

The B.B.S. medium may conveniently be stored in test tubes in quantities of about 20 c.c. which keep quite well for about 4 weeks or even longer. Sometimes the medium may turn greenish earlier and if used in this state inhibition may be excessive. It is preferable therefore not to use the medium when its colour has changed.

It was found that the change in colour was due to a change resulting from oxidation. The medium need not, however, be discarded on that account, for it can be very easily restored to its original condition by adding about 1 c.c. of the sodium sulphite solution to every 100 c.c. of the medium. This restoration does not affect the results or its keeping properties in any way. Apparently by this procedure the medium may be kept indefinitely, affording a saving in both material and labour.

It is desirable not to overheat the medium when melting it, but to pour it on to the plates as soon as it has liquefied. Poured plates should not be stored at room temperature as oxidation is liable to occur early, causing the agar to turn greenish owing to the larger surface exposed. This process can, however, be retarded to a considerable extent if the plates are stored in a refrigerator.

Selectivity of the medium was tested in the following way:

(a) Several batches of the medium were inoculated with various organisms and the results observed within 24 and 48 hr.

Table 3. *Illustrating the characteristic results on B.B.S. medium*

	Within 24 hr.	Within 48 hr.
<i>B. typhosus</i>	++	+++
<i>B. paratyphosus</i> A	++	++
B	++	++
C	+	+
<i>B. coli</i>	-	-
<i>B. aerogenes</i> (a)	-	-
(b)	-	*
<i>B. carolinus</i>	-	-
<i>B. asiaticus</i>	-	+
<i>B. dysenteriae</i> , Flexner	-	-
<i>B. dysenteriae</i> , Shiga	-	-
<i>B. dysenteriae</i> , Sonne	+	++
<i>B. dysenteriae morgani</i>	+	++
<i>B. proteus</i>	+	+

Table 3 shows that when stock cultures are used: *B. typhosus* and *B. paratyphosus* grow well and are identifiable within 24 hr.; *B. coli* are completely suppressed; whilst many strains of *B. aerogenes* are completely suppressed, some show growth after 24 hr.; of the dysentery group Flexner and Shiga fail to grow, but Morgan and Sonne grow within 24 hr.; *B. proteus* grows but does not spread.

(b) A series of experiments was performed by adding minute amounts of diluted suspension of *B. typhosus* to normal faeces, and inoculating two drops of the mixture on to the medium. In every case *B. typhosus* could be identified within 24 hr. The typhoid colonies, in the early stages, appeared as small, rounded glassy colonies which gradually became pale brownish, and later exhibited a brown pigmented centre and a pale periphery. In fact, the appearance was so characteristic that when a plate showed small glassy colonies, it is only necessary to scrape gently some of them with a platinum loop and inoculate them on to an ordinary agar plate for further tests on sugars and for agglutination. When the growth was scanty, they could be picked out under a low power of the microscope within 24 hr.

As regards the growth of the other organisms, it may be mentioned that usually *B. coli* was completely suppressed, but was less completely suppressed when certain strains of *B. aerogenes* appeared. It was observed that there was an almost complete inhibition of *B. aerogenes* in most plates for about 24 hr., but later a few of these plates showed a slight growth of this organism. In very rare instances, however, it was noted that the growth of *B. aerogenes* was more marked even at an earlier stage although never so prolific as on ordinary agar plates. Even in these cases there was no doubt of the practical value of the medium.

B. aerogenes produces dark brown, convex colonies, larger than those of *B. typhosus*. The pigmentation, which is generally present from the very beginning, later becomes almost blackish. This contrast in the pigmentation is often by itself sufficient to distinguish the colonies of *B. typhosus* from those of *B. aerogenes*.

Application of the medium

Technique adopted. The emulsion of the suspected faeces is prepared in the usual way by taking a small amount about the size of a sixpenny piece and mixing it well in about 25 c.c. of distilled water. If the faeces is already very watery, then no further dilution is necessary.

Three to four drops of the emulsion are spread on the medium in a plate with a sterile glass spreader and the plate is incubated at 37° C. for about 20 hr., or, if necessary, a little longer. The plates are examined after 18–20 hr. for suspicious looking glassy colonies. If such colonies are not discernible to the naked eye, a lens may be used. The suspicious colonies are picked off and plated on ordinary lactose-agar plates containing a suitable indicator such as bromthymol-blue for further tests.

The characteristics of the colonies. In the early stages, the colonies of *B. typhosus* are small, round and ground-glass like in appearance, but gradually acquire a pale brownish colour. This pigmentation increases as the growth gets older, when the colonies develop a brownish centre with a light brown periphery. These changes are noticeable in about 3 days, but later the outline of the colony assumes an irregular vine-leaf shape. It has to be

emphasized that the pigmentation never reaches the dark brownish or almost blackish colour seen in colonies of *B. aerogenes*.

Some plates may turn greenish early, in which case the typhoid colonies also take on the greenish tinge, whereas any stray *B. aerogenes* present develop as usual the dark brown pigment.

Examination of faeces for typhoid bacilli

After the preliminary observations, the efficiency of this medium was tested on a large number of samples of faeces from typhoid cases at different stages, varying from the 12th to the 75th day of illness. Each case was examined only once and then only on a single plate of the B.B.S. medium. It was found that by this procedure 88% of cases gave positive results.

Most of the plates were covered with innumerable colonies of *B. typhosus*, which were easily transferred in pure culture by gently scraping the colonies and inoculating them on to lactose agar plates for further tests. Whenever a few colonies of *B. aerogenes* were present they were easily avoided by recognizing their obvious characteristics.

In a very small percentage of cases the growth of *B. typhosus* was much less prolific and the colonies had to be picked out. The smallest number present in a single case was four, distributed amongst an equal number of colonies of *B. aerogenes*.

In order to compare the value of this medium with Tabet's modification, both media were tested on a number of these faeces. It was found that the results agreed in 84% of the cases. In the remaining 16% Tabet's medium gave negative results, whilst the B.B.S. medium gave positive results.

DISCUSSION

It appears from these experiments that only a few of the ingredients of Wilson & Blair's medium are necessary to produce a selective and stable medium for the isolation of *B. typhosus* and allied organisms. The essential ingredients are brilliant green, bismuth-ammonium-citrate and sodium sulphite, which, when added to the heart-broth agar in the proportions stated, produce a highly satisfactory and selective medium for all general purposes.

The role played by these three ingredients seems to be as follows. Brilliant green plays the part of a selective agent by inhibiting most of the unwanted organisms. To be of any selective value by itself, it has to be finely adjusted. This is by no means easy nor are the results always constant. The bismuth-ammonium-citrate also possesses some inhibitory action which is not continued for long but, as explained in the next paragraph, its presence is necessary for the selective mechanism to function. When sodium sulphite is added to brilliant green, a reduction occurs and the dye is modified, as indicated visually by the disappearance of the green colour. At this stage the inhibitory effect of the brilliant green is kept in abeyance, but this state is not stable as the dye reverts to its original state by oxidation.

In practice, therefore, the mechanism of the completed medium may be explained as follows. At the beginning, the bismuth-ammonium-citrate exerts its inhibitory effect and suppresses many unwanted organisms, but this action is soon exhausted. Meanwhile, the reduced brilliant green becomes gradually oxidized and on its return to its original state is able to exert its selective action, thus continuing the suppression of the unwanted organisms.

The process of reduction and oxidation may be easily exhibited by excluding the air from the surface of one plate by covering the medium with liquid paraffin, and using a normal plate as a control. It will be seen that the plate covered with liquid paraffin retains its original colour, whilst the control plate turns greenish within 24 hr.

Experiments have shown that, to obtain the best results, the medium should not be greenish before use. As has previously been explained, the greenish colour indicates more inhibition. Normally such plates would therefore have to be discarded, but with this simplified B.B.S. medium it should not be necessary to discard the medium when greenish as it may be restored easily without impairing its original selectivity by adding about 1 c.c. of sodium sulphite solution to every 100 c.c. of the altered medium. The keeping properties of this medium are definitely better than those of all the modifications previously proposed, and the restoration method enables the medium to be kept indefinitely.

This B.B.S. medium causes as complete suppression of *B. coli* as Wilson & Blair's medium or Tabet's modification, and its inhibitory action on *B. aerogenes*, the chief interfering organism, is definitely better, and is sufficiently great for all practical purposes. On the other hand, the growth of *B. typhosus* and *B. paratyphosus* is definitely profuse.

No difficulties are encountered in the identification of the colonies and confirmation of the organisms. In fact, the agglutinability of the bacilli, if interfered with at all, becomes easily restored in subculture.

Using the B.B.S. medium it should be possible to give a diagnosis, including the confirmatory tests, within 48 hr.

SUMMARY

A simple modification of Wilson & Blair's medium for the isolation of typhoid and paratyphoid bacilli has been evolved.

1. Instead of the original six ingredients, this modification contains only three, namely, brilliant green, bismuth-ammonium-citrate and sodium sulphite, and may therefore be conveniently termed "B.B.S. medium".

2. The glucose, sodium phosphate and ferrous sulphate of the original medium are eliminated. The bismuth-ammonium-citrate solution is made without caustic soda.

3. The B.B.S. medium keeps well for several weeks. Deterioration can easily be detected and readily rectified.

4. The inhibitory action towards *B. aerogenes* is greatly improved in the B.B.S. medium. This is particularly advantageous in countries where this organism is frequently encountered.

5. *B. typhosus* and *B. paratyphosus* grow readily. The colonies are sufficiently characteristic and can be picked out early for confirmatory tests.

6. Using B.B.S. medium a complete diagnosis should be possible within 48 hr.

REFERENCES

- TABET, F. (1938). Modification of Wilson and Blair's bismuth medium suitable for both typhoid and paratyphoid bacilli. *J. Path. Bact.* **46**, 181-93.
- WILSON, W. J. (1938). Isolation of *Bact. typhosum* by means of bismuth sulphite medium in water- and milk-borne epidemics. *J. Hyg., Camb.*, **38**, 507-19.
- WILSON, W. J. & BLAIR, E. M. McV. (1927). Use of a glucose bismuth sulphite iron medium for the isolation of *B. typhosus* and *B. proteus*. *J. Hyg., Camb.*, **26**, 374-91.
- — (1931). Further experience of the bismuth sulphite media in the isolation of *Bacillus typhosus* and *B. paratyphosus* from faeces, sewage and water. *J. Hyg., Camb.*, **31**, 139-61.

(*MS. received for publication 6. III. 40.—Ed.*)