Improved Laboratory Enrichment for Enterohemorrhagic *Escherichia coli* by Exposure to Extremely Acidic Conditions

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Analysis of food samples for *E. coli* O157:H7 using the standard U.S. Food and Drug Administration procedure is frequently complicated by overgrowth of nontarget microorganisms. A new procedure was developed for enrichment of enterohemorrhagic *E. coli* (EHEC) which utilizes exposure to pH 2.00 for 2 h. This procedure yielded larger populations of EHEC than the standard method by factors ranging from 2.7 to 7.7 and, when age-stressed cultures were used, by factors ranging from 2.7 to 11.5. Cultures of competing enterics were more effectively inhibited by the new enrichment protocol as well.

Escherichia coli capable of producing toxin with a cytopathic effect on Vero cells were originally designated Verotoxin-producing *E. coli* (VTEC). As similarities in the structure of Verotoxin and Shiga toxin were elucidated, the term Shiga toxin-producing *E. coli* (STEC) came into usage (3). There are many serotypes of STEC, but only those clinically associated with hemorrhagic colitis are designated enterohemorrhagic *E. coli* (EHEC) (6). EHEC are thought to be capable of causing illness if as few as 10 to 100 viable cells are ingested (19). Detection in food and water samples is difficult, because innocuous microorganisms may be present at high levels and may mask small numbers of potentially hazardous EHEC cells. For example, leaf surfaces average 10^7 to 10^8 epiphytic bacteria/cm² in the field, creating a potential problem for pathogen detection in fruits and vegetables, especially because microbial populations typically increase during storage (12, 13). Consequently, public health organizations worldwide use an initial enrichment step to minimize competitors and to increase the number of any target cells. These enrichment methods typically utilize a rich medium, such as modified tryptic soy broth (TSB) or modified *E. coli* broth (EC broth) supplemented with antibiotics, bile salts, and, in some instances, elevated incubation temperatures. Following enrichment, selective isolation steps are conducted by using selective agars, such as tellurite cefixime sorbitol MacConkey agar (TCSMAC) or hemorrhagic coli agar (HC). Finally, detection and confirmation steps utilize enzyme-linked immunosorbent assay, agglutination, or DNA methods (4, 6, 14, 17).

This paper describes a new enrichment procedure that yielded larger populations of EHEC cells and more efficiently eliminated competing microorganisms. This new enrichment procedure utilizes an innate ability of *E. coli* to withstand exposure to high levels of acid for extended periods. It does not require use of antibiotics or other inhibitory compounds or elevated incubation temperatures. Samples were exposed to pH 2.00 for 2 h at room temperature and then were transferred to growth medium devoid of inhibitors. This dual approach of

acid selection followed by growth in noninhibitory media resulted in larger final EHEC populations than the standard procedure used by the U.S. Food and Drug Administration (FDA).

For acidification enrichment experiments with pure cultures, Bacto TSB (Becton Dickinson, Sparks, Md.) was adjusted to pH 2.00 with concentrated HCl (approximately 38%), sterilized by filtration through a membrane filter (pore size, 0.45 μ m), and aseptically dispensed in 9-ml volumes in 18- by 150-mm test tubes. TSB formulations produced by some other manufacturers could not be used, because large amounts of precipitate formed when the pH was lowered to 2.00. Cultures to be exposed to acidic conditions in these tubes were initially incubated in TSB plus 0.6% yeast extract (TSBYE; pH 7.2) for 24 h at 35°C. These were individually diluted in Butterfield's buffer (16) to obtain approximately 10^3 CFU ml⁻¹ (range, 910 to 1,660). One-milliliter volumes of this dilution were placed into 9-ml volumes of pH 2.00 TSB, vortexed briefly, and allowed to stand at room temperature (20 to 22°C) for 2 h. After 2 h, 1-ml volumes were removed and introduced into TSB at standard pH (pH 7.3 ± 0.2). These tubes were vortexed briefly and were incubated at 35°C for 24 h. After 24 h all TSB cultures were decimally diluted and enumerated by spread plating on tryptic soy agar plus 0.6% yeast extract (TSAYE). The same procedure was used to compare growth of pure cultures after exposure to pH 2.00 for 1 to 5 h, except that 24-h TSB cultures were evaluated turbidimetrically rather than by spread plating on TSAYE.

For comparison to the standard FDA enrichment procedure, 9-ml volumes of sterile EHEC enrichment broth (EEC) (6, 16) were placed in 18- by 150-mm tubes. When 24-h TSBYE cultures were diluted to approximately 10^3 CFU ml⁻¹ as described above, an additional 10-fold dilution was made in Butterfield's diluent, and 1 ml of this was placed into 9 ml of EEC for 24 h of incubation at 35°C. Both the experimental TSB cultures and the control EEC cultures therefore received approximately the same number of cells at the onset of their respective 24-h growth periods. Incubation at 35°C is a slight modification of the FDA procedure, which calls for incubation at 37°C. Cultures used in this study are indicated in Table 1.

For experiments with age-stressed cultures, strains were grown in TSBYE for 24 h at 35°C. The screw-cap closures on

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TABLE 1. Cultures used in this study

Strain	Serotype	Source
<i>E. coli</i> 6424	O157:H7	FDA ^a
<i>E. coli</i> 6443	O157:H7	FDA
<i>E. coli</i> 6457	O157:H7	FDA
<i>E. coli</i> M3579	Orough:H2	P. Fey ^b
<i>E. coli</i> G550637	O103:H2	P. Fey
<i>E. coli</i> M3039	O26:H11	P. Fey
<i>E. coli</i> 6321	O157:H7	FDA
<i>E. coli</i> 6347	O157:H7	FDA
<i>E. coli</i> 6396	O157:H7	FDA
<i>E. coli</i> 178190	O111:NM	P. Fey
<i>Morganella morganii</i> MG01	ND ^c	FDA
<i>C. freundii</i> 8090	ND	FDA
<i>E. aerogenes</i> 13048	ND	FDA
<i>K. pneumoniae</i> 13883	ND	FDA
<i>Proteus mirabilis</i> 6264	ND	FDA
<i>Enterobacter sakazaki</i> MG05	ND	FDA
<i>H. alvei</i> MG04	ND	FDA
<i>Ewingella americana</i> MG02	ND	FDA
<i>Acinetobacter calcoaceticus</i> 19606	ND	FDA

^a Bothell, Wash.^b University of Nebraska Medical Center, Omaha, Nebr.^c ND, not determined.

these tubes were sealed, and the cultures were held on the lab bench at room temperature for 44 days to mimic stress induced by cell starvation. For experiments summarized in Fig. 2 and Table 2, these aged cultures were used directly in a comparison of enrichment methods with no resuscitation.

For comparative enrichment of a sample representing a complex mixture of enteric and other bacteria, a wastewater sample was used (see Table 4). This was an untreated influent sample obtained at the Edmonds, Wash., wastewater treatment plant, and it was refrigerated until analyzed in the laboratory approximately 1 h later. *E. coli*, total heterotrophic population, and sorbitol-positive and -negative populations were measured after comparative enrichments of this sample with m-ColiBlue24, TSAYE, and TCSMAC, respectively (8, 16). The four enrichments were also analyzed for *stx1* and *stx2* amplicons by using the multiplex PCR procedure of Grant et al. (9).

When fresh cultures of 10 EHEC strains were selectively enriched by both the standard method and the experimental acidification method, resulting populations of all 10 strains were larger via the experimental method by factors ranging from 2.7 to 7.7 (Fig. 1). When the same strains were stressed by aging for 44 days and were similarly enriched, all 10 strains again yielded larger populations with the experimental method, this time by factors ranging from 2.7 to 11.5 (Fig. 2).

In addition to producing larger populations of target cells, the experimental method was also more effective in eliminating non-*E. coli* species that could compete with the target cells or otherwise serve as sources of interference in subsequent isolation procedures (Table 2). By using fresh cultures the acidification method yielded lower population levels, with five of the nine non-EHEC cultures used. With two additional species the experimental and standard enrichments gave comparable results, and with two other species the standard enrichment yielded lower numbers of cells. When age-stressed cultures were used, six of nine species were more effectively inhibited with the experimental method, two were comparably inhibited, and one was slightly more inhibited by the standard method.

As shown in Table 3, both pathogenic and nonpathogenic strains of *E. coli* demonstrated greater capacity for surviving acid shock than the other species tested. All four *E. coli* strains grew well after exposure to pH 2.00 for as much as 5 h. In contrast, *Citrobacter freundii*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* were killed by as little as 1 h of exposure. *Hafnia alvei* and *Shigella flexneri* were slightly more tolerant of acid shock; *H. alvei* survived 2 h but not longer, and *S. flexneri* survived 1 h but not longer. None of the strains, including *E. coli*, survived exposure to pH 1.00 in TSB for 2 h, and when the exposure temperature was raised from approximately 22 to 35°C, some *E. coli* strains were inhibited (data not shown).

Present FDA procedures for detection of *E. coli* O157:H7 utilizes enrichment in modified TSB with cefixime, cefsulodin, and vancomycin, followed by streaking onto TCSMAC (6). This procedure was initially described in 1995 (18) based on earlier studies in the United Kingdom (2, 20). This method is used by the FDA to analyze foods such as produce, cheese, and sprouts, which are of heightened concern because they are typically consumed without heating or other final antimicrobial barriers. Such samples may contain high levels of microorganisms which are harmless but which are present in such large numbers that they may conceal small numbers of EHEC. TCSMAC plates streaked after this standard enrichment are commonly covered with sorbitol-positive colonies to the extent that it is difficult to see whether small numbers of target sorbitol-negative colonies are present. The nine cultures of potential competitors examined in this study are typical of species appearing on TCSMAC plates at the completion of the Bacteriological Analytical Manual (BAM) procedure. No gram-positive strains were tested in these initial experiments, as they do not routinely cause interference on TCSMAC.

The improved enrichment procedure described in this paper

TABLE 2. Improved reduction of potential competing species by acidification enrichment method

Expt and species ^a	Population (CFU/ml) after:	
	Standard enrichment	Acidification enrichment
Expt 1		
<i>Morganella morganii</i>	7.2×10^7	7.8×10^8
<i>C. freundii</i>	3.3×10^8	<10
<i>E. aerogenes</i>	3.1×10^8	<10
<i>K. pneumoniae</i>	2.1×10^3	<10
<i>Proteus mirabilis</i>	<10	<10
<i>Enterobacter sakazaki</i>	7.0×10^8	<10
<i>H. alvei</i>	5.6×10^7	1.7×10^9
<i>Ewingella americana</i>	<10	<10
<i>Acinetobacter calcoaceticus</i>	9.13×10^8	<10
Expt 2		
<i>Morganella morganii</i>	9.8×10^7	<10
<i>C. freundii</i>	2.1×10^8	<10
<i>E. aerogenes</i>	2.3×10^8	<10
<i>K. pneumoniae</i>	1.0×10^2	<10
<i>Proteus mirabilis</i>	<10	<10
<i>Enterobacter sakazaki</i>	9.6×10^7	<10
<i>H. alvei</i>	9.8×10^8	1.3×10^9
<i>Ewingella americana</i>	<10	<10
<i>Acinetobacter calcoaceticus</i>	4.3×10^8	<10

^a Experiment 1 used fresh cultures, while experiment 2 used age-stressed cultures.

FIG. 1. Growth of 10 strains of EHEC with either standard selective enrichment (S) or experimental acidification enrichment (A). Numbers 1 to 10 indicate strains 6424, 6443, 6457, M3579, G550637, M3039, 6321, 6347, 6396, and 178190, respectively.

is based on the inherent ability of *E. coli* to withstand extremely low pH. Numerous studies have been conducted on acid stress in food pathogens, typically to determine whether they retain the ability to grow to infective levels after transient exposure to acidic conditions (1, 11, 15). However, this is apparently the first demonstration that extreme acid shock, followed by growth in noninhibitory media, is an effective method for selective enrichment of *E. coli*.

Ideally, an EHEC enrichment would eliminate all other strains, including nonpathogenic *E. coli*. However, pathogenic and wild-type *E. coli* are very similar physiologically. Even the two traits frequently used to differentiate them, possession of a functional β -glucuronidase gene and rapid fermentation of

sorbitol, are not greatly different on close examination. EHEC strains contain a *uidA* gene that differs from the wild type by a single base substitution (5). Similarly, even *E. coli* O157:H7 will ferment sorbitol slowly, and other EHEC serotypes rapidly ferment sorbitol (6, 7). Given the physiological similarity of EHEC and wild-type *E. coli* it is unlikely that any enrichment will yield only EHEC.

Although some species other than *E. coli* survived exposure to pH 2.00 for 2 h, it was encouraging that by increasing the exposure time to 3 h, *H. alvei* would have been eliminated (Table 3). If certain sample types are found to contain recalcitrant competitors, the length of acid exposure can likely be modified to enhance enrichment of *E. coli*, including EHEC. It is also encouraging that when cultures had been stressed by aging, the comparative growth of EHEC and inhibition of

FIG. 2. Growth of 10 strains of age-stressed EHEC with either standard selective enrichment (S) or experimental acidification enrichment (A). Numbers 1 to 10 indicate strains 6424, 6443, 6457, M3579, G550637, M3039, 6321, 6347, 6396, and 178190, respectively.

TABLE 3. Ability of *E. coli* to withstand prolonged exposure to highly acidic conditions^a

Species	Length of exposure to pH 2.00 at room temp (h)				
	1	2	3	4	5
<i>C. freundii</i>	NG	NG	NG	NG	NG
<i>H. alvei</i>	G	G	NG	NG	NG
<i>S. flexneri</i>	G	NG	NG	NG	NG
<i>E. aerogenes</i>	NG	NG	NG	NG	NG
<i>K. pneumoniae</i>	NG	NG	NG	NG	NG
<i>E. coli</i> 6347	G	G	G	G	G
<i>E. coli</i> 6396	G	G	G	G	G
<i>E. coli</i> 6424	G	G	G	G	G
<i>E. coli</i> 6268	G	G	G	G	G

^a Cells of each species were introduced into pH 2.00 TSB for indicated lengths of time and then were transferred to neutral-pH TSB and incubated at 35°C for 24 h. Growth or no growth indicates the status at the end of the 24-h period. Cultures demonstrating growth were all heavily turbid, containing approximately 10^9 CFU mL⁻¹. *E. coli* strains 6347, 6396, and 6424 are all O157:H7. Strain 6268 is a nonpathogenic wild-type *E. coli*. NG, no growth. G, growth.

TABLE 4. Improved selection of *E. coli* from wastewater by using acid enrichment

Enrichment method ^a	Count (CFU/ml) ^b		
	<i>E. coli</i> ^c	Total cell count ^d	Sorbitol-positive colonies ^e
Acidic enrichment 1 (growth in TSB)	4.87×10^8 (0.49×10^8 ; 81% of total)	5.98×10^8 (0.79×10^8)	1.07×10^7 (0.13×10^7)
Acidic enrichment 2 (growth in TSBYE)	6.00×10^8 (0.85×10^8 ; 98% of total)	6.15×10^8 (0.77×10^8)	1.37×10^7 (0.38×10^7)
TSB	4.67×10^7 (0.47×10^7 ; 9% of total)	5.40×10^8 (0.63×10^8)	1.27×10^7 (0.35×10^7)
EEB	2.03×10^8 (0.47×10^6 ; 61% of total)	3.35×10^8 (0.17×10^8)	3.16×10^7 (0.49×10^8)

^a Wastewater sample contained 6.1×10^4 *E. coli*/ml prior to any enrichments, as determined by m-ColiBlue24 analysis. Acidic enrichment 1: 5 ml of wastewater was placed in 45 ml of pH 2.00 TSB for 2 h at room temperature, and then the entire 50-ml volume was placed in 450 ml of pH 7.3 TSB for 24 h of incubation at room temperature. Acidic enrichment 2 was the same as enrichment 1, except that after 2 h of acid exposure, the entire 50-ml volume was placed into 450 ml of pH 7.3 TSBYE. TSB enrichment: 5 ml of wastewater was placed directly into 495 ml of pH 7.3 TSB and was incubated for 24 h at 35°C. EEB enrichment: 5 ml of wastewater was placed directly into 495 ml of EEB broth and was incubated for 24 h at 35°C.

^b Standard deviations are in parentheses. *stx1* and *stx2* genes were not detected by multiplex PCR analysis (9).

^c *E. coli* was enumerated by using m-ColiBlue24 medium.

^d Total cell count was determined by using TSAYE.

^e Sorbitol-positive and -negative colonies were enumerated on TCSMAC. The count of sorbitol-negative colonies for each enrichment method was $<10^6$.

competitors was superior to results with fresh cultures. It is probable that cells in an actual food, water, or environmental sample would have undergone some stress induced by aging and/or starvation and may therefore respond to the acid enrichment process in a fashion analogous to that of the aged cells used in this study.

Further studies will be undertaken to determine the effect of the acid enrichment procedure in food and other complex samples. However, an illustration of the possible utility of this approach is demonstrated in Table 4. A wastewater sample was chosen to provide a wide variety of competing microorganisms. Five-milliliter volumes of this wastewater were enriched by four procedures, as described in the footnotes to Table 4. Two of these methods utilized 2 h of exposure to pH 2.00 TSB prior to unrestricted growth. In method 1 unrestricted growth was in pH 7.3 TSB (16), and in method 2 unrestricted growth was in pH 7.3 TSBYE (16). The third enrichment was direct addition of wastewater to pH 7.3 TSB. The fourth enrichment, the present BAM method, involved addition of wastewater to EEB. As shown in Table 4, the two acid enrichment procedures were more effective than the BAM method, as determined by the larger percentage of *E. coli* recovered and by lower numbers of colonies on TCSMAC. Acid selection followed by growth in TSB resulted in a total population containing 81% *E. coli*. When acid selection was followed by growth in TSBYE, 98% of the resulting total population was *E. coli*. By comparison, the BAM procedure yielded 61% *E. coli* in the total population, and the TSB-only procedure yielded 9%.

Multiplex PCR analysis did not reveal *stx1* or *stx2* amplicons in any of the four enrichments. This finding is not remarkable, as Grant et al. previously reported that Shiga-like toxin II genes could only be detected in one of six sewage concentrates (10).

Probably the greatest drawback to the present BAM method is the large number of sorbitol-positive colonies appearing on TCSMAC that potentially obscure small numbers of target colonies. Although regular (biotype 1) *E. coli* are sorbitol positive, the potential advantage of acidic enrichment is seen in the approximately threefold reduction in nontarget colonies on TCSMAC at the same time a larger overall percentage of *E. coli* is generated, presumably because of elimination of more non-*E. coli* competitors. This example indicates that additional experiments coupling the acidic enrichment approach with numerous existing methods for EHEC isolation is warranted and could be useful. Additionally, its effectiveness as an enrichment

for other pathogenic *E. coli* strains, such as enterotoxigenic and enteropathogenic *E. coli*, should be examined.

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