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EVALUATION OF ENTEROCOCCI AND COLIFORM ORGANISMS AS WATER POLLUTION INDICATORS

TUSLOG
DET 36
APO 224

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ABSTRACT

The utilization of enterococci and coliform organisms as water pollution indicators was studied using the Izmir water supply system and other local sources. The individual merits of each organism are discussed in detail with particular application to the Izmir findings. Recommendations are included insofar as current literature research and field application will allow.

This publication has been reviewed and approved.

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FOREWORD

This publication was prepared by Captain William J. Robertson based on studies performed by the following personnel of the USAFE Epidemiological Flight (TUSLOG Detachment 36):

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EVALUATION OF ENTEROCOCCI AND COLIFORM ORGANISMS AS WATER POLLUTION INDICATORS

INTRODUCTION

In the United States tests for coliform organisms are still used in determining the sanitary quality of water. Standard Methods[1] defines the coliform group to include --- "all of the aerobic and facultative anaerobic, Gram-negative, nonsporeforming, rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 37 C." Because of their preponderance in feces, Escherichia coli strains are the most significant coliforms in water pollution. Other members of the coliform group including Aerobacter, Klebsiella, and Citrobacter (Escherichia) freundi are often found in feces in small numbers. These forms, however, are widely distributed in nature and may also gain access to water from nonfecal sources, e.g., soil and vegetation. According to Taylor,[2] the presence of E. coli indicates true fecal contamination, but the presence of coliforms other than E. coli may reflect beginning or past fecal pollution, thus requiring further investigation.

In any event it is often difficult to ascertain the significance of positive coliform results with waters in the absence of E. coli. Mallmann[3] has outlined the assets and limitations of the coliform test in determining fecal pollution. In his opinion the test is reliable for checking potable waters originating from known sewage contaminated sources, as well as in measuring sewage loads on streams where a health hazard exists. Conversely, he believes the test becomes questionable in examining relatively clean streams, shallow lakes with high biological activity, shallow wells, swimming pools, and products other than water.

Although it appears unnecessary to sanction any organisms even remotely of potential fecal origin in waters for human use, occasions do arise when more precise definition of contamination is necessary to resolve pollution problems.

For many years the enterococci or fecal streptococci have been given varying degrees of consideration as indicators of fecal pollution. Greatly improved laboratory methods for isolation and identification of these organisms have stimulated interest.

Bartley and Slanetz[4] believe the use of enterococci as indicators would obviate the question of fecal or nonfecal origin encountered through use of coliform tests for water pollution. Other investigators believe fecal streptococcus examinations should be employed to supplement, not substitute for coliform analyses. The detection of enterococci in water would definitely confirm fecal pollution when only equivocal fecal-soil coliforms such as Aerobacter, Klebsiella, or Citrobacter (Escherichia) freundi are present.

The enterococci are defined in Bergey's Manual of Determinative Bacteriology[5] as Streptococcus faecalis, S. faecalis var. liquefaciens, S. faecalis var. zymogenes and S. durans. These are Gram-positive, coccoid organisms usually occurring in pairs or short chains. Enterococci are not encapsulated, spore-forming, or motile. Culturally these organisms exhibit rather unique properties, growing well aerobically and anaerobically on ordinary media at temperatures ranging between 10 and 45 C (37 C optimum). Enterococci grow in 6.5 percent sodium chloride broth, at pH 9.6 and in 0.1 percent methylene blue milk, and are able to survive 60 C for 30 minutes.

Since Houston[6] reported enterococci as being associated with human and animal wastes, voluminous information has been accumulated regarding their identification and use as fecal indicators. For the sake of brevity, reference is made to reviews and works of Taylor,[2] Mallmann,[3] Kenner et al,[7] and Litsky and Mallmann.[8]

Literature reviews of numerous investigations concerning fecal streptococci as indicators have evolved the following conclusions:

1. Enterococci, like E. coli, are natural inhabitants of the intestinal tract of man and animals.

2. They remain viable in feces, sewage, and soil polluted with human and animal wastes for varying periods of time.

3. They are absent from unpolluted water, virgin soil, and areas free from human and animal contacts.

4. Although enterococci apparently outlive coliforms in fecal material and sewage waters, they may die more rapidly when these wastes are introduced into soil and relatively clear waters containing little organic material. If this is true, their presence in water can be regarded as evidence of more recent fecal pollution.

5. Enterococci are only found at polluted sites, yet some coliform organisms are recovered from areas not associated with fecal pollution.

As fecal indicators, enterococci have particularly shown promise in analyzing well waters. Ritter et al [9] found enterococcus-coliform counts in close agreement after examining seventy-two wells of different construction. A study of fifty-two wells by Morris and Weaver [10] revealed that coliform and enterococci were of almost equal value in detecting pollution of underground water.

Slanetz and Bartley [11] employed the millipore filter technique using M-Enterococcus agar in examining water and sewage samples as well as fecal specimens from man and animals. On the basis of arithmetic mean counts of samples tested, the ratio of enterococci to coliforms was 1.9:1 for water and 1:1.7 for sewage. Fecal samples from human beings and animals were 1:1.6 and 15:1, respectively. These results indicate that enterococci may well prove their value as indicators of fecal contamination.

The author's concern for more precise definition of fecal pollution is best understood when considering water sanitation problems in Turkey. In most rural areas rainwater reservoirs, shallow wells, small lakes, and mountain streams--all subject to surface pollution at one time or another--are utilized by the populace. Sources for metropolitan areas are usually pure or relatively pure waters from artesian wells, rivers, springs, or large lakes. In the past extensive studies throughout Turkey conducted by the USAFE Epidemiological Flight at Izmir have allowed certain broad conclusions to be drawn. On the basis of repeated coliform analyses, a rather large percentage of water systems is unsatisfactory.[12] Either the main sources are contaminated, which particularly holds true for surface waters and shallow wells, or fundamentally pure water sources become contaminated upon

passing through faulty distribution systems. The latter more appropriately applies to the metropolitan water systems. Although chlorination is practiced in the larger cities, residues are quickly dissipated by organic material gaining access through breaks in the pipes.

Under the above existing conditions, the composite picture reflected by coliform tests may not be entirely reliable. For reasons previously stated, some of the contamination might represent soil-vegetation pollution rather than fecal. Conversely, the poorly constructed water systems and multitude of human and animal sources from which fecal contamination can originate in the cities and villages raise the question of whether or not coliform tests are sufficiently sensitive.

In order to more fully assess the value of enterococci as fecal indicators, it is important to study their frequency of association with E. coli and other coliforms in water and sewage water from various sources. This was the basic approach used for the present investigation. Water from different sources in and around Izmir, Turkey, was examined for enterococcus and coliform count. This included tap water from various sources of the municipal artesian well system along with samples from shallow wells, creeks, and streams utilized by a much smaller percentage of indigenous inhabitants. Water from obvious sewage sources was also examined.

MATERIALS AND METHODS

Beginning in January 1962, 19 water samples were collected from the Izmir distribution system and examined for enterococci and coliform organisms. In most instances 1 sample each was taken from seven different sources located within five districts. Three such collections were made at weekly intervals. During the same period, enterococcus-coliform examinations were also performed on water samples secured directly from 11 shallow wells and 19 creeks and streams without duplication.

A more comprehensive survey of the Izmir system was initiated in January 1963. Enterococcus-coliform examinations for this study were performed on 83 water samples collected from 15 different tap water sources which also included 5 city districts.

To allow monitoring of pollution variations in relationship to time, most sources were examined one or two times every seven to fourteen days over a 10-week period. Four samples of sewage water were also examined for fecal streptococci and coliform organisms. To determine the relative viability of both fecal groups, counts were made on sewage water after storage for different time intervals at 26 C.

All samples were collected in sterile, screw-capped bottles (130 ml capacity) containing sodium thiosulfate equal to 100 mg per liter of sample. They were usually processed within six hours following collection and always within the 24-hour acceptable limit. Precautions were taken regarding storage temperature of water samples as imposed by Standard Methods. Following collection of a water sample at any given site, an on-the-spot check for residual chlorine was performed using the orthotolidine method [1] as adapted to a Filter Photometer Test Kit.

For isolation of fecal streptococci appropriate dilutions of all samples were passed through millipore filters (47 mm Type HA) which were subsequently cultured on M-Enterococcus agar. [11] A detailed outline of the procedure is given in Standard Methods for Examination of Water and Wastewater. M-Enterococcus agar was prepared by dissolving the following ingredients in distilled water to yield 1 liter:

Tryptose	20 grams
Yeast Extract	5 grams
Glucose	2 grams
Dibasic Potassium Phosphate	4 grams
Sodium Azide	0.4 grams

The pH was adjusted to 7.2, and after adding 10 grams (1 percent) of agar the mixture was brought to a boil with frequent agitation. Upon cooling slightly, 1 ml of a 1 percent filter sterilized solution of 2, 3, 5 - Triphenyltetrazolium chloride was added for each 100 mls of medium. The medium was dispensed in approximately 12 to 15 ml quantities to sterile, 58 mm diameter ointment tins. Following agar solidification, the tins were stored in the refrigerator. Sterilization of the medium was not necessary; however, it was found to lose its selective properties after a 2-week period. After sample filtration the filters were aseptically placed on the surface of the agar medium. The pink to maroon colored

colonies of enterococci were counted after 48 hours' incubation at 35 C.

Analysis of water from the main Izmir system was accomplished by filtering 100 ml samples through millipores. To insure countable plates, it was necessary to filter 10 and 100 ml quantities of water from the shallow wells and surface streams. In most cases sewage waters were diluted 1:100 and 1:1000, and 10 ml quantities of each dilution were filtered.

After fecal streptococcus counts had been made, at least 3 typical colonies were picked from a millipore culture of each sample and inoculated to sectored areas of a blood agar plate. The latter was incubated for 18 hours under increased carbon dioxide, and fecal streptococci were verified on the basis of colony appearance, hemolysis, catalase testing, and subsequent growth in 6.5 percent NaCL broth.[5] Atypical appearing cultures were Gram-stained.

The samples were examined for coliforms following filtration as described above. Millipores were cultured on 47 mm absorbent pads saturated with Difco Modified Endo Medium [13] in sterile ointment tins. Counts of typical dark (purplish-green) colonies with metallic sheening were made following 20 hours of incubation. To determine the presence or absence of E. coli, at least 3 typical colonies were subcultured to Eosine Methylene Blue agar. The organisms were subsequently identified from reactions obtained by growth in Triple Sugar Iron agar and IMViC media.[1] Identification of coliforms was accomplished on all specimens except those shown in Table II.

RESULTS AND DISCUSSION

In Table I the results of the January 1962 Izmir tap water survey are shown. Of the 19 samples examined, 5 (26.4 percent) were positive exclusively for enterococci, while only 2 samples (10.5 percent) yielded coliform organisms only. Four samples (21 percent) contained organisms of both groups. In all instances coliform positive samples were found to contain Escherichia coli. Both E. coli and Citrobacter freundi were recovered from 4 water samples. Employing enterococci as indicators, approximately 47 percent of the water samples were nonpotable, as opposed to about

32 percent using coliforms. Therefore, enterococcus analyses yielded one-third more positive samples with a 10.5 percent error manifested in the 2 samples which contained coliforms only. These results indicate that fecal streptococci were more sensitive indicators, at least for the water system examined in this study. On the basis of arithmetic mean counts of the samples shown to contain both groups, the ratio of enterococci to coliforms was 1:2.8.

TABLE I

ENTEROCOCCUS-COLIFORM RECOVERIES
IZMIR WATER SYSTEM
(Jan-Feb 1962)

No. of Samples	Mean Count/100 mls		Percent	Coliform Species	
	Enterococci	Coliforms		E. coli only	E. coli and C. freundi
8	0	0	42.1	-	-
5	11.2	0	26.4	-	-
2	0	21.0	10.5	1 sample	1 sample
4*	2.5	7.0	21.0	1 sample	3 samples

19 Total Samples Examined

*Enterococcus-Coliform ratio - 1:2.8

In Table II the enterococcus-coliform recoveries from 83 water samples collected during the January 1963 Izmir tap water survey are shown. Enterococci were isolated from 19 or 22.9 percent of the specimens which failed to yield coliforms. Coliform organisms, in the absence of fecal streptococci, were demonstrated in 10 (12.0 percent) of the samples. Both groups were recovered from 11 (13.3 percent) of the water samples. On the basis of enterococcus isolations, about 36 percent of these samples were nonpotable, while approximately 25 percent were unacceptable by coliform standards. Enterococcus recoveries closely paralleled those of the January 1962 survey (Table I) in that

almost one-third more of the samples were positive for organisms of this group than for coliforms. In this case a 12 percent error was manifested by 10 samples that contained coliform organism only. Based on arithmetic mean counts the enterococcus-coliform ratio was 1:2.5 for samples containing both groups.

TABLE II

ENTEROCOCCUS-COLIFORM COUNTS
IZMIR WATER SYSTEM
(Jan-Mar 1963)

No. of Samples	Mean Count/100 mls Enterococci	Coliforms	Percent
43	0	0	51.8
19	41.0	0	22.9
10	0	8.8	12.0
11*	10.0	25.0	13.3

83 Total Samples Examined

*Enterococcus-Coliform ratio - 1:2.5

The city districts where fecal organisms were isolated from the water system are shown in Tables III and IV. Despite the fact that chlorination is routinely practiced at the main water source, residuals were not detectable at any of the collection sites. Although water from certain collection points was less consistently polluted, the overall number of contaminated samples was alarmingly high. Water in these districts cannot be safely consumed over any appreciable period of time without prior boiling or chemical purification in the home.

Repeated examinations of samples taken directly from the main reservoir fed by artesian wells have been negative for fecal organisms and chlorine residues. Apparently, the water becomes contaminated upon passing through the antiquated distribution system.

As was anticipated, the surveys presently cited failed to implicate a common pollution source within the distribution system. Water pressure within the mains of many areas is known to fluctuate considerably from time to time. Pressure variations are due to a combination of factors such as increased water consumption during the dry months, pump failures, and breaks in the system. Drops in water pressure undoubtedly result in back-siphoning of contamination into the system at various break-points. Fecal pollution very likely occurs when the latter are located in proximity to leaking, subterranean sewer pipes. Under these conditions it would be impossible to maintain a chlorine residual in most branches of the system.

TABLE III

ENTEROCOCCUS-COLIFORM COUNTS BY AREA
IZMIR WATER SYSTEM
(Jan-Feb 1962)

Tap Water Source	Total Samples	Enterococci Only	Coliforms Only	Positive both Groups	Total Positive
Basmane	3	1	1	0	2
Karabaglar	3	1	1	1	3
Alsancak	2	0	0	0	0
Konak	3	0	0	1	1
Hali Rifat					
Location 1	3	1	0	1	2
Location 2	3	1	0	0	1
Location 3	2	1	0	1	2

TABLE IV

ENTEROCOCCUS-COLIFORM COUNTS BY AREA
IZMIR WATER SYSTEM
(Jan-Mar 1963)

Tap Water Source	Total Samples	Enterococci Only	Coliforms Only	Positive both Groups	Total Positive
Karantina					
Location 1	8	2	0	0	2
Location 2	9	1	0	1	2
Alsancak					
Location 1	8	1	1	1	3
Location 2	10	3	3	1	7
Location 3	2	0	0	0	0
Location 4	1	0	1	0	1
Location 5	1	0	0	0	0
Location 6	3	0	2	0	2
Location 7	1	0	0	0	0
Tepecik	6	3	0	1	4
Mt. Pagus-Konak Area					
Location 1	8	1	1	2	4
Location 2	9	3	0	3	6
Location 3	9	2	1	1	4
Bornova	1	0	0	1	1
Kahramhnlar	7	3	1	0	4

In Table V the enterococcus-coliform recoveries from 11 shallow wells in and around Izmir are shown. In this case all samples were contaminated with fecal organisms, and enterococcus isolations accounted for 2 (about 18.2 percent) more polluted samples. The remaining 9 samples were shown to possess both fecal groups in a mean count ratio of approximately 1:1. Four of these samples contained Escherichia coli only; 1 contained Citrobacter freundi; and 4 contained both E. coli and C. freundi.

TABLE V

ENTEROCOCCUS-COLIFORM RECOVERIES
SHALLOW WELL WATER

No. of Samples	Mean Count/100 mls		Percent	Coliform Species		
	Enterococci	Coliforms		E. coli Only	C. freundi Only	E. coli and C. freundi
2	99.0	0	18.2	-	-	-
9*	135.0	137.0	81.8	4 samples	1 sample	4 samples

11 Total Samples Examined

*Enterococcus-Coliform ratio - approximately 1:1

The enterococcus-coliform isolations from 19 creeks and streams are summarized in Table VI. Again all samples were contaminated, but coliform organisms rather than streptococci accounted for one more sample (5.3 percent) being positive. However, only C. freundi was recovered from the millipore of this specimen, which may or may not represent fecal contamination. The 18 samples which contained both groups yielded a mean count ratio of 1.1 enterococci to 1 coliform. Fourteen samples contained E. coli and C. freundi. E. coli exclusively was isolated from 3 samples, while 2 samples contained only C. freundi.

TABLE VI

ENTEROCOCCUS-COLIFORM RECOVERIES
CREEKS AND STREAMS

No. of Samples	Mean Count/100 mls		Percent	Coliform Species		
	Enterococci	Coliforms		E. coli Only	C. freundi Only	E. coli and C. freundi
1	0	12	5.3	-	1 sample	-
18*	182	155	94.7	3 samples	1 sample	14 samples

19 Total Samples Examined

*Enterococcus-Coliform ratio - approximately 1.1:1

Results of the sewage water examinations are set forth in Table VII. Enterococcus-coliform ratios of about 1:22.5, 1:2.2, 1:1.25, and 1:1 were demonstrated in 4 specimens. Counts obtained on 1 of these samples after storage at 26 C for different time intervals are shown in Table VIII. Although this sample initially contained 10,000 more coliform organisms per 100 mls than enterococci, only the latter organisms survived the entire storage period. A rather abrupt increase in numbers of organisms of both groups was detected in the sewage water after three days' storage. Maximum counts for enterococci and coliform organisms were obtained at three and twelve days, respectively. By the forty-first day of storage enterococcus counts had decreased 10-fold, and coliform counts about 26-fold from their greatest concentrations. From this point on enterococci died more rapidly than coliforms until nearly equal counts were obtained on the ninety-eighth day. After one-hundred and seven days of storage, only enterococci in a concentration of 20 organisms per 100 mls of sample were demonstrated.

TABLE VII

ENTEROCOCCUS-COLIFORM COUNTS
SEWAGE WATER

Sample Number	Enterococci Per 100 mls	Coliforms* Per 100 mls	Ratio
1	40,000	900,000	1:22.5
2	3,700	8,200	1:2.2
3	1,100	1,100	1:1
4	40,000	50,000	1:1.25

*Including Escherichia coli

TABLE VIII

SURVIVAL OF ENTEROCOCCI AND COLIFORMS IN SEWAGE
WATER STORED AT 26 C

Days	Enterococci/100 mls	Coliforms/100 mls*
0	40,000	50,000
3	910,000	670,000
6	280,000	950,000
9	200,000	890,000
12	240,000	1,800,000
27	250,000	150,000
41	90,000	69,000
63	10,000	7,500
72	2,200	6,600
75	3,000	6,200
78	100	7,000
98	30	50
107	20	0
112	20	0

*Including Escherichia coli

CONCLUSIONS

In the foregoing studies, fecal streptococci proved to be more sensitive fecal indicators than coliforms in examinations of the Izmir water system. The combined results of two surveys revealed almost one-third more samples to be positive for fecal streptococci. An error of approximately 11 percent was exhibited by samples which were negative for enterococci, yet positive for coliform organisms. Some investigators have reported that fecal streptococci tend to die more rapidly than coliforms when fecal material or sewage is introduced into waters relatively free of organic material.[6, 14, 15] In most instances these workers dealt with river waters. The presently reported studies of Izmir water diverge from the above concept. Harrocks,[16] however, found streptococci to live longer in sewage than E. coli and reported similar experience with polluted waters. Taylor's results[17] suggest that fecal streptococci have a greater potentiality for survival than E. coli, both under conditions of passing through soil and in jute yarn used in the joints of water mains. In examining river water, Litsky et al[8] found an E. coli to enterococcus ratio of 1:7.6, on the basis of median values of all samples taken. The reasons for the differences in findings are not known. Perhaps they are due to inadequate definition of the kind of water examined and the environmental states under which such waters exist when samples are taken. Jute yarn is most certainly a common item used in the repair and construction of water mains in the city of Izmir, and may account for the greater number of enterococcus isolations. The greatly improved methods for isolation of fecal streptococci must also be considered in this connection.

It has been stated that enterococci may be more resistant to chlorine than coliform bacteria.[1] This being true would have explained the greater number of fecal streptococcus recoveries, had any level of chlorine been demonstrable in the city reservoir or water mains. The need for further studies concerning survival of fecal streptococci in water from various sources is indicated.

The enterococcus to coliform ratios reported in Tables I and II fall within the range reported for natural waters in Standard Methods. It was interesting to note that water samples from shallow wells, creeks, and streams were nearly equal both in enterococcus-coliform count and isolations of each group (Tables V and VI). These results match those of other workers.[9, 10]

Moreover, the enterococcus-coliform counts obtained on examination of the sewage waters (Table VII) yielded ratios comparable to those frequently reported in the literature for such specimens. The survival of enterococci over coliform organisms upon storage at room temperature (Table VIII) generally supports the findings of earlier investigations made by Prescott and Baker,[18] Harrocks,[16] and Leiguarda.[19]

Escherichia coli was demonstrated in about 92 percent of of the 38 millipore filter cultures subcultured to Triple Sugar Iron agar and IMViC media. Although other coliforms were also present in some of the specimens, only the metallic sheening colonies typical of E. coli and Citrobacter freundi were selected for identification. This revealed a close correlation between typical sheening colonies isolated using Modified Endo's medium and the presence of E. coli.

M-Enterococcus agar was found to be almost 100 percent effective in allowing only fecal streptococci present in the samples to grow. Contaminants resembling these organisms were very rarely encountered on the millipore filter cultures using this medium. It was discovered that other organisms may grow, however, if the medium is used after two to three weeks' storage in the refrigerator.

The author strongly recommends that both fecal streptococcus and coliform examinations be performed on all drinking water samples collected. This will insure greater accuracy in detection of pollution problems, regardless of the water source.

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