Impact of microbial diversity on rapid detection of enterohemorrhagic *Escherichia coli* in surface waters

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Received 25 January 2006; revised 19 May 2006; accepted 25 May 2006.
First published online June 2006.

doi:10.1111/j.1574-6968.2006.00334.x

Editor: Rob Delahay

Keywords
enterohemorrhagic *Escherichia coli*; *Escherichia coli* O157:H7; enteropathogenic *Escherichia coli*; verotoxigenic *Escherichia coli*; water-borne *Escherichia coli*.

Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) are a physiologically, immunologically and genetically diverse collection of strains that pose a serious water-borne threat to human health. Consequently, immunological and PCR assays have been developed for the rapid, sensitive detection of presumptive EHEC. However, the ability of these assays to consistently detect presumptive EHEC while excluding closely related non-EHEC strains has not been documented. We conducted a 30-month monitoring study of a major metropolitan watershed. Surface water samples were analyzed using an immunological assay for *E. coli* O157 (the predominant strain worldwide) and a multiplex PCR assay for the virulence genes stx_1, stx_2 and *eae*. The mean frequency of water samples positive for the presence of *E. coli* O157, stx_1 or stx_2 genes, or the *eae* gene was 50%, 26% and 96%, respectively. Quantitative analysis of selected enriched water samples indicated that even in samples positive for *E. coli* O157 cells, stx_1/stx_2 genes, and the *eae* gene, the concentrations were rarely comparable. Seventeen *E. coli* O157 strains were isolated, however, none were EHEC. These data indicate the presence of multiple strains similar to EHEC but less pathogenic. These findings have important ramifications for the rapid detection of presumptive EHEC; namely, that current immunological or PCR assays cannot reliably identify water-borne EHEC strains.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as a serious gastrointestinal pathogen in many countries. Although the predominant mode of EHEC transmission to humans is via consumption of contaminated meat and produce (Mead *et al.*, 1999), outbreaks associated with water-borne EHEC have also been documented. For example, one of the largest water-borne outbreaks of EHEC occurred in 2000 in Walkerton, Ontario, Canada (Hrudey *et al.*, 2003). It is likely that the incidence of infection because of water-borne EHEC is underreported. Confirmation of exposure to water-borne EHEC is difficult due to the transient nature of bacterial strains in water; organisms are readily transported away from the site of exposure, are diluted below detectable levels, or die.

Despite the potential public health threat from water-borne EHEC, owing to either accidental or intentional contamination, there are no accepted methods for the rapid, accurate detection of EHEC in surface waters. Current measures of microbial water quality rely exclusively on 'indicators' of fecal pollution (e.g., fecal coliform bacteria or generic *E. coli*). However, there are no established correlations between the prevalence/concentration of these 'indicators' and specific pathogens, including EHEC. Recently, a variety of immunological and PCR assays have been developed for the rapid, sensitive detection of EHEC that are

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potentially suitable for water analysis. However, the ability of these assays to consistently detect the most prevalent EHEC strains (i.e., no false negatives) while excluding closely related non-EHEC strains which pose less of a threat to human health (i.e., no false positives) has not been documented.

A variety of rapid immunoassays have been developed for the detection of *E. coli* O157:H7, the most commonly reported EHEC serotype worldwide (Kaper *et al.*, 2004). In fact, in both the scientific and popular literature *E. coli* O157:H7 is frequently used synonymously with EHEC. Note, however, that several other EHEC serotypes are also responsible for human illness and that the prevalence of these strains varies from country to country (WHO, 1998; Bettelheim, 2003). The common feature of all immunoassays is the binding of antibodies to specific cell surface O antigens (e.g., O157 serogroup). These assays, however, detect the serogroup (e.g., O157), not the serotype (e.g., O157:H7). Therefore, all strains within the serogroup, including less virulent or nonpathogenic strains, will be detected (false positives); while EHEC strains belonging to other serogroups will be missed (false negatives).

An alternative approach to the immunological detection of presumptive EHEC is the use of PCR assays. As the stx_1 and/or stx_2 genes (encoding for the shiga-like toxins) are the critical virulence factors for EHEC, these genes are uniformly included in PCR assays for EHEC (Sharma *et al.*, 1999; Wang *et al.*, 2002). However, the stx_1/stx_2 genes are widely distributed among *E. coli* [referred to as STEC (Shiga-toxin producing *E. coli*) or VTEC (verotoxin-producing *E. coli*)] and *Shigella* strains, as well as other water-borne bacteria, due to their dissemination via bacteriophages (James *et al.*, 2001). Consequently, the presence of stx_1/stx_2 genes in water samples may be presumptive, but is not definitive for EHEC. An alternative gene target is the *eae* gene encoding for intimin, a component of the locus of enterocyte effacement (LEE). The *eae* gene, however, is not unique to EHEC; it is also a critical virulence factor for the enteropathogenic *E. coli* (EPEC), which are similar to EHEC but lack *stx* genes (Kaper *et al.*, 2004). Previous research indicates that there are several alleles of the *eae* gene and that certain alleles are characteristic of certain serotypes (Zhang *et al.*, 2002). PCR assays have been designed to detect the different serotypes (e.g., O157:H7); however, these can result in false negatives depending on the prevalence of other EHEC strains. Alternatively, generic PCR assays can be designed to detect all *eae* alleles (including both EHEC and EPEC); however, these can result in false positives depending on the prevalence of EHEC vs. EPEC strains.

We are unaware of any previous studies systematically documenting the prevalence of both serogroups and genes typical of EHEC in surface waters from which the reliability of rapid PCR assays or immunoassays can be evaluated. In the present study, we report the results of a 30-month monitoring study of Baltimore, MD metropolitan area watersheds to assess the prevalence of *E. coli* O157 and stx_1/stx_2 genes, and to a lesser extent, *eae* genes. In addition, pure cultures of *E. coli* O157 were isolated from selected water samples and characterized. The goals of this study were to evaluate the reliability of PCR assays and immunoassays in detecting water-borne presumptive EHEC by comparing the prevalence of *E. coli* O157 and stx_1/stx_2 genes throughout the watersheds, and elucidating the diversity of *E. coli* O157 strains present.

Materials and methods

Watersheds

The Baltimore, MD metropolitan area watersheds sites are part of the Baltimore Ecosystem Study (BES), which is a component of the Long-Term Ecological Research Network (LTER) funded by the US National Science Foundation. Maps of the Baltimore metropolitan watersheds, with site locations and brief descriptions, can be found at the BES website (http://www.beslter.org/shelton_et_al_map.). For additional information, see Higgins *et al.* (2005).

Sample collection and processing

Weekly samples were collected manually over a span of 30 months (March 2002 to August 2004) from up to 19 sites in the Baltimore metropolitan area watersheds. Sample collection was somewhat variable because of the periodic absence of flow because of no rainfall or freezing conditions. Samples were transported to the Beltsville Agricultural Research Center (BARC), Beltsville, MD and processed within 24 h. Briefly, 100 mL of water were filtered and the filter placed in 10 mL of enrichment broth for overnight incubation at 37 °C (Shelton *et al.*, 2004). The enrichment culture was used for all subsequent analysis and strain isolations.

Microbial analysis

Enriched broth cultures were analyzed for the presence of *E. coli* O157 using the immunomagnetic-electrochemiluminescence (IM-ECL) methodology (Shelton *et al.*, 2004). Previous work indicated that, in general, the detection limit of this method is one viable organism per water sample (Shelton *et al.*, 2004). Enriched broth cultures were also analyzed for the presence of stx_1, stx_2 and *eae* genes using real-time PCR (Higgins *et al.*, 2005). The primer/probe sets for stx_1 and stx_2 genes have been described previously (Sharma *et al.*, 1999). A novel TaqMan primer/probe set for the detection of a broad range of *eae* genes was designed using Primer Express software (Version 1.0, Perkin Elmer/Applied Biosystems, Foster City, CA). The *eae* gene sequence was extracted from an *E. coli* O157:H7 genome fragment in GenBank (Accession no. AF071034) and is the reverse

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complement of bases 16482 to 19286. The extracted O157:H7 sequence was compared with *eae* sequences from other *E. coli* serotypes using CLUSTAL W. The primer/probe set was selected for a conserved region of *eae* in order to detect a broad range of enterohemorrhagic and enteropathogenic *E. coli*. The primers JKP11 (GGCGATTACGCGAAAGATACC) and JKP12 (CCAGTGAACTACCGTCAAAGTTATTACC) are located at bases 550–570 and 685–658, respectively, relative to the first base of the ATG codon representing the site of initiation of translation of the gene. The TaqMan probe, JKTM10 (CAGGCTTCGTCACAGTTGCAGGC) is located at bases 592–614 and was labeled with 6-FAM at the 5′ end and TAMRA at the 3′ end. All TaqMan assays were run on an ABI7700 instrument using a real-time PCR master mix supplied by Applied Biosystems.

Strain isolation and characterization

Pure cultures of *E. coli* O157 or 'O157-like' *Citrobacter freundii* were isolated from selected enriched water samples using immunomagnetic separation (IMS) techniques (Higgins *et al.*, 2005). Samples were plated onto MacConkey Agar and incubated at 44.5 °C, and Sorbitol MacConkey Agar amended with cefixime and tellurite (CT-SMAC) and incubated at 37 °C (Zadik *et al.*, 1993). *Escherichia coli* isolates were distinguished from *C. freundii* isolates using the BBL Enterotube II (Becton Dickinson, Sparks, MD), and based on the presence of the *lacZ* gene. In addition, the ECL signal obtained from *E. coli* O157 cells was *c.* fivefold greater than that obtained from 'O157-like' *C. freundii* cells. *Escherichia coli* O157 isolates were probed for the following genes: heat labile toxin (LT; DebRoy & Maddox, 2001), heat stable toxins a & b (STa/STb; DebRoy & Maddox, 2001), shiga-like toxins 1 & 2 (*stx*1/*stx*2; DebRoy & Maddox, 2001), cytotoxin necrotizing factors 1&2 (*cnf*1/*cnf*2; DebRoy & Maddox, 2001), intimin (*eaa*; DebRoy & Maddox, 2001), capsule (K1; Tsukamoto, 1997), bundle forming pilus (*bfp*; Gunzburg *et al.*, 1995), and pilin (*sfpA*; Brunder *et al.*, 2001). These analyses were performed at the *E. coli* Reference Center (The Pennsylvania State University, University Park, PA).

Results

The mean frequency of water samples positive for the presence of *E. coli* O157 was 50% ($n = 1303$). A seasonal trend was observed in the frequency of *E. coli* O157 with percentages substantially higher during the summer and fall months (Fig. 1). The mean frequency of samples positive for either stx_1 or stx_2 genes was 26% ($n = 1293$). No distinct trend was observed in the monthly percentages of stx_1/stx_2 genes (Fig. 1). The overall frequency of samples positive for both *E. coli* O157 and stx_1/stx_2 genes was 15%. Water samples collected from 2002 (March–October) and 2004 (March–August) were also analyzed for the presence of the *eae* gene; 96% were positive ($n = 596$).

Enriched water samples from selected watershed sites (collected July 22 through October 8, 2002) were quantitatively analyzed for the presence of *E. coli* O157 cells, stx_1 and stx_2 genes, and the *eae* gene. Enriched water samples consistently contained the *eae* gene, generally at higher

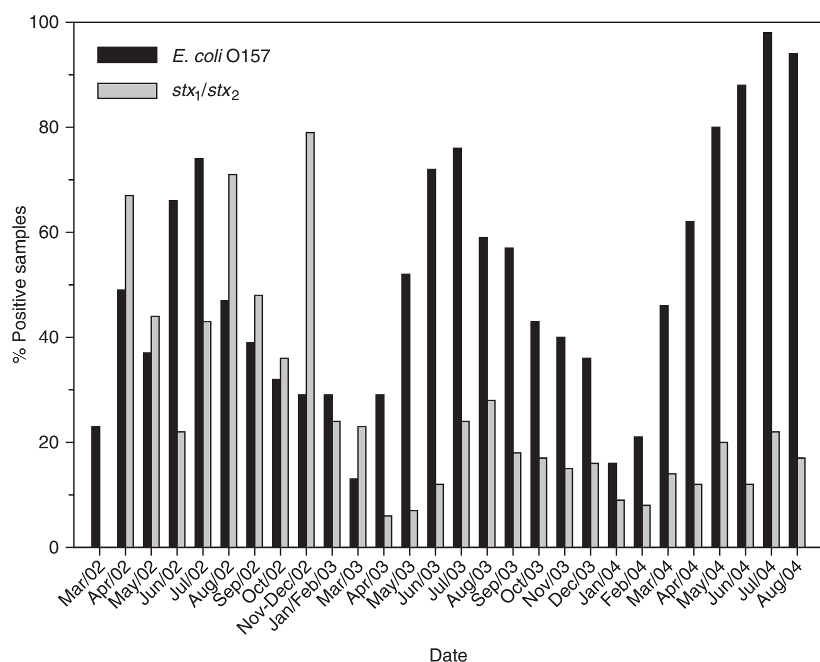

Fig. 1. Prevalence of *Escherichia coli* O157 and stx_1/stx_2 genes in enriched water samples from the Baltimore Metropolitan Area, MD watersheds.

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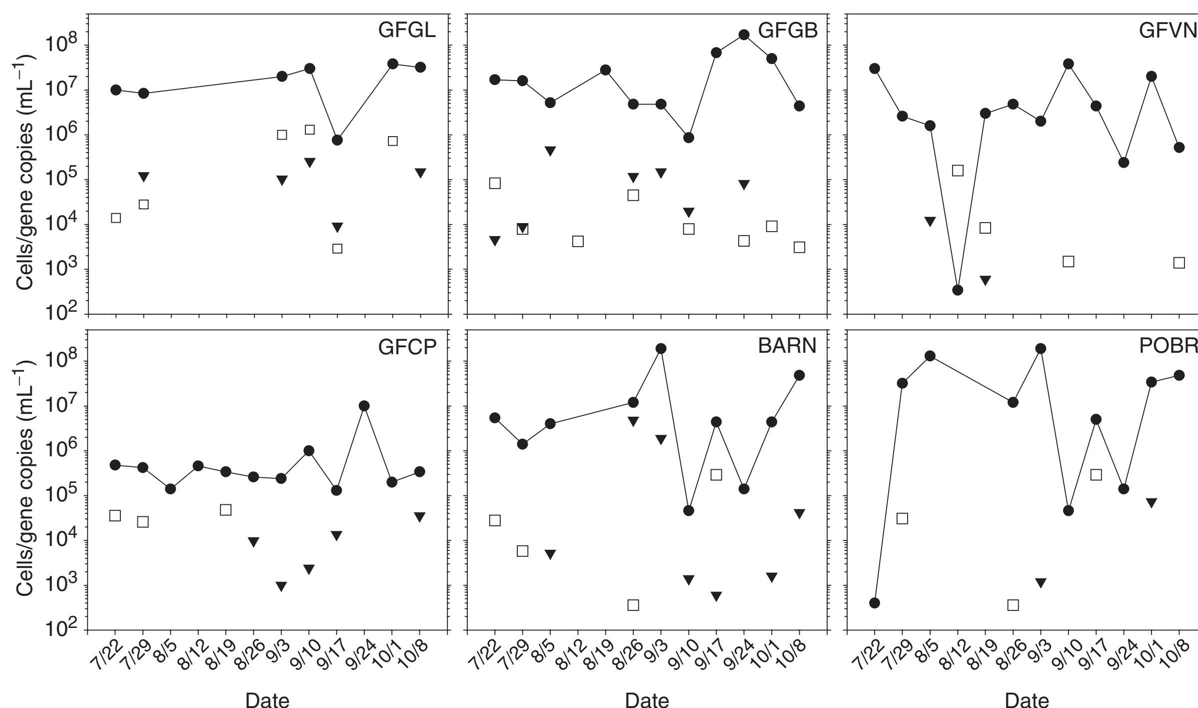

Fig. 2. Concentrations of *Escherichia coli* O157 cells (squares), stx_1/stx_2 genes (inverted triangles), and *eae* genes (connected circles) in selected enriched water samples from Baltimore Metropolitan Area, MD watersheds. The absence of any symbols indicates that water samples were not analyzed on that date; the absence of individual symbols indicates that cell/gene concentrations were not detected.

Table 1. Genetic characterization of *Escherichia coli* O157 strains isolated from Baltimore metropolitan area watersheds

Sample #	ECRC	LT	STa	STb	*stx*1	*stx*2	*cnf*1	*cnf*2	*eae*	K1	*bfp*	*sfpA*	O157	H type
GFGR (7/22/02)	3.2303	–	–	–	–	–	–	–	+	–	–	–	+	–
SBCR (7/29/02)	3.2305	–	–	–	–	–	–	–	+	–	–	–	+	–
SBCR (8/7/02)	3.2306	–	–	–	–	–	–	–	+	–	–	–	+	–
GFGB (8/24/02)	2.4171	–	–	–	–	–	–	–	+	–	–	–	+	–
HBR (9/3/02)	3.2309	–	–	–	–	–	–	–	+	–	–	–	+	–
GFGL (9/3/02)	3.2311	–	–	–	–	–	–	–	+	–	–	–	+	–
WR (10/2/02)*	2.4166	–	–	–	–	–	–	–	–	–	–	–	+	12
WR (10/2/02)	2.4168	–	–	–	–	–	–	–	–	–	–	–	+	12
GFGL (2/4/03)	3.2315	–	–	–	–	–	–	–	+	–	–	–	+	–
LANV (4/27/04)	4.2122	–	–	–	–	–	–	–	+	–	–	–	+	–
LANV (5/11/04)	4.2123	–	–	–	–	–	–	–	+	–	–	+	+	–
RGHT (5/18/04)	4.2124	–	–	–	–	–	–	–	+	–	–	–	+	–
GFCP (6/9/04)	4.2125	–	–	–	–	–	–	–	+	–	–	–	+	–
GFGL (6/9/04)	4.2126	–	–	–	–	–	–	–	+	–	–	–	+	–
GFGR (6/9/04)	4.2127	–	–	–	–	–	–	–	+	–	–	–	+	–
GFUGR (6/9/04)	4.2128	–	–	–	–	–	–	–	+	–	–	+	+	–
JBNW (8/3/04)	4.2129	–	–	–	–	–	–	–	+	–	–	–	+	–

*Cefixime/tellurite resistant.

ECRC, *E. coli* Reference Center strain number.

concentrations than either *E. coli* O157 cells or stx_1/stx_2 genes (Fig. 2). Many enriched water samples contained stx_1/stx_2 genes but no *E. coli* O157, or *E. coli* O157 but no stx_1/stx_2 genes. In those samples that contained both stx_1/stx_2 genes and *E. coli* O157, with only a few exceptions, there was no apparent relationship between the concentrations of *E. coli* O157 and stx_1/stx_2 genes.

Seventeen *E. coli* O157 strains were isolated and characterized from April 2002, through July 2004 (Table 1). All strains fermented sorbitol and grew well at 44.5 °C; with one

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exception, all strains failed to grow on CT-SMAC which is widely used for the selective isolation of 'typical' *E. coli* O157:H7 from environmental and clinical samples (Chapman *et al*., 1997; Willshaw *et al*., 2001; Johnson *et al*., 2003). Fifteen strains were classified as 'atypical' EPEC based on the presence of an *eae* gene and lack of a bundle forming pilus (*bfp*) gene. Two strains were classified as potentially nonpathogenic based on the absence of any genes encoding for virulence attributes. The EPEC O157 strains were isolated from ten different urban/suburban sites, while the potentially nonpathogenic O157 strains were isolated from an agricultural/forested site. Several presumptive *E. coli* O157 strains were subsequently identified as *C. freundii*, consistent with previous work documenting the cross-reaction of anti-O157 antibodies with *Citrobacter* spp. (Bettelheim, 1993; Park *et al*., 1998). Although numerous sorbitol nonfermenting colonies were obtained on CT-SMAC giving the 'typical' *E. coli* O157:H7 colony morphology, none were confirmed as *E. coli* O157:H7.

Discussion

These data indicate that the Baltimore, MD metropolitan area watersheds are uniformly contaminated with *eae*-positive (presumptive EPEC) strains and, to a lesser extent, *stx*-positive (STEC or STEC-like) strains. Based on the observations that (i) the prevalence of the *eae* gene was substantially higher than stx_1/stx_2 genes and (ii) there was no apparent relationship between the concentrations of *eae* vs. stx_1/stx_2 genes in selected enriched water samples, it is clear that independent populations were being detected. No conclusions can be drawn as to what percentage of bacteria (if any) possessed both *eae* and *stx* genes. This is consistent with previous studies documenting the prevalence and diversity of EPEC and STEC strains worldwide. EPEC is one of the major etiologic agents responsible for causing infant diarrhea in both developed and developing countries (Kaper *et al*., 2004). A wide diversity of EPEC serogroups (including O157) have been described that were isolated from patients in urban/suburban areas, including Australia (Robins-Browne *et al*., 2004), Brazil (Gomes *et al*., 2004), and the United States. (Bokete *et al*., 1997). In addition, limited data indicates that EPEC are also excreted by rabbits (Blanco *et al*., 1996), dogs (Beutin, 1999), and wild birds (Pennycott *et al*., 1998). Similarly, several studies conducted worldwide, including in Australia (Djordjevic *et al*., 2004), France (Rogerie *et al*., 2001), Japan (Kobayashi *et al*., 2001), and the United States. (Arthur *et al*., 2002) have documented the wide diversity of STEC serogroups excreted by ruminants. In each study, it was observed that the majority of STEC isolates did not possess an *eae* gene and therefore were unlikely to be EHEC, despite the fact that several isolates belonged to typical EHEC serogroups. Diverse STEC strains have also been isolated from dogs (Beutin *et al*., 1993) and from human sewage (Garcia-Aljaro *et al*., 2004).

Escherichia coli O157 was frequently detected in Baltimore metropolitan area watersheds, although the prevalence may have been overestimated due to the cross-reaction observed with 'O157-like' *C. freundii* strains. The predominant *E. coli* O157 strain isolated was 'atypical' EPEC, although presumptive nonpathogenic *E. coli* O157 strains were also isolated. This is consistent with previous research documenting the existence of non-EHEC O157 from human and animal sources. For example, EPEC O157 (*stx*-negative, *eae*-positive) have been isolated from humans (Willshaw *et al*., 2001; Gomes *et al*., 2004); STEC O157 (*stx*-positive, *eae*-negative) have been isolated from cattle (Rogerie *et al*., 2001); while presumptive nonpathogenic O157 (*stx*-negative, *eae*-negative) have been isolated from cattle (Rogerie *et al*., 2001), pigs (Chapman *et al*., 1997), and human sewage (Garcia-Aljaro *et al*., 2005).

The source(s) of EPEC, STEC, and *E. coli* O157 strains in the Baltimore metropolitan area watersheds are unknown. Primary sources presumably are wildlife, waterfowl and/or companion animals (cats, dogs, etc.) resulting from direct fecal deposition or runoff. As no wastewater treatment plants are located in the watersheds, any input from humans would be due to leaky septic systems in the upstream reaches of watersheds or intermittent sewer line leaks in the mid- to lower reaches of watersheds. Given the limited number of farm animals or land area receiving manure applications in the Baltimore metropolitan area, agriculture likely accounts for very little of the microbial contamination. This may account for our inability to find *E. coli* O157:H7 in these urban/suburban watersheds. It is well established that beef and dairy cattle are major reservoirs for EHEC, including *E. coli* O157:H7, and that watersheds containing high cattle densities can be contaminated with *E. coli* O157:H7 (Johnson *et al*., 2003). On the other hand, the prevalence of EHEC among wildlife or companion animals is largely unknown. In a related study, Higgins *et al*. (2005) observed that 53% of water samples from the Gwynns Falls watershed (Baltimore County, MD) contained an allele of the translocation intimin receptor (*tir*) gene (also a component of the LEE). Forty *tir* amplicons were subsequently sequenced, the majority of which showed a high degree of homology to *E. coli* O157:H7, suggestive of the presence of *E. coli* O157:H7. However, repeated attempts to isolate *E. coli* O157:H7 strains from enriched water samples were unsuccessful; although it was prohibitively expensive and time consuming to screen all colonies giving the 'typical' colony morphology. Consequently, no conclusions can be drawn regarding either the presence or absence of *E. coli* O157:H7, or other EHEC strains, in the Baltimore metropolitan area watersheds.

Based on these results and previous literature, the relationships between EHEC, EPEC, STEC and *E. coli* O157 are

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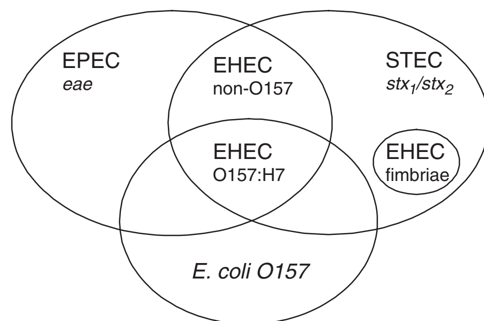

Fig. 3. Venn diagram showing relationships between populations of EPEC, STEC and *Escherichia coli* O157. EHEC O157:H7 and *E. coli* O157:H⁻ occur at the intersection of all three populations while non-O157 EHEC occur at the intersection of EPEC and STEC. Note that strains of EHEC have been reported which do not possess an *eae* gene, but attach to the intestinal epithelium via fimbriae (Paton *et al.*, 1999).

summarized in Fig. 3. Although other EHEC serotypes were not analyzed in this study, previous literature suggests that each is similarly diverse. Note that there is no single criterion that distinguishes EHEC O157:H7 from other closely related strains. These findings have important ramifications for the rapid detection of EHEC; namely, that current immunological or genetic assays targeting specific serogroups or virulence genes cannot reliably identify water-borne presumptive EHEC strains. Ultimately, the prevalence of EHEC can be ascertained through the exhaustive isolation and characterization of water-borne bacteria. However, such protocols are not applicable to routine water monitoring where near real-time information is needed to assess microbial water quality. Future research may reveal the existence of EHEC-specific genes, although the frequency of pathogenic gene transfer among enteric bacteria suggests that this approach is likely to have limited success (Reid *et al.*, 2000). Alternatively, the incorporation of immunological and genetic assays into an integrated detection system may help to reduce the incidence of false positives, thereby providing an acceptable level of reliability.

Acknowledgements

We thank Valerie McPhatter for her invaluable assistance throughout this project, as well as Justine Beaulieu, Fatima Cardoso, Dan Dillon, Tara Krebs, Tiara Brown, Denise Messier and Katie Zoller. Some technicians were supported by NSF/EPA grants.

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