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NOTES

Enumeration of Enterotoxigenic *Bacteroides fragilis* in Municipal Sewage†

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Of 237 isolates of *Bacteroides fragilis* from sewage influent at the Bozeman, Mont., wastewater treatment plant, 22 (9.3%) were enterotoxigenic, as indicated by the ability to elicit fluid accumulation in the lamb ileal loop test. It appears that enterotoxigenic *B. fragilis* is endemic in the human population at a moderately high level.

Enteric disease is a serious problem of complex and often unclear etiology. While a number of infectious agents are recognized causes of enteric disease, approximately 50% of cases are etiologically undefined (11). Recent studies have implicated enterotoxin-producing isolates of an obligately anaerobic bacterium, *Bacteroides fragilis*, in human diarrheic disease (8; R. B. Sack, L. L. Myers, J. Almeido-Hill, and D. S. Shoop, abstract, United States-Japan Joint Conference on Cholera, **25**:33, 1989). The latter study, involving persons in Arizona, indicated that the enterotoxigenic bacterium colonized significantly ($P = 0.036$) more diarrheic persons (33 of 275) than nondiarrheic (control) persons (11 of 185). In addition, the enterotoxigenic bacterium was enteropathogenic in laboratory rabbits (7, 8; L. L. Myers, D. S. Shoop, and J. E. Collins, abstract, United States-Japan Joint Conference on Cholera, **25**:34, 1989) and gnotobiotic pigs (J. R. Duimstra, J. E. Collins, L. L. Myers, and D. A. Benfield, abstract, Conference of Research Workers in Animal Disease, **67**:50, 1986).

In the present study, enterotoxigenic *B. fragilis* was enumerated in municipal sewage and regarded as an indicator of the carriage rate of this enteropathogen (as indicated by studies in animal models) in the human population.

(This report represents part of an M.S. thesis by D. S. Shoop entitled "The Occurrence of Enterotoxigenic *Bacteroides fragilis*, a Newly Recognized Enteropathogen, in Sewage." The thesis was accepted by the Environmental Engineering and Natural Science Division, Industrial Hygiene Program, Montana College of Mineral Science and Technology, Butte.)

Sewage influent was studied at the wastewater treatment plant that services Bozeman, Mont., a city of approximately 23,000 persons. The plant receives approximately 2×10^7 liters of sewage per day. Nine samples (100 ml each) were collected aseptically into sterile glass bottles on different days during April and May of 1989. A portion (3 ml) of each sample was vortexed (Vortex Jr. mixer; Scientific Industries Inc., Queens Village, N.Y.) with glass beads (3-mm diameter) for 1 min to ensure a homogeneous suspension. For enumeration of *B. fragilis*, five suspensions were each diluted 1:100 with isotonic saline solution, and bacteria (in 0.1 ml of suspension) were grown anaerobically (GasPak Anaerobe System; BBL Microbiology Systems, Cockeysville, Md.) on duplicate plates (100 by 15 mm) of a solid selective medium (PINN medium [1]) for 48 h at 37°C. The numbers of CFU per ml of influent were determined by using the viable count-spread plate method (10). Colonies were considered to be *B. fragilis* if they were at least 2 mm in diameter and if they had the internal mottled appearance characteristic of *B. fragilis* (5). Approximately 80% of the colonies on PINN medium appeared to be *B. fragilis*. For determination of enterotoxin production, a total of 237 colonies (8 to 52 colonies from each of nine sewage samples [Table 1]) with the appearance of *B. fragilis* were transferred from PINN medium to plates of a nonselective medium (tryptose blood agar; Difco Laboratories, Detroit, Mich.) to ensure purity. The 237 isolates were confirmed as *B. fragilis* by using selected biochemical tests (8) and were evaluated for enterotoxin activity in the lamb ileal loop test (6).

The mean number of *B. fragilis* CFU per ml of sewage influent was 7.4×10^4 (standard deviation, 2.4×10^4) (Table 1). Of 237 *B. fragilis* isolates, 22 (9.3% ± 1.9%) were enterotoxigenic in the lamb ileal loop test (Fig. 1). From these data, it is calculated that sewage influent contained approximately 6.9×10^3 CFU of enterotoxigenic *B. fragilis* per ml. The ratio of enterotoxigenic *B. fragilis* to nonenterotoxigenic *B. fragilis* in each of the nine sewage samples was similar (Table 1), indicating that enterotoxigenic *B. fragilis* was endemic in the population at a relatively constant level during the 2-month sampling period.

We previously isolated enterotoxigenic *B. fragilis* from the feces of 4 of 10 individuals with diarrhea of unknown etiology in Bozeman (8). We also found that, of 32 cultures of *B. fragilis* isolated from eight diarrheic individuals (four cultures from each person) with enterotoxigenic *B. fragilis*, 28 (87%) were enterotoxigenic (8). This observation indicates that individuals with enterotoxigenic *B. fragilis* excreted relatively few nonenterotoxigenic *B. fragilis*. In the Arizona study, *B. fragilis* was isolated from approximately 70% of individuals over 1 year of age, and of 130 isolates of *B. fragilis* (from 44 persons), 109 (84%) were enterotoxigenic (unpublished data).

In the present study, each sample of sewage influent collected was homogeneous and contained *B. fragilis* from many individuals owing to extensive mixing of large volumes of sewage in the sewage collection system prior to receipt at

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TABLE 1. Enumeration and enterotoxigenicity of *B. fragilis* in sewage influent

Sewage sample no.	*B. fragilis* CFU per ml of sewage influent	No. of *B. fragilis* tested for enterotoxin/no. enterotoxigenic
1	5.5×10^4	15/2
2	6.2×10^4	21/1
3	1.1×10^5	22/2
4	6.4×10^4	8/1
5	8.0×10^4	52/5
6	ND[a]	30/3
7	ND	30/2
8	ND	29/3
9	ND	30/3

[a] ND, Not determined.

the treatment plant. While direct sampling of individuals must be used to accurately determine the carriage rate of enterotoxigenic *B. fragilis* in the population, an estimate of this rate can be calculated by analysis of sewage. On the basis of the assumptions that 70% of individuals were colonized with *B. fragilis*, that persons were colonized with either enterotoxigenic *B. fragilis* or nonenterotoxigenic *B. fragilis* (both of these assumptions were supported by observations made in the Arizona study), and that individuals excreted equal numbers of enterotoxigenic *B. fragilis* or nonenterotoxigenic *B. fragilis*, the estimated carriage rate of enterotoxigenic *B. fragilis* in the population was 6.5%. On the basis of direct sampling of individuals, less than 1% of the persons in the United States were infected with *Shigella* or *Salmonella* spp. (4, 9), rates of infection with enteropathogenic *Escherichia coli* varied from 1.2 to 15.5% (2, 12), and 5.9% of nondiarrheic persons were colonized with enterotoxigenic *B. fragilis* (Arizona study).

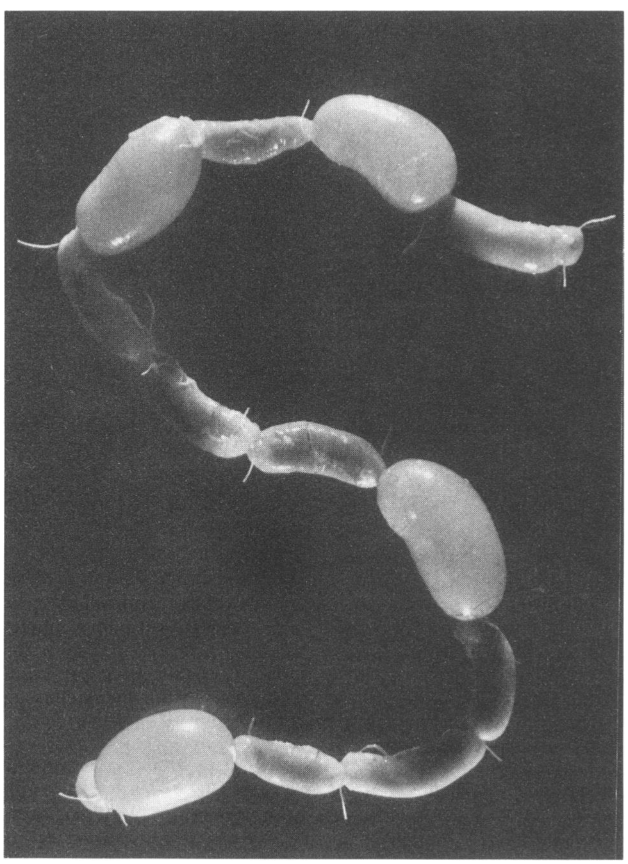

FIG. 1. Accumulation of fluid in the lamb ileal loop test. Each intestinal loop (n = 12) was inoculated with a different isolate of *B. fragilis*; four of the isolates were enterotoxigenic.

The source of enterotoxigenic *B. fragilis* in colonized individuals is unknown. Although *B. fragilis* is obligately anaerobic, it is quite aerotolerant, indicating that fecal-oral transmission may occur. Interperson spread (by an unknown mechanism) of *B. fragilis* in hospitals has been reported (3). Our preliminary studies (D. S. Shoop, M.S. thesis) indicated that less than 2% of enterotoxigenic *B. fragilis* in sewage influent survived the sewage treatment process and were discharged into a nearby recreational stream. There appeared to be little loss in viability of *B. fragilis* in the stream for up to 48 h, indicating that infection of persons via water is also a possibility. In comparison, sewage influent contained approximately twice as many *E. coli* as *B. fragilis*, and slightly more *E. coli* survived the sewage treatment process than did *B. fragilis*.

While the role of enterotoxigenic *B. fragilis* in the etiology of enteric disease is undefined, its association with diarrhea in the Arizona study, its relatively high level of endemicity in the Arizona and Bozeman studies, and its enterovirulence in animal models indicate that the bacterium may be important in the etiology of enteric disease.

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