Effect of Temperature on Survival of *Bacteroides fragilis* subsp. *fragilis* and *Escherichia coli* in Pus

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Approximately 40 to 60% of *Bacteroides fragilis* subsp. *fragilis* in pus from experimental intra-abdominal abscesses lost their viability within 24 h when stored at refrigeration temperature (4°C) either aerobically or anaerobically. No viability loss of *B. fragilis* was noted when pus was stored at 25°C. Only slight loss of viability of *B. fragilis* was observed at 15°C. *Escherichia coli* coexisting in pus with *B. fragilis* increased several 100fold in 24 h when stored at 25°C, but no significant growth occurred when they were kept at 15°C. Approximately 20 to 40% of *E. coli* lost their viability when such pus was stored at 4°C. We suggest that 15°C may be an alternative temperature for storage of anaerobic specimens in laboratories where some delay in routine processing is unavoidable.

Ideally, specimens for anaerobic culture should be processed immediately after collection from patients. However, this may not be practical or feasible for the small hospital laboratory. Finegold et al. (4) recommended that specimens suspected of containing anaerobic bacteria that cannot be processed within 2 h after collection should be refrigerated to suppress the undesirable proliferation of coexistent facultative anaerobes. Authors of other clinical laboratory manuals (3, 8) cautioned not to store such specimens at low temperature because of possible detrimental effects of chilling on anaerobic bacteria.

It was shown in our laboratory (6) and others (1, 9, 12) that *Bacteroides fragilis* survived prolonged exposure to 25°C but was rapidly killed when exposed to chilling under most conditions tested (6). Other investigators have reported similar findings when certain obligate and facultative anaerobic bacteria were suddenly exposed to chilling (5, 7, 11, 13). Thus far, there has been no critical research dealing with the effect of low temperature on the survival of obligately anaerobic bacteria in such clinical specimens. This paper examined the survival of *B. fragilis* and *Escherichia coli* in pus stored at 4, 15, and 25°C under both aerobic and anaerobic conditions.

MATERIALS AND METHODS

Organisms. *B. fragilis* subsp. *fragilis* strain 8044 and *E. coli* strain 8011 (clinical isolates at Loyola University Medical Center) were used throughout this investigation.

Media. Enriched Trypticase soy broth (TSB) contained TSB (Baltimore Biological Laboratory

[BBL]), 0.5% yeast extract (BBL), 0.05% hemin (Eastman Organic Chemicals Div., Eastman Kodak Co.), and 0.0005% menadione (Sigma Chemical Co.). Enriched Trypticase soy agar (TSA) was prepared by adding 1.5% agar (Difco Laboratories) to the enriched TSB. Selective media to isolate *B. fragilis* from mixed cultures containing *E. coli* were prepared by adding kanamycin (Sigma Chemical Co.) to enriched TSA to a final concentration of 100 µg/ml.

Production of pus in an experimental animal model of intraperitoneal abscess formation. A gelatin capsule containing artificial fecal material and designated bacterial species was surgically implanted into the peritoneal cavity of rats.

(i) **Animals.** White rats (Scientific Small Animal Lab and Farm, Inc., Arlington Heights, Ill.) ranging from 250 to 300 g in weight were used.

(ii) **Preparation of artificial fecal inoculum.** Artificial fecal material consisted of minced beef and cellulose fiber (Whatman filter paper, Ward R. Balston, Limited) at a ratio of 1:1.

A small piece (0.35 to 0.4 g, wet weight) of autoclaved artificial fecal material was placed inside a no. 0 gelatin capsule, which was then inserted into a no. 1 gelatin capsule. Gelatin capsules (Eli Lilly & Co.) had been sterilized by exposure to ultraviolet light (20 cm from a Westinghouse Sterilamp 782L-20) for 48 h before use. The effectiveness of this sterilization method was confirmed by the absence of growth of aerobic or anaerobic organisms in enriched TSB inoculated with irradiated capsules. Bacteria grown at 37°C for 18 to 24 h in enriched TSB were diluted to the desired concentration. Immediately before implantation, *B. fragilis* alone, or *B. fragilis* and *E. coli*, were impregnated into capsules containing the artificial fecal material by the use of needle and syringe. To produce infections by anaerobes alone, 10⁸ cells of *B. fragilis* were placed in the artificial feces. To produce mixed infections, 10⁸ cells of *B. fragilis* and 10⁶ cells of *E. coli* were impregnated into the artificial

fecal material. For each type of infection 15 to 20 rats were used.

(iii) **Surgical procedures.** Animal surgery was performed by the procedure used by Deysine et al. (2) and Weinstein et al. (15). Rats were anesthetized by intramuscular injections of 0.05 to 0.07 ml of Inovar (Pitman-Moore, Inc., Washington Crossing, N.J.). The capsule was placed aseptically into the peritoneal cavity. The peritoneum was closed with four or five continuous sutures of 3-0 silk. The skin was closed with four or five continuous sutures of 3-0 monofilament.

(iv) **Collection of pus.** Under our experimental conditions, no animal died before day 17 to 21 after implantation of the capsule. On day 17 to 21, animals were sacrificed, and abscesses were removed under sterile conditions. Abscesses were then placed in a chamber filled with anaerobic gas (10% CO₂ + 90% N₂, anaerobe grade; Benster Specialty Gas Co.). Pus pooled from a number of abscesses was thoroughly mixed under anaerobic conditions. Approximately 0.1 g of pus was then transferred to tared screw-cap tubes and stored under various conditions as specified below.

Storage of pus and survival of *B. fragilis* and *E. coli* in pus stored under various conditions. Three sets of tubes, each containing 0.1 g of pus, were thoroughly flushed with anaerobic gas. One set was placed at room temperature (25°C), the second set at 4°C, and the third set at 15°C. Another three sets of tubes were flushed with air. Again, one set was placed at room temperature and the others were placed at 4 and 15°C. All tubes were tightly sealed to avoid desiccation. Duplicate sample tubes were removed from each set at designated time periods and again weighed. Viable counts of *B. fragilis* in pus were determined by plating samples, after appropriate dilution in saline, on the enriched TSA containing kanamycin and incubating in GasPak jars (BBL). Viable counts of *E. coli* were determined by plating samples, diluted in saline, on enriched TSA under aerobic conditions. All plates were incubated for 24 h at 37°C, colonies were counted, and number of viable organisms per gram of pus was calculated. Data were presented as percent survival relative to those present in pus at 0 h of storage.

RESULTS

Percent survival of *B. fragilis* in pus, in which *B. fragilis* was the only infectious agent, is shown in Fig. 1A and B. *B. fragilis* in the pus lost viability slowly but progressively at 4°C whether it was stored under aerobic or anaerobic conditions. However, when it was stored at 25°C, no significant loss of viability occurred within 24 h under both aerobic and anaerobic conditions.

Since most anaerobic infections are mixed infections containing both strict and facultative anaerobes (10), we investigated the survival of *B. fragilis* and *E. coli* in pus in which both microorganisms coexisted. The results of these experiments are summarized in Fig. 2. Whereas the survival pattern of *B. fragilis* in such pus (Fig. 2A and C) was similar to that seen above

FIG. 1. Survival of *B. fragilis* in pus. Pus containing *B. fragilis* was collected from experimentally produced intraperitoneal abscesses in rats. Samples of pus were placed under aerobic (A) or anaerobic (B) conditions at 25°C (●) or 4°C (○) for varying lengths of time. Number of surviving cells was determined as described in Materials and Methods. Points and bars represent mean ± standard error of at least three values. Absence of a bar indicates that the standard error is smaller than the area covered by the point itself.

(Fig. 1A and B), there was a significant increase in the number of *E. coli* when pus was stored at 25°C (Fig. 2B and D). Apparently, chilling was also detrimental to *E. coli* whether pus was stored under aerobic or anaerobic conditions. In an effort to find an alternative condition for storage of pus that allows the maximum number of *B. fragilis* to survive while suppressing the overgrowth of coexistent *E. coli*, we tested the survival of both organisms in pus stored at 15°C. The results of such an experiment are summarized in Table 1. It is apparent that, at this temperature, most *B. fragilis* remained viable for at least 24 h, whereas the overgrowth of coexistent *E. coli* was minimized.

DISCUSSION

Although the recent advancement in anaerobic bacteriological techniques has considerably facilitated the isolation and identification of causative agents from anaerobic infections, certain fundamental aspects of culture processing still remain to be further clarified. One such problem concerns the storage of clinical samples suspected of containing anaerobic bacteria. This is a particularly serious problem in hospitals where the limitation in both facilities and staff does not always allow the processing of clinical specimens immediately after their collection.

The present investigation pursued this unsettled problem systematically and has provided

FIG. 2. Survival of *B. fragilis* and *E. coli* in pus. Pus containing *B. fragilis* and *E. coli* was collected from experimentally produced intraperitoneal abscesses in rats. Samples of pus were placed under aerobic (A and B) or anaerobic (C and D) conditions at 25°C (●) or 4°C (○) for varying lengths of time. Number of surviving cells was determined as described in Materials and Methods. Points and bars represent mean $\pm$ standard error of at least three values. Absence of a bar indicates that the standard error is smaller than the area covered by the point itself.

TABLE 1. Survival of *B. fragilis* and *E. coli* in pus at 15°C

Time (h) of storage	% Survival $\pm$ standard error					
	<i>B. fragilis</i> (monoinfection)		<i>B. fragilis</i> (mixed infection)		<i>E. coli</i> (mixed infection)	
	Aerobic ^a	Anaerobic ^b	Aerobic	Anaerobic	Aerobic	Anaerobic
0	100 $\pm$ 3	100 $\pm$ 3	100 $\pm$ 2	100 $\pm$ 2	100 $\pm$ 10	100 $\pm$ 10
2	84 $\pm$ 6	116 $\pm$ 8	86 $\pm$ 6	66 $\pm$ 9	106 $\pm$ 6	86 $\pm$ 9
24	76 $\pm$ 13	87 $\pm$ 5	94 $\pm$ 11	73 $\pm$ 3	129 $\pm$ 30	130 $\pm$ 7

^a Stored aerobically.

^b Stored anaerobically.

the experimental data which allow evaluation of various conditions for storage of clinical samples containing both strict and facultative anaerobes. Undoubtedly, chilling (4°C) had a detrimental effect on *B. fragilis* as well as on *E. coli* contained in experimental pus, although it was still possible to isolate *B. fragilis* even after 24 h of storage at 4°C (Fig. 1, 2A and C). Ueno (14) also stated that *Bacteroides convexus* (*B. fragilis* subsp. *fragilis*) could be recovered from pus many weeks after storage at 4°C. The presence of *E. coli* did not protect against loss in viability of *B. fragilis* at 4°C (Fig. 2A and C). Our earlier work (6) indicates that death from chilling was due to the low temperature, not increased oxygen absorption by media. Pure cultures of *B. fragilis* were shown to be similarly susceptible to chilling both under aerobic and

anaerobic conditions (6). It should be noted that, in all cases, loss in viability of *B. fragilis* stored in liquid media was significantly greater than that observed when the organisms were stored in pus. It is possible that the pus itself is a good transport medium (1) and somehow protects the anaerobe against the lethal effects of low temperature.

Our data (Fig. 2B and D) clearly support the contention of Finegold et al. (4) that the storage of clinical samples containing both strict and facultative anaerobes at room temperature (25°C) results in the overgrowth of the latter organisms, making it difficult, if not impossible, to successfully isolate coexistent strict anaerobes.

The data obtained from storage of pus containing both *B. fragilis* and *E. coli* at 15°C

(Table 1) suggest an alternative temperature at which clinical specimens can be stored for delayed processing. This can be easily achieved by the addition of a small refrigerated incubator adjustable to 15°C to the existent setting in any clinical laboratories. Although the viability of *B. fragilis* in the pus was not fully maintained (94 to 66% after 24 h of storage at 15°C), the overgrowth of *E. coli* was so suppressed that it created no serious problems in isolating the coexistent anaerobes. These observations may be of practical significance, because the organisms tested in our study are two of the most frequent isolates from intra-abdominal abscesses (10). Undoubtedly the feasibility of the use of 15°C for storing clinical samples suspected of containing both strict and facultative anaerobes needs further experimentation on other anaerobes and clinical samples.

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