SELECTIVE MEDIA FOR THE ISOLATION, CULTIVATION AND DIFFERENTIATION OF *BACILLUS COLI* AND *B. LACTIS AEROGENES.*

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This paper records the results of experiments made by me in an endeavour to devise media which would (1) allow of the direct estimation of the numbers of coliform bacilli in a material, and which would (2) be selective either for the growth of faecal *B. coli*, or for that of *B. lactis aerogenes*.

Selective media of this nature employed in the direct examination of soil, water and milk, should assist the analyst in appraising the sanitary condition of such material.

Agar media for direct count of coliform organisms.

Agar media containing various constituents were prepared, melted, cooled to 50° C. and then mixed with equal volumes of cold water containing strains of *B. coli* and *B. lactis aerogenes*, and poured out into plates. Colonies developing after incubation were counted and differences between their appearances were sought. It proved possible to find some difference as, for instance, where sodium citrate was the only fermentable substance in the medium, the colonies of *B. lactis aerogenes* were larger than those of *B. coli*, but I failed to obtain a medium which permitted excellent growth of both types of colonies and at the same time allowed of their differentiation.

It was found that in the presence of glucose, sodium thiosulphate was reduced by both *B. coli* and *B. lactis aerogenes*, and that in the presence of bismuth or lead hydrate a black or brown colony was formed. To prevent the growth of other organisms sodium sulphite was added. A medium with which the numerous experiments were carried out consisted of 100 c.c. nutrient agar, 5 c.c. each of 20 per cent. solutions of glucose, sodium sulphite anhydrous, and sodium thiosulphate crystals and 2 c.c. of lead hydrate suspended in water. The lead hydrate was prepared by dissolving 10 g. lead acetate in 100 c.c. water, precipitating the hydrate by the addition of caustic soda, washing the precipitate and making up the volume to 100 c.c. When this medium was melted, cooled to 50° C., and mixed with equal volumes of water, poured out into plates and test-tubes and then incubated, a count of the black coliform colonies could be made.

The distinction between colonies of *B. coli* and of *B. lactis aerogenes* in the plates was not clear, but it was observed that the colonies of *B. lactis aerogenes*

in the tubes developed only near the surface. It was found that a simple method of distinguishing between colon and aerogenes cultures was to plant out in Petri dishes in the way indicated above, and after the agar had set to cover the surface with an additional layer of agar. The covering layer gave equally good results, whether it consisted of plain 2 per cent. agar dissolved in water without any peptone or meat extract, or with the addition of glucose and sulphite.

To get a suitable number of bacteria in the water sample, the procedure was to add one large drop of a 24-hour broth culture to 100 c.c. water and from this to take 0·5 or 1 c.c. and add to 2 litres of water contained in Winchester quart bottles and to shake vigorously. The water in amounts of 1 and 10 c.c. was found to contain the organisms in suitable numbers.

Experiments showed that with glucose agar the colonies of *B. lactis aerogenes* came up equally well in the covered and uncovered plates, but that the thiosulphate or sulphite alone or combined brought about the suppression of the growth of *B. lactis aerogenes* in the plates whose surface had been covered over with an additional layer of agar.

The lead plates were moderately satisfactory, but eventually a simpler medium was devised for the direct enumeration of coliform organisms in water. The composition of this medium which, for convenience, I shall call A is: water 1000 c.c., peptone 10 g., lactose 10 g., sodium citrate 10 g., sodium taurocholate 10 g., Bacto agar 20 g., and 10 c.c. of 1 per cent. solution of neutral red in water.

In the examination of a water sample amounts of 1 c.c. and 10 c.c. are taken in wide test-tubes made up to 20 c.c. by the addition of sterile water and then each mixed with 20 c.c. of the medium which has been melted and cooled to 50° C. and the mixture immediately poured out into Petri dishes.

In this medium, colonies of *B. coli* and *B. lactis aerogenes* appear as deep red and spindle-shaped, and occasionally the colony of the *B. lactis aerogenes* can be distinguished from that of *B. coli* in becoming larger and yellowish in colour.

The relative proportion of *B. coli* and *B. lactis aerogenes* in the water can, however, be determined by the use of this medium modified by the omission of the neutral red and the addition to every 100 c.c. of 1 g. sodium sulphite anhydrous and of 0·5 c.c. of a solution of 1 g. basic fuchsin in 60 c.c. absolute alcohol.

In plates inoculated with the water in the method already mentioned, colonies of *B. coli* and *B. lactis aerogenes* appear, but in similar plates the surface of which had been covered with agar, the growth of *B. lactis aerogenes* is suppressed.

The suppression of growth of colonies of *B. lactis aerogenes* can more conveniently be secured, as will appear from subsequent sections of this paper, by the addition to medium A of 1 c.c. and 3 c.c. of a 40 per cent. solution of hexamine in water.

Medium A has been employed in the examination of many samples of water and the number of coliform organisms thus directly determined has been found to be similar to the number found by the examination in MacConkey's lactose broth tubes.

Selective media for differentiation of *B. coli* and *B. lactis aerogenes*.

Koser's synthetic citrate medium is an excellent example of a medium which is most useful for the differentiation of pure cultures of *B. coli* and *B. lactis aerogenes*. My endeavour has been to secure a medium which might be useful in the examination of water, milk, etc., before the contained organisms had been isolated in pure cultures.

An enrichment medium for *B. lactis aerogenes* and *B. paratyphosus* B.

When sodium sulphite in amounts of 1 or 2 per cent. is added to peptone water or broth, the growth of *B. coli* and *B. lactis aerogenes* is less profuse or is suppressed, but immediately starts on the addition of glucose, lactose or some other substance which can be fermented by the micro-organisms. The addition of sodium citrate which is fermented by *B. lactis aerogenes* and not by most strains of *B. coli* gives the former an advantage and in many cases allows it to outgrow its competitors. *B. paratyphosus* B which is a citrate fermenter flourishes under these conditions.

A medium consisting of 100 c.c. water, 1 g. peptone, 1 g. sodium sulphite anhydrous and 0·5 g. sodium citrate was inoculated with 20 consecutive stools from an institution where an outbreak of paratyphoid fever had occurred, and subcultures made on bismuth sulphite glucose brilliant green plates, and on MacConkey plates. In 13 instances *B. paratyphosus* B was isolated on the bismuth plates, whereas on the bismuth plates directly inoculated there were 12 successes. At the same time 14 strains of citrate and lactose fermenters were isolated by this enrichment process, two of these were *B. lactis aerogenes*, and 12 belonged to Koser's intermediate group, *i.e.* they were V.P. – M.R. + and, apart from being fermenters of citrate, corresponded exactly to faecal colon bacilli.

It is interesting to note that these intermediate strains are present in faeces, and that in a water they may have been derived from excreta and are not necessarily mere saprophytes as is sometimes believed.

For the isolation of *B. paratyphosus* B and *B. lactis aerogenes* the addition of 0·5 c.c. of a 1 per cent. solution of brilliant green in water to each 100 c.c. of the enrichment medium may be an advantage.

In the course of my work I observed that lactose or glucose peptone water containing 0·5 per cent. sodium citrate and 1 c.c. per 100 of a 1 per cent. solution of neutral red in water, allowed of a richer growth of *B. lactis aerogenes* than of *B. coli* and of a reduction or bleaching of the neutral red. In fact most strains of *B. coli* failed to grow in the medium.

The inhibiting action of the medium was found to be due to the combined action of neutral red and sodium citrate—separately these had no inhibiting action.

Neutral red with citrate in the medium inhibited the growth of *B. coli* in a concentration lower than that necessary to inhibit the growth of *B. lactis aerogenes*. The presence of lactose allows of growth with a larger amount of neutral red.

I found that the following media were enrichment media for *B. lactis aerogenes*.

	B	C	D
Water	100 c.c.	100 c.c.	100 c.c.
Peptone	1 g.	1 g.	1 g.
Lactose	0·5 g.	—	—
Sodium citrate	0·5 g.	0·5 g.	0·5 g.
Neutral red 1 per cent. in water	1 c.c.	1 c.c.	0·3 c.c.
Sodium sulphite anhydrous	—	—	0·5 g.

Medium D containing 0·5 g. sodium sulphite is selective for intestinal bacteria and the citrate and neutral red tend to inhibit the growth of *B. coli*; and neutral red in the presence of sulphite cannot be tolerated to the extent of 1 c.c. of a 1 per cent. solution to 100 c.c. of the medium, but probably 0·3 c.c. is about the optimum for selective purposes.

With these media I have studied 42 strains of *B. coli* isolated mainly from the faeces of different individuals and also some strains from water and milk, and have found that either no growth occurs or that there is slight growth, and that the medium remains red. These *B. coli* strains were citrate methyl red + and V.P. −. On the other hand 26 strains of *B. lactis aerogenes* which had been isolated from faeces, sewage, water and milk and which were citrate methyl red − and V.P. + grew well in the media and bleached the neutral red or reduced it to a yellow colour.

STARCH FERMENTATION.

Although Durham as long ago as 1901 pointed out that fermentation of starch was characteristic of the aerogenes group, and this was adopted by Levine (1918) in his classification, and Levine's classification endorsed by myself (1929), little use has been made of this fact by water bacteriologists.

A medium consisting of 100 c.c. water, 10 g. peptone, 1·25 g. soluble starch sterilised at 115° C. for 15 min. and to it 5 c.c. of Andrade's indicator added, was found useful in the differentiation of *B. coli* and *B. lactis aerogenes*. Of 23 strains of *B. coli* tested, 21 gave entirely negative results, whilst two after a few days' incubation produced an acid reaction. Of these two, one was isolated from urine in a case of cystitis and one from faeces, and both appeared to be pure cultures.

Of 44 strains of *B. lactis aerogenes* tested, 38 produced acid and gas, and six were negative.

It was thought that the addition of starch to the citrate neutral red peptone water medium would be an advantage, and so it proved in practice.

In a medium E containing 1000 c.c. water, 10 g. peptone, 1·25 g. soluble starch, 5 g. sodium citrate and 10 c.c. of a 1 per cent. solution of neutral red, 29 strains of *B. coli* failed to grow whereas 48 strains of *B. lactis aerogenes* gave good growth, resulting often in the bleaching of the medium.

Enrichment media for *B. coli*. Sulphite lactose peptone water with addition of absolute alcohol.

In peptone water containing in every 100 c.c., 1 g. of sodium sulphite anhydrous and 0·5 g. of glucose or lactose, the growth of *B. coli* is profuse, and also of most strains of *B. lactis aerogenes*, but the addition of 2 c.c. of rosolic acid dissolved in absolute alcohol was found to suppress the growth of the aerogenes strains and not to interfere with that of genuine excretal *B. coli*. In one experiment 22 strains of *B. coli* produced a dense turbidity, whilst with 48 strains of *B. lactis aerogenes* the fluid remained clear, or showed a mere film on the surface. With a few strains of *B. lactis aerogenes* turbidity throughout the tube is produced, especially after long incubation. A few experiments demonstrated that the inhibition was due to the absolute alcohol contained in the rosolic acid solution, and not to the rosolic acid itself.

Selective action of hexamine.

In hexamine I discovered that I had a substance which in a certain concentration allowed a rich growth of *B. coli* and completely arrested the growth of *B. lactis aerogenes*.

From many experiments I arrived at the following simple selective medium: 1000 c.c. water, 10 g. peptone, 5 g. lactose, and 20 c.c. of a 40 per cent. solution of hexamine (hexamethylene-tetramine). The peptone lactose water is sterilised, and to it, when cool, the hexamine solution is added.

The addition of 5 c.c. of Andrade's indicator does not interfere with the selective action and shows the formation of acid by the *B. coli*. The amount of acid formed is evidently at first not sufficient to liberate formaldehyde, and so bring about arrest of growth.

It is interesting to note that hexamine itself, apart from liberated formaldehyde, has an antiseptic action, and that this is very marked with strains of *B. lactis aerogenes*. In cystitis and pyelitis it is often strains of *B. lactis aerogenes* that are present, and in these cases hexamine may be an effective antiseptic, even when the urine has not been rendered strongly acid.

My conclusion as to the selective action of hexamine in the above medium is based on the study of 101 strains of *B. coli* isolated from separate samples of human faeces, 11 from urine, 27 from water, 6 from cows' milk and 10 from the excreta of cattle. In my experience, the use of hexamine (urotropin) does not allow of a differentiation of colon strains of human and bovine origin, although Calisti (1931) claimed that it had this action. In carrying out the test the peptone water lactose hexamine tubes are lightly inoculated from agar slopes

and the readings taken after 18 hours' incubation at 37° C. Similarly MacConkey's lactose bile salt neutral red broth tubes can be employed, but in this case only 1 c.c. of the 40 per cent. hexamine solution is added to every 100 c.c. of the medium.

In the tubes inoculated with *B. coli* there is produced uniform turbidity, usually dense, but occasionally only moderately dense, whilst the tubes inoculated with *B. lactis aerogenes* remain clear throughout or clear with a film on the surface or with a sticky deposit. The results are as a rule quite clear cut. Hexamine may therefore be employed to suppress any *B. lactis aerogenes* when water or milk is being tested for the presence of genuine faecal strains of *B. coli*. The addition of 1 or 3 c.c. of 4 per cent. hexamine solution to 100 c.c. of medium A furnished a medium which readily allows of the determination of genuine *B. coli* in samples of water or milk; it also suppresses the growth of strains of alcaligenes and other organisms which may tend to obscure the colonies of *B. coli* in the plates. As a routine in water examination, it is useful to compare the number of coliform colonies which develop when 10 or 20 c.c. of the water are mixed with equal volumes of medium A which has been melted and cooled to 50° C. and in the same medium containing in every 100 c.c. 1 or 3 c.c. of a 40 per cent. solution of hexamine. The colonies developing in the hexamine plates will be found to contain few, if any, *B. lactis aerogenes* colonies but only or mainly true excretal *B. coli*.

SOLID SELECTIVE MEDIA.

The fluid media mentioned may be converted into solid media by the addition of 2 per cent. Bacto agar. In the solid media a larger amount of the inhibiting reagents must be employed.

For *B. lactis aerogenes* 100 c.c. water, 1 g. peptone, 0·5 g. sodium citrate, 0·25 g. soluble starch, 2 g. agar and 6 c.c. of a 1 per cent. solution of neutral red was found suitable, and for *B. coli* the following: 100 c.c. water, 1 g. peptone, 0·5 g. lactose, 2 g. Bacto agar and 4 c.c. of a 40 per cent. solution of hexamine.

SUMMARY.

1. An agar medium is described which enables a direct estimation of the numbers of coliform bacilli in a water or milk sample to be made.

2. An enrichment medium for *B. lactis aerogenes* is one containing in every 100 c.c. of peptone water 0·5 g. sodium citrate, 0·25 g. soluble starch and 1 c.c. of a 1 per cent. watery solution of neutral red.

3. Enrichment media for *B. coli* are: (*a*) lactose peptone water containing in every 100 c.c., 1 g. sodium sulphite anhydrous and 2 c.c. of 1 per cent. rosolic acid in absolute alcohol; (*b*) lactose peptone water containing in every 100 c.c., 2 c.c. of a 40 per cent. solution of hexamine in water.

4. Importance is attached to the capacity to ferment starch possessed by *B. lactis aerogenes*.

5. The application of these media to the examination of water and milk is indicated.

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