IS THE EIJKMAN TEST AN AID IN THE DETECTION OF FECAL POLLUTION OF WATER?

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Received for publication April 16, 1930

INTRODUCTION

The present standards of the American Public Health Association (1925) have designated Gram-negative non-spore-forming aerobic rods which ferment lactose with the production of gas as the organisms to be searched for to detect fecal pollution. From time to time other organisms have been proposed wholly or partially to replace these bacteria, but, as stated so definitely and convincingly by Levine (1921), *Bacterium coli* is the organism which best fulfills the requirements for an index of fecal pollution. It is constantly present in the feces of warm blooded animals, is seldom obtained except from feces or from substances recently polluted, is slightly more resistant than intestinal bacterial pathogens, but is not sufficiently resistant to remain long in water after the water has been purified enough to kill pathogens.

However, as shown by many studies summarized by Levine and since added to by others, the related organism, *Bacterium aerogenes*, included in the above description, is in no way an index of fecal pollution. Neither *B. aerogenes* nor its very close relative, *Bacterium cloacae*, is present in feces in very large numbers. They are present in fairly large numbers in soil, plant residues and other places not associated with feces, and are more resistant to storage than *B. coli*. *B. aerogenes* is undoubtedly a separate species, differing from *B. coli* in several correlating characters, namely the production of acetyl-methyl carbinol (Voges-Proskauer test), the production of a higher CO_2/H_2 ratio, the utilization of uric

acid as a sole source of nitrogen, and the production of a higher pH in the methyl-red test. Although the American Public Health Association standards do not distinguish between *B. coli* and *B. aerogenes* in the "completed test," there can be little doubt that *B. aerogenes* has no value as an index of fecal pollution. It can be fairly readily distinguished from *B. coli* on the eosine-methylene blue (Levine (1918, 1921)) or the triple dye agar plates (Skinner and Murray (1924)), as well as by the more accurate methods summarized by Standard Methods (1925).

B. coli itself, however, is occasionally isolated from soil, is often found in feces of cold blooded animals, and occasionally occurs in streams free from apparent human pollution. Koser (1924) has found that, in general, only such strains as are found in soils are able to utilize citrates as a sole source of energy. Since all strains of *B. aerogenes* also utilize citrates, this medium offers an interesting possibility for the elimination of all members of the coli-aerogenes group which are of no sanitary importance. Although as yet enough work has not been done with strains of *B. coli* isolated from feces to prove absolutely the importance of Koser's findings, it is a distinct probability that only the citrate negative organisms should be considered in water analysis. The results of Pawan (1926) who found that citrate negative strains were present in 98 per cent of the samples of cow and human feces and citrate positive strains in 100 per cent of the samples of unpolluted water, certainly confirm Koser's results, although it is probable that most of his non-fecal organisms were *B. aerogenes*. Other workers have obtained somewhat similar results.

Eijkman (1904) has suggested a means of separating the *B. coli* originating in the feces of warm blooded animals from the strains characteristic of cold blooded animals. This consists in incubating inoculated glucose-peptone broth at 46°. Gas formation is said to indicate the presence of *B. coli* from warm blooded animals. *B. coli* from cold blooded animals, practically all *B. aerogenes*, (and *B. cloacae*), *Bacterium proteus*, and the various fermenting anaerobic rods are said to be completely inhibited as far as gas production is concerned. Although of great importance, (if the above is true) this test has not, so far as

we are aware, been used at all in North America except in a limited way by Skinner and Murray (1926) and especially by Leiter (1929) who made a fairly intensive study of it.

However, even if these organisms of little importance, giving presumptive tests, be eliminated, not a great deal of work has been done to show that all or nearly all of the *B. coli* from feces of warm blooded animal origin ferment glucose at 46°. Leiter isolated 143 strains of *B. coli* from human feces all of which produced gas 46°, although five at times produced less than 0.5 cc. in the Smith fermentation tube. Similar results were obtained by Salus and Hirn (1923). Positive Eijkman tests have rarely been encountered from water of known high sanitary quality by any of the European workers. Most of the work done on the test, which has been studied rather intensively in Europe and summarized by Leiter (1929) and by Barth (1930), indicates that false positive tests are rarer than in the case of the usual methods with lactose broth at 37.5°.

It was decided that further work was necessary to indicate whether or not false negative tests could be expected, that is, if all or nearly all of the *B. coli* from the feces of humans are able to produce gas at 46° from glucose broth. If so, since false positives are said to be rare, it should offer a relatively cheap and simple means of detecting fecal pollution.

First of all, if all the *B. coli* grow at 46°, their numbers in feces should be as large when determined by the Eijkman media at 46° as by the lactose broth at 37.5°. Fortunately, the dilution method offers us a rather accurate means of determining numbers (McCrady (1918), Buchanan and Fulmer (1928)). It was also deemed advisable to determine whether the gas production was due to *B. coli*, *B. aerogenes*, or lactose fermenting anaerobes, although evidence is not convincing that any large numbers of *B. aerogenes* are present in feces.

EXPERIMENTAL

Thirty-seven samples of feces from hospital patients and from persons in normal health were diluted in three or four decimal dilutions, 1 to 10^6 to 1 to 10^9 inclusive, in replicates of five

Durham fermentation tubes. Both the glucose broth (Difco peptone 12.5 grams, glucose 12.5 grams, NaCl 6.25 grams per liter) and lactose broth (peptone 10 grams, lactose 10 grams, beef extract 3 grams, NaCl 5 grams per liter, pH 7.2) were inoculated from the same dilutions, at the same time and with the same pipettes. The glucose medium is the same as that used by Leiter (1929) and is of the same concentration as Eijkman's after being diluted with water. After incubating the lactose broth at 37.5° and the glucose media at exactly 46° in a Central Scientific Company water bath with a DeKhotinsky thermoregulator for twenty-four hours, the tubes with any gas at all were streaked on standard eosine-methylene blue agar plates. Those showing gas in forty-eight hours were streaked the following day. Since the streak plates were all confirmed, and since all plates contained only typical *B. coli* colonies as evidenced by the greenish metallic sheen, the confirmation was discontinued, after 157 streak plates had been made. Like Leiter, we found that the streak plates from lactose broth inoculated with feces showed pure or almost pure cultures of *B. coli.* That they were citrate negative, we cannot say, but at any rate they were obviously fecal. Not a single plate of the 157 cultures streaked from either glucose or lactose broth cultures of feces showed a colony resembling *B. aerogenes* or *B. cloacae.*

These results (table 1), calculated from the tables of McCrady (1918) (Buchanan and Fulmer (1928)), show that not all of the lactose fermenting organisms (exclusively *B. coli* by eosine-methylene blue agar plate appearance) produce gas in Eijkman's broth in forty-eight hours. They further show that twenty-four hours at 46° is not a sufficient time for many cultures. Some cultures have large amounts of gas in twenty-four hours, others show none at all at this period but develop gas in forty-eight hours. On the other hand, it was found that those cultures which produced gas in twenty-four hours contained few or no living organisms in forty-eight hours, the acidity apparently being more toxic at this high temperature. All plates streaked from the Eijkman broth contained fewer colonies than those streaked from the lactose broth. All the lactose broth tubes

TABLE 1

Comparison of lactose broth at 37.5° and glucose broth at 46° (Eijkman method) for detecting B. coli in feces

DATE	LACTOSE BROTH 37.5°		GLUCOSE BROTH 46°			RATIO OF NUMBER OF B. COLI, LACTOSE BROTH METHOD TO NUMBER EIJKMAN METHOD
	Tubes showing gas in dilutions of 1:10^6, 1:10^7 and 1:10^8. 48 hours	Most probable number *B. coli* as shown by gas formation	Tubes showing gas in dilutions of 1:10^6, 1:10^7 and 1:10^8. 24 hours	Tubes showing gas in dilutions of 1:10^6, 1:10^7 and 1:10^8. 48 hours	Most probable number *B. coli* as shown by gas formation	
		millions per gram			*millions per gram*	
December 20, 1929	5-4-1	17.0	1-0-0	3-1-0	1.1	15.5
	5-5-1	35.0	0-0-0	0-0-0	<0.2	175.0†
	5-0-0	2.5	3-0-0	3-1-0	1.1	2.3
December 21, 1929	5-5-1	35.0	4-4-0	5-5-0	25.0	1.4
	5-5-5	>180.0	3-3-1	5-5-3	90.0	2.0
	5-5-5	>180.0	0-0-0	0-0-0	<0.2	900.0†
	5-5-5	>180.0	5-5-1	5-5-2	35.0	5.1
	5-4-0	13.0	5-2-0	5-4-0	13.0	1.0
	5-2-0	5.0	4-1-0	5-2-0	5.0	1.0
December 24, 1929	5-5-3	90.0	Not counted	4-0-0	1.3	69.2
	5-5-5	>180.0	Not counted	5-5-3	90.0	2.0
	5-5-5	>180.0	Not counted	5-5-4	160.0	1.1
	5-5-5	>180.0	Not counted	5-5-4	160.0	1.1
	5-5-5	>180.0	Not counted	5-4-2	25.0	7.2
December 31, 1929	3-0-1*	11.0	0-0-0*	0-0-0*	<2.0	5.5
	1-0-0*	2.0	0-0-0*	1-0-0*	2.0	1.0
January 2, 1930	5-5-4	160.0	0-0-0	3-0-1	1.1	145.5†
	5-5-1*	350.0	0-0-0	3-1-0	1.1	318.2†
	5-5-1*	350.0	4-2-0*	4-2-0*	20.0	17.5
	2-2-0	0.9	1-0-0	1-0-0	0.2	4.5
January 3, 1930	5-3-1	11.0	5-2-0	5-4-0	13.0	0.8
	5-5-1	35.0	5-0-0	5-5-1	35.0	1.0
	1-0-0	0.2	0-0-0	0-0-0	<0.2	1.0
	5-5-3*	900.0	5-2-0	5-5-0	25.0	36.0
January 4, 1930	4-0-0	1.3	0-0-0	0-0-0	<0.2	6.5
	4-2-0	2.0	0-0-0	3-1-0	1.1	1.8
	5-4-0*	130.0	0-0-0	3-1-0	1.1	118.2†
	2-0-0	0.5	2-0-0	2-0-0	0.5	1.0

TABLE 1—*Concluded*

DATE	LACTOSE BROTH 37.5°		GLUCOSE BROTH 46°			RATIO OF NUMBER OF B. COLI, LACTOSE BROTH METHOD TO NUMBER EIJKMAN METHOD
	Tubes showing gas in dilutions of 1:10[6], 1:10[7] and 1:10[8]. 48 hours	Most probable number *B. coli* as shown by gas formation	Tubes showing gas in dilutions of 1:10[6], 1:10[7] and 1:10[8]. 24 hours	Tubes showing gas in dilutions of 1:10[6], 1:10[7] and 1:10[8]. 48 hours	Most probable number *B. coli* as shown by gas formation	
		millions per gram			*millions per gram*	
January 15, 1930	5-5-1*	350.0	4-4-0*	5-4-3*	300.0	1.2
	4-1-0	1.7	0-0-0	0-0-0	<0.2	8.5
	2-0-0	0.5	0-0-0	0-0-0	<0.2	2.5
	2-0-0	0.5	0-0-0	0-0-0	<0.2	2.5
	5-3-0	8.0	0-0-0	0-0-0	<0.2	40.0
January 18, 1930	5-5-3	90.0	0-0-0	0-0-0	<0.2	450.0†
	5-4-0*	130.0	2-0-0	3-2-0	1.4	92.9
	5-3-1*	110.0	0-0-0	0-0-0	<0.2	550.0†
	5-5-1*	350.0	0-0-0	2-0-0	0.5	700.0†
						11.5 mean 5.1 median

* Tubes $1{:}10^7$, $1{:}10^8$, $1{:}10^9$ dilutions.

† Values not counted in determining mean.

were either positive in twenty-four hours or negative in forty-eight hours. The table indicates that the Eijkman test should be expected to give many false negatives. Furthermore, it was discovered that the organisms were quickly killed after gas formation had taken place, which probably also explains Leiter's failure to confirm all his tests. Whether the failure to grow at 46° was due to strain variation or to relatively greater resistance to the high temperatures by some of the organisms at certain physiological ages we cannot say. These conclusions were based upon more than one thousand inoculations.

Similar tests were made also with water. For this purpose water was used from the Mississippi river, above the city of Minneapolis, as it comes into the filtration plant.[1] Three decimal

[1] We are indebted to Mr. I. A. Montank, bacteriologist at the Minneapolis filtration plant, who on several occasions took the samples and brought them to us.

dilutions from 1 cc. to 0.01 cc. were used. It was evident at the first preliminary trial that standard eosine-methylene blue agar could not be used for confirmation tests, since non-lactose fermenters in very many cases prevented isolation and identification of the coli-aerogenes organisms. The addition of 1:100,000 crystal violet was found largely to prevent this, as had previously been found to be the case (Skinner and Murray (1924)). Very possibly the use of crystal violet (Hall and Ellefson (1918)), or other dyes, or NaOH (Janzig and Montank (1928)) in the lactose broth would eliminate some of the unconfirmed tests. However, in our case it was not the lactose fermenting anaerobes which gave the false positive presumptive tests so much as the overgrowth of non-lactose fermenting organisms on the plate, which was prevented by crystal violet. There is ample evidence in the literature that such a concentration of the dye is in far too great dilution to cause marked inhibition of *B. coli.*

After incubation for twenty-four hours, the tubes showing gas were streaked on the triple dye agar. From those developing gas in the 24-to-48-hour period, streaks were made after forty-eight hours incubation. Colonies were picked, one colony most resembling *B. coli*, from each plate, and planted on agar slants. These were then inoculated into lactose broth for confirmation, peptone glucose broth for Voges-Proskauer, peptone broth for indol, and Simmon's (1926) agar for the detection of citrate utilization. In case the cultures showed acid and gas from lactose broth, were negative to the Voges-Proskauer test and did not utilize citrates in 48 hours, they were recorded as *B. coli.* If they showed opposite reactions in the tests, save acid and gas on the lactose broth, they were called *B. aerogenes.* If no gas resulted from lactose broth, they were recorded as not confirmed, as were also plates on which no growth resulted after streaking. All other cultures were reinoculated into lactose broth, reisolated and again planted on the media. In case they checked with the first reading, they were recorded as such, or if *B. coli* or *B. aerogenes* reactions appeared after repurification (as was frequently the case) these were duly recorded in the table. Table 2 shows the results.

The table shows many "skips" due most likely to overgrowth of

TABLE 2

Comparison of lactose broth at 37.5° and glucose broth at 46° (Eijkman method) in their effectiveness in detecting typical B. coli and in repressing B. aerogenes and citrate-utilizing B. coli in water

SAMPLE NUMBER	LACTOSE BROTH 37.5°			GLUCOSE BROTH 46°			RATIO OF NUMBER OF TYPICAL B. COLI LACTOSE BROTH AT 37.5° COMPARED TO NUMBER EIJKMAN METHOD
	Tubes containing *B. coli* inoculated with 1, 0.1 and 0.01 cc. water	Most probable number *B. coli* per 10 cc. water	Other members of colon group than typical *B. coli* isolated	Tubes containing *B. coli* inoculated with 1, 0.1 and 0.01 cc. water	Most probable number *B. coli* per 10 cc. water	Other members of colon group than typical *B. coli* isolated	
1	4–0–0	13	1 *B. aerogenes*	1–0–0	2		6.5
2	3–0–0	8		0–0–0	<2		4.0
3	2–2–0	9	3 *B. aerogenes*	3–0–0	8		1.1
4	4–1–0	17	1 not confirmed	4–1–0	17		1.0
5	3–0–0	8	1 *B. aerogenes*	2–0–0	5		1.6
6	2–2–0	9	4 *B. aerogenes* 1 not confirmed	2–1–0	7	2 *B. aerogenes* 1 not confirmed	1.3
7	3–3–0	17	2 *B. aerogenes*	2–0–0	5	2 *B. aerogenes*	3.4
8	3–2–0	14	2 *B. aerogenes*	0–0–0	<2		7.0
9	4–5–1	50	1 *B. aerogenes*	4–2–0	20		2.5
10	2–3–0	12	1 citrate + *B. coli* 5 *B. aerogenes*	2–1–0	7	1 *B. aerogenes* 1 not confirmed	1.7
11	2–2–0	9	3 citrate + *B. coli* 3 *B. aerogenes*	1–1–0	4	1 citrate + *B. coli* 2 *B. aerogenes*	2.3
12	3–3–1	20	3 *B. aerogenes*	1–1–0	4	4 *B. aerogenes* 1 not confirmed	5.0
13	3–4–0	20	2 *B. aerogenes* 1 not confirmed	1–1–0	4	1 citrate + *B. coli* 1 *B. aerogenes* 2 not confirmed	5.0
14	2–1–0	7	2 *B. aerogenes*	0–0–0	<2	1 citrate + *B. coli* 3 *B. aerogenes*	3.5
15	2–3–0	12	5 *B. aerogenes*	2–1–0	7	2 *B. aerogenes*	1.7
16	4–3–0	25	1 citrate + *B. coli* 1 not confirmed	5–0–0	25	1 *B. aerogenes*	1.0
17	3–2–0	14	3 *B. aerogenes*	2–0–0	5	5 not confirmed	2.8
18	2–0–0	5	2 *B. aerogenes* 1 not confirmed	2–0–0	5		1.0
19	4–3–1	30	1 citrate + *B. coli*	2–0–0	5	2 *B. aerogenes* 1 not confirmed	6.0
20	3–3–0	17	3 *B. aerogenes* 1 not confirmed	3–0–0	8	2 *B. aerogenes* 1 not confirmed	2.1
	103 total			48 total			2.4 mean 3.0 median

B. aerogenes and other bacteria. For this reason the results should not have as much weight as those of table 1. They seem to indicate, however, that the Eijkman test fails to detect all the fecal *B. coli.* The large number of unconfirmed tests from the Eijkman media probably indicate cultures in which *B. coli* had been present but had died, due to acidity at the high temperature. Counting all these "not confirmed" as "fecal *B. coli*," table 3 shows that even so, all the *B. coli* are not always obtained by the Eijkman method. It further shows that a fair number of *B. aerogenes* and atypical

TABLE 3

The types of members of the coli-aerogenes group recovered from the inoculation of the same number of lactose broth tubes at 37.5° and of glucose broth at 46° with water from a polluted stream

TYPE RECOVERED	LACTOSE, 37.5°		GLUCOSE, 46°	
	Number recovered	Per cent	Number recovered	Per cent
B. coli; non-spore-forming rods, acid and gas in lactose broth, Voges-Proskauer −, no utilization of citrates	103	64.8	48	70.6
B. aerogenes; as above except Voges-Proskauer + and utilization of citrates	43	27.0	22	25.9
B. coli, atypical; like *B. coli* above except utilization of citrates	7	4.4	3	3.5
Not confirmed; gas formation in tubes, but lactose fermenting aerobes not isolated	6	3.8	12	—*
Total	159		85	

* Not confirmed included in percentage of *B. coli.* See text.

B. coli (citrate positive, or "non fecal" according to Koser) are apt to be involved in a positive Eijkman test. Thus, we were not able to confirm the work of de Graaff (1922) who found that Voges-Proskauer positive, methyl red negative strains of the colon bacilli (*B. aerogenes*) were repressed in the Eijkman test.

DISCUSSION

Our results indicate that, even if the production of gas at 46° from glucose broth be a fair index of pollution from feces, the lack

of such gas formation does not prove the absence of pollution. The absence of a positive presumptive lactose broth test at 37.5° of course indicates the lack of fecal pollution. Together, the tests may be of benefit. Alone, a positive lactose broth presumptive test needs confirmation and differentiation of *B. coli* from *B. aerogenes* or, probably better still, differentiation of citrate from non-citrate utilizing organisms. Alone, a negative Eijkman test does not appear to the authors to be of much value. The results obtained by us indicate that twenty-four hours is not enough time for the Eijkman test. The fact that *B. aerogenes* is seldom encountered in feces and is, therefore, of no significance in water analysis except in so far as it leads to confusion, has been confirmed. The addition of 1:100,000 crystal violet to the standard eosine-methylene blue agar has made the confirmation of presumptive tests easier and more certain.

The fact that our results are on the whole unfavorable to the Eijkman test detracts in no way from Leiter's work. His results were on isolated strains. Ours were quantitative and on feces. Also, his results, as well as those of others, show conclusively that the Eijkman test does eliminate a great many of the positive tests, the origin of which was cold blooded animals. Our results are more in agreement, however, with those of Hehewerth (1912) who found that only 38.8 per cent of the 36 strains isolated from feces gave an Eijkman reaction, and with those of Barth (1930) who found that only 37 per cent of the 31 strains isolated from human feces grew at 45°. This latter work appeared after our experimental data had been compiled and while the manuscript was being written. In all of the work on the test previous to ours, as far as we are aware, no worker has utilized to any great extent the dilution method. This method seems to us to give the most pertinent results, for unless all *B. coli* from feces will grow at 46° in glucose broth (and our results show they do not) and will grow whether 1 or 100 cells are used as an inoculum, the Eijkman test is of little value in detecting all waters containing *B. coli*, and especially is it without value in obtaining quantitative data as to the amount of pollution.

SUMMARY

1. Only a small percentage of the *B. coli* from human feces produced gas in forty-eight hours in glucose-peptone broth at 46° (Eijkman test).

2. Many strains produced gas in twenty-four hours in the Eijkman test, but forty-eight hours were necessary to include all the gas formers.

3. Many "typical" *B. coli* from water from a polluted steam failed to grow in the Eijkman broth at 46°.

4. The Eijkman test did not eliminate all positive tests due to *B. aerogenes* and citrate positive *B. coli*.

5. Only *B. coli* (never *B. aerogenes*) was found in human feces.

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