COMPARATIVE STUDIES OF PRESUMPTIVE TEST MEDIA FOR THE COLI-AEROGENES GROUP OF BACTERIA

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It has been agreed by most workers in water bacteriology that the plain lactose broth, usually used for presumptive tests, is not altogether satisfactory in that it gives many false positive tests. A number of modifications have been suggested to eliminate, so far as possible, these spurious positive presumptive tests. In most of the modifications dyes are used to restrain the growth of Gram-positive organisms which are the cause of many of the false positive presumptive tests obtained in plain lactose broth. Salle (1930) proposed the use of crystal violet in a phosphate-buffered, lactose, peptone broth with which he has reported excellent results. Brilliant-green lactose peptone bile is recognized by the 1933 edition of Standard Methods of Water Analysis for use in control work in water purification plants. Basic fuchsin in plain lactose broth has been advocated by Ritter (1932) and reported to have given splendid results in a large volume of tests in Kansas. Dominick and Lauter (1929) used, to good advantage, a combination of methylene blue, erythrosine and brom-cresol-purple in a lactose peptone buffered broth.

Objection has been raised by Stark and England (1932, 1933) to the use of crystal violet in presumptive tests since they found that it inhibited the growth of many strains of *Es. coli* of human origin when tested by pure culture inoculation. In commenting on the work of Stark and England, Norton (1933) points out that

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while this study has given an unfavorable report on the use of crystal violet, yet the mixed cultures found invariably in water tests might modify the results.

Horwood and Heifertz (1934) reported on comparative studies of brilliant-green lactose peptone bile, the Dominick-Lauter medium, Salle's crystal-violet broth, and Standard Methods lactose broth. They found crystal-violet broth inferior to plain lactose broth, to brilliant-green lactose peptone bile, and to the Dominick-Lauter medium.

It has been felt by the writer that additional comparative studies involving the use of crystal violet might aid in a better evaluation of its usefulness in presumptive tests. A comparison has therefore been made of crystal-violet broth, fuchsin broth, plain lactose broth and brilliant-green lactose peptone bile.

EXPERIMENTAL

Pure cultures of the Escherichia-Aerobacter group of bacteria were isolated from fresh feces of 30 individuals in apparently normal health. From one individual two specimens were obtained and from the others only one specimen each. In all, 201 strains were isolated. All were found to be Gram-negative rods, and gas producers in lactose broth. Of 200 strains tested for indol production in tryptophane broth, 188 gave positive tests.

Of the 201 strains, 183 were methyl-red positive, Voges-Proskauer negative and failed to produce turbidity in citrate broth and were thus typically *Es. coli*. Twelve strains, ten of them from a single specimen, were typical Aerobacter strains since they were consistently methyl-red negative, Voges-Proskauer positive and grew in citrate broth. The remaining 6 strains, all from one individual, were typical Citrobacter strains being methyl-red positive, Voges-Proskauer negative, and citrate positive.

The crystal violet medium was prepared in accordance with the directions given by Salle (1930) and tubed in 14 ml. amounts so that the addition of 1 ml. of a dilution of bacteria in a water blank would give a dye concentration of 1:700,000 and a composition as follows:

	per cent
Peptone	0.5
K_2HPO_4	0.5
KH_2PO_4	0.1
Lactose	0.5

Crystal violet in a final concentration of 1:700,000

Brilliant-green lactose peptone bile and plain lactose broth were prepared according to the 1933 edition of Standard Methods of Water Analysis. Fuchsin broth was the same as plain lactose broth with 0.8 ml. of a filtered saturated alcoholic solution of basic fuchsin added per 1000 ml., giving a concentration of 1:1250 of saturated alcoholic solution of basic fuchsin.

Following the method advised by Stark and England (1933) small inocula were prepared by diluting broth cultures and the approximate number of organisms per inoculum was determined by pouring agar plates. Different dilutions of the organisms tested were added to Durham fermentation tubes of lactose broth, brilliant-green lactose peptone bile, crystal-violet broth, and fuchsin broth and these tubes incubated at 37°C. Observations were made on all tubes at the twenty-fourth hour and at the forty-eighth hour. Only those inocula containing less than 50 organisms per inoculum were considered. Tests with larger inocula were repeated. Since practically all strains tested grew equally well in plain lactose broth and in brilliant-green lactose peptone bile, any test which did not show gas in both within twenty-four hours of incubation was repeated. Table 1 gives the results of the tests in crystal-violet broth. In every case the same size of inoculum was sufficient to produce 10 per cent or more of gas in both plain lactose broth and brilliant-green lactose peptone bile within twenty-four hours of incubation.

After forty-eight hours of incubation only 49 per cent of the 201 fecal coli-aerogenes organisms were found to produce as much as 10 per cent of gas in crystal-violet broth. In most of the negative tests the crystal-violet broth showed no gas whatever and in a large number showed no visible turbidity. From only 5 of the 30 persons from whom specimens were obtained did all strains produce gas in crystal-violet broth in forty-eight hours, and all

TABLE 1

Production of gas in crystal violet broth

INDIVIDUAL	NUMBER OF STRAINS*	AVERAGE INOCULUM	GAS IN CRYSTAL VIOLET BROTH			
			24 hours		48 hours	
			Number of strains negative	Number of strains positive	Number of strains negative	Number of strains positive
1	10	21	0	10	0	10
1	10	9	9	1	0	10
2	10	11	6	4	5	5
3	10	14	10	0	6	4
4	9	14	9	0	9	0
5	10	6	10	0	3	7
6	2	8	2	0	1	1
7	4	9	4	0	0	4
8	6	12	6	0	4	2
9	3	7	2	1	2	1
10	7	7	7	0	3	4
11	7	15	7	0	7	0
12	7	12	7	0	5	2
13	7	8	6	1	4	3
14	7	10	7	0	7	0
15	6	15	3	3	2	4
16	5	16	5	0	2	3
17	7	9	7	0	6	1
18	7	10	7	0	7	0
19	6	6	6	0	6	0
20	6	6	6	0	2	4
21	6	5	4	2	3	3
22	7	18	7	0	2	5
23	4	13	0	4	0	4
24	6	17	3	3	3	3
25	6	8	6	0	4	2
26	6	10	4	2	4	2
27	5	12	5	0	2	3
28	7	15	6	1	4	3
29	4	10	4	0	0	4
30	4	14	3	1	0	4
Totals....	201		168	33	103	98

* All ten strains from the first sample of Individual 1 were Aerobacter strains, two of the three strains from Individual 9 were also Aerobacter, all six strains from Individual 19 were Citrobacter strains. All other strains were typical Escherichia strains.

strains from 5 other individuals failed to form gas in the same period of incubation.

GAS PRODUCTION IN FUCHSIN BROTH

So few of the strains formed gas in fuchsin broth with the small inocula used in the crystal violet tubes that this part of the experiment was discontinued after 80 strains had been tested with small inocula. Of these 80 strains, only 5 produced any gas in the fuchsin broth in forty-eight hours. Later, all strains except the 5 which did produce gas from small inocula were tested in the fuchsin broth by adding 10,000 to 100,000 organisms. Out of all 201 strains tested by large and small inocula, only 33 or about 17 per cent produced gas in fuchsin broth, and 12 of these strains were from two specimens from the same individual.

EFFECT OF SIZE OF INOCULUM IN CRYSTAL-VIOLET BROTH

Ten strains, found to be quite sensitive to crystal-violet, were inoculated into the violet broth using different numbers of organisms in the inoculum. Of these 10 strains, 8 formed no gas in forty-eight hours with inocula as large as 1000 organisms per tube and in the case of 2 of these strains, an inoculum of approximately 1000 failed to produce any apparent growth within an incubation period of five days at 37°C. When the size of the inoculum was increased, all of these strains gave positive gas tests within forty-eight hours of incubation.

Of the strains which were found to be not sensitive to crystal violet in the concentration used, as few as one to three organisms were sufficient to produce gas within two days.

BACTERIAL SYNERGISM IN CRYSTAL-VIOLET BROTH

It has been known for some time that different pairs of organisms may produce gas in lactose broth while neither alone is able to do so. Sears and Putman (1923) found a number of such pairs of organisms which were able to give gas from lactose, sucrose or mannitol when neither would do so alone. Holman and Meekison (1926) found that in similar cases of bacterial synergism

one organism of the pair must be capable of splitting the test substance, forming acid, while the other must be capable of forming gas from monosaccharides. Having noticed that gas production was apparently more likely to occur in a crystal-violet presumptive test fermentation tube when using a mixed culture found in a surface water, than when using pure cultures, there seemed to be a possibility of a somewhat different type of bacterial synergism than the ones to which reference has just been made. It was noticed when one-tenth of a milliliter of a heavily polluted surface water was added to crystal-violet broth, that not only was gas formed rapidly, but that the color of the dye was almost destroyed within twenty-four hours. An organism that would grow readily in the crystal violet and destroy the color was quite readily isolated and found to be a Gram-negative rod producing a greenish fluorescence on agar slants and abundant greenish pigment when grown in Sullivan's medium (1905). This culture was designated merely as culture "A."

When a loop of a broth culture of this green fluorescent organism was added to a tube of crystal violet, together with a small inoculum of one of the dye-sensitive strains of *Es. coli*, it was found that the fluorescent organism so altered the medium that the small inoculum of the dye-sensitive strain was able to produce abundant gas in an incubation period of thirty to forty hours while a similar inoculum alone in crystal violet did not show even a visible turbidity within forty-eight hours. Culture "A" alone produced no gas from lactose either in plain lactose broth or in crystal-violet broth in both of which media it grew readily and rapidly.

Several tubes of crystal-violet broth were inoculated with a loop of culture "A" and incubated sixteen to eighteen hours. A test made on two of these tubes showed no production of acid, but the purple color had almost disappeared. The remainder of these cultures of "A" in crystal-violet broth were heated at 100 to 103°C. for ten minutes and after cooling were inoculated with one of the most sensitive strains of *Es. coli*, culture 123, in decreasing amounts. The tube receiving approximately 10 organisms and all tubes receiving larger inocula produced abundant

gas within twenty-four hours of incubation, while the set of control tubes of crystal violet inoculated with similar numbers of organisms showed no gas with 1000 organisms even after an incubation period of four days. This experiment is summarized in table 2.

In order to find out whether a small inoculum of a dye-sensitive strain like culture 123 was killed by the dye, or was merely inhibited, a series of tubes of crystal violet were inoculated with decreasing numbers of these organisms. These cultures were placed in the incubator for twelve hours, at the end of which time each tube was inoculated with a loop of a broth culture of "A." After four days further incubation no gas was found in any tube that received an inoculum of 1000 organisms or less, indicating

TABLE 2

Effect of bacterial synergism on gas formation in crystal violet broth

APPROXIMATE INOCULUM OF CULTURE 123 OF ES. COLI	GAS IN CRYSTAL VIOLET IN 48 HOURS	GAS IN CRYSTAL VIOLET PLUS LOOP OF "A" IN 48 HOURS	GAS IN KILLED CULTURE OF "A" IN CYSTAL VIOLET 48 HOURS
10,000	Plus	Plus	Plus
1,000	None	Plus	Plus
100	None	Plus	Plus
10	None	None	Plus

that the organisms in the smaller inocula were all dead before the fluorescent organism had so modified the medium that they might start to grow.

It has been suggested by Ingraham and Fred (1933) and by Ingraham (1933) that the bacteriostatic effect of gentian violet (crystal violet used in the experimentation) is due largely to the poising effect of the dye on the oxidation-reduction potential. It was found that the growth of fluorescent culture "A" did reduce the oxidation potential. However, when the pH of the crystal violet was changed from about 7.4 to 6.0 by the addition of sulphuric acid and the oxidation potential was definitely raised, the dye had practically no inhibitory effect on dye-sensitive strains. On the other hand the addition of $CaCO_3$ to the crystal violet broth to change its pH to about 8.0 lowered the potential

but made the medium much more inhibitory to dye-sensitive strains of *Es. coli*. Just how the growth of culture "A" makes possible the rapid growth and gas production of dye-sensitive strains is uncertain. However similar synergism no doubt plays a considerable part in the production of gas by coli-aerogenes strains in water when added to crystal-violet broth. Hence crystal-violet broth would be expected to show more positive tests from polluted waters than would be expected from its poor showing in studying pure culture strains.

PRESUMPTIVE TESTS ON UNTREATED SURFACE WATERS

Twelve samples of different waters were tested for presence or absence of coli-group organisms by using plain lactose broth, brilliant-green lactose peptone bile, crystal-violet broth, and fuchsin broth. In 2 samples, plain lactose broth showed gas in higher dilution than did brilliant-green bile, and these positives were fully confirmed according to Standard Methods. On the other hand brilliant-green bile gave a higher index for coli-group organisms in 8 samples, 7 of which were confirmed. In the other 2 samples plain lactose broth and brilliant-green bile gave a similar index. The 2 samples which showed superior results for lactose broth gave positive tests in secondary tubes of brilliant-green bile.

Crystal-violet broth gave a coli index equal to plain lactose broth for 10 of the 12 samples of waters and gave superior results in the other two, both of which were confirmed. Fuchsin broth showed results equal to plain lactose broth in only 5 samples and inferior results in 7 samples.

DISCUSSION

Winslow (1934) has suggested that parallel planting in plain lactose broth and brilliant-green lactose peptone bile followed by secondary tubes of brilliant-green bile made from those tubes of plain lactose broth which do not also show gas in the brilliant-green bile primary tubes, be made the standard test on all waters without any attempt at further confirmation. The results of our studies both with pure cultures and the very limited number

of tests made on surface waters are in complete accord with the above suggestion of Winslow. Every one of the pure culture strains of Escherichia, Aerobacter, and Citrobacter grew almost equally well in brilliant-green lactose peptone bile and in plain lactose broth. On the other hand while crystal violet will no doubt give better results in mixed cultures as found in water samples than might be expected from its poor showing in pure culture studies, its use in presumptive tests is not advisable since quite frequently dye-sensitive strains would be likely to escape detection.

The poor showing of fuchsin broth would make its use inadvisable unless it can be shown that other samples of basic fuchsin than the one used (Coleman and Bell Certified No. CF-13) are much less inhibitory to coli-aerogenes strains.

SUMMARY

1. Pure culture strains of Escherichia, Aerobacter, and Citrobacter from 30 individuals, 201 strains in all, have been tested for gas formation in plain lactose broth, brilliant-green lactose peptone bile, crystal-violet buffered broth, and fuchsin broth.

2. When small inocula were used (less than 50 organisms per tube) lactose broth and brilliant-green lactose peptone bile gave positive tests after twenty-four hours of incubation at 37°C. for all 201 strains tested.

3. In crystal-violet broth after forty-eight hours of incubation using similar small inocula, only 49 per cent of these strains gave positive tests. From only 5 persons of the 30 from whom specimens were obtained did all strains produce as much as 10 per cent of gas in forty-eight hours, and all strains from 5 other individuals failed to form gas in the same period of incubation.

4. Very few strains formed gas in fuchsin broth when small inocula were used, and with relatively large inocula only 33 of the 201 strains were able to form gas in forty-eight hours.

5. When small inocula of a dye-sensitive strain of Escherichia were added to the crystal-violet broth and the tubes inoculated also with a loop of a culture of a greenish fluorescent water organism, the fluorescent bacterium so modified the medium that this

strain was able to produce gas in forty-eight hours or less. Hence less failures to get gas formation in the presence of Escherichia-Aerobacter organisms in water samples are to be expected than the poor showing of this medium when tested by pure culture strains would indicate.

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