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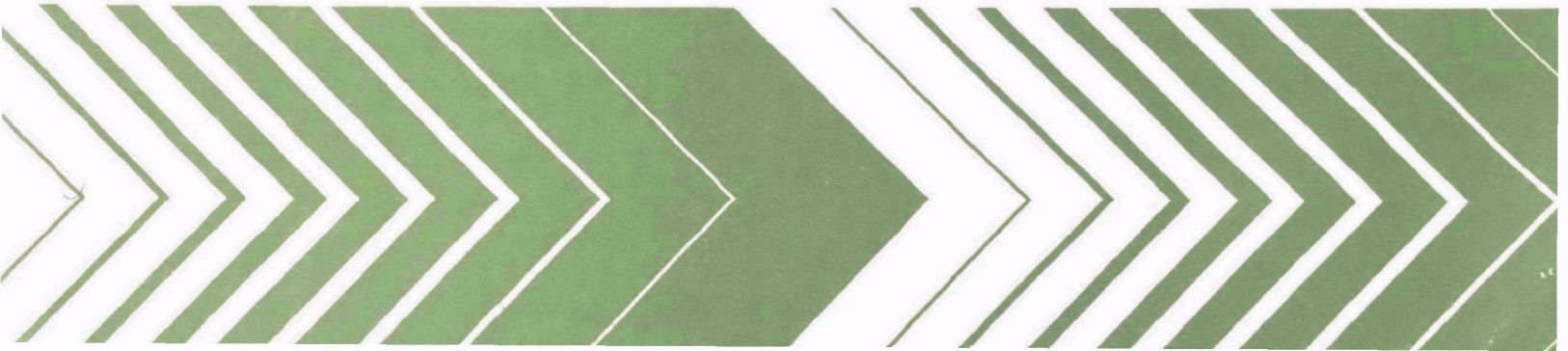
Research and Development

Identification of Fecal Indicator Bacteria Isolates from an Ice-Covered River



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IDENTIFICATION OF FECAL INDICATOR BACTERIA ISOLATES FROM AN ICE-COVERED RIVER

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FOREWORD

Effective regulatory and enforcement actions by the Environmental Protection Agency would be virtually impossible without sound scientific data on pollutants and their impact on environmental stability and human health. Responsibility for building this data base has been assigned to EPA's Office of Research and Development and its 15 major field installations, one of which is the Corvallis Environmental Research Laboratory (CERL).

The primary mission of the Corvallis Laboratory is research on the effects of environmental pollutants on terrestrial, freshwater, and marine ecosystems; the behavior, effects and control of pollutants in lake and river systems; and the development of predictive models on the movement of pollutants in the biosphere. CERL's Arctic Environmental Research Station extends the primary mission to the cold-climate environment; and develops and demonstrates pollution control technology for cold-climate regions.

This report describes the generic composition of the total coliforms, fecal coliforms, and fecal streptococci isolated with the membrane filter technique from sample stations on an ice-covered river downstream from a major source of domestic pollution.

James C. McCarty
Acting Director, CERL

ABSTRACT

The membrane filter technique was used to enumerate the total coliform (TC), fecal coliform (FC), and fecal streptococcus (FS) populations at seven sample stations on an ice-covered river downstream from a major source of domestic pollution. From each membrane filter population (m-TC, m-FC, and m-FS), 210 typical colonies (30 per station) were selected for verification and biochemical differentiation of the component genera. The 210 m-TC isolates were *Klebsiella pneumoniae* (46.2%), *Escherichia coli* (20.5%), *Enterobacter* sp. (18.6%), other total coliforms (5.2%), and 9.5% which did not verify as total coliforms. Among these m-TC cultures, 114 were verified as fecal coliforms (gas production in EC broth at 44.5°C). These 114 fecal coliforms were principally *K. pneumoniae* (53.5%) and *E. coli* (35.1%). In contrast, the 210 m-FC cultures were predominantly *E. coli* (77.6%), with *K. pneumoniae* (10.0%), other fecal coliforms (3.8%), and those not verified as fecal coliforms (8.6%). Of the 210 m-FS isolates, 167 were identified as enterococci, with 165 being *Streptococcus faecalis* biotypes. The results suggested the majority of these indicator bacteria originated from warm-blooded animal feces. Also, within each population, no overall differences in low temperature survival of the component genera were noted. However, the evidence does suggest that generic selectivity of the m-TC and m-FC techniques biases determination of the FC population composition, and that the m-FC technique underestimates the FC population density. Portions of this work were presented at the 78th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, 14-19 May, 1978 [abstract n83].

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SECTION 1

INTRODUCTION

Although indicator bacteria densities are used as a measure of the probable presence of enteric pathogens in water, some of the genera or biotypes comprising the total coliform (TC) and fecal coliform (FC) populations can be found in a variety of environments (1, 2) and may enter surface waters from nonfecal habitats (3, 4, 5). Therefore, important factors in relating indicator bacteria to the presence of enteric pathogens are the influence of specific waste sources on the composition of the population entering the aquatic environment (3), and the genera or biotype survival in this environment (6, 7). The IMViC (indole, methyl red, Voges-Proskauer, and citrate) patterns have been widely used to define biotypes within the coliform population, but lack both generic (2) and source (8, 9) specificity. Thus, as proposed by Dufour and Cabelli (3) differentiation of coliforms at the generic level would probably provide more valuable information than IMViC biotypes. Although emphasis has been placed on the coliforms, the fecal streptococcus (FS) group is also valuable in water pollution studies because some biotypes are associated with specific pollution sources (10, 11). Therefore, survival of FS biotypes in the aquatic environment should also be examined.

While the aquatic environment was noted as being generally unfavorable for maintaining viability of most enteric bacteria (6), water temperature appears to be a factor of major importance influencing survival of these microorganisms (7, 12, 13). Davenport *et al.* (12) recently examined the persistence of the TC, FC, and FS populations at sample stations downstream from a major source of domestic pollution. These authors found a high survival rate and suggested that indicator bacteria populations manifest the greatest resistance to viability loss under natural river conditions when the water has a temperature of 0°C and is ice covered. In the current investigation, these indicator bacteria populations were further examined. Isolates obtained from membrane filters on day five of the survival study (12) were differentiated biochemically to determine the generic composition of the TC, FC, and FS populations and to examine the relative survival characteristics of the component genera under low temperature conditions.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1. The environmental conditions which prevailed during this study, along with the absence of industrial waste sources in the area, precluded the presence of enteric microorganisms in the river from sources other than domestic waste effluents.

2. The coliforms isolated from the river were predominantly fecal coliform-positive (from warm-blooded animal feces) with *Escherichia coli* and *Klebsiella pneumoniae* as the most frequent isolates, while the principal fecal streptococcus was *Streptococcus faecalis* which is generally prevalent in domestic wastes.

3. Among the component genera of the fecal indicator bacteria populations, no overall survival differences in the aquatic environment were observed during this study. Thus, to clearly define whether or not there is differential survival among the component genera, it may be necessary to biochemically differentiate a large number of isolates from any specific sample station.

4. The fecal coliform-positive total coliforms from this domestic waste source have essentially the same generic composition that others have found associated with some industrial wastes.

5. The membrane filter technique for enumerating fecal coliforms appears to have a generic selectivity which suggests this technique may frequently underestimate the fecal coliform population density.

RECOMMENDATIONS

1. All fecal coliform-positive coliform bacteria (either m-FC or EC positive) should continue to be considered valid fecal coliforms indicative of a potential health hazard.

2. Before discarding the methodology currently used for fecal indicator bacteria enumeration, there needs to be a sound basis for recommending the use of new or modified techniques.

3. New or modified techniques for enumerating fecal coliforms should minimize the problem of underestimating the fecal coliform population.

SECTION 3

MATERIALS AND METHODS

The TC, FC, and FS cultures used in this study were collected from seven sample stations located on the Tanana River near Fairbanks, Alaska, downstream from a major domestic pollution source. River characteristics, sample station locations, flow time measurements between stations, domestic pollution sources, field sampling techniques, membrane filter techniques (m-TC, m-FC, and m-FS), and isolate verification methods have been described (12).

Verified TC and FC cultures were streaked on Endo agar (BBL) to obtain well-isolated, typical colonies. These colonies were transferred to Trypticase soy agar (BBL) slants for maintenance, and to improved Enterotubes (Roche Diagnostics, Division of Hoffman-LaRoche Inc., Nutley, NJ) for identification. The cultures were identified using the Encise II system (Roche Diagnostics) and supplemental tests as required. The verified FS cultures were streaked on KF streptococcal agar (BBL), and well-isolated, typical colonies were transferred to brain heart infusion broth (BBL) for identification and to brain heart infusion agar (BBL) slants for maintenance. The identification scheme described by Geldreich (14), with the addition of growth in 40% bile (15), was used to differentiate FS biotypes and identify the isolates showing reactions typical of the enterococcus group.

SECTION 4

RESULTS

The TC, FC, and FS populations at seven sample stations downstream from a major source of domestic pollution were enumerated with membrane filter techniques. For each population, 30 typical colonies per station (210 total) were selected from the membrane filters for verification. The verified isolates were then biochemically differentiated to determine the generic composition of the populations persisting in the ice-covered river. Table 1 shows that station T-700 downstream through T-100 encompassed a river reach having a mean flow time of 7.1 days with a range of 0.8 to 1.9 days between sample stations. It also shows that the indicator bacteria populations exhibited rapid decreases in density during the first 2.9 days with a generally slower rate of decrease thereafter.

Among the 210 m-TC isolates, six genera in the family *Enterobacteriaceae* were identified (Table 2). *Klebsiella pneumoniae* (46.2%) was the predominant total coliform isolated and was found, along with *Escherichia coli* (20.5%) and *Enterobacter* sp. (18.6%), at all sample stations. The other three genera which were verified as total coliforms represented 5.2% of the cultures and were isolated only occasionally, while 9.5% of the isolates did not verify as total coliforms. Further examination of the verified total coliforms revealed that 114 were fecal coliforms as defined by growth and gas production in EC broth at 44.5°C (16). Among the 114 EC-positive cultures, *K. pneumoniae* (53.5%) and *E. coli* (35.1%) were found at all sample stations with *Enterobacter agglomerans* (10.5%) at five stations.

The 210 m-FC cultures were verified as fecal coliforms by being positive in EC broth. Table 3 shows *E. coli* (77.6%) was the predominant isolate and was the only m-FC isolate found at all stations. *K. pneumoniae* (10.0% of the isolates) was found at all but the T-700 station. The other fecal coliforms were isolated infrequently and were 3.8% of the cultures. FC-negative isolates made up 8.6% of the m-FC cultures.

Only 167 of the 210 m-FS isolates exhibited biochemical reactions typical of the enterococcus group. Identification of the 167 enterococcus cultures showed that 165 were *Streptococcus faecalis* and that two were *Streptococcus faecium* (Table 4). *S. faecalis* subsp. *faecalis* was found at all sample stations and represented 74.8% of the enterococcus group. Together, *S. faecalis* subsp. *zymogenes*, *S. faecalis* subsp. *liquefaciens*, atypical *S. faecalis* and *S. faecium* comprised 7.8% of the enterococci. The remaining 17.4% were identifed as *S. faecalis*, but they peptonized litmus milk, did not hydrolyze gelatin and were not beta hemolytic. These isolates may have been *S. faecalis* subsp. *zymogenes* which lost the hemolytic character as a result of serial transfer in the laboratory since this subspecies may peptonize litmus milk and may or may not liquify gelatin (17).

TABLE 1. RIVER FLOW TIMES BETWEEN STATIONS, AND MEMBRANE FILTER DENSITIES OF INDICATOR BACTERIA AT EACH STATION*

Sample station	Days mean flow time between stations	Indicator bacteria densities per 100 ml of river water sample**		
		Total coliforms	Fecal coliforms	Fecal streptococci
T-700		6,200	2,200	78
	1.9			
T-600		1,800	430	65
	1.0			
T-500		700	100	11
	1.3			
T-400		500	200	29
	1.1			
T-300		230	100	17
	1.0			
T-200		180	68	13
	0.8			
T-100		200	90	14

* Samples collected on day five of the survival study (12).

** Mean of three replicate aliquots.

TABLE 2. IDENTIFICATION OF VERIFIED TOTAL COLIFORM ISOLATES FROM EACH SAMPLE STATION*

Genus and species	Reaction in EC broth at 44.5°C	Total isolates verified	Number of isolates from each sample station						
			T-700	T-600	T-500	T-400	T-300	T-200	T-100
Encherichia coli	positive	40	9	1	6	6	5	10	3
	negative	3	1	0	0	0	0	2	0
Klebsiella pneumoniae	positive	61	5	14	10	12	7	5	8
	negative	36	7	5	4	4	7	3	6
Enterobacter cloacae	negative	19	1	3	0	2	4	1	8
Enterobacter agglomerans	positive	12	3	2	3	0	2	2	0
	negative	8	1	1	2	1	1	2	0
Citrobacter freundii	negative	6	0	0	1	2	0	2	1
Serratia liquefaciens	positive	1	0	0	0	0	0	0	1
	negative	3	0	0	1	0	1	1	0
Proteus morganii	negative	1	0	0	0	0	0	1	0
Total isolates verified as TC		190	27	26	27	27	27	29	27
Total isolates verified as FC		114	17	17	19	18	14	17	12

* 210 colonies subcultured from membrane filters: 30 from each sample station, 10 from each replicate aliquot.

TABLE 3. IDENTIFICATION OF VERIFIED FECAL COLIFORM ISOLATES FROM EACH SAMPLE STATION*

Genus and species	Total isolates verified	Number of isolates from each sample station						
		T-700	T-600	T-500	T-400	T-300	T-200	T-100
Escherichia coli	163	27	24	19	22	26	24	21
Klebsiella pneumoniae	21	0	3	7	3	2	1	5
Klebsiella ozaenae	1	0	1	0	0	0	0	0
Enterobacter cloacae	1	0	0	0	0	0	0	1
Enterobacter agglomerans	5	3	0	1	0	0	0	1
Serratia liquefaciens	1	0	0	0	1	0	0	0
Total isolates verified	192	30	28	27	26	28	25	28

* 210 colonies subcultured from membrane filters: 30 from each sample station, 10 from each replicate aliquot.

TABLE 4. IDENTIFICATION OF THE ENTEROCOCCUS GROUP ISOLATES FROM EACH SAMPLE STATION*

Genus and species	Total isolates verified	Number of isolates from each sample station						
		T-700	T-600	T-500	T-400	T-300	T-200	T-100
Streptococcus faecalis subsp. *faecalis*	125	23	19	20	17	15	9	22
subsp. *zymogenes*	7	0	1	1	1	0	2	2
subsp. *liquefaciens*	3	1	0	0	0	0	1	1
other*	29	2	5	7	3	5	7	0
Atypical *Streptococcus faecalis***	1	0	1	0	0	0	0	0
Streptococcus faecium	2	1	0	0	0	0	0	1
Total isolates verified	167	27	26	28	21	20	19	26

* Litmus milk peptonized, gelatin not hydrolyzed, not beta hemolytic.

** Starch hydrolyzed.

SECTION 5

DISCUSSION

The environmental conditions under which the study was conducted were described in an earlier report (12), and precluded the presence of enteric microorganisms from sources other than domestic waste effluent. The generic composition of the m-TC, m-FC, and m-FS populations provided additional confirmation for the domestic waste origin of these enteric bacteria. Overall, *E. coli* was the most frequently isolated coliform, followed by *K. pneumoniae*, and *Enterobacter* sp. (Tables 2 and 3). In addition, the majority of the isolates were EC-positive which indicated that these coliforms were predominantly from warm-blooded feces (8, 9). Furthermore, *S. faecalis* biotypes were the principal FS cultures obtained from the Tanana River (Table 4); these biotypes have been noted as the predominant fecal streptococci in domestic wastes (14).

An earlier report (12) showed continuously decreasing TC, FC, and FS population densities as length of time in the river increased. However, the results in (Tables 2, 3, and 4) revealed no overall differences in persistence among the component genera of the populations. These observations suggest population viability loss as a whole may account for the decreasing fecal indicator bacteria densities in the low temperature receiving water. This is in general agreement with the observations of McFeters *et al.* (6) at warmer temperatures. Although no overall differences in component genera persistence were noted, the number of isolates examined was small and the generic distribution did fluctuate between stations. This suggests that biochemical differentiation of a large number of isolates from any specific sample station may be required to clearly define whether or not there is differential survival among the component genera.

The generic composition of the EC-positive total coliforms isolated in the present study (Table 2) was compared with the reported composition of the EC-positive total coliform populations entering the aquatic environment from sources other than domestic wastes. Dufour and Cabelli (4) showed that 45% of the *Klebsiella* isolates from a textile finishing plant effluent were EC-positive, while 63% of the *Klebsiella* isolates (all *K. pneumoniae*) were EC-positive in the present study. Downstream from a pulp mill, Huntley *et al.* (5) found that the EC-positive isolates consisted chiefly of *Klebsiella* (60%, predominantly *K. pneumoniae*) and *E. coli* (35%). The EC-positive total coliform cultures in the current study also proved to be mostly *K. pneumoniae* (54%) and *E. coli* (35%).

Fecal coliforms are considered to be a heterogeneous group with *E. coli* and *Klebsiella* as principal components (3). However, Geldreich (18) cited a report (L. A. Vinogradova, Hyg. Sanit. 36:157, 1971) in which *E. coli* isolates

from rivers in northern latitudes did not ferment carbohydrates at elevated temperatures, and suggested the elevated temperature test may not be reliable in the far north. In contract, 203 of the 206 *E. coli* cultures isolated from the Tanana River (approximately 65° North Latitude) during this study fermented lactose with gas production at 44.5°C (were EC-positive). Also, the validity of *K. pneumoniae* as an indicator of fecal pollution is being questioned, even though it is a common human and animal intestinal tract inhabitant (19). Part of the problem in establishing the sanitary significance of *K. pneumoniae* was resolved recently when Bagley and Seidler (1) demonstrated that some EC-positive strains give negative results with the m-FC technique. These authors concluded that FC-positive strains (either m-FC or EC-positive) are valid fecal coliforms indicating a potential health hazard. The fecal coliforms isolated during the Tanana River study were a heterogeneous group (Tables 2 and 3) with *K. pneumoniae* the principal EC-positive isolate from the m-TC population and a relatively minor component of the m-FC population. Colonies manifesting the color reactions described by Bagley and Seidler (1) as EC-positive/m-FC-negative were generally present on the m-FC membranes suggesting that *K. pneumoniae* may have comprised a larger percentage of the fecal coliform population in the Tanana River than revealed by the m-FC technique. The m-FC technique and EC test were both developed to differentiate between coliforms of fecal and nonfecal origin (20). However, the apparent generic selectivity of the m-FC techniques suggests that technique frequently underestimates the fecal coliform population density. This may partially explain why the FC population densities previously reported in this survival study (12) were invariabily lower with the m-FC technique than with the multiple-tube method.

It was pointed out previously (21) that various coliform media and procedure combinations may show different generic selectivity patterns, and there appears to be a growing concern that fecal coliforms enumerated with the m-FC technique do not adquately indicate the probable presence of enteric pathogens. Despite these methodology problems, fecal coliforms continue to be, as noted by Dutka (22), "...one of the most important indicators of potential health hazard due to fecal pollution." Thus, before discarding the tools which have had such a significant role in improving and maintaining human health, there must be a sound basis for recommending the use of new or modified techniques.

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Portions of this work were presented at the 78th Annual Meeting of the American Society for Microbiology, Las Vegas, NV, 14-19 May, 1978

16. ABSTRACT

The membrane filter technique was used to enumerate the total coliform (TC), fecal coliform (FC), and fecal streptococcus (FS) populations at seven sample stations on an ice-covered river downstream from a major source of domestic pollution. From each membrane filter population (m-TC, m-FC, and m-FS), 210 typical colonies (30 per station) were selected for verification and biochemical differentiation of the component genera. The 210 m-TC isolates were *Klebsiella pneumoniae* (46.2%), *Escherichia coli* (20.5%), *Enterobacter* sp. (18.6%), other total coliforms (5.2%), and 9.5% which did not verify as total coliforms. Among these m-TC cultures, 114 were verified as fecal coliforms (gas production in EC broth at 44.5°C). These 114 fecal coliforms were principally *K. pneumoniae* (53.5%) and *E. coli* (35.1%). In contrast, the 210 m-FC cultures were predominantly *E. coli* (77.6%), with *K. pneumoniae* (10.0%), other fecal coliforms (3.8%), and those not verified as fecal coliforms (8.6%). Of the 210 m-FS isolates, 167 were identified as enterococci, with 165 being *Streptococcus faecalis* biotypes. The results suggested the majority of these indicator bacteria originated from warm-blooded animal feces. Also, within each population, no overall differences in low temperature survival of the component genera were noted. However, the evidence does suggest that generic selectivity of the m-TC and m-FC techniques biases determination of the FC population composition, and that the m-FC technique underestimates the FC population density.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Coliform Bacteria, Enterobacteriaceae, Escherichia coli, Klebsiella, Microorganisms, Stream Pollution, Streptococcus	Enterobacter, Enterococcus, Fecal Coliform, Fecal Indicator Bacteria, Fecal Streptococcus, Low Temperature Survival, Membrane Filter Technique, Total Coliform	06/F, M 08/H, L

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