BISMUTH SULPHITE MEDIA FOR THE ISOLATION OF *V. CHOLERAE*

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Read (1939), employing various methods for the differential isolation of *V. cholerae* from mixtures of stools and waters inoculated with *V. cholerae*, found that a modification of the bismuth-sulphite enrichment medium of Wilson & Blair gave the best results and enabled him to isolate the vibrio from an inoculum that would only just grow in ordinary broth. Briefly, the modification consisted in the omission of brilliant green, increase of *p*H to 9·2, substitution of mannose for mannitol in 1% concentration, and replacement of broth by peptone water. The new medium, in addition to the inhibitory effect of the sulphite-bismuth complex on the growth of *B. coli* and many other saprophytic organisms, possesses the advantage that the sodium sulphite in it tends to keep the *p*H at a high level and thus at a point favourable to the development of *V. cholerae* and unfavourable to many accompanying organisms.

As the discoverer of the selective action of bismuth sulphite media and their application to the isolation of proteus, typhoid and paratyphoid bacilli, one of us (W. J. W.) decided to study the subject to see if Read's interesting observation could be confirmed.

Whilst we were engaged in this work a paper by Seal (1939) appeared giving the results of a preliminary field trial at Calcutta of Read's modification of the Wilson & Blair medium. Seal found that of the cases of clinical cholera 64·7% yielded *V. cholerae* in this medium as compared with 43·1% in alkaline peptone water and that eight of the 117 tank waters examined yielded positive isolations in the new media, whereas only five of them were positive in alkaline peptone water. Seal also found that compared with alkaline peptone water there was some restriction of growth of the inagglutinable vibrios in the bismuth sulphite mannose medium.

The formula of Read which we made the basis of our study was: peptone 2% 8·8 c.c., sea-salt mixture 1·2 c.c., stool emulsion 10 c.c., liq. bismuthi 0·12 c.c., sodium sulphite 20% 1·2 c.c., mannose 10% 1 c.c., absolute alcohol 0·2 c.c., $HgCl_2$ 1/10,000 0·8 c.c., *p*H 9·2. These 23·3 c.c. were contained in screw-capped bottles. Having had further experience of the use of sulphites and bismuth since 1931 when the paper of Wilson & Blair to which Read refers was published, we did not follow in every detail our old technique. Besides fluid enrichment media we tested solid media and describe in this paper a medium which might be employed in place of that of Dieudonné & Aronson.

Without going into details of the numerous experiments carried out, we may state that the following media have been developed and have proved satisfactory in our hands. Our technique also differed from that of Read in that (*a*) we did not use screw-capped bottles but ordinary test-tubes, and (*b*) we, from reasons of economy, employed glucose, mannitol and saccharose in place of mannose, which latter, according to workers in India, has certain advantages.

FLUID MEDIUM

Peptone 10 g., sodium chloride 20 g., water 1 l. Reaction made *p*H 9·1 by addition of sodium carbonate solution (53 g. sodium carbonate water 400 c.c.). Where Difco peptone was used usually 12 c.c. of sodium carbonate solution was required, with the peptone of Armour, B.D.H. and Witte, the figures were 18, 16 and 8 c.c. Instead of sodium chloride there may be an advantage in the use of sea salt mixture as recommended by Read. In this case we used 950 c.c. water + 50 c.c. of a saline mixture consisting of 135 g. NaCl, 5 g. KCl, 15 g. $MgCl_2 6H_2O$, 5·75 $MgSO_4 7H_2O$ + distilled water 500 c.c.

SULPHITE-BISMUTH MIXTURE

The proportions of sulphite and bismuth are important, and to avoid any variation and for convenience we have found it an advantage to use bismuth ammonio-citrate scales instead of liquor bismuthi. In the sulphite-bismuth solution there is put glucose (if we had had a supply we would have used mannose) which not only serves for the nutrition of the vibrios but also prevents oxidation of the sulphite.

The stock solution is prepared by dissolving 20 g. sodium sulphite anhydrous in 100 c.c. of boiling water and adding to it 0·1 g. bismuth ammonio-citrate scales dissolved in 10 c.c. of water. A precipitate of bismuth hydrate separates out on boiling. A solution of 20 g. of commercial glucose in 100 c.c. of boiling water is made and when cool both solutions are mixed. In place of glucose, saccharose, mannitol or mannose may be employed. The stock solution keeps for months and is added to the saline peptone water just before use: the *p*H of the stock solution is 9·4.

To 100 c.c. of saline peptone water *p*H 9·1, 10 c.c. of stock glucose sulphite bismuth mixture are added and then 1 c.c. of absolute alcohol. We have found little advantage in the addition of 4 c.c. of 1/10,000 $HgCl_2$. Wilson & Blair employed $HgCl_2$ with a view to the suppression of *Proteus* strains, but recently we have found it disappointing for this purpose. In the tubes enterococci often develop, and we have an impression that $HgCl_2$ tends to suppress them.

In our work tubes containing 10 c.c. of the enrichment medium were inoculated with a drop of a peptone water culture of the vibrio. In field work it would probably be found more convenient to employ a medium of double strength and to add to it an equal volume of a saline faecal emulsion.

SOLID MEDIUM

Read and his collaborators appear not to have tried the solid bismuth-sulphite media which we and many others have found useful for the suppression of *B. coli* and the isolation of organisms of the typhoid-paratyphoid group. We have found that the media of Wilson & Blair with slight modifications are very suitable for the isolation of cholera vibrios. Whether best results will be obtained by direct planting of the cholera stool on the solid medium or planting after preliminary enrichment in the fluid medium will only be found out by field trials. We can only state that the following medium has allowed an excellent growth of cholera vibrios and the complete suppression of the *coli-aërogenes* groups.

Peptone 40 g., NaCl 20 g., agar 80 g., water 4000 c.c., sodium carb. sol. (53 g. to 400 c.c. water) 40 c.c. The medium is autoclaved and without being filtered is adjusted to a reaction of *p*H 8·6.

To 100 c.c. of this medium melted and cooled to 50° C. are added 20 c.c. stock mannitol saccharose sulphite bismuth solution, 2 c.c. phenol red 1/1000 watery solution, and 2 c.c. absolute alcohol. No iron salts are added as in the corresponding medium for *B. typhosum*. Plates are poured and the surface inoculated.

The stock mannitol saccharose bismuth sulphite solution is prepared as follows:

(*a*) 100 g. sodium sulphite anhydrous dissolved in 500 c.c. boiling distilled water.

(*b*) 30 g. bismuth ammonio-citrate scales dissolved in 250 c.c. of boiling water.

(*a*) and (*b*) are mixed and boiled for two minutes, cooled and then added to (*c*) which consists of 50 g. saccharose and 5 g. mannitol dissolved in 250 c.c. of water. To the mixture are added 15 g. sodium bicarbonate dissolved in 50 c.c. cold water.

This mixture can be used for preparation of media for the isolation of typhoid bacillus and differs only in the use of 30 g. of sodium bicarbonate in place of 50 g. $Na_2HPO_4 2H_2O$ anhydrous or 100 g. $Na_2HPO_4 12H_2O$.

Through the kindness of Dr St John Brooks, we obtained from the National Collection of Type Cultures a large number of cholera and cholera-like vibrios. We planted out all these cultures in the fluid and solid bismuth sulphite media and found that nearly all genuine cholera vibrios grew well but a few grew sparsely whilst of the cholera-like vibrios some grew well but many were inhibited. Some strains of *El Tor* vibrios grew and some failed to grow.

As the exact position of some of the cultures has not been finally determined we give the results of our examination. We also tested the agglutinative action of a cholera "O" subgroup I serum which Dr Gardner kindly procured for us from the Oxford Standard Laboratory. The titre of this serum was 1/250 against Hikojima and at least 1/100 against Inaba and Ogawa strains and we found that 1/10 dilution in drops on slides enabled us to rapidly test its action on agar cultures of the various organisms. We found that the following (the figures are those of the National Collection of Type Cultures) grew well both in the fluid and on the solid bismuth sulphite media and were agglutinated by specific "O" subgroup I serum: 23, 27, 1633, 2276, 2463, 3241 Zeiss, 3639, 3640, 3642, 3644, 4115, 4117, 4119, 4476, 4479, 4693 (Inaba), 4694 (Hikojima), 4695 (Ogawa), 4897, 4900, 5197, 5198 (Shanghai 10), 5199 (Rangoon Smooth), 5200, 5201 (Kasauli 1800), 5202 (Shillong 610), 5203, 5350 (Rangoon P), 5352, 5353, 5596 (Shanghai 10). These thirty-one cultures were labelled *V. cholerae*, and have given the cultural characteristics and have been agglutinated by "O" Type I specific serum. We received a few other cultures that were labelled *V. cholerae* which did not grow either on the fluid or solid bismuth sulphite media; these were: 29 (Bombay 1279), 290 W (*Vibrio cholerae* Zeiss 1448), 2464 (*V. chol.* 26207), 2465 (*V. cholerae* 26214), 3243 (*V. cholerae* Zeiss 1473). No. 2899 (*V. cholerae* Zeiss 1440) yielded no growth on the fluid medium but fine delicate colonies on the solid medium. As the above cultures were not agglutinated with specific "O" Type I serum it is probable that they were not true cholera vibrios or else had lost their specific character. Of the "cholerae-like" and "paracholera" vibrios the following

failed to grow on both media: 56 W, 559, 561, 686, 689, 717, 718, 2084, 3636, 3646, 3650, 3651, 4708, 4709, 4710, 4713, 4716; of these, only 717 was agglutinated by specific "O" Type I serum. The growth of 262 *Vibrio Metchnikovi* and of 774 *V. Finckler Prior* was inhibited on both media. The following grew on both media: 2236, 3638, 3648, 3649, 4711, 4712, and 3638, 3648 and 3649 were agglutinated by specific "O" Type I serum.

Of the *El Tor* vibrios 3657, 3662, 4714, 4715, 5395 grew on both media; 2890, 3658, 3659, 3660, 3661 failed to grow in the fluid, but grew on the solid medium; 1548 was inhibited on both media.

The colonies of the so-called *El Tor* vibrios on the bismuth sulphite plates were much smaller than those of the genuine cholera vibrios. On the mannitol saccharose sulphite bismuth phenol red alcohol agar plates colonies of the cholera vibrio appeared after one night's incubation and were yellowish brown in colour. In the case of some strains after two days the colonies exhibited a dark metallic lustre. In general the characteristic feature was a yellowish brown growth resulting from the action of the acids produced from fermentation of the mannitol and saccharose on the phenol red. Somewhat similar colonies are formed by various strains of the genus *Proteus*. As is well known, *Proteus* strains on bismuth sulphite plates are unable to form spreading films of growth.

It is possible by microscopic examination of stained films to distinguish between colonies of the *Vibrio* group and those of *Proteus* and other bacilli. The growth of cholera vibrios consists of filaments curved and in spirals, whilst bacilli do not show the characteristic curving and are usually short and thick.

SELECTIVE ACTION OF THE MEDIUM

We have tested faecal emulsions from eight-two healthy individuals on the medium and have found that there is complete suppression of *B. coli* and *B. lactis aërogenes*. Frequently small white colonies of *Streptococcus faecalis* come up but do not interfere with the growth of *Vibrio cholerae* added to the faecal emulsion. Occasionally also *Proteus* organisms develop but these are very seldom found in normal stools.

In stools from cases of suspected dysentery and enteric fever *Proteus* organisms are not infrequent but can readily be rejected as non-vibrios by microscopic examination of stained films; at any rate this was our experience in the examination of thirty such stools.

Twenty laboratory cultures of *B. coli* and nine cultures of *B. lactis aërogenes* planted on the bismuth sulphite plates yielded no growth. The *B. aërogenes* cultures came from India and were kindly supplied to us by Prof. G. S. Wilson; they were strains that grew at 44° C.

Isolation of *Vibrio cholerae* from artificially infected stools

On the solid bismuth sulphite agar medium the yellowish brown colonies are microscopically examined and those found to consist of vibrios are planted out on plain nutrient agar or on lactose phenol red agar and the growth tested as regards agglutination on a slide with 1/10 dilution of specific "O" Type I serum.

Keeping powers of the solid medium

The stock bismuth sulphite solutions keep for months and from them the fluid and solid media can be rapidly prepared and are ready for immediate use. The tubes and plates should be at once inoculated. Although plates and tubes do not keep, it is possible to keep the solid agar bismuth sulphite medium (alcohol being omitted) for weeks if the medium has been poured into screw-capped bottles and the bottles filled right up to the stopper. Such media are boiled for a few minutes, the contents shaken, cooled, and poured out into plates after the addition of alcohol. The ease with which nutrient agar can be converted into the bismuth medium from the stock solutions leads one to prefer freshly made plates, but for field work in the tropics and to save any excess of the prepared medium the use of screw-capped tubes and bottles has advantages. As regards the preparations being available for immediate use and being capable of being kept in full stoppered bottles, the medium has advantages over those of Dieudonné & Aronson.

Discussion

The recognition of *Vibrio cholerae* has been facilitated by the classical serological work of Gardner & Venkatraman (1935) followed by the field work of Taylor (1937) and his colleagues and by further work of Gardner (1937) and Bruce White (1937). The finding of Seal (1939) that the growth of many inagglutinable vibrios is inhibited in sulphite bismuth media is confirmed in this investigation and may be of assistance in the isolation of the true cholera vibrio from accompanying non-agglutinable vibrios. Rich growth on our sulphite bismuth agar medium will be another differential test supplementing serological tests and the finding of Taylor (1937) and others that the true cholera vibrio is (*a*) non-haemolytic of washed goat erythrocytes, (*b*) ferments saccharose and mannose but not arabinose, (*c*) gives a positive cholera red and a negative Voges-Proskauer reaction.

Summary

1. A fluid enrichment medium containing sulphite and bismuth based on Read's modification of one of Wilson & Blair's media has been devised and the claims of Read (1939) and Seal (1939) as to the value of such a medium has been confirmed.

2. A saccharose mannitol sulphite bismuth alcohol phenol red agar medium is described and has been found to allow rich growth of the true cholera vibrio and to inhibit the growth not only of *B. coli* and *B. lactis aërogenes* but of many vibrios liable to be mistaken for the true vibrio cholerae.

3. Thirty-one strains of true cholera vibrios, obtained from the National Collection, grew rapidly and profusely in the media.

4. Of twenty-five cholera-like and para-cholera strains six grew well, whilst the growth of nineteen was scanty or nil.

5. Of eleven *El Tor* vibrios five grew in both fluid and solid bismuth media, five grew only on the solid medium and in one no growth occurred in either medium. The *El Tor* colonies were much smaller than those of the epidemic cholera strains.

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