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# Isolation of Fecal Coliforms from Pristine Sites in a Tropical Rain Forest

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**Samples collected from water accumulated in leaf axilae of bromeliads (epiphytic flora) in a tropical rain forest were found to harbor fecal coliforms. Random identification of fecal coliform-positive isolates demonstrated the presence of *Escherichia coli*. This bacterium was also isolated from bromeliad leaf surfaces. These data indicate that *E. coli* may be part of the phyllosphere microflora and not simply a transient bacterium of this habitat. The isolation of fecal coliforms from these sites was unexpected and raises questions as to the validity of using fecal coliforms as indicators of biological water quality in the tropics.**

Aquatic systems in tropical rain forests are a relatively unstudied subject. Considering the immensity and complexity of the tropical rain forest in terms of the macro- and microflora, this is unfortunate. The aquatic communities found in water-harboring epiphytes may play an important role in nutrient recycling systems. Tropical rain forest soils are heavily leached, water logged, and anaerobic, which puts an even greater emphasis on epiphytic species for retention and transferral of nutrients to other ecosystem components of the rain forest (15).

Bromeliads are one of the most abundant epiphytes in the rain forest and are native to all regions of the Americas (16). Their altitudinal range is from 0 to >4,267 m above sea level. They vary in size from 3 cm to 11 m or more in height. *Guzmania berteroniana* is one of the most abundant bromeliads in the rain forest of Puerto Rico (20). This bromeliad is of medium size with a rosette which is made up of pliable, strap-shaped green leaves about 30 cm long. This tank-type bromeliad has the capacity to collect large amounts of rainwater and forest leachate within its central cup and adjacent leaf axilae. Rainwater carries nutrients from the forest vegetation as it descends through the canopy (N. P. Salamandra, M.S. thesis, Department of Biology, University of Puerto Rico, Río Piedras, 1974). The leachates, which reach the bromeliad under a dense tropical growth, have been shown to have a greater concentration of salts than does rainwater (14). Several studies measuring physicochemical variables in waters present in bromeliad axilae have shown this water to be rich in nutrients (4; Salamandra, thesis). This high concentration of nutrients creates an adequate environment for the development of complex microbial community. A study by Salamandra (thesis) showed the eucaryotic microflora of bromeliad waters to be composed of greater than 200 species. Unfortunately no bacterial isolations were attempted, although it was assumed that bacteria were supporting most of the species present.

Total coliform bacteria are used as a group to indicate the biological quality of treated and untreated drinking waters, whereas fecal coliforms are used for the same purpose in recreational waters. The use of fecal coliforms as indicators of recent fecal contamination of waters is based on the assumption that *Escherichia coli* (the target organism of these analyses) cannot survive for extended periods of time in the environment. It is generally accepted that fecal coliforms, as the name implies, can only originate from fecal (human or other warm-blooded animal) sources. The present study provides evidence that the presence of *E. coli* (a "fecal" coliform) in water collected from bromeliad axilae in tropical areas may not necessarily and exclusively originate from fecal sources and as such may not indicate fecal contamination.

## MATERIALS AND METHODS

**Study site.** The Luquillo Experimental Forest is a cloud rain forest in Northeastern Puerto Rico. Three sites were chosen for sample collection, at elevations of 250, 350, and 700 m above sea level (Fig. 1; sites A, B, and C, respectively).

**Sampling procedures.** Water was collected from leaf axilae of *G. berteroniana* with a sterile pipette. Bromeliads chosen for sampling were at different heights, namely, 1 to 2 m, 2 to 4 m, and 4 to 6 m. Samples of 10 ml were obtained from each bromeliad, placed in sterile Whirl-Pak bags (Nasco, Ft. Wilkinson, Wis.), and stored on ice for transport to the laboratory or analyzed in situ by membrane filtration. Physicochemical analyses were performed for orthophosphates, nitrates and nitrites, and sulfates as indicated in *Standard Methods for the Analyses of Water and Wastewater* (2). For these physicochemical analyses it was necessary to pool samples obtained from several bromeliads at the same altitude. Appropriate preservatives were added to the samples whenever necessary for later assay. Preserved samples were stored at 4°C for later analysis in the laboratory. Concentrations of ammonia were measured spectrophotometrically in situ using Spectro-Kits (Bausch & Lomb, Inc., Rochester, N.Y.). Other variables measured included temperature, pH, and dissolved oxygen. The latter was measured in situ with a Hydrolab Surveyor digital model 4041 (Hydrolab Corp., Austin, Tex.).

**Direct bacterial counts.** Twenty-five microliters of acridine orange ($10^{-6}$ M) was added to 0.5 ml of sample and incubated for 2 min at the ambient temperature. This solution was then filtered through a polycarbonate filter (0.2-μm pore size, prestained with Sudan black B). The total number of fluorescing cells was determined by using an epifluorescence microscope (American Optical Corp., Buffalo, N.Y.), as outlined for acridine orange direct counts by Hobbie et al.

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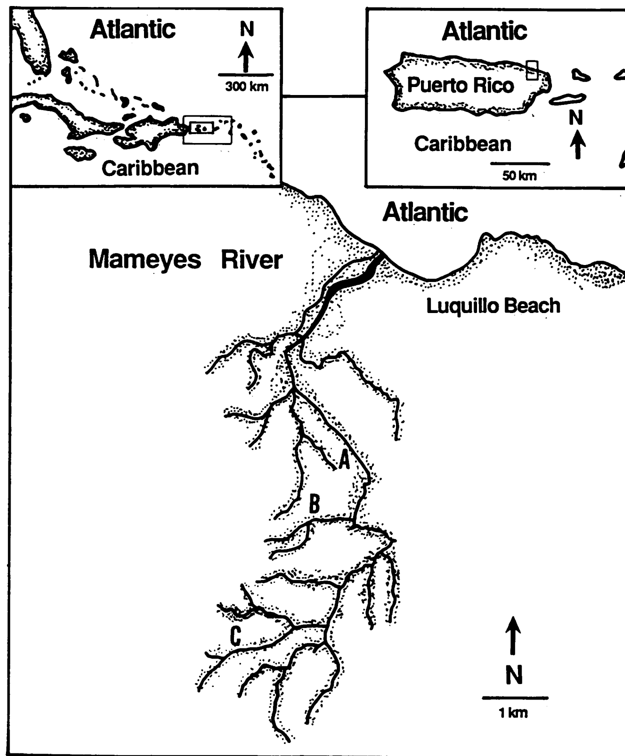


FIG. 1. Map of sites in the Mameyes River watershed.

(10). In addition, the percent of red-fluorescing cells was used in an indirect method to determine those cells actively involved in protein synthesis as outlined by López-Torres et al. (13).

**Bacteriological procedures.** Bacterial analyses were performed by the membrane filtration technique outlined previously (2). For the isolation of total and fecal coliforms, the media used were m-Endo (35°C) and m-FC (44.5°C), respectively. Representative colonies were further confirmed on lactose broth followed by brilliant green-lactose-bile broth (35°C) for total coliforms and lactose broth (45°C) followed by EC broth for fecal coliforms (all media were purchased from Difco Laboratories, Detroit, Mich., and prepared according to the manufacturer's instructions). Random isolates of typical colonies were identified by using API 20E strips (Analytab Products, Plainview, N.Y.). In some cases no water was present in the bromeliad axilae as a result of the lack of recent rains. During this time of dryness, leaves were obtained, placed in sterile Whirl-Pak bags, and transported to the laboratory, where a modification of the RODAC plate (1) (BBL Microbiology Systems, Cockeysville, Md.) was utilized by touching different areas of the leaves to m-Endo and m-FC plates. These plates were then incubated, and the procedures outlined above were used for the identification of typical colonies.

Antibiotic sensitivity patterns of random isolates identified as *E. coli* were conducted as described elsewhere (3). Briefly, a bacterial lawn of the isolate was inoculated onto Mueller-Hinton agar (Difco) and antibiotic sensitivity discs (BBL) were placed onto the surface of the inoculated plate. The bacterial sensitivity to the various antibiotics tested was determined by measuring the zone of inhibition at 24 and 48 h as indicated by the manufacturer. The antibiotics to be tested were those used most often to treat patients afflicted with *E. coli* gastroenteritis (P. A. Rusin and N. A. Sinclair, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, N33, p. 247).

American Type Culture Collection (ATCC) strains *E. coli* C (ATCC 15597) and *E. coli* B (ATCC E11303) were used as controls. These control strains were analyzed in the same manner as the environmental isolates.

**Percent respiration.** A 10-ml sample was mixed in situ with 1.0 ml of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride for 20 min in the dark as outlined by Zimmermann et al. (21). The reaction was stopped by the addition of 0.5 ml of formaldehyde (37%). The cells containing dark granules were considered to be involved in respiration.

**Data analyses.** Programs developed on Apple II Plus and Macintosh Plus computers were used for all statistical analyses. Multiple correlations and regressions were used to determine relationships between densities of bacteria, total bacterial activity (respiration as well as protein synthesis), and water physicochemical properties. Any statistical probability less than or equal to 0.05 was considered significant (20).

## RESULTS

**Physicochemical variables.** Table 1 shows the average values of the physicochemical variables measured throughout the study. No significant differences (as determined by statistical methods) were found in bromeliad waters collected from different altitudes (250, 350, and 700 m). It was interesting to note that phosphates in all cases were below detection levels by the method utilized. The concentration of nitrites-nitrates was 3.4 mg/liter, which is a higher concen-

TABLE 1. Physicochemical variables and bacterial densities and activity measured in bromeliad water[a]

| Elevation (m) | Temp (°C) | Dissolved $O_2$ concn (mg/liter) | pH | Concn (mg/liter) of: | | | AODC[b] (cells/100 ml), $10^9$ | Total coliforms (CFU/100 ml)[c], $10^6$ | % Activity (AODC)[d] | % Respiration (INT)[e] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $NO_3$ | $NH_4$ | $SO_4$ | | | | |
| 250 | 23 | 8.4 | 8.6 | 2.5 | 0.46 | 10 | 1.1 | 1.3 | 45.9 | 51.8 |
| 350 | 24 | 4.6 | 6.9 | 4.8 | 0.42 | 22.1 | 2.5 | 1.1 | 52.4 | 39.3 |
| 700 | 21 | 4.6 | 6.4 | 2.8 | 0.66 | 25.5 | 5.0 | 2.3 | 49.1 | 63.8 |
| Avg. values[f] | 22.7 | 5.9 | 7.3 | 3.4 | 0.51 | 19.2 | 2.9 | 1.5 | 51.6 | 49.1 |

[a] All phosphorus levels were below the level of detection of the method used.
[b] AODC, Acridine orange direct counts.
[c] CFU, CFU on m-Endo medium.
[d] Values indicate the number of red-fluorescing cells over all fluorescing cells multiplied by 100. INT, [2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride].
[e] Values indicate the percentage of cells containing dark granules.
[f] No statistically significant differences (among samples obtained from the indicated elevations) were found for all variables measured.

tration than that normally present in waters (0.064 mg/liter). All other values of the physicochemical variables measured were not significantly different from those values obtained in tropical waters by other investigators (11).

**Bacterial densities.** Table 1 shows data obtained on the bacterial densities in bromeliad water. An average of $1.5 \times 10^6$ coliforms per 100 ml was present in the samples according to viable counts. When acridine orange direct counts were performed, an average of $2.9 \times 10^9$ total bacterial cells per 100 ml was observed. No significant statistical correlations were observed among physicochemical variables and the densities of either total bacteria (as determined by acridine orange direct counts) or total coliforms. Fecal coliform densities ranged from 4 to 400 CFU/100 ml.

**Activity and respiration.** The activity and respiration measurements obtained from bromeliads at different heights and elevations were averaged together since no significant statistical differences were found among these values (data not shown). An average of 51.6% (±10%) of total bacterial cells were involved in respiration, whereas an average of 49.1% (±2.7%) (Table 1) of cells were involved in protein synthesis.

**Bacterial isolates.** All presumptive fecal coliform colonies showed the typical blue color on m-FC agar plates. In some cases fresh m-FC plates were streaked for isolation to ensure single colonies. All isolates which grew and produced gas in EC broth at 44.5°C were inoculated into API 20E strips. All of these isolates were identified as *E. coli*. On several occasions typical colonies were directly inoculated into API 20E strips from m-FC and m-Endo plates. Out of 50 colonies isolated on m-FC agar, 36 (72%) were identified as *E. coli* (Table 2). *Citrobacter freundii* was the next most frequent isolate (12%) which gave a positive presumptive test on m-FC, followed by *Pseudomonas cepacia* (4%), *Kluivera* spp. (4%), and four isolates which were unidentifiable (8%) (Table 2). All samples tested by the modified RODAC m-FC plates yielded a few typical blue colonies, and all of the colonies were isolated from the bottom portion of the leaf. Nonetheless, all blue colonies (18 colonies) were identified as *E. coli* by the API 20E system (data not shown).

Growth on m-Endo agar was profuse, and most of the time several dilutions were necessary to obtain isolated colonies.

TABLE 2. Bacterial species isolated from bromeliad water on selective media[a]

| Medium used | Bacterial species | No. of isolates | % of total |
|---|---|---|---|
| m-Endo | *Citrobacter freundii* | 18 | 13 |
| | *Serratia liquefaciens* | 18 | 13 |
| | *Aeromonas hydrophila* | 11 | 7.9 |
| | *Klebsiella oxytoca* | 9 | 6.5 |
| | *Enterobacter cloacae* | 7 | 5.1 |
| | *Escherichia coli* | 3 | 2.2 |
| | *Pseudomonas* spp. | 3 | 2.2 |
| | Unidentifiable[b] | 69 | 50 |
| | Total no. of colonies identified | 138 | 100 |
| m-FC | *Escherichia coli* | 36 | 72 |
| | *Citrobacter freundii* | 6 | 12 |
| | *Pseudomonas cepacia* | 2 | 4 |
| | *Kluivera* spp. | 2 | 4 |
| | Unidentifiable[b] | 4 | 8 |
| | Total no. of colonies identified | 50 | 100 |

[a] All identifications were done with the API 20E system using colonies randomly picked from several plates representing different elevations and times of the year.

[b] Unidentifiable by the API 20E system.

One hundred and thirty-eight typical green-sheen colonies were randomly picked from several m-Endo plates (from samples obtained from different bromeliads at different altitudes and times of the day and year) and identified as described above. The most commonly isolated bacteria were *C. freundii* and *Serratia liquefaciens* (both 13% of total isolates), followed by *Aeromonas hydrophila* (7.9%); 69 (50%) colonies were unidentifiable by the API 20E method. *E. coli* was identified 2.2% of the time (Table 2). It was also observed that noncoliform bacteria (such as *Pseudomonas* spp. and, as mentioned above, *Serratia* as well as *Aeromonas* spp.) were capable of producing green-sheen colonies.

**Antibiotic susceptibility.** Nineteen colonies isolated on m-FC (and identified as *E. coli* using API 20E strips) were subjected to antibiotic susceptibility tests. For every isolate the average diameter of the inhibition zone was exactly the same as in the controls (both ATCC strains) for all antibiotics tested (except kanamycin). With kanamycin the average diameter of the zone of inhibition of the control strains was larger than with the environmental strains. Nonetheless in both cases the results could be interpreted in the same manner (i.e., susceptibility to the antibiotic). All strains were susceptible to all of the antibiotics tested except tetracycline, to which all isolates (including controls) were intermediately susceptible.

## DISCUSSION

It is generally assumed that members of the genus *Escherichia* cannot exist as part of environmental microflora or even survive for long periods of time extraenterally. Thus, their presence in extraenteral environments is believed to be only as a result of recent fecal contamination. The isolation of *E. coli* from unpolluted sites has been a cause for skepticism, since these findings go against the traditionally accepted idea that this bacterium cannot survive in the environment and can only replicate and survive in the intestines of warm-blooded animals. It can be argued that birds are a likely source for the presence of *E. coli* in the rain forest sampled. However, the geographical location of Puerto Rico (which is an island located at great distances from any other in the Caribbean) and the low population of birds refute the latter hypothesis. Even if the *E. coli* cells isolated in the course of this study originated from birds or tree-climbing mammals (of which only rats exist in Puerto Rico), it is very unlikely that there should be "recent" fecal contamination in every single one of the bromeliads sampled at different times of the day and during different days of the month and year. Thus we could presume either that these bacteria may at some time have originated from fecal sources and are now capable of surviving indefinitely in tropical environments (and have now become part of the normal flora of some vegetation), or that *E. coli* has always been part of the natural microflora of tropical areas. Moreover, the isolation of *E. coli* from dry leaf surfaces by a modification of the RODAC (contact plate) technique further indicates that this bacterium may, in fact, be part of the phyllosphere and not simply a transient bacterium which recently originated from fecal sources. We are currently trying to determine whether *E. coli* is also part of the indigenous microflora of other types of vegetation in the rain forests of Puerto Rico and other Latin American countries.

All of the bacterial strains were isolated on m-FC medium containing rosolic acid (whenever rosolic acid was not used in the medium, the profuse growth of nontypical bacteria masked any typical colonies that may have been present).

Typical colonies were then confirmed by inoculating them on EC broth (incubated at 44.5°C) and further identified by using biochemical tests. This is normally all that is done whenever a presumptive *E. coli* isolate is being identified. To assure ourselves that all of the strains identified as *E. coli* by the above methods were indeed *E. coli*, we ran other confirmatory tests. Antibiotic resistance profiles (which, although not a method for identification of bacteria, can be used as an excluding characteristic) were done. In all cases, the profiles of the strains isolated from bromeliads were identical to those of the control ATCC strains used. We are currently conducting coliphage sensitivity assays as well as plasmid profile analyses and correlating the latter to antibiotic sensitivity in environmental strains (G. T. Toranzos, manuscript in preparation).

In our laboratory we have conducted nucleic acid analyses on DNA extracted from several environmental isolates. These analyses (such as percent guanine-plus-cytosine content and DNA-DNA hybridization with homologous and heterologous nucleic acids isolated from bacterial strains) have confirmed in all cases that the isolates are indeed *E. coli* strains (M. Bermúdez and T. C. Hazen, submitted for publication).

The values obtained from physicochemical measurements (except for nitrates plus nitrites) were not found to be different from those described in the literature in regard to similar aquatic systems (11). Statistical analyses performed on the data did not indicate any significant correlations between any of the variables and the concentrations of total coliforms or total bacterial numbers or activity and respiration. It was interesting to note that the concentrations of phosphorus were below the level of detection of the method used. This and the high bacterial densities in the bromeliad water may indicate that this system has a very high nutrient turnover rate; thus most of the nutrients could be tied up in the microflora. The latter hypothesis is further corroborated by the high rates of physiological activity observed (i.e., respiration and protein synthesis). Thus *E. coli* may be able to survive in other areas with similar nutrient content and temperature.

The enumeration of either total or fecal coliforms in the samples analyzed in the present study was hampered by the varied amounts of sediment present in the bromeliad axilae. We are presently using the most-probable-number technique and correlating fecal coliform densities with sediment dry weights at different times of the year. Nonetheless, it should be noted that the isolation of *E. coli* from these environments is more important than the actual concentrations at which this bacterium is present in this environment. A more extensive study is in progress to determine whether any specific variable is responsible for the indefinite survival of *E. coli* in the rain forest. In addition, we are studying the strains isolated from the environment for the presence of extra- and intracellular enzymes not present in *E. coli* strains isolated from sewage and fecal specimens.

The high numbers of total bacteria observed ($2.9 \times 10^9$/100 ml) with acridine orange direct counting indicate that *E. coli* is able to successfully compete with noncoliform bacteria in the environment. The high number of coliforms ($1.5 \times 10^6$/100 ml) present in bromeliad axilae is to be expected as coliforms are an integral part of the environmental microflora of plants and plant-associated sediment. Fecal coliforms were found consistently throughout the study, although sometimes in relatively low numbers. We should keep in mind that the isolation of *E. coli* from this environment is more important than the actual numbers. We routinely isolate fecal coliforms from pristine rain forest streams; thus the isolation of fecal coliforms is not limited to the unique environment we describe in this communication. The presence of *E. coli* in this unique environment further corroborates the hypothesis that fecal coliforms can indeed survive in extraenteral environments.

We need to reevaluate the use of these bacteria as indicators of fecal contamination. The presence of *E. coli* in waters is immediately assumed to be a result of fecal contamination, even though no such contamination may be apparent. This assumption may lead to erroneous conclusions. This does not mean that currently used (5) bacteriological standards should be abandoned altogether, but rather it should be kept in mind that these bacteria are in fact capable of survival and proliferation under conditions other than those in the gastrointestinal tract of warm-blooded animals. Studies in other tropical areas of the world have also indicated the possible growth and proliferation of *E. coli* in the environment (8, 9; Roger S. Fujioka, personal communication). We have also isolated fecal and total coliforms from treated drinking waters in tropical countries where levels of chlorine were up to 1.0 mg/liter (Toranzos, manuscript in preparation).

Several government agencies have started a shift toward the use of fecal coliforms in lieu of total coliforms to indicate the possible presence of pathogens in water (6). This is a result of the sometimes ambiguous nature of total coliforms (which are part of the environmental microflora) as indicators of biological pollution of waters. There is a growing body of evidence which indicates that the presence (or absence) of coliforms and fecal coliforms does not correlate with biological quality of waters (7, 12, 13, 17–19). Enteric viruses have been isolated from treated drinking waters which were free of indicator bacteria (17–19). Thus the use of these groups of bacteria as indicators of fecal pollution of waters has been questioned. The present study further demonstrates that the isolation of presently used indicator bacteria (total coliforms) does not in fact necessarily correlate with the biological quality of waters. Our laboratory routinely isolates *E. coli* from pristine streams in the rain forests of Puerto Rico. Thus, the presence of naturally occurring fecal coliforms in nonpolluted sites negates the validity of using this group of bacteria to indicate recent fecal contamination. It is very possible that the results described in this communication may represent a phenomenon typical of tropical environments where the average nutrient concentrations as well as ambient and water temperatures do not vary throughout the year. More studies (in temperate as well as tropical environments) are needed to refute or prove the latter hypothesis.

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