THE IMPORTANCE OF SLOW LACTOSE FERMENTERS IN WATER ANALYSIS

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HISTORICAL BACKGROUND

Many men have reported the presence of Bacillus coli in the intestinal and urinary tract of man and in a few instances they have been noted in the feces of horses and cattle. Gilbert and Lion (1893), appear to have been the first to describe paracolon bacilli. Morgan and Bedingham (1909), encountered such types in human feces and divided them into three groups. On the basis of cultural characters, they reported them as numerous in cases of diarrhea but regarded them as of little pathogenic significance. On the other hand, GyÖrgy (1920) suggested that they played a considerable part in diarrhea in both man and calves.

Jones, Orcutt, and Little found, during a study of infectious diarrhea in cows, motile Gram negative rods which failed to attack lactose. Their numbers varied greatly; at times they made up 90 percent of the organisms, at others they were present in relatively small numbers, and in many cases no such colonies were found. In one instance they were found throughout the intestine of a cow slaughtered during an attack of diarrhea.

In earlier work Jones, Orcutt, and Little found many non-lactose fermenters from the intestines of cows having diarrhea. In some epidemics all cows had them, in others very few had them, and in still later epidemics none were found. Upon examination of healthy cows (50) only one atypical colon bacillus was found showing that they are

probably not present in large numbers in the intestinal tract of normal cows although they may be the predominating types during certain intestinal disorders.

Culturally the above three workers found that all strains readily attacked glucose, mannitol, maltose, and xylose, with the production of acid and gas, failed to ferment rafiinose, but fermented lactose slowly. In addition all gave a negative Voges-Proskauer test, positive methyl-red test, produced indole, and failed to liquefy gelatin or produce hydrogen sulphide. Upon cultivation it was found that cetain strains of the atypical form could be built up to fast lactose fermenters by rapid transfer and others could not. It was not possible to again knock them down after they were built up.

When put into Durham lactose fermentation tubes, it was found that the organisms grew rapidly but did not produce acid reactions. This experimental evidence showed that the bacilli utilized the lactose but at the same time produced alkali, for a time at least, in sufficient quantities to mask the acidity of fermentation. The indicator in the tubes remained unchanged for two or more days. Acidity was first noticed at the bottom of the tube, and the reaction gradually changed, accompanied by a moderate gas production. Ten days or more was required to change the color of the indicator completely.

On lactose agar plates these organisms may readily be mistaken for paratyphoid bacilli, especially when

freshly isolated and cultivated in glucose, lactose, and sucrose. They also form smooth transparent colonies on agar plates.

Immunologically it was found that members of the group differ only in degree rather than constitutionally.

CLASSIFICATION

According to Stuart, Mickle, and Lorman, since so much confusion in terminology has occurred in the literature relating to slow-lactose fermenting members of the coliform group, it was suggested that the term "aberrant coliforms" be used to describe all Gram negative, non-sporulating rods which ferment lactose weakly at 37° C.

They also suggested a tenative separation of the aberrant types into four groups based on a study of more than 10,000 coliforms isolated from water, soil, milk, feces, and other sources. The classification proposed is as follows:-

Aberrant Coliforms

Strains differing from typical coliforms in respect to their fermentation of lactose (producing less than 20 percent gas in 48 hours at 37° C.)

I. Micro-aerogenic Coliforms - aberrant coliforms producing gas from lactose slowly or in small amounts at either 37° C. or 20° C.

II. Pseudomicro-aerogenic Coliforms - aberrant coliforms having the characteristics of the true micro-

aerogenic strains at 37° but showing normal lactose splitting activity at 20° C.

III. Papillae-forming Coliforms - aberrant coliforms showing the type of dissociation evidenced by Bact. coli mutabile, but not confined to the genus Escherichia.

IV. Anaerogenic Coliforms - aberrant coliforms producing acid but no gas from lactose.

The existence of a group of non-lactose-fermenting coliforms is recognized, but this group is not sufficiently well delineated for satisfactory discussion.

I. Micro-aerogenic Coliforms - belong to Aerobacter, Intermediate, or Escherichia groups. The produce acid in lactose and gas: a bubble to 10 percent gas in 48 hours at 37° C., some 20 to 100 percent gas in 3 to 9 days at 37° C. Acid and gas are produced more slowly at 20° C. The organisms grow as well in 24 hours in lactose as typical cultures, but at the end of 24 to 36 hours the lactose becomes strongly alkaline. This is followed first by acidification of the broth in the insert, and then (1 to 4 days later) by a general acid reaction and appearance of gas bubbles.

On Eosin-Methylene-Blue agar many produce only white colonies, and when isolated do not ferment lactose any faster than the parent colony. If the plates are left in the incubator, the centers of the colonies become black, but no change occurs in the speed of fermentation of lactose.

These organisms have been isolated from normal and pathological human feces in small numbers and were almost invariably accompanied by coliforms which produced normal gas amounts. The workers had encountered appreciable numbers of all three strains (Aerobacter, Intermediate, Escherichia) in public water supplies, mostly from samples of untreated water.

II. Pseudomicro-Aerogenic Coliforms - comparable to Aerobacter and Intermediate strains. Cultures were isolated from water, soil, and milk, but none from 600 fecal samples. They produce in lactose broth acid and gas: some a bubble to 10 percent in 48 hours at 37° C., others no gas in 21 days at 37° C. On E.M.B. agar at 37° C. they produce colonies from typical coliform down to colonies barely visible to the eye. Large colonies; raised, moist, confluent with dark center, or flat, dry, and discreet with large black center and high metallic luster. Small colonies; similar to large except the smaller the colonies, the less intense the black center, the very small ones being entirely white.

It was discovered that when the plate was left at room temperature for 24 to 48 hours, all colonies became large and typical. When tests (duplicate) were run on cultures at 20° and 37° C., they found a marked variation in results. At 20° C. all cultures produced 20 to 100 percent gas in lactose in 24 to 72 hours, were Methyl-Red negative and Voges-Proskauer positive. At 37° C. they

produced from a bubble up to 10 percent gas in 24 to 48 hours and were positive to some degree in both the Methyl-Red and Voges-Proskauer tests.

On running growth curves;- 37° C. for 18 to 24 hours the growth was stationary or on the decline; 20° C. for 18 to 24 hours the growth was in the logarithmic phase.

They found that cultures put into preheated lactose (37° C.) and incubated at 37° produced acid in only 3 out of 32 in 48 hours and no gas. Others either died or did not produce gas or acid in two weeks. The cultures transferred into lactose broth at room temperature and then placed into the incubator (air) produced a bubble to 10 percent gas. This showed that the organisms began growing before the broth warmed to the 37° temperature.

III. Papillae-forming Coliforms - may belong to Aerobacter, Intermediate, or Escherichia, but the greater majority appear to be Escherichia.

In lactose at 37° C. they may produce acid and small amounts of gas in 48 hours. Later as much as 30 to 40 percent gas may be produced. These organisms, by repeated transplanting, were induced to produce 20 percent gas in 18 to 24 hours.

On E.M.B. agar both black and white colonies develop. The black colonies resemble typical coliforms and when picked produce 20 percent or more gas in 18 to 24 hours. The white colonies in 2 to 5 days form small, black, daughter colonies within the parent colony. The white

colony, when put into lactose, produces acid and gas slowly.

Better than half of the fecal samples collected from 100 cases of gastroenteritis produced this type of aberrant coliform organism. None were isolated from the feces of normal individuals.

Facts show this type to be, probably, the most important of aberrant coliforms. Frequently they are not detected by Standard Methods of Water Analysis since many strains do not produce gas or even acid in 48 hours.

IV. Anaerogenic Coliforms - produce acid in lactose in 1 to 7 days at 37° C. but no gas. They have a close resemblance to Aberthella and Shigella. They were isolated from soil, water, cereals, normal and pathological feces. Several were found in an outbreak of gastroenteritis.

Stuart, Mickle, and Borman stated that their knowledge of these types was so limited that no suggestion of their relationship to the coliform group or of their sanitary significance could be advanced.

IMPORTANCE

Stuart, Mickle, and Borman state that the exact sanitary significance of the aberrant coliforms is not entirely clear. The true microaerogenic coliforms (Group 1) probably have a significance similar to that of comparable Aerobacter, Intermediate, and Escherichia strains showing typical lactose fermentation. They conceded no

significance to the pseudomicro-aerogenic group(Group II) which is made up entirely of low temperature-loving members of the Aerobacter section together with a few Intermediates isolated from soil, water, and milk, and which they had not isolated from feces in their investigation. They found that the papillae forming group (Group III) could be determined only infrequently in routine coliform estimates in which gas production is used as the criterion for their presence, and the anaerogenic group (Group IV) would not be detected at all. However, they think that these two groups may be highly significant because of their frequent association with gastroenteritis and genito-urinary infections. They state that "certainly no aberrant coliform giving a positive completed test in accordance with a strict interpretation of Standard Methods of Water Analysis should be disregarded without a further study of its characteristics".

Ziegler states that non-lactose fermenting and late lactose fermenting organisms have been isolated from polluted water supplies and from diarrhea patients. The agglutination of, at least, two varieties of such organisms occurred in high dilutions of patients' serum which indicates that they are a cause of the infection. During the latter part of a study of an outbreak of diarrhea in Springfield, Ziegler found that typical Esch. coli were not isolated from the water supply, but pathogenic non-lactose fermenting intestinal types of organisms were

present indicating pollution which was not detected by Standard Methods of water analysis.

McCrady states that "since the coliform density of a filtered,treated, or filtered-treated water is a measure of the effectiveness with thich the treatment has eliminated disease organisms from the water, it is reasonable to attribute equal sanitary significance to the presence in such water of either typical or atypical coliform organisms, slow lactose fermenters or rapid.

"Slow lactose fermenters are found, although in small proportion, in fresh feces, in greater proportion in stored feces, and occasionally in discharges from cases of gastrointestinal disturbance; the presence of the organisms, even in natural waters, therefore, cannot always be dismissed as of negligible sanitary significance. Furthermore, since other organisms contained in the sample may reduce the amount of gas produced in lactose broth by typical coliform organisms, the volume of gas produced cannot be accepted as a sure indication of the type of organism present."

Data were collected by McCrady on questionnaires to 25 laboratory workers as to their ideas on the significance of slow lactose fermenters. Fifteen were in favor of including them, six were in favor but did not, one favored including those from filtered and treated waters but not from natural, and three were not in favor of including them at all.

Parr states that Clemsha's "Bacillus P" is a good deal like slow lactose fermenters since it produces only acid in lactose with small amounts of gas if any, and sometimes neither. Bacillus P is said to be common in the feces of gastroenteritis patients.

Dulaney and Smith state that they feel that they "must recognize the widespread distribution of such coliform organisms (slow lactose fermenters) in untreated waters of apparently satisfactory sanitary state. They may or may not be detected in the routine examinations. This finding should invite caution as to interpretation of their presence at any time. The results from sanitary surveys should be carefully applied and such data correlated with bacteriological findings. At the same time we must recognize that their non-fecal origin has not yet been proved and all information regarding their potential pathogenicity should be carefully accumulated and evaluated."

EXPERIMENTAL WORK

As shown in the literature, the incidence of slow lactose fermenters in water, both treated and untreated, is high. This naturally constitutes a serious problem in the bacteriological examination of water. This thesis is for the purpose of trying to find some solution to this problem.

Several cultures have been sent to this laboratory

which were isolated from water samples that had passed the presumptive and partially confirmed tests in the Standard Methods of water analysis as unpotable, but had failed to confirm. In some instances the gas production in the presumptive test had been as high as 90 to 100 percent, but the organisms had still failed to confirm. Upon isolation of some cultures from these water samples, it was found that microscopically they were Gram negative rods, and when put into lactose broth, they produced only a bubble of gas in 48 hours, but produced from a bubble to 10 percent in three to five days. In many cases no gas at all was produced in 48 hours.

Since the atypical reaction of these organisms occurred only after the partially confirmed test, it seemed logical that the Eosin-Methylene-Blue agar had a toxic effect on the gas production. Experiments were then conducted with a pure culture of Escherichia coli to determine if this were true. A suspension of Esch. coli from an agar slant was placed in sterile distilled water and transplants were taken daily and subjected to the standard procedure of water analysis by first placing them in lactose broth, incubating them at 37° for 48 hours, streaking on E.M.B. agar plates, incubating these at 37° for 24 hours, and then picking colonies from the plates and transplanting them back into lactose broth. Readings were taken on both the primary and secondary lactose broth tubes at 24 and 48 hour periods. It was found that there

was no inhibition of gas production using a pure culture of Esch. coli

Since the above method did not produce the desired results, some of the cultures of atypical organisms which had been sent to this laboratory were then subjected to approximately the same procedure. Eight of these cultures were placed in lactose broth and incubated for 48 hours. Transplants from these tubes were then streaked on both E.M.B. agar and plain nutrient agar. Colonies were then picked from these plates at one, two, and three days and placed in lactose broth. Readings were taken on these tubes at 24, 48, and 72 hours, and it was found that there was a definite inhibition, with the exception of a few cases, of their gas production (Table No. I)

An attempt was then made to increase the gas production of thirteen slow lactose fermenters. It was found that their gas production could be increased slightly, but not more than 5 percent at the most. This was accomplished by rapid transfer methods in lactose broth of pH 6.9, i.e., transferring every 24 hours to a new lactose broth tube. When lactose broth of pH 7.8 was used, it was found that there was a definite inhibition of gas production, probably due to the formation of carbonates by the reaction of the medium with the carbon dioxide produced. Lactose broth with a pH of 7.2 was then prepared and used, and it was found that there was still a slight inhibition of gas production.

TABLE NO. I

Eosin-Methylene-Blue Agar

	1 day				2 days			3 days		
Culture	48	24	48	72	24	48	72	24	48	72
1	25%	5%	15%	20%	bub	5%	8%	bub	10%	15%
2	30%	20%	25%	30%	bub	5%	8%	bub	8%	10%
3	10%	no growth			0%	bub	bub	bub	bub	5%
4	25%	bub	15%	20%	0%	bub	bub	0%	bub	8%
5	30%	10%	50%	50%	8%	15%	25%	bub	15%	15%
6	30%	8%	20%	35%	10%	10%	20%	20%	30%	35%
7	10%	bub	8%	8%	bub	bub	8%	bub	bub	bub
8	20%	bub	20%	40%	0%	bub	8%	bub	20%	25%

Plain Nutrient Agar

1	25%	8%	20%	30%	10%	20%	30%	bub	8%	10%
2	30%	5%	20%	25%	bub	8%	10%	8%	25%	30%
3	10%	5%	25%	35%	0%	8%	40%	bub	30%	30%
4	25%	bub	30%	35%	0%	8%	40%	bub	20%	25%
5	30%	8%	50%	60%	5%	10%	25%	bub	10%	25%
6	30%	10%	30%	40%	bub	5%	10%	8%	40%	50%
7	10%	bub	15%	20%	bub	5%	8%	bub	10%	30%
8	20%	8%	30%	40%	0%	bub	25%	bub	30%	40%

Since the attempt to produce typical Esch. coli from the slow lactose fermenters by rapid transfer methods failed, the reverse was tried, and several methods were used to try to attenuate a pure culture (typical) of Esch. coli so that it would take on some of the properties of the slow lactose fermenters that had been isolated. The procedure used was as follows:- a pure typical culture of Esch. coli was grown in nutrient broth for 24 hours, and then put on plain nutrient agar slants. These, in turn, were then incubated for 24 hours so that a good growth would be obtained, and then the cultures were scraped off into flasks containing different dilutions of brilliant green, copper sulphate, sodium arsenate, and lithium chloride. This procedure yielded a good suspension of organisms in each of the flasks. The flasks were then placed at varying temperatures and transplants taken from them at intervals:- brilliant green in 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 20 days; lithium chloride and sodium arsenate in 1, 2, 3, 4, 6, 7 days. Transplants from these flasks were placed in lactose broth and then run through the standard procedure for water analysis. It was found that the copper sulphate was too toxic and killed the organisms in one to two days, but up until the time that the organisms were destroyed they did not show any suppression of gas production. It was also found that a temperature of 42° C. was too high for the organisms, the flasks becoming sterile in one to two days.

The above experiment proved that, by the methods used, slow lactose fermenters could not be produced from a pure typical culture of Esch. coli. However, Hershey and Bronfenbrenner report that they were able to build up the gas production of slow lactose fermenters by "rapid transfer methods" and were also able to repress it again by partially destroying the lactase by the use of sodium succinate.

Shortly after completing the above experiments, a water sample was received in our laboratory for analysis which produced gas in all five primary lactose broth tubes and also in all five tubes of the tryptose-lactose-laurylsulfate broth (T.L.S.) of Mallmann which was run in parallel with the standard lactose broth, upon streaking from the tubes to E.M.B. agar, however, it was found that from the lactose tubes two typical Aero. aerogenes, one typical Esch. coli, and two atypical organisms were obtained; and from the T.L.S. broth three typical Esch. coli and two atypical organisms were obtained. Colonies were picked from all 10 plates and seeded into lactose broth. All colonies produced gas except two which were picked from plates streaked from the T.L.S. broth. These produced small bubbles of gas in 24 hours, and 10 and 15 percent gas in 48 hours.

Later ten atypical colonies were picked from each plate showing the slow lactose fermenters and planted into lactose broth. Of the 20 colonies that were picked,

9 of them produced only a bubble of gas in 24 hours, and in 48 hours one produced 20 percent gas, one 15 percent, and the rest 10 percent or less. All 20 of these lactose tubes were then streaked on E.M.B. agar and incubated for 24 hours. Duplicate colonies were then picked from each plate, and from the 40 colonies picked, 10 produced 8 percent gas or less in 48 hours.

Meanwhile other water samples had been received at the laboratory for analysis that contained many slow lactose fermenters. Four colonies were picked from each plate showing atypical colonies, and of the four, two were placed in lactose broth and the other two in T.L.S. broth. The gas production was recorded at 24 and 48 hours, and if 8 percent gas or less was produced in 48 hours, a slant culture was made from the tube and kept. In this manner 108 slow lactose fermenters were collected from 7 water samples.

These organisms were then identified according to the classification of coliform organisms found in water as stated in Topley and Wilson. No tests were run on gelatin, nor was the Eijkman test run. A summary of the results is as follows:-

Escherichia coli

Variety I - 5 cultures

Variety II - 3 cultures

Intermediates

Variety I - 60 cultures (3 others conformed but did not give M.R. test)

Variety II - none

Aerobacter aerogenes

Variety I - 29 cultures (5 others conformed but gave M.R. test)

Variety II - 2 cultures

One culture could not be identified according to the classification since it gave only a positive Voges-Proskauer test, the rest of the tests being negative.

In running these tests, the Barritt modification of the Voges-Proskauer test was used and run on a 40 hour culture of buffered dextrose broth. The citrate test was read in 60 hours, and the indole and methyl-red tests were run on 64 hour cultures.

In running the routine water analyses, it was noticed that the average gas production of standard double strength lactose was 21.6 percent per tube, while the average gas production of T.L.S. broth was 26.5 percent per tube. It was also noted that of the 35 plates streaked from lactose broth, only 13 of them gave the typical metallic sheen; while of those streaked from the T.L.S. broth, 23 gave the typical metallic sheen.

Another thing of interest which was noted was that where the growth was heavy on the E.M.B. plates there occurred the typical metallic sheen, but where the organisms were more spread out and well isolated colonies were formed, the typical sheen was not present. Upon picking these well isolated colonies, it was found that they were often the slow lactose fermenters, while, on the other hand,

those with the sheen gave typical lactose fermentation.

CONCLUSIONS

From the above experiments and observations it would be logical to conclude that the incidence of slow lactose fermenters in water samples of both treated and untreated water is quite high. However, even though present, these organisms do not show up in the standard method of water analysis, but, as shown in the literature cited, it is quite probable that their presence can mean fecal contamination. Thus they should have all the significance of typical Esch. coli with its regard to water analysis.

These atypical organisms may be missed in the partially confirmed test when the E.M.B. agar plates are streaked from primary lactose tubes rather than from the L.S.B. broth due to the absence of the typical metallic sheen. If, however, the typical reaction is obtained on the heavily seeded partof the plate, it is quite possible that the well isolated colonies are of the atypical variety. Since the Standard Methods of Water Analysis states that well isolated colonies shall be picked for the completely confirmed test, these well isolated colonies, being atypical, will probably produce little or no gas in 48 hours. In addition, these organisms will have been subjected to the toxic effects of the E.M.B. agar, and thus will have their gas production further depressed, Consequently, the chance of detecting the presence of these slow

lactose fermenters is quite small, but they should be detected since they are an indicator of pollution.

SUGGESTIONS

From the above data presented, it would seem that the following procedure for the bacteriological examination of water might be added to the method already given:-

Presumptive Test - Double strength tryptose-lactose-lauryl-sulfate broth should be used since it gives greater gas production, and the typical Esch. coli reaction on E.M.B. agar has a higher incidence.

Partially Confirmed Test - If the typical metallic sheen is present in 24 hours on the E.M.B. agar plates, this could be accepted as the final test since Norman, Robinton, and Stuart state that only 0.05 percent of the colonies giving the metallic sheen fail to confirm. This would also do away with the possible picking of atypical colonies which might have been well isolated.

Confirmed Test - If this test is run, it is advisable to pick colonies that show the typical sheen even though they may not be well isolated.

SUMMARY

In this paper the literature concerning the discovery, classification, and importance of slow lactose fermenters was reviewed. The experimental work was then related.

the toxicity of Eosin-Methylene-Blue agar toward gas production was tested for by streaking plates with a pure typical culture of Esch. coli, but it was found that there was no gas inhibition. Atypical cultures were then used in the same manner, and it was found that their gas production was decreased by the E.M.B. agar.

It was then attempted to increase the fermentation of the atypical cultures by rapid transfer methods, but it was found that it could be increased only slightly. It was also attempted to decrease the fermentation properties of a pure culture of Esch. coli, but this,too, met with failure.

Water samples were then received in the laboratory which contained slow lactose fermenters. From 7 water samples, 108 cultures of slow lactose fermenters were isolated. These were then identified and found to be mostly of the intermediate variety.

Suggestions were given as to the procedure which might be used in the bacteriological examination of water to aid in the detection of slow lactose fermenters that might be present.

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