Standard Methods and New Procedures for the Isolation of Colon Bacilli from Water*

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THE isolation of *B. coli* † from water dates back at least to the work of Theobald Smith (1893) 40 years ago. Glucose broth was used at first but later it appeared that lactose broth was preferable since it cut out many forms unrelated to the colon group which ferment glucose but not lactose. In the second edition of *Standard Methods* (1912) a lactose-bile presumptive test was recommended for the "colon group" as a whole; and preliminary cultivation in glucose or lactose broth with isolation on litmus-lactose-agar and identification by various tests, for *B. coli* itself. In the third edition (*Standard Methods,* 1917) there was outlined the procedure which has been essentially followed up to the present day.

This procedure as set forth 16 years ago involved the inoculation of lactose broth fermentation tubes as a presumptive test with plating on Endo medium or litmus-lactose-agar as a partially confirmed test and with isolation of pure cultures and testing for lactose fermentation and morphology as essential for a completed test. In the fifth edition (*Standard Methods,* 1923) eosin-methylene-blue was substituted for litmus-lactose-agar as an alternative to Endo medium for plating from positive lactose-broth presumptive tests. Otherwise no change was made in basic procedure until the present year. From 1917 to 1933 it was allowable to perform the lactose-broth presumptive test only, in routine examination of raw waters in connection with control of the operation of purification plants and the partially confirmed test only "in the routine examination of water supplies where a sufficient number of prior examinations have established a satisfactory index of the accuracy and significance of this test in terms of the completed test."

Meanwhile numerous efforts had been made to replace lactose broth by some medium which would be so specifically adapted to *B. coli,* and to this organism alone, that a single test for gas formation might serve as the final criterion of its presence. Among the earlier procedures were the use of bile media, of phenol broth, of media containing neutral red, of aesculin media, of liver broth and cultivation at 46° C. References to these earlier studies are given in detail by Prescott and Winslow (1931). Most of them have been abandoned; but the Eijkman test (in-

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† Throughout this paper the term "*B. coli*" is used for the entire colon group, including both Escherichia and Aerobacter. The differentiation between these two forms is not here considered though it has very real importance. Parr and Caldwell (1933) found that the organisms cut out by the use of brilliant-green bile were chiefly of the Aerobacter type.

volving cultivation at 46° C.) has recently been studied by Leiter (1929), Brown and Skinner (1930), and Perry and Hajna (1933); and bile still plays a part in some of the more recent media.

The possibility of utilizing inhibitive dyes in selective media for the isolation of *B. coli* received a powerful impetus from the work of Hall and Ellefson (1918) with gentian violet. Bronfenbrenner, Schlesinger and Soletsky (1920) suggested the use of rosolic acid and Muer and Harris (1920) that of brilliant green. Dunham, McCrady and Jordan (1925) and Jordan (1927) obtained good results with a medium containing both brilliant green and ox gall. Both reported over 90 per cent correlation with the standard procedure. Noble (1928) developed an agar medium containing potassium ferrocyanide, ferric citrate, fuchsin and sodium sulphite. Salle (1929, 1930) experimented with crystal violet and Dominick and Lauter (1929), Salle (1930), and Leahy (1930) with brom-cresol-purple media. Ritter (1932) reported highly encouraging results from Kansas with standard lactose plus 0.8 c.c. per liter of saturated alcoholic basic fuchsin. Out of 33,532 positive presumptive tests in this medium, over 94 per cent were confirmed as *B. coli*. In a series of 533 samples run in parallel the average coli index after confirmation was essentially the same in both media; 17 per cent of the samples showed a higher index in standard broth, 16 per cent a higher index in fuchsin broth. Jordan (1932), a year ago, presented a review of the brilliant green procedure which led to a stimulating discussion.

As a result of all this agitation the Committee on Standard Methods in its seventh edition at last recognized that something had happened since 1917. It provided (*Standard Methods,* 1933) that for " unfinished waters," duplicate inoculation into lactose broth and brilliant-green bile might be employed, with transfer to a secondary tube of brilliant-green bile from any positive primary lactose tube paralleled by a negative primary brilliant-green bile tube. This shortens the test time to between 24 and 96 hours for this class of waters. It is still permissible to rely on a presumptive lactose broth test alone in the case of raw waters subject to purification processes; and it is still necessary to follow out the old routine of isolation and identification for all waters assumed to be potable.

During the past 6 months several important new contributions to this subject have appeared. Nolte and Kramer at St. Louis (1933) found Noble's cyanide-citrate agar unsatisfactory; but Dominick and Lauter's methylene-blue brom-cresol-purple broth gave admirable results. Less than 5 per cent of the lactose-broth presumptives were confirmed as compared with over 76 per cent of the Dominick and Lauter presumptive tests. Furthermore, the Dominick and Lauter medium yielded more confirmed positive isolations than the standard lactose medium (11.4 per cent against 8.9 per cent for unfinished waters and 1.1 per cent against 0.9 per cent for finished waters).

Butterfield (1933), on the other hand, takes a vigorous stand against the use of simplified methods except as permitted by the new edition of *Standard Methods*. He reports new data (for Cincinnati waters) showing that brilliant-green bile gave only 84–90 per cent of the final completions obtained with *Standard Methods* on raw waters and only 35–37 per cent with finished waters. The Dominick-Lauter method was even more unsatisfactory. Butterfield then analyzes the results presented by Jordan (1927) and shows that while on the average for all samples brilliant-green bile (with 2 per cent ox gall) used as a complete test,

gave over 80 per cent of the completed positive tests yielded by *Standard Methods,* the proportion fell to 56 per cent for one source (Cincinnati). Only one other source, however, fell below 70 per cent. The apparent difference between Butterfield's results and those reported by others is, of course, due to the fact that he computes his results in per cent of Standard Method-positives, which are also brilliant-green-bile positive, while most summaries have presented merely the per cent of all samples positive by each of the two methods. Thus, if lactose broth shows 8 per cent of samples positive with lactose broth and 4 per cent with lactose bile, most reports would suggest a close agreement (92 per cent negative by both tests, 4 per cent positive by both tests, 4 per cent divergence). Butterfield would say that the method showing 4 per cent positives was 50 per cent in error.

Still more recently Stewart (1933) has reported favorably on the Dominick-Lauter method, and Parr and Caldwell (1933) have presented a new study of brilliant-green bile. Full data are given for 1,407 samples with the following results: Lactose-broth presumptive tests were positive for 82 per cent of all samples while only 43 per cent of the primary brilliant-green bile tubes were positive. Colon bacilli were confirmed after lactose-broth enrichment in 32 per cent of all samples, after brilliant-green bile enrichment in 24 per cent of all samples. Thus, if the confirmation according to *Standard Methods* be taken as a norm, the brilliant-green presumptive gave, in this instance, results which were 23 per cent too low. These samples (for Alabama wells) contained a large number of anaerobic organisms producing a false-positive reaction in brilliant-green bile.

We may summarize the general situation by saying that the brilliant-green bile medium (which has been most exhaustively studied), when used without any confirmatory procedure, gives us on the average about four-fifths as many positive results as the *Standard Methods* with complete confirmation. Media employing crystal violet, basic fuchsin and, particularly, brom-cresol-purple have also yielded highly encouraging results.

If this were the whole story, the argument for adopting a simplified procedure would be practically conclusive. It sounds impressive to say that the brilliant-green bile test yields only three-quarters or half as many positive results as *Standard Methods;* but such a difference as this has no very great practical significance when interpreted from a practical quantitative standpoint. No sanitarian can rationally condemn a water which shows 8 per cent positive results, and pass one which shows 4 per cent. Actually, of course, we work in powers of 10 in making our dilutions, and even such extreme variations as those recorded by Butterfield would only influence one's decision in a few borderline cases. The loss of one-fifth of the colon bacilli present could not conceivably have any real influence on the practical conclusions to be drawn.

Furthermore, if there were even a greater difference than anyone has suggested, this difference—if it were reasonably uniform—could be allowed for by a change in standards of interpretation. Neither the colon test nor the Treasury Department Standards form part of any law handed down on tables of stone from Sinai. If it seemed more convenient to use a procedure which gave only half as many colon bacilli as the present *Standard Methods* we could quite easily set a limit of one positive 10 c.c. portion in 20 (instead of 1 in 10) as our limit of safety.

The only aspect of the situation which gives one pause is the possibility that marked and really significant differences may exist between different

waters. If it be true that Cincinnati treated waters tested with brilliant-green bile show only 35 per cent as many positives as *Standard Methods,* while with other waters the ratio rises to nearly 100 per cent, the problem is more serious.

Even so, however, the question is by no means closed. We need to know the reason for the differences. Does the Cincinnati treated water contain a large proportion of attenuated colon bacilli which are really of slight sanitary significance? Do the Alabama wells contain anaerobic spore-formers of the *Cl. Welchii* type, which may be much more important than attenuated *Esch. coli?*

We have used the colon test in its standardized form so long that it has become something sacred and untouchable; and that is always a dangerous frame of mind smacking of religion rather than of science. Let us remember that this is a test devised by plain human beings like ourselves and that it was designed to differentiate between safe and unsafe water supplies. The colon bacillus in itself has no inherent significance except as an index of sanitary quality. The isolation of all the colon bacilli present is of no importance except as an index of sanitary quality. What we want is a test which shows the greatest possible difference between good waters and bad waters; and that is all we want. It is quite possible that where the newer bile-dye media yield results different from those of *Standard Methods,* they may be more significant than *Standard Methods* and not less. They appear to give higher results with more polluted waters, and lower results with less polluted waters, which is exactly what we want in a yardstick of this kind.

The standard procedure for isolating *B. coli* has two serious defects. In the first place, it permits the use of lactose-broth fermentation as a final and complete test for raw waters in connection with the operation of purification plants. This is a test which yields results which are often far too high, since certain waters rather regularly contain spore-formers or combinations of symbiotic organisms which are of no sanitary significance but yield positive results. Thus, the efficiency of purification processes is greatly overestimated.

In the second place, *Standard Methods* requires for all waters in a potable state the old complex process of isolation and identification which consumes 6 or 7 days and an enormous amount of time and materials. The procedure was the best available in 1917. It seems to the writer to be archaic and indefensible in 1933.

There seem to be two possible alternatives which are clearly suggested by the progress made in the study of new methods during the past 10 years. The first of these alternatives is the use of simultaneous inoculation into lactose broth and brilliant-green bile with secondary inoculation into brilliant-green bile from positive primary lactose tubes paralleled by negative primary brilliant-green bile tubes. This is the procedure now permitted for unfinished waters; but there seems no valid reason why this type of procedure should not be accepted for all classes of waters. Even Butterfield's analysis shows that this procedure yields on the average 95 per cent of the positive results obtained by *Standard Methods.* He is disturbed by the fact that in results from 4 out of 15 cities the ratio falls below 90 per cent—the figures being 89 per cent for Omaha, 87 per cent for Indianapolis, 86 per cent for Montreal, and 71 per cent for Chicago. It is really difficult to see how such results can be considered as unsatisfactory if one lifts his eyes for a moment from the laboratory table and considers what the colon test really means, the actual uses to which it is put, and the limits of accuracy

within which it can conceivably be interpreted.

Personally, I hope and believe it should be possible to go even further than the general adoption for all waters of the double inoculation into lactose broth and brilliant-green bile. There are several of the new tests (particularly the brilliant-green-bile and brom-cresol-purple media) which seem to offer excellent promise of a single primary fermentation test which could be accepted as final for all classes of waters. For supplies from most regions it seems possible by such procedures to obtain four-fifths as many colon bacilli as by the use of *Standard Methods;* and such a result is well within the range of practical interpretation.

The fact that certain waters when tested by such procedures show divergent results, some higher than when tested by *Standard Methods,* some considerably lower, may be a real disadvantage. On the other hand, it may be an advantage. The use of lactose-broth tubes followed by isolation and identification has, after all, no Divine sanction. It may be that the newer procedures correspond more closely than the old ones to actual sanitary conditions.

My plea is for a fundamental re-examination of this whole problem with an open mind, and with a clear view of the practical results to be obtained, and of the margin of error associated with their interpretation. The pressing need for economy and efficiency makes such a reëxamination peculiarly imperative at the present time. If we can cut down the cost of an examination we can make more examinations; and a fuller measure of control should vastly overweigh any 5 or 10 per cent loss in accuracy.

I would therefore urge upon the Laboratory Section the adoption of definite steps along somewhat the following lines.

1. The bacteriologists associated with the Standard Methods Committee should first make a preliminary survey of the field and select three or four of the most promising of the new procedures (such as the use of brilliant-green bile, brom-cresol-purple, crystal violet and basic fuchsin media or cultivation at 46°). The methods selected should include 1 or 2 which involve the use of a single confirmatory medium following lactose-broth fermentation, and 1 or 2 which involve the use of a single primary selective medium by itself.

2. A considerable group of collaborators should be enlisted to make comparative tests of water samples from various sections of the country, raw waters and finished waters, filtered and chlorinated waters, well waters and surface waters. These collaborators should employ for each sample: (a) *Standard Methods* with full confirmation, (b) one or more methods employing a single confirmatory test following lactose-broth fermentation, and (c) one or more methods employing a single primary fermentation test alone. The work involved need not be very great. For instance, each sample might be inoculated into lactose-broth, brilliant-green-bile and brom-cresol-purple broth. All positive lactose-broth cultures which were negative in either of the other media should be inoculated into a secondary tube of that medium; and all positive lactose-broth cultures should be followed through by *Standard Methods.* The procedure set forth by the central committee for each test should be strictly followed. Records of sanitary inspection should be available to give an idea of the probable sanitary quality of each water.

3. The Central Committee of Bacteriologists associated with the Committee on Standard Methods should assemble all results, and compute comparative averages in various ways for

various classes of waters. Those waters which yield markedly divergent results by various methods should then be studied in detail by obtaining data as to their real sanitary quality.

Such a program, if vigorously prosecuted, should make it possible in 2 years' time to arrive at reasonably definitive results. I believe it would open the way to new *Standard Methods,* in accord with the recent progress in water bacteriology, and not only more efficient and more economical but more significant than those now employed.

The Laboratory Section is the logical body to take such a step. If it fails to do so, some other group is likely to take the initiative, for, to many of us, the present situation seems well nigh intolerable.

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DISCUSSION

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AT the 1932 meeting of this Section I had the opportunity to say a few words on the merits of the MB–BCP medium, which I wish to discuss more fully. I am looking at the isolation of the colon-aerogenes group in water works practice, purely as a laboratory worker and representative of the thousands of water works laboratories and superintendents where the bacteriological analysis of the processed water is necessarily only a part of the