The Coliform Group

I. The Boric Acid Lactose Broth Reaction of Coliform IMViC Types

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Received for publication June 7, 1957

Several methods have been suggested for the accurate, rapid, and convenient detection and enumeration of *Escherichia coli* in potable waters, stream samples, sewage, and in certain food products. The first of these procedures was described by Eijkman who proposed a "fecal coli" test that he believed differentiated between coliforms from the gut of warm-blooded animals and strains originating from cold-blooded ones. The method was based on the assumption that only fecal types of coliforms from warm-blooded animals could grow in glucose broth at 46 C with the production of gas. Twenty-five years later a close relationship between the positive Eijkman reaction and indole production was observed by Leiter (1929) and he suggested that the combination of the two positive tests was practically specific for *E. coli*.

Many procedures have been proposed which claim to have provided a more suitable environment for the growth of *E. coli* and at the same time to have suppressed to a large degree the growth of other coliform types along with the majority of noncoliform bacteria. Most of the procedures have required the addition of one or more selectively inhibitory substances to lactose broth with some peptone derivative and have used an incubation temperature with a narrow tolerance between 43 C and 46 C. Such procedures were usually recommended as a "direct test" with the original sample inoculated into the selective medium, without any preliminary enrichment. Most investigators considered that their positive tubes represented quantitative data with the multiple tube (MPN) procedures. There were differences in opinion concerning the productivity of the selective media, the chief criticism being the possible failure to secure growth when minimal numbers of coliforms were planted, as in the MPN method. At the same time, the sensitivity of the medium was confused with the specificity of the reaction.

The specificity of these procedures should be related to the positive reactions with one or more IMViC types of coliforms and is quite a distinct problem from the quantitative recovery rates based on minimal number of coliforms. Other considerations which may have contributed to differences in observations between investigators were development and evaluation of methods with laboratory strains of coliforms and the differences in bacterial flora from one geographical area to another.

This report compares the reactions of the boric acid lactose broth test described by Vaughn *et al.* (1951), and Levine *et al.* (1955) with strains of each coliform IMViC type as they appeared in polluted surface waters at 14 geographical areas in the United States.

Materials and Methods

The coliform organisms were isolated from samples of untreated surface waters from the Missouri River at Omaha, Nebr., and Kansas City, Kan.; Mississippi River at Minneapolis, Minn., Quincy, Ill., and Chalmette, La.; Elm Fork River near Dallas, Tex.; Rio Grande at El Paso and Laredo, Tex.; Schuylkill River at Philadelphia, Pa.; Big Cottonwood Canyon at Salt Lake City, Utah; Colorado River aqueduct water at LaVerne, Calif.; Detroit River at Wyandotte, Mich.; Chattahoochee River at Atlanta, Ga.; and Oradell Reservoir at New Milford, N. J.

The cooperating laboratories at each location filtered appropriate quantities of surface water through membrane filters, placed the filters on preservative (benzoated endo) medium (Geldreich *et al.*, 1955) in 1-ounce sterile ointment tins, and mailed them to the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio. When received in this laboratory, the tests were completed as described by Geldreich *et al.* (1955) for the delayed incubation test for coliforms. Between 100 and 200 coliform colonies were isolated in a series from the membranes at each location and, generally, there were three series at regular intervals. The coliforms were immediately identified and classified by IMViC types according to the procedures described in *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes* (A.P.H.A., 1955a).

Simultaneously the boric acid lactose broth test, as devised by Levine *et al.* (1955), was used as a confirmatory procedure on each coliform strain. Any noncoliform bacteria that may have been picked up in the isolation procedures were discarded as they failed in confirmatory or identification tests. The technical methods recommended by the authors of the boric acid lactose broth test were followed as exactly as possible.

The medium was prepared from boric acid broth (Difco, B439[1]), sterilized as recommended by Vaughn *et al.* (1951), and the hydrogen ion concentration of each sterile lot was determined on a Beckman[2] pH meter, model H-2. The tubes were usually inoculated from 24-hr coliform cultures and were immediately placed in a constant-temperature water bath with the water height above the level of the medium inside the tubes. Temperature of the water bath was 43 C with less than 0.5 C variation and was regularly checked with a Bureau of Standards certified mercury thermometer.

[1] Difco Laboratories, Inc., Detroit, Mich.
[2] Beckman Instruments, Inc., Fullerton, Calif.

TABLE 1

Reactions in boric acid lactose broth (BALB) of coliform IMViC types

IMViC Type	No. of Strains Examined	Percentage of Coliforms Isolated	Positive BALB Tests		Negative BALB Tests	
			No. of strains	Percentage positive	No. of strains	Percentage negative
−−++	1414	31.97	284	20.1	1130	79.9
−+−+	764	17.28	41	5.4	723	94.6
++−−	731	16.53	682	93.3	49	6.7
−+++	384	8.68	33	8.6	351	91.4
++++	332	7.51	9	2.7	323	97.3
++−+	323	7.30	37	11.5	286	88.5
+−++	203	4.59	9	4.4	194	95.6
−+−−	139	3.14	21	15.1	118	84.9
+++−	74	1.67	60	81.1	14	18.9
−++−	31	0.70	0	0.0	31	100.0
−−+−	20	0.45	0	0.0	20	100.0
−−−+	8	0.18	6	—	2	—
Total..	4423		1182	26.7	3241	73.3

— = Insufficient number of cultures studied.

Inverted vials in the boric acid lactose broth were examined at 24-hr and 48-hr periods for the presence of gas. The presence of gas in any quantity in the inverted vial within a 48-hr incubation period was recorded as a positive reaction and the absence of gas in the same time was considered a negative result. All tubes were discarded at the end of incubation for 48-hr.

Results

During the period of this study, 4423 strains of coliforms were examined by the boric acid lactose broth test and classified as to IMViC types. Between 216 and 450 coliform strains were examined from each sample area. Almost one-third were −−++ IMViC type, approximately another third were −+−+ and ++−− types, and the remaining coliform strains were distributed among nine IMViC types. Data for types studied are summarized in table 1. The assigned numbers for the sample areas were arbitrary and were not according to any order of listing in this report.

In percentage of positive reaction in boric acid lactose broth (BALB), the ++−− type was highest with 93.3 per cent of the strains tested. Next in descending percentage of positive reactions were +++− type with 81 per cent; −−++ type with 20 per cent; −+−− type with 15 per cent; and ++−+ type with almost 12 per cent. The BALB positive strains in the next seven types varied between 8.6 per cent for −+++ type to none for the −++− and −−+− types.

A comparison by sample points of the BALB positive reactions for ++−− type with the BALB negative reactions for the remaining IMViC types is made in table 2.

TABLE 2

Positive tests with ++−− type and negative tests with the remaining types (excluding ++−− type) by sample locations

Location	++−− IMViC Type				Other IMViC Types (Excluding ++−− Type)			
	No. of strains	No. of positive tests	Percentage positive tests	Order*	No. of strains	No. of negative tests	Percentage negative tests	Order*
1	44	41	93.2	7th	230	210	91.3	4th
2	28	24	85.7	12th	422	366	86.7	6th
3	22	19	86.4	11th	275	254	92.4	2nd
4	81	76	94.8	5th	199	179	90.0	5th
5	61	59	96.7	4th	194	166	85.6	10th
6	72	71	98.6	2nd	282	243	86.1	8th
7	69	65	94.2	6th	147	122	83.0	11th
8	37	31	83.8	13th	302	287	95.0	1st
9	81	75	92.6	9th	326	198	60.7	13th
10	60	53	88.3	10th	171	147	86.0	9th
11	49	49	100.0	1st	383	310	80.9	12th
12	55	51	92.7	8th	203	175	86.2	7th
13	4	2	—		285	285	100.0	
14	68	66	97.1	3rd	273	250	91.6	3rd
Total...	731	682	93.3		3692	3192	86.5	

— = Insufficient number of cultures studied.
* "Order" represents position of data arranged in a descending series by percentages.

Of the total 731 ++−− type coliform strains examined, 682 strains or 93.3 per cent were positive. There were variations between 83.8 per cent positive reactions at location 8 and 100 per cent at location 11. For the remaining 3692 IMViC types of coliforms (excluding ++−− type), negative reactions resulted with 3192 or 86.5 per cent of the strains examined. Variations were also observed between locations in the percentage of negative reactions with this group. Location 9 was the lowest with 60.7 per cent negative tests and location 13 was highest with 100 per cent negatives.

In order to present the data as completely as possible, table 3 presents the number of strains examined, with the percentage of positive reactions by each IMViC type for the individual sample areas. The marked variations of type reactions between sample areas are emphasized in this table. For example, the −−++ type was 48 per cent positive at location 9; 23 to 26 per cent positive at locations 4, 6, and 11; and 1 per cent or less at locations 7 and 13. The −+−+ type was positive with 22 and 23 per cent of the strains at locations 9 and 10 but had only 2 per cent positives at locations 1, 2, and 8, and none at locations 4, 6, 12, and 13. The same variation can be observed for each IMViC type with the possible exception of the +++− type. In the small number of strains examined for this type (74), the proportion of positive reactions appeared to be about the same in each location where they were present.

Discussion

The ++−− type gave a reasonably good percentage of positive reactions with the BALB test in many locations, but the large number of positive reactions with such types as −−++, ++−+, and −+−− strains introduces a considerable classification error in its over-all application. These three latter IMViC types represented 42.4 per cent of the coliform strains isolated from polluted surface waters. The +++− type yielded 60 positives or 81 per cent of 74 strains, but as a reasonably rare type representing only 1.67 per cent of the total coliform strains in these waters, it would scarcely introduce sufficient error to change the interpretation on a series of results.

Application of the tentative coliform group classification described in *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes* (A.P.H.A., 1955b) would result in the following data: Assuming *E. coli* to include types ++−− or −+−−, the number of strains in this study would be 870 with 703 positive reactions or 80.8 per cent. Using the same classification, the "commonly designated source" of the remaining IMViC types are usually considered nonfecal strains. There were 3553 strains in this group which gave 475

TABLE 3

Number of strains examined and percentage of positive strains for each IMViC type by individual sample points

IMViC Type		Sample Points Listed Numerically														Totals
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
−−++	No. strains	84	129	164	31	113	107	76	40	201	79	146	97	16	131	1414
	Percentage	13	16	10	23	17	25	1	17	48	14	26	15	0	11	20.1
−+−+	No. strains	41	92	35	21	27	38	21	117	40	31	92	34	149	26	764
	Percentage	2	2	6	0	7	0	5	2	22	23	15	0	0	4	5.3
++−−	No. strains	44	28	22	81	61	72	69	37	81	60	49	55	4	68	731
	Percentage	93	86	86	94	97	99	94	84	93	88	100	93	*	97	93.3
−+++	No. strains	33	30	12	59	2	23	12	37	37	10	47	19	27	36	384
	Percentage	0	7	8	0	*	9	0	3	24	20	23	16	0	3	8.6
++++	No. strains	21	32	17	37	9	55	8	25	14	20	43	17	1	33	332
	Percentage	0	9	6	0	*	2	*	0	7	0	0	0	*	0	2.7
++−+	No. strains	25	76	13	25	21	27	13	37	20	12	21	13	None	20	323
	Percentage	12	3	8	4	9	4	100	3	30	17	9	8		5	11.5
+−++	No. strains	19	17	30	13	18	18	6	32	2	11	12	7	9	9	203
	Percentage	5	6	0	15	11	5	*	3	*	0	8	*	*	*	4.4
−+−−	No. strains	2	18	2	7	1	9	5	6	8	4	8	5	62	2	139
	Percentage	*	5	*	*	*	*	*	*	*	*	*	*	0	*	15.1
+++−	No. strains	4	25	None	6	None	3	5	2	3	2	7	7	2	8	74
	Percentage	*	88		*		*	*	*	*	*	*	*	*	*	81.8
−++−	No. strains	None	1	None	None	None	None	1	1	1	None	1	4	17	5	31
	Percentage		*					*	*	*		*	*	0	*	0.0
−−+−	No. strains	1	1	None	None	2	2	None	2	None	2	6	None	1	3	20
	Percentage	*	*			*	*		*		*	*		*	*	0.0
−−−+	No. strains	None	1	2	None	1	None	None	3	None	None	None	None	1	None	8
	Percentage		*	*		*			*					*		*(25.0%)

* Insufficient number of cultures studied.

(13.37 per cent) BALB positive reactions. Even using this liberal tentative classification of "usual sources," the reaction does not appear sufficiently precise for group separation on the basis of common fecal or non-fecal types.

Comparison of locations by positive-reaction percentages for ++−− type with negative-reaction percentages for remaining types, as listed in table 2, reveal a peculiar distribution. Locations 14, 4, 12, and 10 appear in similarly relative positions for positive reactions with ++−− type and for negaive reactions with remaining types when examined by percentages in descending order. There is marked disagreement in relative positions for the remaining locations, as for example, location 11 at the top of the series for ++−− type and next to lowest for the remaining types, and location 6 in second and eighth positions respectively. Further disagreement in results by locations is shown in table 3. The −−++ type gave 48 per cent positives at location 9; values between 23 and 26 per cent at locations 4, 6, and 11; and 1 per cent or less at locations 7 and 13. IMViC type −+−+ gave more than 20 per cent positives at locations 9 and 10 and 2 per cent or less at locations 1, 2, 4, 6, 8, 12 and 13. Similar variations in percentages of positives by locations were evident in all the types investigated except +++−, −++− and −−+−. This lack of uniformity in data by individual locations suggests differences in the coliform flora found in waters and may account for some controversial data and interpretations. The possibility cannot be eliminated that the IMViC system of classification does not adequately divide the coliforms found in waters into groups consistent with their common sources.

These data will not recommend the BALB procedure for accurate and rapid classification of the ++−− IMViC type. However, the method appears applicable for survey work where an approximate value for the ++−− coliform type would meet the need. In this investigation, 4423 coliform strains gave 1182 positive BALB reactions or 26.7 per cent when BALB was the only test considered. By IMViC classification, 731 strains of the ++−− type or 16.53 per cent were present. Inclusion of the indole reaction, which has been used by Hoather and Dewey (1954), Thomas, *et al.* (1955), and Bicknell, *et al.* (1954) by direct inoculation of a suitable medium from positive presumptive tubes, would greatly increase accuracy in survey procedures. Using a combination of a positive indole test with a positive BALB reaction, the total positives by this combination (from table 1) would be 797 strains or 18.0 per cent. Of course, this total will not include the 49 BALB negative strains of ++−− type nor eliminate the 115 positive strains in types ++++, ++−+, +−++, or +++−.

In evaluation of these data, all comparisons were made from tests on freshly isolated strains with a minimum of cultivation on laboratory media to make the results applicable for survey work and practical water bacteriological investigations. Some of the reactions might change after continued cultivation and stabilization on laboratory media in a controlled environment.

Acknowledgments

Our grateful appreciation and sincere thanks is expressed to the following organizations for their technical aid and other assistance in the various phases of this study: Water Purification Plant, Dept. of Water Works, Atlanta, Ga., Water Purification Plant, Dept. of Water Works, Dallas, Tex., Water Treatment Plant, Public Service Board, El Paso, Tex., Dept. Filtration and Sanitation, Hackensack Water Co., New Milford, N. J., Kaiser Aluminum and Chemical Corp., Chalmette, La., Quindaro Station, Board of Public Utilities, Kansas City, Kan., Water Prod. and Purification, Laredo Waterworks System, Laredo, Tex., Division of Laboratories, Louisiana State Board of Health, New Orleans, La., Fridley Softening Plant, Water Dept., Minneapolis, Minn., Florence Filter Plant, Metropolitan Utilities District, Omaha, Nebr., Belmont Laboratory, Water Dept., Philadelphia, Pa., Water Plant, Water Works Commission, Quincy, Ill., Dept. Water Supply and Water Works, Salt Lake City, Utah, Laboratory, City Board of Health, Salt Lake City, Utah, Metropolitan Water Dist. Y of Southern California, LaVerne, Calif., Water Plant, Department of Municipal Services, Wyandotte, Mich.

Summary

Coliforms from 14 geographical areas of the United States were divided into 12 types by their IMViC reactions. The 4423 strains were examined for their reaction in boric acid lactose broth (BALB) which was incubated at 43 C for 48 hours. There were 682 positive cultures (93.3 per cent) in the 731 strains of the ++−− type and 500 positive cultures (13.5 per cent) in the 3692 strains belonging to the remaining eleven types. The lack of sensitivity (6.7 per cent negative reactions) for the ++−− type and poor specificity (13.5 per cent positive reactions) for the other IMViC types do not recommend the BALB as a single specific test for the accurate detection of ++−− IMViC type.

However, these data vary within relatively wide limits by locations and even the sensitivity and specificity may not agree at each location. These variations between sample areas may account for the controversial data and interpretations for this and other methods for the detection of ++−− IMViC types.

The possibility cannot be eliminated that these variations may result from differences in coliform flora for which the IMViC system of classification is not adequate.

Using the BALB procedure as a single test, 1182 positive reactions or 26.7 per cent would have been considered as ++−− type although only 731 strains or 16.5 per cent were actually ++−− by IMViC classification. The BALB test alone could be used in survey work where an approximate value would be sufficient. However, by using both a positive indole and positive BALB test as a survey procedure, the elapsed time would not be increased and, by elimination of 385 false reactions, the positives would be reduced to 797 strains or 18.0 per cent of the 4423 strains tested.

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Studies on the Mode of Action of Chlortetracycline in the Preservation of Beef[1,2]

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Received for publication June 7, 1957

It is now well established that chlortetracycline and certain other antibiotics are effective at very low concentrations in the preservation of meat (Deatherage, 1956, 1957). This is true despite the fact that much of the bacterial flora of a given type of meat is very often resistant to the antibiotic when examined by the usual laboratory methods for testing sensitivity. Previous studies carried out in this laboratory (Jay *et al.*, 1956, 1957), showed that certain strains of *Proteus vulgaris* which were inhibited by chlortetracycline at concentrations as high as 100 ppm were actually inhibited in beef by as little as 3 ppm in 24 to 48 hr.

In an attempt to explain this unusual phenomenon, it has already been shown that the inhibition in beef by the sub-bacteriostatic concentration of chlortetracycline could be reversed by adding Mn^{++} ions to the meat (Jay *et al.*, 1956). Earlier workers (Saz and Slie, 1953; Weinberg, 1954) had shown that Mn^{++} was able to counteract the effectiveness of chlortetracycline. On this basis, the hypothesis was advanced that this phenomenon was a nutritional one in which the chlortetracycline competed with the organisms for essential ions.

This report deals with other substances that were tested for their ability to reverse this chlortetracycline inhibition of resistant *Proteus* in beef.

Experimental Methods

Twenty gram samples of freshly slaughtered beef, either top or bottom round, were taken by an aseptic searing technique previously described (Lepovetsky *et al.*, 1953; Jay *et al.*, 1956) and placed into sterile 100-ml beakers. The removal and transfer of the beef

[1] Published with the approval of the Associate Director, The Ohio Agricultural Experiment Station, as Journal Article No. 53-57.

[2] Presented in part at the 57th Annual Meeting of the Society of American Bacteriologists, Detroit, Mich., 1957.