United States
Environmental Protection
Agency

Water Engineering
Research Laboratory
Cincinnati OH 45268

Research and Development

EPA/600/S2-87/010 Apr. 1987

EPA

# Project Summary

# Evaluation of *Bacteroides* as Indicator Bacteria in Drinking Water

James T. Staley, Thomas T. Moench, N. Robert Ward, and Donald L. Johnstone

**Research was undertaken to examine the feasibility of using the *Bacteroides fragilis* group of intestinal bacteria as indicators of fecal contamination in drinking water. This group surpasses *Escherichia coli* in fulfilling criteria for an ideal microbial indicator of fecal contamination.**

**Two immunological approaches—fluorescent antibody (FA) and enzyme-linked immunosorbent assay (ELISA)—were used to detect and quantify intestinal *Bacteroides* spp. in the laboratory and in simulated contaminated drinking water supplies that were seeded with fecal material from raw sewage.**

**FA procedures did detect intestinal *Bacteroides* spp. in sewage-contaminated waters, but full quantitative recovery was not attained. Although an extensive effort was made to develop an ELISA test, considerable difficulty was encountered in the nonspecific adsorption of goat anti-rabbit peroxidase to filters. Thus this approach is not advisable until this problem can be overcome.**

**Viable counting of intestinal *Bacteroides* spp. from simulated raw-sewage-contaminated drinking waters indicates that there is a need for better selective and differential med[illegible] for this purpose.**

***This Project Summary was developed by EPA's Water Engineering Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).***

## Introduction

The ability to determine the quality of drinking water is essential to protecting the public health. In particular, it is necessary to know whether drinking water contains fecal material from humans or animals, since its presence indicates the possibility of intestinal pathogens in the water. Because pathogens would be expected to occur in low numbers relative to the total bacteria from feces, tests have been designed to detect *Escherichia coli,* a normally nonpathogenic bacterium that is indigenous to the intestinal tract of humans and other animals. Unfortunately, no simple and rapid test exists to determine whether *E. coli* is present in a water sample. Instead, lengthy cultivation tests (requiring at least 24 to 48 hr of incubation) must be used to determine the presence of total and fecal coliform species, which are only presumptive indicators of the presence of *E. coli.*

The ideal microbial indicator of fecal contamination should have the following features: (a) it should occur in large numbers in the intestinal tract of humans, (b) it should grow only in the intestinal tract and not in the habitat, and (c) it should be sufficiently distinctive so that it can be identified quickly with simple tests that are inexpensive.

This research project examined the feasibility of using the *Bacteroides fragilis* group of intestinal bacteria as indicators of fecal contamination because this

group surpasses *E. coli* in fulfilling the three criteria outlined above. First, members of the enteric *Bacteroides* group *(B. fragilis, B. vulgatus, B. ovatus, B. thetaiotaomicron,* and *B. distasonis)* occur in much higher numbers (100 to 1000-fold greater concentrations) in the normal human intestinal tract than *E. coli.* Second, because the *B. fragilis* group comprises obligate rather that facultative anaerobes, they cannot grow in aerobic receiving waters. Finally, unlike *E. coli,* there are specific immunological tests for this group that could potentially be used to quantitate them rapidly in water samples.

This project examined the use of two immunological approaches—fluorescent antibody (FA) and enzyme-linked immunosorbent assay (ELISA) procedures—to detect and quantify intestinal *Bacteroides* spp. in the laboratory and in simulated contaminated drinking water supplies that were seeded with fecal material from raw sewage.

## Materials and Methods

Pure cultures of *Bacteroides* spp., primarily *B. fragilis* and *B. vulgatus,* were grown anaerobically in either Gas Pak containers* (BBL Laboratories) or in an anaerobic glove bag (Coy Laboratories, Ann Arbor, Michigan). Several media were used for pure culture work and also for viable enumeration of environmental *Bacteroides* spp., including brain heart infusion (BHI), which is a nonselective medium, as well as a variety of selective and differential media, including the medium of Wilkins and Chalgren (WC), *Bacteroides* bile esculin (BBE), and kanamycin bile (KB). Facultative anaerobes were grown aerobically on R2A medium.

Antisera against *Bacteroides* spp. were tested. These included a polyvalent commercial preparation, Fluorotec-F (General Diagnostics, Morris Plains, New Jersey), as well as specific rabbit preparations against *B. fragilis* and *B. vulgatus*. The Fluorotec-F serum was used for direct fluorescent microscopic counting (dFA), whereas antisera for *B. fragilis* and *B. vulgatus* were labeled with goat anti-rabbit serum conjugated to fluorescein (Miles-Yeda Ltd., Israel) for indirect fluorescent microscopic counting (iFA). These latter two sera were used for the ELISA testing in which goat anti-rabbit peroxidase (Miles-Yeda) was used as the enzyme.

FA microscopic counting was performed on 0.2-μm pore size Nuclepore filters that were prestained with irgalen black to reduce background fluorescence. After filtration of the sample, 1.0 mL of a 2% hydrolyzed gelatin solution was added before exposure of the filter to Fluorotec-F (dFA) or anti-*Bacteroides* serum (iFA). Following appropriate incubation, washing, and mounting procedures, dFA counts were made using an epifluorescence microscope. For iFA counting, an additional incubation with fluorescent goat anti-rabbit serum was included.

ELISA tests were performed using a variety of filters and pore sizes, including polysulfone (0.45 μm), polypropylene (10 μm), polycarbonate (0.2 μm), polyvinylidine-fluoride (0.45 μm), cellulose acetate (0.45 μm), cellulose nitrate (0.45 μm), and teflon (0.45 μm). To prevent the nonspecific binding of goat anti-rabbit peroxidase (GARP) to filters, a variety of proteins (including bovine serum albumin, gelatin, and goat normal serum) were tested under a variety of conditions before exposure to GARP.

To simulate the contamination of a drinking water supply, some experiments were performed, including the addition of one part of raw untreated wastewater to nine parts of lake water samples. In some experiments, pure cultures of *B. vulgatus* and/or *B. fragilis* were seeded in addition. Samples were withdrawn at periodic intervals and enumerated by iFA and viable plating procedures. Oxygen concentrations were measured, and all flasks were found to be aerobic (near oxygen saturation) shortly after initiation of the experiment and 24 hr later.

## Experimental Results

### *Fluorescence Microscope Counting of Intestinal Bacteroides spp.*

Pure culture studies of *B. vulgatus* showed that the iFA counts remained high throughout the growth curve, paralleling culture turbidity even after viability declined appreciably (Table 1). This result indicates that the iFA procedure closely follows the optical density and total microscopic count in cultures of this numerically important intestinal *Bacteroides* spp.

Recovery experiments were performed in which *B. fragilis* was added to buffered water, tap water, or primary wastewater and recovered by viable plating and dFA counting. Although high recoveries (36% to 96% of the viable count) were obtained from buffered water suspensions, lower recoveries were obtained from tap water (8% to 32%) and primary wastewater (5% to 11%). The low recoveries from wastewaters were partly due to the large amount of detritus that interfered with the fluorescence microscope counting.

In another experiment, a pure culture of *B. vulgatus* was inoculated into filter-sterilized lake water and incubated aerobically at 17°C. Cell counts by iFA indicated there was no decline in numbers following 3 days of incubation.

These experiments demonstrate that fluorescent antibody counts of *Bacteroides* spp. can be made with pure cultures in media and when inoculated into natural environmental samples of lake water, tap water, and wastewater.

### *ELISA Test for Bacteroides spp.*

The ELISA methodology was thought to be a more effective means of assessing intestinal *Bacteroides* spp. in natural samples than fluorescent antibody procedures for several reasons. First, the microscopic procedure is tedious and requires considerable time to perform. Also, it was thought that the problem of detrital interference might be partly circumvented by the use of the ELISA procedure.

Because drinking water samples that would be tested for *Bacteroides* would have to be concentrated before examination, efforts of this study were directed toward the development of an ELISA procedure that would use a membrane filtration step. However, problems were encountered in preventing the goat anti-rabbit peroxidase from nonspecifically binding to filters. Despite the use of various types of pore sizes of filters and several pretreatment procedures, this problem could not be resolved.

### *Fluorescent Antibody Enumeration of B. fragilis and B. vulgatus in Simulated Contaminated Drinking Waters*

Because of the difficulties encountered in the development of an ELISA test, efforts were directed toward examining fluorescent antibody and viable counting procedures for recovering *Bacteroides* spp. from drinking waters. Since there was no assured source of

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

drinking water that was contaminated by *Bacteroides* spp., a simulated one was used. Raw wastewater was added to lake water and incubated aerobically. In some experiments, a pure culture of *B. vulgatus* (the most common human intestinal species) or a combination of *B. vulgatus* and *B. fragilis* was seeded into the contaminated water. *Bacteroides* spp. were enumerated on several selective and differential media and also by use of iFA techniques.

The data in Figure 1 show the results of one of the experiments using artificially contaminated water. In this experiment, pure cultures of *Bacteroides* were not seeded, so the counts that are shown are representative of what would be found in a natural situation. First note that the iFA counts were the highest of all, remaining in excess of $10^5$/mL at zero time on days 1 and 2, and declining only slightly below that level on day 3. This result indicates that the FA technique can detect *Bacteroides* spp. (a pooled antiserum of *B. vulgatus* and *B. fragilis* was used) in a simulated contamination of drinking water.

Viable counts of presumptive *Bacteroides* spp. on WC medium were higher than total coliforms at zero time, whereas other media gave results comparable with those found for total and fecal coliform bacteria (Figure 1). However, when cultures of the presumptive *Bacteroides* were isolated and characterized, it was discovered that most could not be *Bacteroides* because they were facultative anaerobes rather than obligate anaerobes. Thus the actual levels of viable *Bacteroides* spp. in this experiment (as well as another that was performed) indicated that this group died off much more quickly than would have been predicted from pure culture studies.

Thus no adequate selective and differential procedure presently exists for viable counting of *Bacteroides* spp. for water samples contaminated with raw sewage.

## Conclusions

The results of this study indicate that FA procedures can detect intestinal *Bacteroides* spp. in waters that have been contaminated with raw sewage. However, full quantitative recovery was not attained by this procedure for two reasons: a) problems occurred with detrital interference, which obscured fluorescing cells and gave rise to background fluorescence, and b) several serotypes

**Table 1.** *Comparison of Various Enumeration Procedures during Growth of* B. vulgatus

| | | Number of Cells/mL ($\times 10^7$) | | |
|---|---|---|---|---|
| *Hours After Inoculation* | *Optical Density (660 nm)* | *Direct Microscopic Count* | *iFa Count* | *Viable Count* |
| 7.5 | 0.08 | 5.6 | 6.1 | 3.7 |
| 11.5 | 0.21 | 29 | 27 | 22 |
| 25 | 0.46 | 63 | 42 | 35 |
| 36 | 0.46 | N.D.* | 64 | 54 |
| 218 | 0.49 | 58 | 45 | 0.07 |

*Not determined.

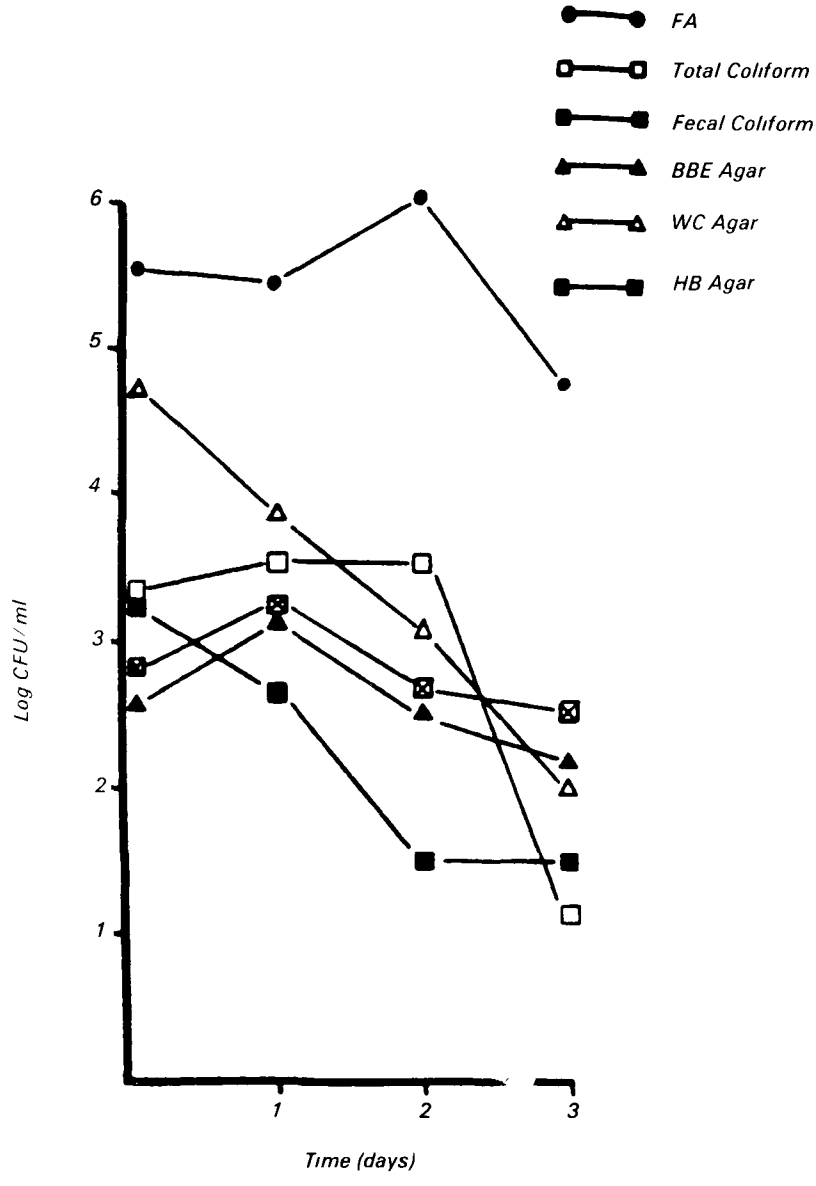


**Figure 1.** *Comparison of various techniques for enumeration of bacteria from a simulated contaminated drinking water source at daily intervals following contamination FA is the indirect fluorescent antibody count using pooled* B vulgatus *and* B fragilis *sera. Total coliform and fecal coliform bacteria were enumerated by standard methods, and three media (BBE, WC, and KB agar) were used for plating of viable* Bacteroides *spp. (see text for details).*

existed among the intestinal *Bacteroides* spp., and a better polyvalent antiserum (comparable to or better than the Fluorotec-F, which is no longer commercially available) would be needed to obtain better recovery. Furthermore, microscopic counting procedures are tedious and laborious and therefore not as desirable as other procedures such as ELISA testing.

Although an extensive effort was made to develop an ELISA test, considerable difficulty was encountered in the nonspecific adsorption of goat anti-rabbit peroxidase to filters. Until this problem can be overcome, it is inadvisable to consider using this approach.

Viable counting of intestinal *Bacteroides* spp. from simulated raw-sewage-contaminated drinking waters indicates that there is a need for better selective and differential media for this purpose. However, even if a better medium were to be developed, its usefulness is questionable for two reasons: a) it would be no more rapid than current methods already developed for the enumeration of coliform bacteria, and b) *Bacteroides* spp. appear to die off quickly in raw sewage (i.e., much more quickly than *Escherichia coli*). One possible advantage of such a test would be to assess how recently a water supply had been contaminated by raw sewage. Thus if a high ratio of *Bacteroides* spp. to fecal coliforms was found, it would suggest recent contamination, whereas a low ratio would suggest earlier contamination.

The full report was submitted in fulfillment of Cooperative No. CR-810477-01-3 by the University of Washington under the sponsorship of the U.S. Environmental Protection Agency.

*James T. Staley, Thomas T. Moench, and N. Robert Ward are with University of Washington, Seattle, WA 98195; and Donald L. Johnstone is with Washington State University, Pullman, WA 99164.*

***Donald Reasoner** is the EPA Project Officer (see below).*

*The complete report, entitled "Evaluation of Bacteroides as Indicator Bacteria in Drinking Water," (Order No. PB 87-145 892/AS; Cost: $11.95, subject to change) will be available only from:*

*National Technical Information Service*
*5285 Port Royal Road*
*Springfield, VA 22161*
*Telephone: 703-487-4650*

*The EPA Project Officer can be contacted at:*

*Water Engineering Research Laboratory*
*U.S. Environmental Protection Agency*
*Cincinnati, OH 45268*

United States
Environmental Protection
Agency

Center for Environmental Research
Information
Cincinnati OH 45268

BULK RATE
POSTAGE & FEES PAI
EPA
PERMIT No G-35

Official Business
Penalty for Private Use $300

EPA/600/S2-87/010