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Survival and Detection of *Bacteroides* spp., Prospective Indicator Bacteria

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Preliminary experiments were performed to assess the use of intestinal *Bacteroides* spp. as indicators of fecal contamination of water. Viable counts of *Bacteroides fragilis*, an anaerobic bacterium, declined more rapidly than those of *Escherichia coli* and *Streptococcus faecalis*. However, a fluorescent antiserum prepared against *B. fragilis* successfully detected high proportions (18 to >50%) of *B. fragilis* cells suspended for 8 days in aerobic water in dialysis bags at the ambient temperature. These percentages were higher than the percent viable recoveries of the two indicator bacteria used for comparison. Thus, the fluorescent antiserum test for *B. fragilis* might serve as a useful indicator of fecal contamination of water. An advantage of this approach over coliform analysis is the rapidity at which the test can be performed.

Escherichia coli is the primary indicator bacterium used for the assessment of fecal contamination of drinking water and wastewater. The two most common methods for its detection, i.e., total and fecal coliform analyses by most-probable-number and membrane filter methods, require 24 to 74 h to complete. In this study intestinal *Bacteroides* spp. were tested as alternative indicator bacteria for detection of fecal contamination. In addition to a viable plating procedure, a rapid fluorescent antibody (FA) procedure was examined for the detection of this prospective indicator group. The development of an FA procedure for detection of fecal contamination is desirable because it would reduce the time required for assessing water quality.

Intestinal *Bacteroides* spp. appear to be ideal indicator bacteria because they are found in much higher concentrations than is *E. coli* in the human intestinal tract. Slanetz and Bartley (11) found the coliform count per gram of human feces to range from 1×10^4 to 2.6×10^8 in the 20 individuals they examined. The arithmetic mean for these samples was 1.9×10^7. In contrast, the *Bacteroides fragilis* group (including what were earlier described as five subspecies, which are now classified as five separate species: *B. fragilis*, *B. vulgatus*, *B. thetaiotaomicron*, *B. ovatus*, *B. distasonis*) accounts for 20 to 30% of the total cultivable bacteria from the human intestinal tract. The concentration of *B. vulgatus* alone is 3×10^{10} to 6×10^{10} cells per g of feces in humans (5, 8). *B. thetaiotaomicron* and *B. distasonis* are also found in similarly high concentrations. Thus, it appears that there are ca. 1,000-fold-higher numbers of the *B. fragilis* group per gram of human feces than there are fecal coliform bacteria. Therefore, from a purely numerical standpoint, intestinal *Bacteroides* spp. could potentially serve as more sensitive indicators of fecal contamination compared with fecal coliform bacteria. A recent relevant report examines the recovery of viable *Bacteroides* spp. from sewage and natural waters with a selective medium (1).

MATERIALS AND METHODS

Survival experiments. The organisms used during the survival experiments were pure cultures of *B. fragilis* (ATCC 25285), *E. coli*, and *S. faecalis*. The *E. coli* and *S. faecalis* strains were isolated from the Lake Washington Ship Canal water in which the field experiments were performed. Before the survival experiments, *B. fragilis* was grown in PYG broth (12) at 35°C for 48 h; *E. coli* and *S. faecalis* were also grown in PYG medium at 35°C for 24 h. The cells were centrifuged (5,000 × *g*, 15 min) and washed three times with sterile 0.1% peptone buffer (1a). The cell pellet was then suspended in 10 ml of sterile 0.1% peptone buffer, and 1.5 ml of this suspension was used to inoculate 150 ml of filter-sterilized canal water in 300-ml Erlenmeyer flasks. The filter-sterilized canal water was filtered sequentially through membrane filters (pore sizes, 0.45 and 0.22 μm; Millipore Corp., Bedford, Mass.). After inoculation with the stock suspensions, the canal water was incubated under aerobic conditions at 12°C in the dark.

In the field survival experiments, the bacterial suspensions were processed in the same manner as the laboratory experiments except that filter-sterilized canal water was used for washing cells and the final suspension, instead of the 0.1% peptone buffer. Unfiltered, raw canal water (70 ml) was inoculated with 0.7 ml of each bacterial suspension (i.e., *B. fragilis*, *E. coli*, and *S. faecalis*) which filled the pretreated dialysis bags (no. 20 size; VWR Scientific, Seattle, Wash.). The dialysis bags were boiled for 10 min in 10% $NaCO_3$, rinsed and soaked in distilled water five times, suspended in 0.1 M EDTA (pH 7.5), boiled 10 min, and again rinsed and soaked in distilled water five times before filling. Dialysis bags were suspended out of direct sunlight in the canal at a depth of ca. 0.5 m.

Bacterial enumeration. (i) CFU counting. *B. fragilis* was enumerated during survival studies by spreading 0.1-ml sample dilutions (diluted with sterile, filtered canal water) on PYG-agar plates which were then incubated for 3 days at

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35°C under anaerobic conditions in GasPak anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.).

E. coli was enumerated during survival studies in the field by the membrane filter method (1a) with m-Endo medium (Difco Laboratories, Detroit, Mich.) designed for enumeration of total coliforms. Plates were incubated 2 days at 35°C before enumeration. *S. faecalis* was enumerated during survival studies in the field by using the mE-filtration method (7) with a modified mE-medium, which differs from mE-medium in that it contains, in addition, 0.75 g of indoxyl-β-D-glucoside per liter, lacks esculin, and has only 0.02 g of triphenyl tetrazolium chloride per liter (A. Dufour, personal communication).

(ii) Fluorescence microscope counting. *B. fragilis* was enumerated in samples preserved in formaldehyde (final concentration 1%) by the acridine orange (AO) epifluorescent procedure as described by Hobbie et al. (4) and an FA technique combining elements of the methods described by Holland et al. (6) and Dahle and Laake (3), with the commercially available Fluorotec F fluorescent antiserum (Pfizer Inc., New York). Fluorotec F was prepared against a strain of *B. fragilis*. It has been tested against *Bacteroides* spp. and has been found to react with the fecal *Bacteroides* group (i.e., 98.2% of 223 strains of *B. fragilis*, 98% of 100 strains of *B. thetaiotaomicron*, 71% of 31 strains of *B. vulgatus*, 55.6% of *B. distasonis* strains, and 61.5% of *B. ovatus* strains) (10). There was no cross-reactivity with a variety of other genera, including *Escherichia*, *Enterobacter*, *Proteus*, *Pseudomonas*, *Serratia*, *Salmonella*, *Shigella*, *Acinetobacter*, *Citrobacter*, *Yersinia*, *Aeromonas*, *Flavobacterium*, *Providencia*, *Alcaligenes*, *Moraxella*, *Streptococcus*, *Staphylococcus*, *Fusobacterium*, *Propionbicaterium*, and *Clostridium* (10). Also, it did not react with the oral species of *Bacteroides*, *B. melaninogenicus*. Therefore, the antiserum appeared to be fairly specifically directed toward the intestinal tract species of *Bacteroides*.

For the FA technique, a fixed volume of subsample was filtered through a Nuclepore filter (0.2-μm pore size; 25-mm diameter) prestained with Irgalan black (4). The filter was washed three times with 5 ml of 0.01 M phosphate-buffered saline (pH 7.2, 0.15 M NaCl) in distilled water. The vacuum was removed, and 1.0 ml of gelatin (2% in phosphate-buffered saline, base hydrolyzed at pH 11.0 and 120°C for 15 min and neutralized) was added for 20 min to reduce background fluorescence (2, 3). Excess gelatin was removed by washing twice with 5 ml of phosphate-buffered saline, and the filter was transferred to a glass slide and allowed to air dry (ca. 10 min). A drop of prestain (rhodamine-conjugated globulin) was added to the filter, which was then placed in a moisture chamber in the dark for 15 min. The filter was again placed on the filter column, and excess prestain was removed by vacuum through the filter. The filter was again placed on a glass slide, and one drop of the fluorescein-labeled anti-*B. fragilis* conjugate was placed on top of the filter and incubated in the moisture chamber for 15 to 30 min. The filter was transferred back to the filter column, washed twice with 5 ml of phosphate-buffered saline, and air dried on a slide. A drop of mounting fluid and a cover glass were added, and cells were then enumerated by epifluorescence microscopy (Zeiss standard GFL microscope adapted for epifluorescence using ×1,000). At least 10 fields of view and 300 cells were counted per sample. Some cells fluoresced more brightly than others, and these were counted again, in addition to a total count. All fluorescence faded within several minutes, so bright counts were enumerated within 1 min, and total counts were enumerated shortly thereafter.

Water analysis. Dissolved oxygen concentrations were measured by the method of the American Public Health Association (1a), using the azide modification.

TABLE 1. Enumeration[a] of *B. fragilis* cells in filtered canal water

Time (h)	No. of CFU	No. (10^6) of cells (±SE) by:		
		AO	FA_{total}	FA_{bright}
0	3.4×10^6	4.67 (0.02)		
1.0	3.3×10^6	4.72 (0.17)	3.07 (0.29)	1.29 (0.11)
1.5	1.7×10^6	4.50 (0.20)	3.07 (0.25)	1.24 (0.20)
2.0	1.0×10^6	4.43 (0.32)	2.87 (0.21)	1.19 (0.15)
2.5	1.0×10^6	4.85 (0.13)	2.76 (0.17)	1.31 (0.12)
20.0		4.87 (0.14)	2.15 (0.21)	0.98 (0.11)
192.0	2.1×10^4	4.66 (0.14)	1.75 (0.15)	0.81 (0.07)

[a] Mean counts per milliliter at 12°C.

TABLE 2. Enumeration[a] of *B. fragilis* cells in dialysis bags

Time (h)	CFU (10^6) (±SE)	No. (10^6) of cells (±SE) by:		
		AO	FA_{total}	FA_{bright}
0	2.70 (0.34)	2.42 (0.14)	1.63 (0.09)	0.87 (0.07)
2	2.76 (0.64)	2.39 (0.14)	1.70 (0.04)	0.82 (0.04)
5	2.53 (0.61)	1.98 (0.10)	1.67 (0.09)	0.82 (0.07)
20	1.36 (0.06)	2.04 (0.11)	1.43 (0.16)	0.73 (0.06)
27.5	1.13 (0.19)	2.10 (0.11)	1.42 (0.11)	0.74 (0.06)
192	<0.0001	NR[b]	0.29 (0.03)	0.08 (0.01)

[a] Mean counts per milliliter from survival experiments in the Ship Canal. See Table 3 for temperatures and dissolved oxygen concentrations.
[b] NR, Not reliable.

RESULTS

Different enumeration techniques were used to assess the survival of *B. fragilis* in flasks containing filtered canal water (Table 1). The decline in viable cell numbers (from 3.4×10^6 to 2.1×10^4 CFU/ml) was greater than the reduction in FA counts. AO counts remained unchanged throughout the experiment. Both brightly fluorescing cells (FA_{bright}) and cells with weak fluorescence were found. They were counted separately, and both FA_{bright} and FA_{total} cells are reported in Table 1. The declines in FA counts were approximately equal, 57% (from 3.07×10^6 to 1.75×10^6/ml) and 63% (from 1.29×10^6 to 0.81×10^6/ml) for FA_{total} and FA_{bright}, respectively.

The survival of *B. fragilis*, *S. faecalis*, and *E. coli* was investigated by incubating bacterial suspensions in dialysis bags in the Ship Canal, Seattle (Tables 2 and 3). Water was supersaturated with dissolved oxygen during the experiment

TABLE 3. Enumeration[a] of *E. coli* and *S. faecalis* CFU in dialysis bags

Time (h)	Temp (°C)[b]	Dissolved oxygen (mg/liter)[b]	*E. coli* CFU (10^5)/ml	*S. faecalis* CFU (10^4)/ml
0	12.0		5.6 (0.5)	5.0 (0.7)
5	11.9	12.4	6.7 (0.9)	11.0 (0.7)
192	11.5	12.0	0.13 (0.02)	1.00 (0.04)

[a] Mean counts per milliliter from survival experiments in the Ship Canal. Raw canal water water contained <1 *B. fragilis* CFU/ml, 82 total coliforms per ml, and 2.7 enterococci per 100 ml.
[b] Temperatures and dissolved-oxygen concentrations were measured in the canal water where the bags were positioned.

(9). Almost no reduction was observed in *B. fragilis* CFU during the first 5 h (Table 2). After 20 h the CFU was reduced by 50%, and after 192 h the CFU was less than 10^2/ml. The AO count was reduced by 20% during the first 5 h and then remained constant during the next 22 h. The FA counts remained constant during the first 5 h and then declined by 13% (FA_{total}) after 20 h and by 84% (FA_{total}) after 192 h (Table 2).

Both *E. coli* and *S. faecalis* multiplied during the first 5 h; the CFU then declined 98% (from 6.7×10^5 to 0.13×10^5/ml) and 91% (from 11×10^4 to 1.0×10^4/ml) for each species, respectively, after 192 h (Table 3).

DISCUSSION

The major purpose of this investigation was to assess *B. fragilis* as a potential indicator organism for detecting fecal contamination of water. The primary focus of this initial study was to evaluate various ways to detect *B. fragilis* and compare its survivability with other fecal indicator bacteria, including *E. coli* and *S. faecalis*.

The results of this study indicate that viable *B. fragilis* cells do not survive as well in aerobic freshwaters as do *E. coli* and *S. faecalis* cells. This result was not surprising inasmuch as *Bacteroides* spp. are obligate anaerobes. Nonetheless, for short periods of time (up to 1 day at 12°C), ca. 50% of the cells of *B. fragilis* remained viable. Less than 1% of *B. fragilis* cells could be detected after a week compared with ca. 2% for *E. coli* and 9% for *S. faecalis*. Thus, it appears that detection of large numbers of viable fecal *Bacteroides* spp. in an environmental sample indicates recent or extensive fecal contamination.

Although the results from the viable cultivation of *B. fragilis* in the survival experiments indicate that it dies rather quickly in aerobic waters, it is evident that it serves as an antigen long after its death. In the laboratory survival experiment, over 50% of the initial population of this bacteria could be detected by the fluorescent antiserum after 192 h (8 days). A similarly high percentage of persistence (17.8%) was found in the fluorescent antiserum counts of *B. fragilis* that were suspended in dialysis bags in a natural freshwater source for 192 h.

The results of this study suggest that *B. fragilis* might be useful as an indicator bacterium. It is noteworthy that the antigen can be detected for a long time after it has been exposed to aerobic waters and the organism has died. An advantage of the FA procedure for detection of indicator organisms is that the indicator organism can be enumerated without growing the organism. Thus, it is possible to enumerate the organisms within a short time (1 to 2 h) after collection of the water sample. Because there are at least 160 known serotypes of *E. coli* and because it occurs in lower concentrations in the human digestive tract than do fecal *Bacteroides* spp., *E. coli* appears to be less satisfactory for serological detection.

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