THE FAILURE OF BRILLIANT GREEN AND TELLURIC ACID AS SELECTIVE AGENTS FOR THE ISOLATION OF *BACILLUS TYPHOSUS* FROM FAECES

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For some years past considerable dissatisfaction has been felt in these laboratories over the disappointing results obtained with McConkey's medium in the isolation of *B. typhosus* from faeces. In a recent outbreak of typhoid in Omdurman and Khartoum it was noted that positive cultures from the faeces of cases, proved by blood culture, were very rare, and in the attempt to remedy this unsatisfactory position recourse was had to brilliant green-telluric acid enrichment methods.

The results of using these substances, either separately or in combination, were with one exception uniformly negative in a series of twenty-five proved cases of typhoid, the faeces from each case being examined on at least three occasions. These findings were so unexpected that suspicion was cast on the sample of brilliant green used (Grübler, obtained in 1932) and a pre-war sample of Grübler was also tried but with equally poor results. The McConkey's medium used was made up from the standard formula and had always given excellent results in the isolation of dysentery bacilli.

As a further test samples of faeces were inoculated with several loopfuls of a fairly heavy suspension of *B. typhosus* and a loopful (4 mm.) of each mixture was inoculated into brilliant green-telluric acid peptone water tubes. The dilutions of the dye ranged from 1/125,000 to 1/500,000 with the telluric acid in a concentration of 1/12,500; the *p*H reaction of the peptone water tubes was 6·5. In no dilution nor from any of the mixtures was *B. typhosus* isolated. Practically all the tubes showed good or even heavy growth after 24 hours and when plated on to McConkey's medium gave a mixture of *B. coli*, *B. aerogenes*, other atypical coliform strains, *B. asiaticus* and *B. pyocyaneus*, the latter being a most ubiquitous organism in the Sudan.

Of these organisms the Proteus group and *B. pyocyaneus* are known to be more resistant to brilliant green than *B. typhosus* (Wilson), and it is generally admitted also that *B. aerogenes* is more resistant than *B. coli* (Browning, Gilmour and Mackie, 1913; Rakieten and Rettger, 1927). Krumwiede and Platt (1914), however, state that *B. coli* and *B. aerogenes* are more susceptible to the action of the dye than *B. typhosus*.

FAECAL FLORA IN THE TROPICS

A discussion of the types of coliform organisms found in the tropics is outside the scope of this paper. It is sufficient to note that in the more recent literature on the subject there is a tendency to discount the value formerly placed on minor fermentative differences, and to rely instead on the Methyl red, Indol, Voges-Proskauer and Citrate tests as a basis of primary classification.

On such a basis the majority of coliform strains in the tropics as elsewhere are of the *B. coli* type, viz. Methyl red +, Voges-Proskauer −, Citrate − (type 88·9, Taylor, 1927; 89·4, Hicks, 1927), but the author is unable to find any references in the literature to the action of brilliant green or telluric acid on these tropical strains. Consequently it was considered desirable in the first place to test the resistance of the more common local types.

Sixteen samples of normal faeces were plated on McConkey's medium and two colonies picked at random from each plate. Twenty-seven strains were of the *B. coli* type—including saccharose fermenters which are very common in Khartoum (Archibald, 1911). Three strains were *B. aerogenes*, Methyl red −, Voges-Proskauer +, Citrate +; two strains intermediates, Methyl red +, Voges-Proskauer −, Citrate +.

Owing to the small number of samples this result scarcely gives a true picture of the complexity of faecal flora of the Sudan which is known to undergo seasonal variation. Such variation was observed also by Clemesha (1912) in India. Further, under the stimulus of a mild intestinal disturbance, an abrupt and complete change from *B. coli* to *B. aerogenes* and other atypical strains very commonly occurs.

Organisms tested. The organisms used included the above strains as well as other local strains of *B. coli*, *B. aerogenes*, *B. pyocyaneus*, *B. asiaticus*, *B. morgani* No. 1, and eight local strains of *B. typhosus*.

Method. The technique followed was that of Rakieten and Rettger (1927). All results were read after 24 hours.

Brand of dye used. To obtain an independent opinion, samples of the two batches of dye in stock were sent to Prof. J. W. Bigger, Trinity College, Dublin, who tested them on suspensions of faeces inoculated with *B. paratyphosus* B. He reported that the pre-war brand was definitely the superior, and in addition sent another sample of Grübler, 1934, which he had found very satisfactory. A few tests showed that there was nothing to choose between these two samples, and all further work was carried out with the pre-war brand.

Telluric acid was a fresh sample obtained from British Drug Houses Ltd.

The chief points to be noted are:

(1) Of the twenty-nine strains of *B. coli* isolated from normal faeces nine were inhibited by dilutions of 1/250,000 or lower, six showed growth in 1/250,000 but not in 1/165,000, while fourteen showed growth in 1/165,000.

(2) The intermediate strains from faeces and urines with one exception grew in dilution of 1/125,000.

(3) *B. aerogenes* is by far the most resistant of the intestinal organisms examined, which would suggest a positive correlation between Voges-Proskauer positive organisms and their resistance to brilliant green. The reverse is not true.

(4) The other organisms—*B. asiaticus*, *B. pyocyaneus*, *B. morgani* No. 1—all showed equal if not greater resistance than *B. typhosus*.

Table I. *Effect of varying dilutions of brilliant green on intestinal organisms—24 hours*

Organism	No.	Isolated from	Dilutions of brilliant green				
			1/100,000	1/125,000	1/165,000	1/250,000	1/500,000
B. typhosus	W 1378	Blood	±	+	+	+	+
,,	18	,,	0	+	+	+	+
,,	2687	,,	0	±	+	+	+
,,	10	,,	0	±	±	+	+
,,	25	,,	0	0	±	+	+
,,	F 31	Faeces	0	±	+	+	+
,,	U 4	Urine	0	0	±	+	+
B. coli	693, MAI, A 1, A 2, E 2, K 1, K 2, L 1, M 2	Faeces	0	0	0	0	±
,,	520, W 1, B 1, C 2, E 1, I 1	,,	0	0	0	±	+
,,	Dr 2, F 1, F 2, L 2, M 1, 688	,,	0	0	±	± (or +)	+
,,	D 1, ES 1, Dr 1, G 1, H 1, H 2, I 2, J 2	,,	0	±	+	+	+
Intermediates	B 2	,,	0	0	0	+	+
,,	D 2	,,	0	±	+	+	+
,,	W 19, W 20, WM 2	Urine	0	±	+	+	+
B. aerogenes	7 strains	Faeces	+	+	+	+	+
B. asiaticus	2 ,,	,,	0	±	+	+	+
B. morgani No. 1	2 ,,	,,	0	±	+	+	+
B. pyocyaneus	2 ,,	,,	±	±	+	+	+

0 = no growth.
± = moderate clouding.
\+ = growth as in controls.

(5) The inhibiting dilutions for *B. typhosus* are in agreement with those of most authors and show that the failures with brilliant green cannot be attributed to the existence of local strains of *B. typhosus* abnormally sensitive to the dye.

Judging from these results it seems legitimate to conclude that a large proportion of coliform and similar bacteria found in the Sudan are definitely resistant to the action of brilliant green; consequently it is hopeless to expect any selective action of the dye for *B. typhosus* in most specimens of faeces. The results further indicate the fallacies in applying standard methods, worked out in temperate climates, to tropical conditions.

EXPERIMENTS WITH TELLURIC ACID

Following the work of Browning, Mackie and Smith (1914), telluric acid in connection with brilliant green has been frequently recommended by the text books. These authors found that the inosite fermenters (*B. aerogenes*) were more susceptible to the antiseptic action of potassium tellurate than *B. coli* or *B. typhosus*.

Apart from this early work the writer has been unable to find references in the available literature to any recent work on the value of telluric acid as a selective agent.

The following table shows the effect of the acid on the local strains of *B. typhosus* and *B. aerogenes*.

Technique. To 5 c.c. of peptone water, *p*H 6·5, varying dilutions of a 1 per cent. solution of the acid in distilled water were added. Each tube was inoculated with one loopful (4 mm.) of a 24-hour broth culture of the organism to be tested and the results read after 24 hours' incubation at 37° C. The results were controlled by plating on to McConkey's medium:

Table II

	Telluric acid		
Organism	1/12,500	1/25,000	1/50,000
B. typhosus (7 strains)	0	0	0
B. aerogenes ESH 2	0	+	+
A 1	0	±	+
B 1	0	±	±
C 1	0	±	+
Mc 2	0	±	+

0 = no growth.
± to + = slight to profuse growths.

A few strains were tested at *p*H 7·4, but the results were identical with the above.

The results are entirely at variance with those of Browning, Mackie and Smith (1914), showing a marked resistance of all strains of *B. aerogenes* with complete inhibition of *B. typhosus* in the three dilutions used and no explanation can be given of this discrepancy. Under the circumstances it was considered useless to pursue further investigation with telluric acid.

Discussion. It is obvious from recent literature that many workers have become increasingly critical of the claims of brilliant green as an enrichment agent for *B. typhosus* while still admitting its value in the isolation of paratyphoid bacilli. In their monograph on Chronic Enteric Carriers, Browning *et alii* (1933) found that "from 479 specimens of faeces from excreters of *B. typhosus* 311 positive results were obtained by direct plating and 167 by the brilliant green method." Garrod has also drawn attention to the unsatisfactory results of brilliant green in the isolation of *B. typhosus* as compared with *B. paratyphosus* B.

The practice of using varying dilutions of the dye is followed by most workers and seems to be based essentially on the conception that there is a narrow margin of safety between the inhibiting concentrations of brilliant green for *B. typhosus* and *B. coli* respectively, "The margin of safety with the differential antiseptics is not very wide...the practical problem is how to arrange conditions so as to secure a preponderance of the specific organism" (Browning, 1918).

Under optimal conditions in temperate climates it is no doubt possible to strike such a margin of safety; in other cases, judging from the frequent failures reported, it would appear to be a matter of considerable difficulty, while under conditions as mentioned above, where a majority of coliform strains are equally resistant, the expression is meaningless.

It would be desirable to abandon the use of the term and to recognise instead that an ill-defined zone exists in which strains of *B. typhosus* and coliform bacteria are equally resistant to the dye, and that the narrower this zone, the greater the chances of successful isolation of the former.

In seeking an explanation for the comparative failure of the brilliant green enrichment method in carriers of *B. typhosus* Browning *et alii* (1933) refer to Smith's results (1923). It was found that "Carrier strains of *B. coli* possessed in marked degree the property of inhibiting the vitality of *B. typhosus*." Further it was shown that even a stock strain of *B. coli* had some inhibitory effect on *B. typhosus*.

The inhibitory effect of *B. coli* on *B. typhosus*

It is curious that such little attention has been paid by other workers to this phenomenon.

The above-mentioned papers give full details, but the phenomenon can be easily demonstrated by mixing suspensions of *B. coli* and *B. typhosus* in the proportions of 1 : 1, 1 : 2, 1 : 4, and plating out immediately on to McConkey's medium and again after 24 hours. In the mixture of equal proportions colonies of *B. typhosus* are present on immediate plating, but absent in the other mixtures. After 24 hours' incubation *B. typhosus* cannot be recovered from any of the mixtures. Smith's results have been confirmed by the writer using *B. aerogenes* and other atypical coli strains as well as organisms like *B. asiaticus* and *B. morgani* No. 1.

In the attempts to elucidate this inhibiting effect, it is obvious that some more selective medium than McConkey's must be used for plating, and from preliminary tests Wilson's recent modification (1933) of his bismuth medium was found very suitable for this purpose. The brilliant green recommended as an ingredient is not essential.

Method. Saline suspensions of 24-hour agar cultures of *B. typhosus* and *B. coli* were mixed in the proportions of 1 : 4 and 1 : 8 respectively, and a loopful from each was plated on to Wilson's and McConkey's media im-

mediately and after varying periods of incubation of the mixtures at room temperature (*circa* 34° C.). The following is a typical result:

Proportion of B. typhosus *and* B. coli

	1 : 4		1 : 8	
Time	McConkey	Wilson	McConkey	Wilson
Immediate	Coli + + + Ty 0	Ty + + + Coli 0	Coli + + + Ty 0	Ty + + + Coli 0
24 hours	Ditto	Ditto	Ditto	Ditto
48 ,,	,,	,,	,,	,,
96 ,,	,,	,,	,,	,,

As the antagonistic effect of certain strains of *B. coli* on *B. typhosus* has long been known (Browning *et alii*, 1933), it was thought advisable to test this point with the available local strains—using the above technique and Wilson's medium.

The results briefly summarised are as follows:

With 29 strains of *B. coli*, *B. aerogenes*, etc. and *B. typhosus*. No inhibitory effect noted up to 48 hours.

B. coli (688) and *B. morgani* No. 1 (A). Complete inhibition of all dilutions on immediate plating.

Both these latter strains were isolated from normal faeces and appeared typical in every respect.

These results confirm the accepted belief that certain coliform strains have a specific inhibitory or lethal action on *B. typhosus*. According to Browning *et alii* (1933) some strains of *B. coli* from typhoid carriers appear to be antagonistic to a marked degree, but from their paper it would appear that only McConkey's medium was used. Unfortunately no carrier strains were available, but it would be interesting to test such out on Wilson's medium. It would be reasonable to infer from the above findings that, in the majority of cases, the so-called antagonism of *B. coli* on *B. typhosus* is due to the practice of using McConkey's medium, whilst admitting the existence of occasional strains of *B. coli* or similar organisms which have a specific inhibitory or lethal effect on *B. typhosus*.

Further investigations by plating on to Wilson's medium decimal dilutions of the mixtures of *B. coli* and *B. typhosus*, with corresponding saline controls, show a slight but definite diminution in the number of colonies of *B. typhosus* as compared with the controls. This diminution is scarcely noticeable until after 4 or 5 days' incubation of the mixture.

APPLICATION OF THE ABOVE FINDINGS TO THE BRILLIANT GREEN ENRICHMENT METHOD

Two factors are involved in the success or failure of the method: (1) the proportion of resistant strains of *B. coli* or similar bacteria normally present in the faeces; (2) the relative numbers of such organisms and *B. typhosus* present in the brilliant green tubes after 24 hours' incubation.

For reasonable chances of success when plated on to McConkey the proportions should be about equal. With certain dilutions of the brilliant green one frequently obtains under optimal conditions a pure culture of *B. typhosus* while the next dilution may give negative results or at the most a few colonies of *B. typhosus*. That such dilutions also contain large numbers of *B. typhosus* has been repeatedly proved by the use of Wilson's medium.

The following is an illustration:

Technique. The faeces from a case of typhoid, proved by blood culture, were plated directly on to McConkey's and Wilson's media. Also two loopfulls of the suspension were inoculated into three dilutions of brilliant green, and after 24 hours' incubation at 37° C. all dilutions were plated on to the two media.

			Brilliant green enrichment method, dilutions		
	Direct plating		1/165,000	1/250,000	1/500,000
McConkey	*B. coli*	+ + +	*B. aerogenes* + + +		all dils.
	B. typhosus	0	*B. typhosus* 0		all dils.
Wilson	*B. aerogenes*	±	*B. asiaticus* ±	*B. asiaticus* +	*B. asiaticus* +
	B. asiaticus	±	*B. typhosus* + +	*B. typhosus* +	*B. typhosus* ±
	B. typhosus	+			

0 =no colonies.
± =scattered colonies.
+ to + + =numerous colonies to confluent growth.

There is no doubt that McConkey's medium is unsatisfactory for the isolation of *B. typhosus* either used directly or in conjunction with brilliant green. One is really employing an excellent selective medium for coliform types. To quote Wilson and Blair (1931): "With media depending for the differentiation between colonies of *B. typhosus* and *B. coli* on the presence of lactose, the dice are loaded against *B. typhosus* since an extra supply of energy in a utilisable form is being supplied to the *B. coli*."

By the use of Wilson's medium a higher percentage of successes would undoubtedly be obtained with the brilliant green method but as the results of direct plating on to this medium have been so satisfactory it is doubtful whether preliminary enrichment is necessary and it seems likely that it will be generally applied as the medium of choice for the isolation of *B. typhosus*. In my experience it is proving especially valuable under tropical conditions.

It is of course recognised that numerous successes are obtained by direct plating of typhoid faeces on to McConkey's medium and that the above conclusions (obtained from mixtures in liquid media) need not necessarily apply to faeces.

Probably, however, such successes are governed by two considerations: (1) the relative proportion of *B. typhosus* to coliform strains present in the faeces (it seems certain that in many positive cases *B. typhosus* is present almost in pure culture), and (2) the tendency to overgrowth, even in freshly passed faeces, of certain bacteria such as *B. aerogenes* or *B. asiaticus*.

This would appear to be responsible for many failures obtained in the Sudan and in other tropical parts.

SUMMARY AND CONCLUSIONS

1. The causes of failure of the brilliant green enrichment method for isolation of *B. typhosus* are discussed.

2. It has been shown that the negative results obtained depend partly on the number of resistant *B. coli* strains normally present in faeces, and partly on the use of McConkey's as a plating out medium.

3. The alleged selective action of telluric acid could not be confirmed.

4. The so-called antagonism between *B. coli* and *B. typhosus* has been investigated, and by the use of a selective bismuth medium it is shown that this is more apparent than real in most cases.

5. However, the existence of certain strains of *B. coli* or similar bacteria with a specific inhibitory effect on *B. typhosus* has been confirmed.

6. The value of Wilson's bismuth medium for the isolation of *B. typhosus* has been demonstrated.

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