STUDIES ON LACTOSE FERMENTING BACTERIA*

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THE FUNDAMENTAL investigations of Rogers, Clark and Evans [1] on the classification of members of the colon-aerogenes group and the subsequent studies of Clark and Lubs,[2] and Levine [3] on the application of the methyl red and Voges-Proskauer reactions, added an important chapter to our conception of this group of bacteria. Doubtless there was considerable hope among water analysts that these results might prove of much practical value in estimating the sanitary quality of water supplies. It is doubtful, however, notwithstanding the fact that the Standard Methods Committee [4] included the tests in their 1917 report and have continued them in later reports, whether, with the exception of research studies, any extended application has been made of the tests in laboratories required to make routine analyses and to pass opinion on the sanitary quality of individual samples of water.

Principal among the reasons why there has not been a more general adoption of differential tests may be mentioned: first, the lack of close agreement in the results of different investigators and the failure of these results to correlate with the sanitary survey of water supplies; second, failure of the various cultures isolated from water to conform to the two groups defined as fecal (MR+ VP—) and soil (MR— VP+). The lack of close correlation with sanitary surveys would seem to be fundamentally the most important objection.

Levine [5] in an excellent review of this subject discusses the results of all except a few studies of this kind and summarizes the situation as follows: "If . . . the coli and aerogenes sections are so characteristically of different origin, should we not expect a correlation of the sanitary survey with the type of colon bacillus found in a water? Reliable information in this matter would certainly be a great aid in the interpretation of water analyses." And again, "It is felt that the relation of the sanitary survey to the type of colon bacilli present in water, particularly well supplies, is in need of considerable investigation. The lack of correlation in the vicinity of New Haven reported by Winslow and Cohen is difficult to reconcile with the observations of Stokes in Maryland and Wood in England. Possibly local conditions, if taken into proper consideration, may explain the seeming discrepancies."

Subsequent to Levine's summary [5] several investigations have been made with results which do not differ markedly from those in previous studies. For example, Bahlman and Sohn [6] in their studies of 1,223 cultures at the Cincinnati Water Purification Works found no apparent correlation of "rainfall and high river stages with the relative abundance of coli and aerogenes," nor "did the degree to which the water was purified have any bearing upon the incidence of these two sub-groups." These investigators found that 53 of their 1,223 cultures, "survived the invigoration and replating processes and remained atypical in apparently pure culture." This failure to reduce all cul-

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tures to the recognized groups was also emphasized by Perry and Monfort,[7] who thought that "further study of these variants prior to rejuvenation is essential to their correlation with known pollution." Cunningham and Ragavachara[8] in a very extensive study of the waters in India report 30.6 per cent, 25.5 per cent, and 31.9 per cent "MR— VP+" types in 2,455 cultures from rivers, impounded surface water, and springs and wells respectively. They state, "there is no substantial difference to be found in the distribution of the two groups if the samples are divided according to their source." Koser[9] in a comparison of the methyl red and Voges-Proskauer reactions with growth in his citrate medium as applied to cultures from polluted river water, and water of high sanitary quality, found but a slight difference in the percentage of "MR+ VP—" cultures in the two types of water. He remarks "If we are to consider all methyl red positive and Voges-Proskauer negative cultures as indicating intestinal origin then we must conclude that about the same proportion of fecal cultures may be found in both polluted and unpolluted samples."

Although a wide variety of water samples have been included in many of these investigations, there have been apparently few studies attempting to correlate the sanitary survey and distribution of coli and aerogenes on a representative group of wells or an attempt to apply these tests to particular types of wells used as private or public supplies except the investigations by Wood[10] and Stokes.[11] The former felt that there was a correlation between the aerogenes and sanitary survey in 66 samples but he does not state definitely how the differential tests correlated with sanitary survey in the remainder of his samples. Wood thought his results were significant and suggests that this differentiation be employed in routine examinations of water and food products. Stokes unfortunately was unable to complete his studies by correlating his results with the sanitary survey of wells. He found only one low-ratio organism in 14 samples from artesian wells, but he was doubtful whether the differential tests would be practical in routine analyses, principally because it would be necessary to study so many cultures from each sample.

In view of the foregoing, we are presenting the results of two studies of this type which have been made on carefully selected samples. The two series consist of samples from ground water supplies in an urban and in a rural community and these are representative of such supplies in Ohio. The laboratory studies vary slightly in the two series because several years elapsed between them.

The differential tests applied to the first or urban series were confined to the methyl red and Voges-Proskauer reactions. In the rural series we gave first attention to these two reactions because we thought the correlation of these with the sanitary survey was of prime importance. Later, the cultures isolated in the rural series were tested for indol production. This test was used in preference to Koser's uric acid medium because the latter had shown very close correlation with the methyl red and Voges-Proskauer reactions, and, with a limited time available for testing the freshly isolated cultures, it seemed more desirable to utilize the indol test since so much importance has been attached to it by English analysts.

Subsequent to our studies of the two series just described, Koser recommended his citrate medium,[12] and we began a similar study of a much larger number of wells with special reference to the correlation of the citrate medium with the methyl red and Voges-Proskauer reactions and the sanitary survey of the wells. The latter results are the subject of a separate report.

SAMPLES FROM URBAN SUPPLIES

The 198 samples in this series were collected in a village with a population of

TABLE I

DISTRIBUTION OF "MR+ VP—" CULTURES IN WELL WATERS OF DIFFERENT SANITARY QUALITY

Field grade	Total No. wells	Wells giving confirmed test for Bact. coli		Total cultures	MR+	Per cent	VP—	Per cent
		Number	Per cent					
Good	78	20	25.7	40	33	80.2	31	77.5
Fair	90	20	22.2	41	33	80.4	31	75.6
Poor	30	13	43.3	27	24	88.8	25	92.5

about 1,000 and they represent all except 4 wells in the village. All samples were collected by R. S. Durrell, Assistant Engineer of this department, after a careful sanitary survey in which he classified the supplies as "good," "fair," and "poor," on the basis of their location and development. Samples were delivered to the laboratory as promptly as possible. Whenever the interval between the collection and the examination was more than a few hours, the samples were kept on ice. In several instances check samples were collected from supplies in question.

METHODS OF EXAMINATION

Bacteriological examinations were made according to Standard Methods. In addition to the routine tests, a separate set of presumptive tests were made in lactose peptone-bile, and an Endo plate was made, using 1 c.c. of the sample. In selecting colonies from the Endo plates made either from the sample or from the presumptive tubes, an effort was made to select representatives of the different types of colonies. First, attention was given to colonies which gave a typical *Bact. coli* reaction and appearance, but whenever other types were present, one of each was selected for subsequent study. These colonies were fished into lactose broth for confirmatory gas. Agar slants were prepared from the confirmatory tubes which proved positive, and at the same time dextrose broth was inoculated for testing the methyl red and Voges-Proskauer reactions.

Table I shows the total number of samples under each of the groups established by sanitary survey, also the number which gave a confirmed positive test for the colon group, and the relative distribution of the so-called fecal types (MR+ VP—) in the different grades of wells.

It will be seen that the percentage of MR+ and VP— cultures show a fairly close correlation of these two tests. Further, it will be noted that there is no great difference in the distribution of the fecal (MR+ VP—) type in the three classes of waters. One cannot, of course, make direct comparisons between these results and those found in studies where grossly polluted supplies and waters of excellent quality were used as extremes, because even though some of our wells were classed as "poor" they still were within reasonably safe limits for drinking water, and were being used for that purpose at the time of sampling. It would not be expected, therefore, that one would find quite so high a percentage of fecal types in any of our samples in this series as would be found in rivers or other grossly polluted water. It is surprising, however, to note the high percentage of this type (MR+ VP—) in well waters classed as "good" and "fair" by the sanitary survey.

We found very little difference in the percentage of coli and aerogenes types isolated from presumptive tubes positive in 24 hours, and those positive in 48 hours. This would indicate that there is not so much tendency to overgrowth of one type or another as has been suggested by some investigators.

COMPARATIVE TESTS WITH LACTOSE BROTH AND LACTOSE BILE

Ritter [13] in a comparison of lactose bile and lactose broth on surface waters found when lactose broth alone was positive in the presumptive test about 70 to 78 per cent of the tubes were "proved not to contain *B. coli*." Our results in numerous

routine samples of ground waters have shown that a very much smaller per cent of the positive lactose broth presumptive tests fail to confirm, and it was found in this series of well waters that Ritter's percentages were practically reversed. We found that while a higher per cent of the presumptive-positive bile tubes were confirmed, 36 out of 42, as compared with 44 out of 66 positive lactose broth presumptives, the latter actually gave a larger number of positive colon tests in the "good," "fair," and "poor" quality waters; a total of 44 positive results being obtained in broth and 36 in bile. Furthermore, the relative number of "MR+ VP—" cultures was about the same in both mediums. It appears, therefore, that lactose broth detects more colon group organisms in ground waters of "good," "fair," and "poor" quality, and that this excess of positive colon results is not altogether due to non-fecal varieties, a condition which we thought might be found. Our results seem to further confirm Jordan's conclusion that bile is inhibitive to a certain per cent of colon bacteria. Also our experience in finding about the same number of fecal and non-fecal types on plates from broth or bile apparently conforms with the experience of others. For example, English investigators, Wood,[10] and Cunningham and Ragavachara,[8] using bile-salt media report about the same general percentages of the two types as were found by Winslow and Cohen and other American investigators in similar waters but using media containing no bile.

RURAL SUPPLIES

The work included in this series was part of a program of water supply survey in a county health demonstration. A careful survey was made of the wells in one township and samples were collected in duplicate from each well. All bacteriological examinations were made according to Standard Methods.

A total of 89 wells were examined. These were classified by field sanitary survey and type of well as follows:

TABLE II

Field Survey	Total wells	Drilled		Dug	
		No.	Per cent	No.	Per cent
Good	30	21	72.0	9	15.0
Fair	29	4	13.7	25	41.6
Poor	30	4	14.3	26	43.3
		29		60	

Examined in the routine manner for the colon group, the samples from these supplies gave the following results:

TABLE III

Colon group	Drilled		Dug	
	No.	Per cent	No.	Per cent
Pos. 1 and 10 c.c...	6	21.4	43	70.4
Pos. 10 c.c. only...	6	21.4	14	22.9
Neg. 1 and 10 c.c...	16	57.2	4	6.7
	28		61	

These figures show about what one would expect in the two types of wells, namely, a relatively high percentage classed as "good" in the drilled wells, both by sanitary survey and the colon test, and the reverse of this condition in the dug wells which are often poorly located and constructed.

DIFFERENTIAL TESTS

A correlation of colon-group results, and methyl red and Voges-Proskauer reactions with the sanitary survey is shown in Table IV. The result of the indol tests on these cultures is also included in this table. The figures represent combined results from the drilled and dug wells.

It is apparent from these results that there is about the same correlation of the methyl red and Voges-Proskauer tests with source of the samples, as was found in the urban series (Table I). Also it

TABLE IV

Field grade	Total samples	No. containing colon bacteria in 10 c.c. or less	Per cent	Total cultures isolated	MR+		VP—		Indol+	
					No.	Per cent	No.	Per cent	No.	Per cent
Good	60	14	23.3	53	36	67.9	36	67.9	22	41.5
Fair	58	23	39.6	76	61	80.2	59	77.6	34	44.7
Poor	60	28	46.6	100	92	92.0	86	86.0	62	63.0

will be noted that while the total percentages of indol-positive cultures are considerably lower than the percentages of methyl red positive and Voges-Proskauer negative cultures, the degree of correlation of the three tests with sanitary survey is about the same. The actual differences in the percentages of fecal types in "good" and "poor" wells as determined by methyl red, Voges-Proskauer, and indol tests are 24.1, 18.1, and 20.5 per cent respectively. Approximately the same difference is noted between the confirmed *Bact. coli* tests on the "good" and "poor" types of water.

Somewhat more striking figures were obtained when we considered the results on the drilled wells alone, for in these we presumably had few waters that were seriously polluted. Especially in the drilled well supplies graded "good" in the field we should expect a minimum of fecal types. Actually, however, we found that 16 of the 21 cultures isolated from drilled wells graded "good" by field survey and found to contain colon-group organisms, were "MR+ VP—." This would seem to be the strongest evidence in this study against the specificity of either of these tests in representing fecal contamination.

COLON GROUP RESULTS COMPARED WITH COUNTS AT 20° AND 37° C. AND WITH SANITARY SURVEY

It was customary in this laboratory until recently to make counts on agar at both 20° and 37° C. on all water samples. However, we noted so many instances in which there was such a complete lack of correlation between these counts and the colon results that we discontinued the 20° C. counts on samples from private supplies, believing that the results were of little value in estimating the quality of the water. Considerable attention was given to this question and while it does not seem necessary to include the tabular comparison of these figures, it may be of interest to note the general summary from these tables.

We found that in 233 samples from urban private supplies and 178 samples from rural supplies, the percentage of low counts (less than 100 per c.c.) at 37° C. in the "good" wells containing colon group bacteria was about the same as the percentage in the same type of wells which were negative for colon group organisms, viz., 83 per cent and 88 per cent respectively. Also in the "poor" type wells the percentage of low counts (less than 100 per c.c.) at 37° C. was rather high even in samples containing *Bact. coli.* Furthermore the percentage of samples giving less than 100 bacteria per c.c. on agar at 20° C. was practically the same in "good," "fair," and "poor" wells. Although each sample must be interpreted individually, and totals or averages of groups of samples may be misleading in some respects, we believe, nevertheless, that in examining private supplies neither the 20° nor 37° C. counts add much to our knowledge of the sanitary quality of the water, and that the time spent in making such counts could be used to better advantage in the estimation and study of any lactose fermenting types present in the water.

SUMMARY AND DISCUSSION

A total of 287 well waters varying in sanitary quality, as determined by careful survey of the source, from "good" to "poor," were examined for colon bacilli with particular reference to the value of the methyl red and Voges-Proskauer reactions. A majority of these wells (198) were located in a village and therefore were probably subject to more direct contamination with human discharges because of the proximity of outdoor toilets on the same or neighboring lots. The remainder of the wells (89) were in a rural district where the danger of contamination with human discharges would probably be less but the danger from animal discharges greater than in the village wells. The percentages of methyl red positive and Voges-Proskauer negative cultures in the urban and rural wells were

practically the same. It is understood, of course, that no claim has ever been made that these two tests would distinguish between human and animal discharges, but it was thought that possibly some difference might be found in the relative numbers of fecal and soil types in the two groups of wells.

There was an appreciable difference in the percentage of so-called "fecal" types in the "good" and "poor" wells in each series. This difference was apparent to about the same degree on the basis of the methyl red, the Voges-Proskauer, and the indol test, and it agreed rather closely with the difference in percentage of confirmed colon tests in the respective wells. It is difficult to place a strict interpretation on these figures because there is no established scale of expected frequency of the fecal and soil types as we progress from a grossly polluted water to one of excellent quality. The important point is whether too many "good" wells show fecal types as established by the differential tests. The percentage of fecal types in the "poor" wells is of minor importance, for these supplies would be condemned on the sanitary survey alone.

The striking feature of the results in both rural and urban wells was the number of "MR+VP—" cultures in the "good" wells, and particularly in the "very good" drilled wells. It is scarcely conceivable that 70 per cent of the colon group bacteria in these supplies are actually of fecal origin. The only satisfactory explanation which conforms with our conception of safe well water, as determined by field sanitary survey, is that many of the cultures constituting this 70 per cent are soil types and are therefore of slight sanitary significance. This confirms Koser's conclusion based on his sanitary survey of the waters he examined and on comparative tests with his citrate medium.

Without attempting to attach too much importance to the indol results in this series it is perhaps worth noting that the lower percentage of indol positive cultures correlates more closely with the sanitary survey than does either the methyl red or Voges-Proskauer reaction.

CONCLUSION

Absence of methyl red positive and Voges-Proskauer negative members of the colon group in ground waters of good sanitary quality is significant, but the presence of this type in such waters cannot be regarded as conclusive evidence of fecal contamination. These differential tests, therefore, have very limited value in the analysis of well waters.

REFERENCES

1. Rogers, Clark and Evans. *J. Infect. Dis.*, 15:100, 1914; *ibid.*, 17:137, 1915.
2. Clark and Lubs. *J. Infect. Dis.*, 17:160, 1915.
3. Levine. *J. Infect. Dis.*, 18:358, 1916.
4. Standard Methods of Water Analysis. American Public Health Association, 1917, 1920, 1923.
5. Levine. Iowa State College, 1921, *Bulletin 62:* 78, 81.
6. Bahlman and Sohn. *J. Am. W. W. Assn.*, 11:416, 1924.
7. Perry and Monfort. Illinois Water Survey, *Bulletin No. 16*, p. 208.
8. Cunningham and Ragavachara. *Indian J. M. Research*, 12:75, 1924.
9. Koser. *J. Infect. Dis.*, 35:14, 1924.
10. Wood. *J. Hyg.*, 18:46, 1919.
11. Stokes. *A. J. P. H.*, 9:571 (Aug.), 1919.
12. Koser. *J. Bact.*, 8:493, 1923; *ibid*, 9:59, 1924.
13. Ritter. *J. Bact.*, 4:609, 1919.