The Fallacy of Refined Readings of Gas Percentages in the Fermentation of Lactose Peptone Bile and Lactose Broth

William W. Browne and Benjamin Ackman

From the College of the City of New York

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The detection of the B. coli group in water, milk, oysters, and sewage effluents is of the utmost importance to the sanitary bacteriologist, for knowing its normal habitat to be the intestine of man and the higher animals, he at once infers that its presence must indicate pollution of intestinal origin. It is now firmly established that the B. coli group is an index of fecal pollution. Immediately after its discovery by Escherich in 1885, workers in this field of science began to devise methods of identifying the colon organism; as a result of their investigations we have various tests proposed and used quite extensively down to 1906, which aimed primarily at determining the presence or absence of the Bacillus coli group. The following tests were used:

A. Litmus lactose agar plate method (Wurtz, 1892)

This test is based on the principle that B. coli has the property of fermenting lactose with the production of acid. B. coli may be identified by the red colonies on the blue litmus lactose agar plates. The chief objections to this method are these: (1) Other microorganisms, notably the streptococci, also produce red colonies on litmus lactose agar plates. (2) Too much time is taken up in making supplementary tests for further identification.
B. Glucose broth-enrichment test (1901)

This test depends upon the fact that *B. coli* in glucose broth produces gas, which can be measured. In the early years of water bacteriology, this test was merely the first step in a long scheme of analysis; experience soon showed that 25 per cent or more of gas in the fermentation tube or in the small inverted vial was sufficient to justify the presumption of the presence of *B. coli*, and it now took the name “presumptive test.” The chief advantage of this test is that only twenty-four to forty-eight hours are required to determine the presence of *B. coli*. The great disadvantage, however, is that it sometimes fails to show the presence of the colon group, since the culture medium is particularly suited to the development of other bacteria, which inhibit the colon group. Furthermore, it has been found that the factors of temperature, storage and reaction of the medium have much to do with this test.

C. Phenol broth (Reynolds, 1902)

The problem of the struggle for supremacy in the glucose broth tube was soon solved. By the addition of a small amount of a weak solution of phenol the ordinary water bacteria could be inhibited in growth to the advantage of the colon bacilli. Again, however, disadvantages arose even more serious than those affecting the pure glucose tube, since, in waters of fairly pure quality, phenol inhibits the growth of the *B. coli* group.

D. Eijkman test (1904)

Eijkman suggested that water forms might be eliminated in this test if the temperature of incubation was made 46°C. It was soon pointed out that weak strains of colon bacilli are also inhibited at that temperature.

E. Neutral red reaction (Rothberger, 1898)

This test depends on a color reaction. Objections have been made to its use because of the relatively large number of organisms unrelated to the colon group giving this reaction.

F. Lactose broth test

Experimental evidence is not lacking to show the usefulness of lactose broth as a medium for making the presumptive test for *B. coli*. 
It has the decided advantage over the glucose broth medium that most water bacteria are inhibited to the advantage of the colon group; the fact to be emphasized here is that both these tests depend on the fermentation of sugars with the production of acid and gas.

**G. Lactose bile test (Jackson, 1906)**

It was not until 1906 that Jackson, working on this problem, suggested the use of a fermentation test such as had been used before in the case of the glucose and lactose broths but substituted, for these media, lactose bile. He believed that bile would most effectively represent conditions as the colon bacilli find them in the intestinal tract. Experiments soon proved the value of this test because: (1) The test can be performed in twenty-four to forty-eight hours. (2) Bile salts inhibit the ordinary water forms, and at the same time offer an ideal culture medium for typical *B. coli* (selective action). (3) Bile salts inhibit the growth of the glucose positive, lactose-negative fermenting organisms.

Of all these tests which have been offered, the lactose broth presumptive test and the lactose bile presumptive test have been used most extensively in the last decade. Against the test as a means of identification of the colon group the writers have no protest, but in its application in the laboratory, and in its interpretation, a change seems necessary.

It is a common practice in laboratories to read gas production in fermentation tubes and inverted vials very accurately, sometimes to the fractional part of a unit, thus: 33⅓ per cent gas = + *B. coli*, 66⅔ per cent gas = + *B. coli*, 75 per cent gas = + *B. coli*, etc. Of what value are these numbers, especially fractions to the left of the equal sign to the practical laboratory man? He is primarily interested in what follows the equal sign. It is the purpose of this paper to show that the percentages of gas produced in lactose peptone bile and lactose broth have little quantitative value, and that there can be only one accurate way of reading and recording gas in these media, and that is, by positive (+) and negative (−) signs respectively. Among the factors influencing the percentage of gas production we have found: (1) Temperature, (2) time, (3) initial re-
action of the medium, (4) length of the inverted vial, (5) source of bile, (6) absorption of formed gas.

The changes and variations produced by these factors are so well-defined, as will become apparent to the reader, that refined gas readings are absolutely valueless in performing these presumptive tests for the colon group and results could be expressed more scientifically by the plus (+) and minus (−) signs. Our investigation was begun with the aim of studying only the lactose peptone bile test, but toward the end of the experiments it was deemed best to show that the same factors influence the readings in the lactose broth test, which is now being used in many laboratories. (Lactose peptone bile gives a precipitate of acid protein, which makes gas reading very difficult or impossible, and for this reason it is being dropped in making water analyses; lactose broth is taking its place.)

CULTURES USED IN THE INVESTIGATION

1. B. coli. Members of the B. coli group were isolated from Hudson River water, and cultures were kept in stock in the laboratory. The following characteristics are assigned to this organism: (1) short bacillus with rounded ends, (2) Gram negative, (3) non-liquefaction of gelatin in sixteen days, (4) grayish white growth on agar 20° C., (5) facultative anaerobe, (6) non spore-forming, (7) gas in lactose peptone bile, (8) fermentation of glucose and lactose with the production of acid and gas.

2. Hudson River water (taken from the river as it was needed in the experiments—from January 25 to June 10, 1916). Along with pure cultures of B. coli, Hudson River water was used, the purpose being to compare the action of pure and mixed cultures. Throughout the experiments, reference will be made to pure and river cultures. Various dilutions were used expressed as follows: 0 = 1 cc., 2 = 0.01 cc., 4 = 0.0001 cc. dilution.

METHODS

The culture media were prepared according to the Standard Methods of Water Analysis of the Laboratory Section of the
American Public Health Association (1912). The reaction, unless otherwise stated, was neutral to phenolphthalein. The greatest care was taken to have all the tubes uniform in size as well as in the amount of medium each contained. All were sterilized in the same manner, using 15 pounds steam for fifteen minutes.

METHODS OF DETERMINING THE AMOUNT OF GAS PRODUCED (PER CENT)

Vials of uniform size (2 inches long) were used in the greater part of the work. In accordance with the custom of reading gas percentages, a vial filled completely with gas, was read 100 per cent, and other readings made in proportion to the amount of gas in the inverted vial. In order to facilitate reading and at the same time to obtain a more accurate reading, a scale was constructed on a small card, which showed all the necessary subdivisions to enable the writers to read the percentages accurately; the card was held against the tube and the reading made.

RELATION OF TEMPERATURE TO THE AMOUNT OF GAS PRODUCED

Fifty 1 cc. portions of the 0, 2, and 4 dilutions of Hudson River water were inoculated into lactose peptone tubes. Twenty-five 1 cc. portions of a twenty-four hour nutrient broth of *B. coli* (0, 2, 4 dilutions) were also inoculated into another series of tubes. Four such sets of tubes were inoculated, making the total 900 tubes, and 225 were subjected to each temperature, there being four temperatures studied: 31°C. incubator temperature, 35°C. incubator temperature, 37°C. incubator temperature, 39°C. incubator temperature.

At the end of the twenty-fourth hour, the tubes were removed from the incubator and the percentages of gas recorded.

Reference to the plot of these results clearly brings out the variations caused by the temperature factor. The reader will
TABLE 1

Amount of gas produced at different temperatures by members of the B. coli group in pure and mixed cultures

| TEMPERATURE °C | RIVER | | | | RIVER | | | | | | RIVER | | | | | | RIVER | | | | | | RIVER | | | | | |
|----------------|-------|---|---|---|-------|---|---|---|---|-------|---|---|---|---|-------|---|---|---|---|-------|---|---|---|---|-------|---|---|---|---|
|                | 0     | 2 | 4 |    | 0     | 2 | 4 |    | 0     | 2 | 4 |    | 0     | 2 | 4 |
| 31             | 56.2% (20-95) | 17.9% (0-55) | 0% | 77.8% (50-90) | 73.0% (55-85) | 51.6% (15-80) |
| 35             | 67.5% (30-90) | 12.0% (0-100) | 0% | 80.2% (65-90) | 83.6% (70-90) | 84.2% (60-90) |
| 37             | 79.0% (35-100) | 42.5% (0-90) | 0% | 87.5% (65-100) | 79.3% (95-100) | 77.9% (65-95) |
| 39             | 79.9% (15-100) | 45.6% (0-95) | 0% | 81.8% (65-100) | 86.2% (75-100) | 73.3% (60-90) |

The results are the average of 50 readings in the case of the river cultures, and the average of 25 readings in the case of the pure cultures.
FALLACY OF REFINED READING OF GAS PERCENTAGES

note the results under river 2, which are specially significant in that they show the widest range in gas readings when the same media and cultures were used.

RELATION OF TIME TO THE AMOUNT OF GAS PRODUCED

In this experiment, one cubic centimeter portions of the various dilutions of the pure and river cultures of the B. coli group were inoculated into lactose peptone bile tubes. Gas readings were made at the end of twenty-four, forty-eight and seventy-two hours. Incubation temperature, 37°C.

![Diagram showing the relation of time to gas production](http://jb.asm.org/Downloaded from plot 2)
TABLE 2

Amount of gas produced in different periods of time, by the B. coli group in pure and mixed cultures

<table>
<thead>
<tr>
<th>Hours</th>
<th>River</th>
<th>Pure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>79.0% (35-100)</td>
<td>42.5% (15-90)</td>
</tr>
<tr>
<td>48</td>
<td>97.1% (50-100)</td>
<td>63.9% (0-100)</td>
</tr>
<tr>
<td>72</td>
<td>89.9% (75-100)</td>
<td>64.3% (0-100)</td>
</tr>
</tbody>
</table>

River culture results are the average of 50 tubes. Pure culture results are the average of 25 tubes.

It will be noted that the members of the B. coli group produce the maximum amount of gas at the end of the forty-eighth hour. The results show an absorption of gas beginning at the end of the forty-eighth hour. It will be seen that gas production is a variable, depending upon the factor of time.

THE RELATION OF THE LENGTH OF VIAL TO GAS PRODUCTION

The purpose of this experiment was to determine the difference in gas production (gas readings) when vials of different lengths were used. Vials from 1 inch to 5 1/2 inches in length were used, and the same amount of culture medium was added to each tube no matter what vial was placed in the tube. All the tubes were inoculated with 1 cc. of Hudson River water and incubated at 37°C. for twenty-four hours.

TABLE 3

Amount of gas produced in vials of different lengths

<table>
<thead>
<tr>
<th>Vial length, in inches</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>2.25</th>
<th>3</th>
<th>3.5</th>
<th>4</th>
<th>4.5</th>
<th>5</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Readings in per cent</td>
<td>95</td>
<td>70</td>
<td>61</td>
<td>62</td>
<td>51</td>
<td>43</td>
<td>58</td>
<td>61</td>
<td>70</td>
<td>41</td>
</tr>
</tbody>
</table>

The results in each case represent the average of five tubes.

The results should not be surprising; such have been the comparative results obtained continually in sanitary bacteriology. What we have done has been to choose arbitrarily a 2 inch vial to measure gas production—but is it not clear and evident that the length of the vial is an important factor in the end result?
FALLACY OF REFINED READING OF GAS PERCENTAGES

RELATION OF THE INITIAL REACTION OF THE MEDIUM TO THE AMOUNT OF GAS PRODUCED

Tubes of lactose peptone bile and lactose broth, to which had been added varying amounts of acid and alkali were inoculated with 1 cc. of a twenty-hour nutrient broth culture of \textit{B. coli} and 1 cc. of Hudson River water, and incubated at 37°C. Readings were made at the end of twenty-four and forty-eight hours.

\begin{table}
\centering
\caption{Amount of gas produced in lactose peptone bile, with varying initial reactions, by \textit{B. coli}, in pure and mixed cultures}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{REACTION} & \textbf{RIVER} & \textbf{48 hours} & \textbf{FURE} & \textbf{48 hours} \\
\hline
+1\% & 55.9\% (35-90) & 71.1\% (60-80) & 99.2\% (60-100) & 81.8\% (70-95) \\
+2\% & 39.4\% (5-80) & 55.8\% (30-75) & 94.9\% (30-100) & 74.0\% (65-90) \\
+3\% & 34.2\% (5-70) & 34.7\% (5-60) & 95.2\% (50-100) & 68.0\% (55-80) \\
\hline
\end{tabular}
\end{table}

Results are the average of 35 readings.

\begin{table}
\centering
\caption{Amount of gas produced in lactose broth, with varying initial reactions, by \textit{B. coli}, in pure and mixed cultures}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{REACTION} & \textbf{RIVER} & \textbf{48 hours} & \textbf{FURE} & \textbf{48 hours} \\
\hline
+2.0\% & 0.0\% & 15.0\% (10-20) & 0.0\% & 0.0\% \\
+1.5\% & 10.0\% (5-20) & 21.4\% (10-30) & 5.0\% (5-10) & 11.4\% (10) \\
+1.0\% & 19.3\% (15-35) & 24.3\% (20-45) & 17.9\% (10-30) & 20.0\% (15-30) \\
+0.5\% & 15.7\% (10-20) & 23.6\% (20-35) & 22.1\% (20-30) & 24.3\% (20-35) \\
0.0\% & 20.7\% (10-25) & 29.9\% (20-40) & 20.2\% (25-35) & 30.8\% (25-40) \\
-0.5\% & 21.4\% (15-30) & 29.3\% (25-35) & 27.5\% (20-30) & 33.3\% (30-40) \\
-1.0\% & 23.3\% (15-30) & 32.5\% (20-45) & 35.8\% (30-40) & 46.6\% (40-50) \\
-1.5\% & 16.4\% (15-25) & 23.8\% (20-30) & 32.7\% (25-40) & 45.6\% (40-55) \\
-2.0\% & 11.4\% (5-20) & 39.2\% (30-60) & 22.9\% (10-30) & 55.8\% (50-70) \\
\hline
\end{tabular}
\end{table}

These results are the average of 6 readings.

INDIVIDUAL VARIATION

The next point taken into consideration was individual variation. The best way to show that gas readings are fallacious
and have no real quantitative value is to present the variations not caused by any particular constant factor, but rather variations within a given group under similar conditions. Tubes of lactose peptone bile and lactose broth were inoculated with

TABLE 6

Individual variation within the group (variation curve)

<table>
<thead>
<tr>
<th>PER CENT OF GAS</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>45</th>
<th>50</th>
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<th>70</th>
<th>75</th>
<th>80</th>
<th>85</th>
<th>90</th>
<th>95</th>
<th>100</th>
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<td>1</td>
<td>2</td>
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<td>1</td>
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<td>Pure (0)</td>
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<td>85</td>
<td>90</td>
<td>95</td>
<td>100</td>
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<tr>
<td>Lactose broth, medium</td>
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<tr>
<td>Pure (0)</td>
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<tr>
<td>(2)</td>
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</tbody>
</table>

Temperature, 37° C.

(VARIATION CURVE) INDIVIDUAL VARIATION
the pure and river cultures (various dilutions) and incubated at 37°C. for twenty-four hours, after which readings were made.

Reference to the table and to the plot showing the variation curve will indicate the wide range in the amount of gas produced. These results, perhaps give the most convincing evidence as to the utter uselessness of gas readings as quantitative values, since the same substance inoculated with the same organism gives such a wide variation.

RELATION OF THE SOURCE OF BILE TO GAS PRODUCTION

It was believed that bile from different cows might produce variations in gas production, and therefore, culture media were prepared using the bile from ten different cows. The culture tubes were inoculated with the pure and river cultures of the colon bacillus, and readings made at the end of twenty-four and forty-eight hours.

As soon as the bile was received from the slaughter-house, the following characteristics were noted: (1) acidity, (2) color, (3) consistency.

<table>
<thead>
<tr>
<th>BILE NUMBER</th>
<th>CONSISTENCY</th>
<th>COLOR</th>
<th>REACTION per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Clear, translucent</td>
<td>Light green</td>
<td>+0.5</td>
</tr>
<tr>
<td>2</td>
<td>Clear, translucent</td>
<td>Light green</td>
<td>+0.7</td>
</tr>
<tr>
<td>3</td>
<td>Heavy liquid, opaque</td>
<td>Green—black</td>
<td>+0.8</td>
</tr>
<tr>
<td>4</td>
<td>Slimy, slight yellow precipitate, opaque</td>
<td>Black</td>
<td>+0.5</td>
</tr>
<tr>
<td>5</td>
<td>Clear, translucent</td>
<td>Dark green</td>
<td>+0.8</td>
</tr>
<tr>
<td>6</td>
<td>Heavy liquid, green precipitate</td>
<td>Dark green</td>
<td>+0.5</td>
</tr>
<tr>
<td>7</td>
<td>Very heavy yellow precipitate</td>
<td>Dark green</td>
<td>+0.5</td>
</tr>
<tr>
<td>8</td>
<td>Slight precipitate</td>
<td>Dark green</td>
<td>+0.55</td>
</tr>
<tr>
<td>9</td>
<td>Slight cloudy precipitate</td>
<td>Green—black</td>
<td>+0.55</td>
</tr>
<tr>
<td>10</td>
<td>Clear liquid</td>
<td>Light green</td>
<td>+0.55</td>
</tr>
</tbody>
</table>
### TABLE 8

**Amount of gas produced in the various biles**

<table>
<thead>
<tr>
<th>BILE NUMBER</th>
<th>RIVER</th>
<th>PURE</th>
<th>RIVER</th>
<th>PURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Twenty-four hours</td>
<td>Forty-eight hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39.0% (20-65)</td>
<td>63.5% (50-70)</td>
<td>Readings could not be made</td>
<td>72.0% (60-80)</td>
</tr>
<tr>
<td>2</td>
<td>70.6% (60-80)</td>
<td>81.0% (75-90)</td>
<td>on account of a heavy yellow precipitate of acid</td>
<td>82.0% (75-90)</td>
</tr>
<tr>
<td>3</td>
<td>53.0% (5-85)</td>
<td>72.0% (55-75)</td>
<td>81.0% (75-85)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40.0% (20-65)</td>
<td>69.0% (35-80)</td>
<td>72.0% (65-75)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29.0% (15-45)</td>
<td>65.0% (60-75)</td>
<td>84.0% (80-100)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>40.3% (10-75)</td>
<td>84.0% (75-100)</td>
<td>86.0% (80-90)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>42.5% (10-75)</td>
<td>82.5% (80-85)</td>
<td>86.0% (80-85)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25.6% (10-45)</td>
<td>48.0% (40-65)</td>
<td>73.0% (65-80)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>29.4% (5-45)</td>
<td>68.0% (65-70)</td>
<td>79.0% (75-85)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>21.3% (5-45)</td>
<td>42.5% (40-50)</td>
<td>63.0% (60-70)</td>
<td></td>
</tr>
</tbody>
</table>

### CONCLUSIONS

From the data presented in this paper, it will be seen that:

1. The following factors cause variations in the gas readings in lactose peptone bile and lactose broth.
   a. Temperature.
   b. Time of incubation.
   c. Initial reaction of the culture medium.
   d. Length of inverted vial.
   e. Source of bile.
   f. Absorption of formed gas.

2. The gas readings in a given group vary within a wide range.

Since all these factors play an important part in the final gas readings, which necessarily must vary considerably, and since no real quantitative value can be assigned to any observation of gas production, under such conditions it seems that refined readings of gas percentages have no scientific justification in sanitary bacteriological analyses and that gas production could be recorded more conveniently and scientifically by positive (+) and negative (−) signs respectively.
REFERENCES


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