Simultaneous Determination of Coliform and *Escherichia coli* Indices

MAX LEVINE, R. H. TANIMOTO, H. MINETTE, J. ARAKAKI, AND G. B. FERNANDES

Laboratories of the Hawaii Territorial Department of Health, Honolulu, Hawaii

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Whether all members of the coliform group of bacteria should be accorded equal sanitary significance has been debated for at least a generation. English and German authorities are disposed to assign special significance to the presence of *Escherichia coli*, whereas it is the American practice, particularly as applied to water analyses, to consider all coliform bacteria of equal sanitary significance. The latter concept has been challenged from time to time, especially as to its applicability to various foods and, under some conditions, even to certain water supplies. With respect to foods, a strong tendency has developed to distinguish and to ascribe different sanitary significance to the presence of various species, or types, of coliform bacteria, as, for example, in the case of oysters and clams.

Irrespective of which of the foregoing views one may hold, there is undoubtedly agreement that until a relatively simple, dependable, expeditious, and economical procedure for ascertaining the *E. coli* and coliform indices, respectively, are available, the data necessary to evaluate properly the sanitary significance of the presence of various species, or types, of the coliform group of bacteria will not be forthcoming. This report will deal with a procedure for the selective enrichment and detection of *E. coli* in the presence of other coliform bacteria in water, and this should be equally applicable to other foods.

MATERIALS AND METHODS

For the purpose of this report, the organisms of the coliform group will be allocated to three categories, as based upon the Voges-Proskauer, Koser citrate reactions (table 1).

A Selective Medium for Detection of Escherichia coli

In the course of some studies on the use of boric acid in home canning (Levine, 1921, 1923) it was demonstrated that this compound was not suitable for canning; but it was observed that the concentration of boric acid recommended (about 0.65 per cent), if present in nutrient agar, prevented the growth of the genus *Aerobacter*, whereas the *E. coli* strains employed grew luxuriantly. In 1935, Vaughn and Levine reported that a lactose broth medium containing 0.325 per cent boric acid and incubated at a temperature of 43 C could be employed for selective isolation of *E. coli*, and Vaughn *et al.* (1951) presented a detailed report verifying these findings. The medium in question has the following composition.

Boric Acid Lactose Broth

Proteose peptone, Difco	10.0 g
Lactose	5.0 g
K_2HPO_4	12.2 g
KH_2PO_4	4.1 g
Boric acid	3.25 g
Water	1000 ml
Reaction of medium pH 7	

Growth of Coliform Bacteria in Boric Acid Lactose Broth

In table 2 are shown the results obtained with 1078 strains of coliform bacteria when a 4-ml loopful from 24-hr lactose broth cultures was inoculated into boric acid lactose broth and incubated at 43 C.

It will be noted that 223 (55.6 per cent) of 401 strains of the genus *Aerobacter* failed to grow at all, and only 20 (5.0 per cent) produced gas in 48 hr. The medium was even more restrictive for the strains falling into the intermediate group of coliform bacteria. Thus, of 280 such strains, 195 (69.8 per cent) failed to grow, and only 2 (0.7 per cent) formed gas. In contrast to the foregoing, only 6 (1.5 per cent) of 397 strains of *E. coli* failed to grow, whereas 390 (98.3 per cent) grew luxuriantly with gas production. A few strains (7, or 1.7 per cent) of *E. coli* did not produce gas, thus indicating that the boric acid lactose broth medium, under conditions employed, exerts an inhibitory action against some *Escherichia coli* strains. However, as will be shown later, this inhibitory effect is insignificant in comparison with the proportion of *E. coli* strains that are not detected, as a result of overgrowth by other coliform bacteria, when standard lactose broth is employed. It appears quite evident that gas production, within 48 hr at 43 C, in the boric acid lactose medium may be considered a reliable index of the probable presence of *Escherichia coli*.

Lactose Broth and Boric Acid Lactose Broth as Primary Media for Detection of Coliform Bacteria in Water

In the foregoing observations with pure cultures, the inocula were, of course, large. Similar results were obtained with small inocula, for example, those encoun-

TABLE 1. *Characteristics of the primary subgroups of coliform bacteria*

Group Designation*	Voges-Proskauer Test	Koser Citrate Test	Bergey Classification
Escherichia coli	−	−	*Escherichia coli*
Intermediate	−	+	*Escherichia freundii* *Escherichia intermedium*
Aerobacter	+	+	*Aerobacter aerogenes* *Aerobacter cloacae*

* For the purposes of this report.

TABLE 2. *Growth and gas production of 1078 strains of the coliform group of bacteria in boric acid lactose broth**

Group	Strains					
	Growth − *Gas* −		*Growth* + *Gas* −		*Growth* + *Gas* +	
	No.	%	No.	%	No.	%
Escherichia coli (397)†	6	1.5	1	0.2	390	98.3
Intermediate (280)†	195	69.7	83	29.6	2	0.7
Aerobacter (401)†	223	55.6	158	39.4	20	5.0

* Incubation 48 hr, 43 C ± 0.5 C.
† Total number of strains employed.

tered in routine water analysis, as may be seen from the following.

Lactose broth (LB) and boric acid lactose broth (BALB) were inoculated in duplicate with various quantities of water (10, 1, 0.1, 0.01, 0.001 ml, and so on). The lactose broth was incubated at 35 C, and the boric acid lactose broth at 43 C. Every tube showing gas (after 24 or 48 hr) was streaked on to Levine's eosin-methylene-blue agar (EMB). Representative colonies from each plate were fished for identification and allocated to the coliform group as indicated in table 1 (*E. coli*, intermediate, and *Aerobacter*). The *E. coli* and coliform indices (MPN) were then obtained from the appropriate tables of *Standard Methods for the Examination of Water and Sewage* (A.P.H.A., 1946).

It was observed that in some instances the *E. coli* index was significantly lower when BALB was employed as the primary medium, but the reverse was even more frequently the case. These two apparently conflicting phenomena are attributed, on the one hand, to inhibitory properties of boric acid on some *E. coli* strains and on the other (in the case of standard lactose broth) to the effect of overgrowth by *Aerobacter* and intermediate strains of the coliform group.

In table 3 are shown some representative data for water specimens in which the *E. coli* indices obtained with LB as the primary medium were either significantly higher or significantly lower than when BALB was employed.

TABLE 3. *Some coliform and Escherichia coli indices (MPN) of waters planted into standard lactose broth and boric acid lactose broth as primary enrichment media*

Lactose Broth (LB) 35°C			Boric Acid Lactose Broth (BALB) 43 C			Ratio *E. coli* Indices B:L†
Index (MPN)		Ratio C/E*	Index (MPN)		Ratio C/E*	
Coliform	*E. coli*		Coliform	*E. coli*		
E. coli index in LB greater than in BALB						
7,000	1,300	5.4:1	240	240	1.0:1	1:5
500	230	2.2:1	4.6	4.6	1.0:1	1:50
24,000	1,300	18.0:1	62	62	1.0:1	1:21
24,000	24,000	1.0:1	600	600	1.0:1	1:40
E. coli index in LB less than in BALB						
24,000	62	390.0:1	2,400	700	3.4:1	11:1
7,000	62	110.0:1	2,400	2,400	1.0:1	39:1
700	9.4	74.0:1	240	240	1.0:1	26:1
10,000	1,400	50.0:1	24,000	24,000	1.0:1	17:1

* Ratio of coliform to *E. coli* index.
† Ratio of *E. coli* index of primary BALB to primary LB.

Considering those specimens in which the *E. coli* indices employing LB (at 35 C) were greater than when BALB (at 43 C) was used as the primary enrichment medium, it will be noted that the ratio of the coliform to the *E. coli* indices for the lactose broth series was (except for one instance when it was 1:1) not very high, 2.2:1 to 18:1. On the other hand, in the instances in which the *E. coli* indices in lactose broth were less than those obtained with the boric acid medium, the ratios of the coliform to the *E. coli* indices in the lactose broth series were very high, 74:1 to 390:1. These high ratios, it is believed, are indicative of overgrowth of *E. coli* by, or antibiotic effects of, coliform strains of the *Aerobacter* and intermediate groups. The fact that the ratios of the coliform to *E. coli* indices when lactose broth was used as the preliminary enrichment medium were practically always greater than 1:1 indicates that, as is of course generally recognized, gas production in primary lactose broth is not a dependable criterion of the probable presence of *E. coli*. In contrast to the foregoing, it will be noted that when the boric acid medium at 43 C was used for preliminary enrichment, the ratios of the coliform to the *E. coli* indices were practically always 1:1, a phenomenon which further attests to the value of boric acid lactose broth as a presumptive test for *E. coli*.

Overgrowth of Escherichia coli by Other Coliform Bacteria in Standard Lactose Broth

That *E. coli* present in a water sample may not be detected by the standard procedure of lactose broth enrichment followed by streaking positive presumptive tubes onto EMB may be demonstrated by planting a drop from all positive presumptive lactose broth tubes

TABLE 4. *Overgrowth or failure to detect Escherichia coli by lactose broth enrichment in presence of other coliform bacteria and detection of E. coli by secondary boric acid lactose broth*

	Volume Water Seeded (ml)										Index (MPN)	
	10.0		1.0		0.1		0.01		0.001			
	Tube										Coliform	*E. coli*
	a	b	a	b	a	b	a	b	a	b		
Watercress Pond												
Primary lactose broth	A	A	A	A	I	I	I	I	—	—	24,000	<4.5
Secondary boric acid lactose broth	E†	E	E	E	E	*	*	*			700	700
Palolo Stream												
Primary lactose broth	EA	A	EA	A	A	A	A	—	—	—	7,000	60
Secondary boric acid lactose broth	EA	E	EA	E	E	E	A				7,000	2,400
Wahiawa Irrigation Reservoir												
Primary lactose broth	I	I	IA	I	I	I	E	E	E	—	70,000	1,400
Secondary boric acid lactose broth	E	E	E	E	E	E	E	E	E		70,000	70,000

* Gas not produced in secondary BALB.

† A, *Aerobacter*; I, Intermediate; E, *E. coli*.

into boric acid lactose broth, which is then incubated at 43 C, by streaking all tubes showing gas (both primary LB and secondary BALB) onto EMB, and then by allocating the colonies that develop to their appropriate coliform groups. In table 4 are shown the results obtained for three types of waters examined in this manner.

In the water sample obtained from a watercress pond which showed a coliform index of 24,000, *E. coli* was not detected in any of the positive LB presumptive tests, but, with the aid of the BALB as a secondary test medium, an *E. coli* index of 700 was obtained. Similarly, in a stream sample which had a coliform index of 7000, with an *E. coli* index of only 60 by the LB technique, utilization of BALB as a secondary test medium disclosed an *E. coli* index of 2400.

In both of the foregoing instances *E. coli* constituted a minority of the coliform organisms present. The next example, however, indicates that the same phenomenon, loss of *E. coli* with LB as a presumptive test medium followed by streaking on EMB, may occur when *E. coli* is present in larger numbers than the *Aerobacter* or intermediate types. Thus, in the sample of water obtained from an irrigation reservoir, *E. coli* was present in each of two 0.01-ml, and one of two 0.001-ml portions of water planted, but it was not detected in the 0.1-, 1.0-, or even 10-ml water portions with standard LB preliminary enrichment followed by streaking EMB agar. However, with BALB as a secondary medium it was demonstrated that *E. coli* was actually present in each of the standard LB positive presumptive test tubes.

The phenomenon of loss, or failure to detect *E. coli*, with standard lactose broth is attributed to overgrowth and the antibiotic properties of some coliform strains, especially of the intermediate group against *E. coli*. Fredericq and Levine (1947) reported that a group of 13 intermediate coliform strains studied were antibiotic against *E. coli* in over 25 per cent of trials. Recently we encountered a strain of *Aerobacter aerogenes* which was similarly very antagonistic to a large proportion (22 out of 23) of *E. coli* strains against which it was tested.

Boric Acid and Standard Lactose Broth as Primary Media, and Boric Acid Lactose Broth as a Secondary Medium, for Detection of Escherichia coli

Various portions of water were inoculated simultaneously into LB and BALB as primary enrichment media and were incubated at 35 and 43 C, respectively. A drop from each positive presumptive LB tube was planted into secondary BALB. All tubes showing gas, in either the primary or secondary test media, were streaked onto EMB, and several of each coliform type of colony that developed were fished and examined for allocation to their appropriate coliform group.

The results for about 400 water samples in which 3323 tubes were planted into primary LB and BALB, and positive LB presumptive tests inoculated into secondary BALB, are summarized in table 5. On comparison of LB and BALB when employed as primary media, it will be noted that members of the coliform group were detected in 1213 (36.4 per cent) of the water portions planted into LB as compared with only 474 (14.2 per cent) of the water portions when BALB was used, a loss of 61 per cent of coliform isolations with BALB. However, as to the frequency of isolation of *E. coli*, it will be noted that positive results were obtained from 499 (14.9 per cent) of the water portions

TABLE 5. *Relative efficiency of various procedures for detection of coliform bacteria and Escherichia coli in water*

	Primary Lactose Broth	Secondary BALB* from Positive Lactose Broth Presumptive Tests	Primary BALB*
Number of tubes			
Planted	3,323	1,359†	3,323
Gas produced (48 hr)	1,359†	710	475
Confirmed as			
Coliform	1,213	697	474
E. coli	499	669	447
Per cent of tubes planted with water portions			
Confirmed as			
Coliform	36.4	21.0	14.2
E. coli	14.9	20.1	13.5
Per cent of tubes showing gas			
Confirmed as			
Coliform	89.3	98.2	99.8
E. coli	36.7	94.2	94.1

* BALB, boric acid lactose broth.

† The 1359 positive primary lactose broth cultures were planted into secondary boric acid lactose broth.

with BALB, a loss of only 10 per cent *E. coli* isolations with BALB. The boric acid medium was a far better presumptive test for *E. coli* than LB, but, as is so frequently the case with good presumptive tests, the actual *E. coli* yield is apt to be somewhat lowered.

Considering the positive LB presumptive tests, it will be noted that 89.3 per cent was confirmed for the coliform group, but only 36.7 per cent for *E. coli*, whereas, if gas was produced in BALB, when used either as a primary or secondary medium, 98.2 to 99.8 per cent was confirmed for the presence of the coliform group and 94.2 per cent for the presence of *E. coli*.

A perusal of the data in table 5 shows that planting water portions directly into the BALB yielded 447 tubes with *E. coli*, as compared with 499 tubes with standard LB, a loss of 10 per cent, which may well be within the limits of error incident to distribution of organisms in the samples. However, when BALB was employed as the secondary test medium for positive LB presumptive tests, *E. coli* was detected in 669 tubes or 34 per cent more frequently than was the case with standard lactose broth.

It might be mentioned, in this connection, that these studies were conducted independently by four laboratories on different islands. Three of the laboratories, water portions being planted simultaneously into LB and BALB, reported fewer recoveries of *E. coli* with the boric acid medium, but in one laboratory *E. coli* was isolated from about 8 per cent more tubes when BALB planted into LB as compared with 447 (13.5 per cent) was used. In all instances, however, with BALB as a secondary medium for confirmation of positive LB tube presumptive tests, the results in each laboratory showed appreciably greater numbers of successful detection of *E. coli* than were obtained by the standard LB-EMB technique.

Considering that over 94 per cent of all tubes showing gas in BALB were confirmed for *E. coli*, it is apparent that the relatively simple and inexpensive procedure of confirming positive or doubtful LB presumptive tests by planting both into brilliant-green bile and boric acid lactose broth may be employed for obtaining simultaneously, economically, and relatively expeditiously both the coliform and *E. coli* indices, respectively. The *E. coli* indices thus obtained will frequently be appreciably higher than those of LB to EMB followed by the Voges-Proskauer and Koser citrate tests on isolated colonies.

SUMMARY

With pure cultures of coliform bacteria, only 0.7 per cent of intermediate and 5 per cent of *Aerobacter* strains, as compared with over 98 per cent of *Escherichia coli* strains, produced gas in boric acid lactose broth. When water portions, or a drop from positive lactose broth presumptive tests, were planted into boric acid lactose broth, 94.2 per cent of all tubes showing gas were confirmed for *E. coli*. Gas production in boric acid lactose broth in 24 to 48 hr at 43 C may therefore be considered a reasonably reliable criterion of the probable presence of *E. coli*.

Data are presented which show that *E. coli* may frequently be overgrown, if present together with other coliform bacteria, in positive lactose broth presumptive tests, so that they are missed by the procedure of streaking on eosin methylene blue agar, but that *E. coli*, if present, may be detected in such positive presumptive tests by transferring a drop to boric acid lactose broth and then streaking onto eosin methylene blue agar.

When water portions were planted into standard lactose broth at 35 C and boric acid lactose broth at 43 C as primary enrichment media, *E. coli* was isolated from 10 per cent fewer tubes with the boric acid lactose broth medium. However, by employing the boric acid lactose broth as a secondary test medium for positive lactose broth presumptive tests, on the same water samples, 34 per cent more tubes showing gas in primary lactose broth were demonstrated to contain *E. coli* than by streaking such tubes directly onto eosin methylene blue agar.

Confirmation of positive, or doubtful, lactose broth presumptive tests by planting both into brilliant-green bile and boric acid lactose broth and observing gas production in these secondary confirmatory media constitutes a simple, economical, and relatively expeditious procedure for obtaining, simultaneously, the coliform

and *E. coli* indices, respectively, of water samples, and this method should be equally applicable to food products.

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Pustule Formation by *Lactobacilli* on Fermented Vegetables

T. L. THOMPSON, W. E. ENGELHARD AND HILLIARD PIVNICK

Department of Bacteriology, University of Nebraska, Lincoln, Nebraska

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Genuine dill pickles are the product resulting from the fermentation, predominantly lactic, of cucumbers immersed in a brine containing 3 to 4 per cent salt. The brine also contains dill weed and may contain spices and garlic or extracts of their essential oils. Genuine dill tomatoes are manufactured from green tomatoes by a fermentation similar to that used for pickles (Fabian and Wadsworth, 1944).

Lactobacillus plantarum is commonly associated with the fermentation of pickles (Pederson, 1936), although other lactobacilli and some yeasts undoubtedly play a role. A survery of the literature failed to uncover any reports concerning the microbial flora of fermenting dill tomatoes. Pederson (1936), however, mentions that *L. plantarum* has been found in fermenting and spoiled tomato products.

The formation of white pustules on fermented olives has been investigated by Vaughn *et al.* (1953). These workers found that the pustules were actually massive, subepidermal growths of *L. plantarum*. They also noted, but did not investigate, the presence of such pustules on fermented Italian peppers and pickled green tomatoes.

We have noted the presence of white pustules on commercially packed dill tomatoes and pickles and on sweet pickles prepared from salt stock. This report deals with microbiological and histological studies of pustules on dill tomatoes and pickles.

MATERIALS AND METHODS

Isolation and Study of Microorganisms

Isolations were made from home-canned dill pickles and dill tomatoes 2 to 3 months after active fermentation had ceased. The tomatoes or pickles were washed in tap water, immersed for 10 sec in a solution containing 50 ppm of chlorine, and rinsed in tap water. Pustules were lanced with a sterile scalpel, and a small amount of the pustular material was shaken thoroughly in sterile water. The aqueous suspension was streaked on tomato juice agar (Difco tomato juice agar, with the agar content increased to 2 per cent), and the plates were incubated at 30 C either aerobically or in an atmosphere of 10 per cent carbon dioxide and 90 per cent hydrogen. Controls were carried out on the efficiency of the surface sterilization and sterility of the tap water.

Well-isolated colonies were picked from the streaked plates and their purity ensured by successive streaking on tomato juice agar. Forty-four cultures were studied for gram reaction, catalase production, and ability to ferment sugars and polyalcohols. The following compounds were added aseptically to 0.5 per cent sterile yeast extract broth containing brom cresol purple indicator in order to yield a final concentration of 0.5 per cent: glycerol, inulin, maltose, mannitol, arabinose, xylose, fructose, galactose, glucose, mannose, lactose, sorbitol, sucrose, rhamnose, and raffinose.

Yeast extract or 2 per cent malt sprout extract containing 3 per cent glucose was employed as the medium for the production of acid by representative cultures. Qualitative and quantitative studies were made on the fermented medium 4 to 7 days after inoculation.

Tests for volatile acids were made by steam distillation of the acidified fermented medium and titration of the distillate with standard base (Neish, 1952). Lactic acid was recovered as zinc lactate by the method of Brin, Olson, and Stare (1952), and the rotation determined polarimetrically.

Quantitative determinations of acid were made by