

REVIEW ARTICLES

Abstract: High numbers of total and fecal coliform bacteria have been detected in pristine streams and in water samples collected from epiphytic vegetation 30 feet above ground in the rain forest of Puerto Rico. Identification of fecal coliform isolates demonstrated the presence of *Escherichia coli* in these samples. Nucleic acid (DNA) analysis indicated that the guanosine + cytosine content of the environmental isolates was identical to that of clinical isolates of *E. coli*. Diffusion chamber studies with *E. coli* at several freshwater sites revealed that this bacterium can survive indefinitely in most freshwaters in Puerto Rico. An evaluation of methods for the enumeration of fecal coliforms showed that currently used media have poor reliability as a result of large numbers of false positive and false negative results when applied to Puerto Rican environmental water samples. Based on these findings total and fecal coliform bacteria may not be reliable indicators of recent biological contamination of waters in Puerto Rico or other tropical areas.

The role of drinking and other types of water as disease vectors has been well documented.¹ Many pathogens and opportunistic pathogens can be found in polluted and unpolluted waters (some of the most common ones are shown in Tables I and II), although not all of these have been linked to waterborne disease many of them have been found in Puerto Rico. It has been estimated that up to five million people die each year from waterborne diseases worldwide.² Many of these deaths may be as a result of drinking biologically contaminated water. Many waterborne outbreaks of gastroenteritis and

Table I
Bacterial Pathogens and Opportunistic Pathogens Present in Waters

Organism	Disease	Infectious Dose
<i>Acinetobacter calcoaceticus</i>	Nosocomial Infections	?
* <i>Aeromonas hydrophila</i>	Enteritis, Wounds	?
* <i>A. sobria</i>	Enteritis, Wounds	?
* <i>A. varius</i>	Enteritis, Wounds	?
* <i>Chromobacterium violaceum</i>	Enteritis	?
* <i>Citrobacter</i> spp.	Nosocomial Infections	?
* <i>Enterobacter</i> spp.	Nosocomial	?
* <i>Escherichia coli</i> serotypes	Enteritis	>10 ⁶ CFU*
<i>Flavobacterium meningosepticum</i>	Nosocomial, Meningitis	?
<i>Francisella tularensis</i>	Tularemia	10 CFU
<i>Haemophilus parainfluenzae</i>	Liver abscesses	10 ⁶ CFU
* <i>Klebsiella pneumoniae</i>	Nosocomial, Pneumonia	?
* <i>Legionella icterohaemorrhagiae</i>	Leptospirosis	?
* <i>Legionella pneumophila</i>	Legionellosis	>10 CFU
* <i>Morganella morganii</i>	Urethritis, Nosocomial	?
* <i>Mycobacterium tuberculosis</i>	Tuberculosis	?
<i>M. marinum</i>	Granuloma	?
* <i>Naeromonas thalassialis</i>	Enteritis	?
<i>Pseudomonas pseudomallei</i>	Melioidosis	?
* <i>Salmonella enteritidis</i>	Enteritis	>10 ⁶ CFU
* <i>S. montevideo</i> II	Salmonellosis	?
<i>S. paratyphi</i> A&B	Paratyphoid Fever	?
* <i>S. typhi</i>	Typhoid Fever	10 ⁵ CFU
* <i>S. typhimurium</i>	Salmonellosis	>10 ⁶ CFU
* <i>Serratia marcescens</i>	Nosocomial	?
* <i>Staphylococcus aureus</i>	Wounds, Food poisoning	?
<i>Vibrio alginolyticus</i>	Wounds	?
* <i>V. cholerae</i>	Cholera dysentery	10 ³ CFU
* <i>V. parahaemolyticus</i>	Enteritis	10 ⁶ CFU
* <i>V. vulnificus</i>	Wound Infections	?
* <i>Yersinia enterocolitica</i>	Enteritis	10 CFU

*Found in Puerto Rico, adopted from Dufour³¹ and Grimes³²

* CFU - Colony Forming Units

hepatitis A have been described in the literature.⁴ Since many pathogenic organisms can occur in water at very low densities (< 1 per liter) and still be infective, their actual presence may be undetectable or assessed only through very expensive and time consuming tests. Nearly all of these pathogens are transmitted to water by fecal contamination. For these reasons, bacteria that are found exclusively and universally in feces at very high densities are used as indicators of fecal contamination. Thus the presence of a certain group of bacteria in water is used to demonstrate the possibility of biological contamination. In the late 1800's Houston proposed the idea of

Microbial Ecology Laboratory, Department of Biology, University of Puerto Rico, Rio Piedras, Puerto Rico 00931

All correspondence to: Dr. Terry C. Hazen, Department of Biology, College of Natural Sciences, University of Puerto Rico, Rio Piedras, Puerto Rico 00931. Phone: 809/764-0000 x2404, x2569

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Table II
Other Pathogens Present in Sewage Contaminated Waters

Organism	Disease	Infectious Dose
Viruses		
Adenovirus	enteritis, pharyngitis	1-10 PFU*
Calicivirus	enteritis	1-10 PFU
Norwalk virus	enteritis	1-10 PFU
Coronavirus	enteritis	1-10 PFU
Coxsackievirus A & B	meningitis	1-10 PFU
Echovirus	enteritis, meningitis	1-10 PFU
*Hepatitis A virus	hepatitis	1-10 PFU
*Poliovirus	poliomyelitis	1-10 PFU
*Rotavirus	enteritis	1-10 PFU
Astrovirus	enteritis	1-10 PFU
Fungi		
* <i>Candida</i> spp.	candidiasis	?
<i>Rhizosporidium zoeberi</i>	rhizosporidiosis	?
Protozoa		
* <i>Cryptosporidium</i> spp.	cryptosporidiosis	?
* <i>Giardia lamblia</i>	giardiasis	1 cyst
* <i>Entamoeba histolytica</i>	dysentery	1 cyst
* <i>Narcelesia fowleri</i>	meningoencephalitis	?
<i>Acanthamoeba</i> spp.	meningoencephalitis	?
Helminths		
* <i>Schistosoma mansoni</i>	schistosomiasis	1 cercariae
<i>S. haematobium</i>	schistosomiasis	1 cercariae
<i>S. japonicum</i>	schistosomiasis	1 cercariae
<i>S. intercalatum</i>	schistosomiasis	1 cercariae
<i>S. mekongi</i>	schistosomiasis	1 cercariae
* <i>Fasciola hepatica</i>	fascioliasis	1 metacercariae
<i>Paragonimus westermani</i>	paragonimiasis	1 metacercariae
<i>Clonorchis sinensis</i>	chinese liver fluke	1 metacercariae
<i>Diphyllobothrium latum</i>	pernicious anaemia	1 plerocercoid

*Found in Puerto Rico, adapted from Dufour²¹ and Grimes²²

*PFU = Plaque Forming Units

using three groups of bacteria (i.e. coliforms, fecal streptococci, and the gas-producing clostridia which are commonly found in the feces of warm blooded animals) as indicators of fecal pollution of waters.⁴ He argued that since these groups could only come from fecal sources their presence would indicate recent fecal pollution.⁴ For nearly eighty years the coliform group of bacteria has been used as such indicators.

The term "coliform" is used to indicate certain bacteria which resemble the bacterium *Escherichia coli* (coliform, or *E. coli*-like). The coliform group is further divided into two subgroups; total coliforms and fecal coliforms. The total coliform group are Gram-negative, facultatively anaerobic, non-sporulating rods, which ferment lactose with the production of gas at 35°C, more than 50 species of bacteria have been shown to give a positive coliform reaction.⁵ The fecal coliform group (though *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* may give positive fecal coliform reactions, *E. coli* is the target organism in this assay) has the same properties as mentioned above with the added property of thermotolerance, i.e. capability to ferment lactose at 44.5°C. *Escherichia coli* was first described by Escherich in 1855 and is found in high densities in the feces of warm blooded animals. As the term "fecal coliform" implies, it has not been found to replicate or survive extraenterally (i.e. in the environment). The presence of fecal coliforms in water, therefore indicates recent fecal contamination. On the other hand, the total coliform group is used to indicate the possible presence of *E. coli* in water. Even though total coliforms are found as part of the normal environmental microflora, they meet several criteria which make them

desirable as indicators of bacterial pollution. Bond¹ enumerated these criteria as follows:

1. The indicator must be present whenever pathogens are present.
2. It must be present only when the presence of pathogenic organisms is an imminent danger.
3. It must occur in much greater number than the pathogens.
4. It must be more resistant to disinfectants and to aqueous environments than the pathogens.
5. It must grow readily on relatively simple media.
6. It must yield characteristic and simple reactions enabling as far as possible an unambiguous identification of the group or species.
7. It should preferably be randomly distributed in the sample to be tested.
8. Its growth on artificial media must be largely independent of any other organism present.

History

The presence of pathogens in water was suggested by the earliest microscopists. In 1678 Leeuwenhoek described organisms found in canal water that resembled vibrios, though he did not make any connection between these observations and disease. Snow is generally acknowledged to be the first in describing a waterborne disease outbreak.^{1, 6} By removing the pump handle from a public well in Broad Street, London and a subsequent decrease in disease incidence he demonstrated the connection between water consumption and a cholera epidemic. In 1856 Budd showed a relationship between typhoid fever and fecal contamination.^{1, 6} However, it was not until the work of Koch and coworkers that procedures were established for examining sources and pathways of infections.⁴ For more than one hundred years we have looked for reliable methods to determine whether water is fit for human consumption.¹

Regulations

The first drinking water regulation for microbial contamination in the U.S. was published in 1914. This was the first "Public Health Service Drinking Water Standards" regulation. Subsequently, this regulation was replaced by U.S. Public Service Acts of 1915 and 1962. The current U.S. regulation comes from the Safe Drinking Water Act (Public Law 93-523, 1974). The U.S. Environmental Protection Agency proposed changes that are now being implemented (Federal Register 48:45502-45521, October 1983). The law was approved in July 1986 and is currently in its first phase of implementation. The new regulation requires that there be 0 coliforms/100 ml by any method, for any sampling frequency for drinking waters. Potable water in Puerto Rico is currently controlled by Regulation Number 50 of the Secretary of Health, June 21, 1983 (Fig. 1). This repeals the previous regulation of the Secretary of Health Number 44, November 29, 1979. Both of these regulations are pursuant to the provisions of Law Number 5, July 21, 1977 known as "Law to Protect the Purity of the

Membrane Filter Technique

- 1 coliform CPU^b/100 ml as a mean of all samples/month
 or
 4 coliform CPU/100 ml in more than 1 sample when less than 20 or more samples are examined per month

MTF Technique^c

- (10 ml standard portions)
 not more than 10% of tubes in any one month
 or
 3 or more tubes in more than one sample when less than 20 samples examined per month
 or
 1 or more tubes in more than 5% of the samples when 20 or more samples examined per month

a MCL - Maximum Contaminant Level

b CPU - Colony Forming Units

c MTF - Multiple Tube Fermentation

Source - Regulation of the Secretary of Health Number 50, Commonwealth of Puerto Rico

Article IV 6, June 21, 1983

Figure 1. Puerto Rico Microbiological MCL^a Regulations for Potable Water.

Potable Waters of Puerto Rico." Article VI of Regulation 50 deals with the Maximum Microbiological Contaminant Levels (MCL's).

During the last 20 years there has been a shift by government agencies to use fecal coliforms for water-monitoring purposes (instead of the currently used total coliforms), even though the regulations are still based on total coliform levels. Fecal coliform enumeration is less ambiguous than total coliform enumeration.⁷

Fecal Coliforms in Puerto Rico

Monitoring of Puerto Rican waters by the U.S. Geological Survey⁸ reported that 54 out of 67 water sampling stations on rivers in Puerto Rico exceeded the recommended MCL's for recreational waters (i.e. <1,000 fecal coliforms per 100 ml) during 1984. Thus only 19% of all sites sampled met the recommended MCL for recreational waters. These findings have resulted in condemnation of sewage treatment facilities in Puerto Rico as a source of fecal pollution of natural waters.

Studies in our laboratory over the past seven years^{9, 10, 11} have shown that even pristine sites in the Caribbean National Forest are in "violation" of recreational water MCL's for fecal coliforms. Identification of more than 300 fecal coliform isolates from these sites showed that less than 40% of these isolates were actually *E. coli*.¹² Similar studies using the same methods in the continental U.S.A., Canada, and England have demonstrated that more than 90% of fecal coliform positive isolates are identified as *E. coli*.^{3, 4, 13} This suggests that Puerto Rican water harbour bacteria capable of giving false positive reactions when fecal coliform assays are used. Even more surprising is the

routine isolation of *E. coli* by our laboratory from water collected from bromeliads (epiphytic vegetation) 30 ft. above ground level in the rain forests of Puerto Rico. The isolation of *E. coli* from these sources indicates that this bacterium may be a naturally-occurring bacterium in the rain forest. In order to confirm the latter hypothesis, analyses were performed on the nucleic acid (DNA) extracted from these environmental isolates. The thermal melting point (T_m) of the nucleic acid strands was determined as described elsewhere¹⁴⁻¹⁶ and compared to that of *E. coli* B (ATCC 13706) obtained from the American Type Culture Collection. The thermal melting points were found to be identical. The T_m is linearly related to the average DNA base composition and it can be related to its guanine + cytosine (G+C) content, since this base pair confers extra thermal stability to the molecule.¹⁷ The importance of the G+C content in bacterial taxonomy is that it can be an excluding characteristic. If two organisms have DNA with differing G+C content it can be concluded that they belong to different genera. We are currently conducting nucleic acid hybridization analyses with these bacteria in order to determine with certainty if in fact the environmental isolates are members of the genus *Escherichia*.

Studies in our laboratory on the *in situ* survival of an ATCC strain of *E. coli* have demonstrated that this bacterium is capable of long term survival in rain forest rivers.^{9, 10, 18} In these studies pure cultures of *E. coli* (ATCC 11775) were inoculated into diffusion chambers^{19, 20} which were then exposed to aquatic environmental conditions in the river and sampled for 5-6 days. Results from these studies indicated that this clinical strain of *E. coli* is capable of surviving tropical environmental conditions for extended periods of time (Fig. 2).

Pagel et al.¹¹ compared four fecal coliform assays in various types of freshwaters in Southern Canada. They found that while these assays were somewhat variable in their abilities to detect fecal coliforms from environmental samples, they were all acceptable in terms of their specificity and selectivity. In similar studies in our laboratory¹² using the same methodology to detect fecal coliforms from freshwaters in Puerto Rico we found that

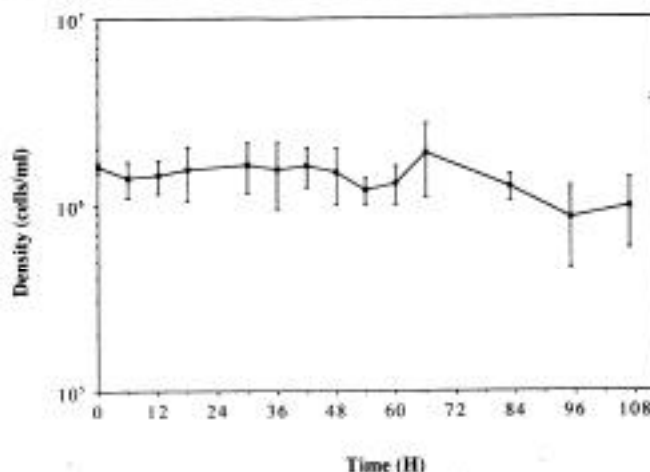


Figure 2. *In situ* survival of *Escherichia coli* in diffusion chambers in the Mameyes River, Puerto Rico (all values are means \pm one standard error, N = 8).

the specificity of the media (determined by the ability of the medium to restrict growth of organisms other than the target bacterium) was at least 20% less than the specificity claimed by the Canadian investigators (Table III). Thus all the methods gave significantly higher false positive and false negative errors. Controls using known strains of *E. coli* indicated the accuracy of the methods to be the same in both studies (Table III).

Table III

Comparison of Fecal Coliform Assay Methods in Temperature and Tropical Waters

Performance Parameter	mFC		mFC1		mFC2		mTEC	
	Temp	Trop	Temp	Trop	Temp	Trop	Temp	Trop
Accuracy ^a	58	57	89	84	100	100	94	93
Specificity ^b								
False positive	11	39	16	38	18	18	11	16
Undetected	4	25	1	28	2	11	2	21
Sensitivity ^c	88	65	83	72	80	82	86	86
Comparability ^d								
FC recovery	18	81	11	75	88	84	45	73
Non-FC recovery	4	33	11	29	13	18	7	18
Overall rank ^e	2	4	3	2	1.5	1	1.5	3

^a All data labeled temp is from Pagei et al.¹¹
^b Reported as: Mean number of colonies per ml medium/mean number of colonies on a non-selective medium) X 100. % of Accuracy and stated for an *E. coli* ATCC culture.
^c Reported as: (False positive error + false negative error / False positive error + number of presumptive target number of verified target colonies/total presumptive target colonies) X 100. False negative error = False negative counts/verified + undetected target counts) X 100.
^d Sensitivity Index = Presumptive Typical Colonies/Presumptive Typical or Target Colonies + Presumptive Non-Typical Colonies) X 100.
^e Reported as: Fecal Coliforms (FC) Recovery and Non-Fecal Coliforms (NFC) Recovery.
^f Best Overall Efficiency of the Method is Given by the lowest Overall Rank.

These studies indicate that currently used criteria to determine the degree of fecal contamination or the microbiological quality of the waters in Puerto Rico may be unrealistic. The presence of high concentrations of naturally-occurring total and fecal coliform bacteria means that bacterial pollution is grossly overestimated in Puerto Rico. In fact, fecal contamination may be indicated when none is present. Two possibilities are open: 1) change the indicator system and determine which bacterial genus would most closely indicate fecal contamination in tropical waters or, 2) directly enumerate pathogens, establishing standards based on the most environmentally resistant species.

Research in other tropical areas²¹⁻²⁶ indicates that the situation in Puerto Rico is analogous to that found in other tropical areas of the world. Nowhere is the importance of accurate determination of recent human fecal contamination greater than in the tropics. The diversity of waterborne diseases and their severity greatest in tropical environments. Since most of the countries in tropical climates are underdeveloped, with large populations that are undernourished, ill housed, with poor medical services, waterborne diseases have a much greater effect on public health in the tropics than in temperate areas. Surprisingly few studies have examined the efficacy of total coliform and fecal coliform standards in the tropics. Lavoie²⁵ compared isolates from fecal coliform and total coliform assays of well water in the Ivory Coast, and found a high proportion of *E. coli* isolates from fecal coliform assays. Although in the latter study high densities of fecal coliforms (51 CFU/100 ml) were isolated from untreated groundwater it was assumed this reflected a high degree of fecal contamination even though none was apparent. Fujioka et al.^{24, 26} also showed that *E. coli* may be a normal inhabitant of fresh and marine waters in Hawaii. Thus

the use of total and fecal coliforms as indicators of biological quality of water seems to be of limited value according to the various studies reviewed in this communication. Hagler and Mendonca-Hagler²² showed that even marine waters in Brazil can have high densities of coliforms. Other studies by our lab have shown that marine environments around Puerto Rico can also support high densities of coliforms when hydrocarbon concentrations are also high.²⁷⁻³⁰ In view of these findings, the isolation of fecal coliforms from waters in tropical countries may not necessarily need be a cause for health concern. Yet tropical countries have a greater need for accurate determination of the presence of recent fecal contamination due to the greater number and diversity of waterborne diseases. This exacerbates the need for alternate indicators of fecal pollution which are more unambiguous than those presently in use.

Resumen: Altas concentraciones de coliformes totales y fecales fueron aislados tanto en muestras de arroyos no contaminados como de vegetación epifítica en árboles a de 30 pies de altura en bosques de Puerto Rico. La identificación de coliformes fecales aislados de estas fuentes indicaron la presencia de *Escherichia coli*. El análisis de ácidos nucleicos (DNA) indicó que el contenido de guanosina + citosina de estas cepas del medio ambiente es idéntico al de las cepas clínicas de *E. coli*. Estudios en *habitats* de agua dulce usando cámaras de difusión conteniendo cepas clínicas de *E. coli* demostraron que esta bacteria puede sobrevivir en las aguas dulces de Puerto Rico. Los estudios de evaluación de los medios de cultivo actualmente usados para el aislamiento de estas bacterias a partir de agua demostraron que estos medios tienen una eficiencia baja debido al gran número de falsos positivos y falsos negativos cuando se utilizan para la detección de coliformes totales y fecales en aguas de Puerto Rico. Estos estudios indican que los coliformes totales y fecales no son los más apropiados indicadores de contaminación biológica reciente de aguas en áreas tropicales tales como Puerto Rico.

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