Detection of Salmonellae in the Environment

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The incidence of salmonellae in contrasting environments was compared in this study. Samples collected from or near surface waters in a lush hardwood forest yielded four salmonellae serotypes from six culturally positive samples. A total of 76 samples collected from the top of a granite outcrop over a 3-month period yielded 10 positive samples. Only two salmonellae serotypes were isolated, and one of these was isolated only once. The nature of the sample material had no significant effect on the detection of salmonellae from the two sampling sites. However, the presence or absence of visible moisture in the sample significantly affected the recovery of salmonellae. The results showed that even a harsh environment such as that found on top of Stone Mountain may serve as an ecological niche for the survival and transmission of salmonellae.

Recent studies (2, 3) have shown that the salmonellae are widely distributed in the environment and pose a potential threat to the health of humans and other animals. Salmonellae have been recovered from rivers and streams in remote areas devoid of any apparent human fecal contamination, as well as from urban streams and recreational lakes subjected to fecal pollution from humans and other animals. Fair and Morrison (4) concluded that unpolluted, potable surface water sources do not exist. Cherry et al. (2) demonstrated the ease of recovering salmonellae from urban and rural streams and suggested that these organisms may be a better indication of a health hazard than the standard coliform count. It has also been suggested by Cherry et al. (3) that these organisms, whose habitat has been thought to be the intestinal tract, may in fact be free-living in nature.

The present study was conducted to determine the incidence of salmonellae in a harsh and unfavorable environment as compared with that of a lush hardwood forest characterized by abundant moisture and moderate temperatures.

MATERIALS AND METHODS

Sampling sites. Environmental samples were collected from two locations within the metropolitan Atlanta area. The first area sampled was Lullwater Estate, which is a mature hardwood forest located on the Emory University campus. It is well protected against casual human intervention. The forest provides an excellent habitat for diverse populations of both warm- and cold-blooded vertebrates, as well as many invertebrates. Samples were taken from three small streams, a spring, and a pond edge.

The second sampling site was the top of Stone Mountain, which is the largest of a series of granite outcrops near Atlanta, Ga. The top of the mountain receives heavy human usage as the central attraction in a state park. The total number of feral animals which the environment will support is small in comparison to a forest. Samples were collected from 13 different weather pools, 10 of which were sampled several times over a 3-month period. Only one of the pools remained wet during the entire sampling period, and it was created from the runoff from a large air-conditioning unit. All other pools exhibited intermittent drying.

Collection of samples. Environmental samples were collected in or near standing or running water and from weather pools. Samples consisted of water, algae, mosses, tree roots, grasses, and soil. These were collected in sterile, screw-capped, 35-mm film canisters which held either 30 ml of water, 50 g of soil, or 5 to 25 g of vegetation depending upon its type and moisture content. The samples were returned to the laboratory within 3 h of collection and placed in enrichment broth for isolation of salmonellae.

Cultural methods. For culturing, the samples were placed in 0.5-liter refrigerator jars containing 100 ml of tetrahionate broth and a 1:10,000 concentration of brilliant green dye. They were incubated at 41.5 ± 1.0 C for 48 h. The broths then were streaked on brilliant green agar and on bismuth sulfite (Difco) agar plates and incubated at 35 C. Colonies resembling salmonellae were fished from the plates after both 24 and 48 h of incubation and inoculated onto triple sugar iron agar, lysine iron agar, and urea agar slants (all from Difco). All salmonellae isolates were confirmed by further biochemical and serological tests. Representative isolates of each O group found at each collection site were definitively serotyped by the National Salmonellae Reference Center at the Center for Disease Control.
FA methods. Smears of the tetrathionate broth cultures were made on multiwell slides and fixed and stained with Salmonella polyvalent OH conjugates as described previously (8). The enrichment broths were examined at the end of 24 h, and those found negative by fluorescent antibody (FA) were reexamined after 48 h of incubation. Representative colonies from specimens found positive by FA but negative by culture were suspended in sterile 0.85% NaCl and stained with the FA reagent to detect organisms that cross-reacted with the Salmonella conjugate.

RESULTS

Lullwater Estate. A total of 24 samples of soil and vegetation were collected. The vegetation consisted of mosses, leaves, emergent plants, and algae. Eleven of the 24 samples were positive for salmonellae by FA staining tests. Six of these samples, which were collected from three separate sites, were confirmed as Salmonella by cultural methods. Four Salmonella serotypes, S. typhimurium, S. give, S. infantis, and S. bern, were isolated. No samples were culture positive and FA negative.

When the results obtained from examination of soil were compared with those derived from the testing of vegetation, neither showed any apparent advantage for harboring salmonellae (Table 1). However, when the samples were classified as to their visible moisture content and the results were compared, a decided difference between the wet and dry samples was noted (Table 2). No salmonellae were isolated from the dry samples, whereas 10 of the 12 wet samples were positive for salmonellae by FA, and salmonellae were isolated from six of these.

Stone Mountain. A total of 76 environmental samples were collected. Of these, 19 were positive for salmonellae by FA tests. All isolates but one were S. bariety and S. vestlaco. S. bariety was isolated from one or more samples from 6 of the 10 sites sampled repeatedly. No samples were culture positive and FA negative.

The Stone Mountain samples were classified into the categories of soil, vegetation, and water (Table 3). Of the 58 soil samples, 15 were positive by FA staining and eight of these also were culturally positive. Only one of seven vegetation samples was positive by either test. Three of 11 water samples were positive by FA tests and one was positive by culture. Although the number of vegetation and water samples is small, there is no apparent difference in the isolation rate among the three types of samples. When the samples are classified according to their moisture content, the results are striking (Table 4). No FA- or culture-positive results occurred among the 20 dry samples, but 19 of the 56 wet samples were positive by FA, and 10 of these were confirmed by cultural methods. When the wet samples were classified by type (Table 5), soil and vegetation samples were found to yield almost twice the percentage of positive culture results as did the samples of water, even though the difference was not significant by the chi-square test.

The weather pools differed biologically by the presence or absence of macroinvertebrates. Three different types of aquatic microinvertebrates were observed. One was a clam shrimp, another, the endemic fairy shrimp (both in the order Eubranchiopoda), and the third, a single species of water mite of the order Hydracarenida. The isolation rate of salmonellae from specimens collected in pools containing macroinvertebrates was not significantly higher than the

### Table 1. Salmonellae detected in soil and vegetation samples from Lullwater Estate

| Sample material | No. of tests | FA + culture | FA + culture | FA – culture |
|-----------------|--------------|--------------|--------------|--------------|
| Soil            | 5            | 2            | 1            | 2            |
| Vegetation      | 19           | 4            | 4            | 11           |

### Table 2. Salmonellae detection in wet and dry samples from Lullwater Estate

| Sample | No. | FA + culture | FA + culture | FA – culture |
|--------|-----|--------------|--------------|--------------|
| Wet    | 12  | 6            | 4            | 2            |
| Dry    | 12  | 0            | 1            | 11           |

* The two proportions are significantly ($P < 0.01$) different by the chi-square test.

### Table 3. Salmonellae detection in soil, vegetation, and water samples from Stone Mountain

| Sample | No. | FA + culture | FA + culture | FA – culture |
|--------|-----|--------------|--------------|--------------|
| Soil   | 58  | 8            | 7            | 43           |
| Vegetation | 7 | 1            | 0            | 6            |
| Water  | 11  | 1            | 2            | 8            |

* The associated proportions are not significantly different by the chi-square test.
enteric bacteria were adsorbed on the surface of collection bottles, thereby reducing the enteric population within the liquid. Absorption of organisms to soil particles could account for negative results on water samples taken from weather pools where salmonellae were isolated with ease from substrate samples.

The two sampling sites were selected because of their dichotomous nature. At the Lullwater site, the forest canopy reduces the range of fluctuation of air movements, and of humidity, temperature, and photogenic effects. At Stone Mountain, the intensity and range of fluctuation of environmental effects on the bare rock are profound. Also, wildlife associated with the two sites is quite different, being much more numerous and diverse in the Lullwater forest than on Stone Mountain. Another difference is the abundance of flowing water within the Lullwater site, whereas the summit of Stone Mountain is characterized by pools of stagnant water which are subjected to intermittent drying.

A major difference noted between the results obtained from the two sites was the diversity of salmonellae isolated from Lullwater as compared to Stone Mountain. Four different *Salmonella* serotypes were isolated from six culturally positive samples obtained during a single survey of the Lullwater area. A total of 76 samples were collected from the top of Stone Mountain on five different occasions over a 3-month period. Only two *Salmonella* serotypes were isolated from the 10 positive samples. One of these, *S. welshemi*, was isolated only once from a single sample. All other isolations were *S. barielli*.

Only two sample characteristics were noted at the time of collection: the predominant material of which the sample was composed and its moisture content. The composition of the sample material appeared to have no significant effect on detection or isolation of salmonellae in samples from Lullwater (Table 1) or Stone Mountain (Table 3). However, the presence or absence of water in the sample had a significant effect on the detection of salmonellae (Tables 2 and 4). Only one of 32 dry samples from both locations was positive by FA staining, whereas 25 of 68 wet samples were FA positive. Salmonellae could not be cultured from any of the dry samples, whereas these organisms were isolated from 18 of the 25 wet samples which were positive by FA staining.

The appearance and disappearance of the salmonellae during the wet and dry periods provokes speculation on what occurs in these small weather pools. Do the salmonellae die off during the drying process and are they reintroducible?

### DISCUSSION

In previous studies (2, 3) of urban and mountain streams, salmonellae were associated with the water, stream vegetation, and stream bottom substrates. They were not found in other soil samples, forest litter, aquatic vertebrates or invertebrates, or terrestrial invertebrates. Cherry et al. (3) found that salmonellae survived on contaminated vegetation taken from Lullwater streams for at least 1 week, and they suggested that the surface of aquatic plants may provide a good substrate for salmonellae multiplication. In 1943, Zobell (9) demonstrated that certain aquatic bacteria adhere so tenaciously to solid surfaces that they are not dislodged by washing with running water or by staining procedures. Hendricks (5) suggested that the increased recovery rate of salmonellae from stream bottom sediments versus surface water may be due to adsorption of organisms to sand and clay particles. More recently, Hendricks (6) confirmed that significant numbers of
duced each time it rains, or do they somehow survive the desiccation and reappear once they are rehydrated? Recently, Mossel et al. (7) emphasized the importance of resuscitating stressed Enterobacteriaceae before inoculating them into a selective medium. Bissonnette et al. (1) have shown that Escherichia coli exposed to river water for varying periods of time acquired a nonlethal injury that prevented their growth on selective media. This injury, however, could be rapidly repaired by placing them in a nutritionally rich, nonselective medium. The absence of salmonellae in dry samples should be investigated further by rehydrating the samples and incubating them in nonselective pre-enrichment broth before placing them in the inhibitory tetrathionate broth.

Generally, microbiologists have assumed that Salmonella-contaminated water supplies were the result of fecal contamination by either humans or other animals. It appears that most creeks, lakes, and remote mountain streams contain these organisms. Consumption of the contaminated water by wildlife, domestic animals, or by humans presumably completes one phase of the cyclic movement of these organisms in nature. Our studies suggest that this view may be a simplism, and our results indicate the need to define the role of aquatic vegetation, benthic sediments, and, perhaps, other factors in maintaining salmonellae in the environment.

The studies reported here have shown that even a harsh environment such as that found in small weather pools at the summit of a granite outcropping may serve as an ecological niche for the survival and transmission of salmonellae.

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