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Occurrence of virulence genes associated with enterohemorrhagic *Escherichia coli* in raw municipal sewage

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Abstract

Municipal sewage influent was screened for the presence of the virulence genes encoding Shiga-like toxins SLT-I and SLT-II (*slt-I* and *slt-II*) and intimin (*eaeA*) and those involved in biosynthesis of O157 (*rfbE*) and H7 (*fliC*) antigens by multiplex PCR to simultaneously identify the enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and its virulence factors in a single reaction. The screening was carried out monthly from October 2004 to September 2005. Direct PCR analysis using total DNA from sewage concentrate showed the presence of at least one virulence gene in 100% samples ($n = 12$). Sixty six percent of these samples were also positive for *rfbE* (O157) gene and *fliC* (H7) gene. The PCR amplification of these genes was possible when the concentration was above 20 cells ml^{-1}. From the multiplex PCR of the isolates following plating on Cefixime-Tellurite Sorbitol MacConkey (CT-SMAC) agar to detect non-sorbitol fermenting (NSF) colonies ($n = 600$), one *E. coli* strain carrying *slt-II* gene and two strains of *E. coli* O157:H7 carrying *slt-I* were detected. The results show that municipal sewage represents a potential reservoir of EHEC. CT-SMAC agar was proved to have limited *E. coli* O157:H7 selectivity and only 0.005% (3/600) sensitivity for sewage samples due to the high frequency (43%) of NSF strains in sewage. The enrichment of sewage sample in modified *E. coli* broth (mEC) increased the sensitivity of PCR resulting in the clearer amplification of five genes. Amplification of target cell type in mEC broth implied that EHEC were present in sewage in a culturable and hence potentially infectious state. However, pre-enrichment did not affect the selectivity of CT-SMAC because frequency of NSF colonies remained the same as that obtained without enrichment. The study, therefore, underscores the need for more sensitive screening techniques that can be routinely employed for the regular monitoring of sewage influent.

Keywords: Enterohemorrhagic *E. coli* (EHEC); *E. coli* O157:H7; Municipal sewage; Sorbitol MacConkey agar; Modified *E. coli* broth

1. Introduction

Enterohemorrhagic *E. coli* (EHEC) has emerged as the leading cause of hemolytic colitis (diarrhea) [17] and hemolytic uremic syndrome (HUS) [19] in humans. EHEC strains are characterized by the ability to form attaching and effacing (A/E) lesions on the surface of epithelial cells in the gastrointestinal tract [6], and the production of shiga-like toxins (SLTs). The first gene to be associated with A/E activity was the intimin gene, *eae*, and its presence is often used as a marker for the infections caused by EHEC [15,31]. Shiga-like toxins are categorized into two main groups, SLT-I and SLT-II [12]. The majority of *slt* genes are bacteriophage-borne, which may be important for the spread of shiga toxin-producing *E. coli* (STEC) [2,22,23]. *E. coli* O157:H7 was the first serotype associated with hemorrhagic colitis [11,17] although more than 100 STEC serotypes have since been isolated from different sources, such as food [28] and recreational [33] and drinking water [4,14,30]. However, not all pathogenic STEC strains have been shown to produce intimin [10]. EHEC pathotypes, therefore, constitute a subset of STEC.

Usually the isolation of pathogens from the environment is difficult due to the low proportion of pathogens relative to higher concentration of general microbes. Selective media for the isolation and identification of *E. coli* O157:H7 from feces were developed to increase the detection sensitivity. Differences in sugar fermentation are used to differentiate *E. coli* O157:H7 from other coliforms. Sorbitol was initially added to MacConkey medium [29] in place of lactose to differentiate *E. coli* O157:H7 strains, most of which do not ferment sorbitol, from other *E. coli* strains which are predominantly sorbitol fermenters. Further sensitivity improvements resulted from the addition of potassium tellurite

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Table 1
Primer sequences used in the multiplex PCR and the expected sizes of the products [27]

Target	Size (bp)	Primer	Sequence
O157	292	RfbF	5′-GTGTCCATTTATACGGACATCCATG-3′
		RfbR	5′-CCTATAACGTCATGCCAATATTGCC-3′
H7	625	$FLIC_{h7}$-F	5′-GCGCTGTCGAGTTCTATCGAGC-3′
		$FLIC_{h7}$-R	5′-CAACGGTGACTTATCGCCATTCC-3′
Intimin	368	Int F	5′-GACTGTCGATGCATCAGGCAAAG-3′
		Int R	5′-TTGGAGTATTAACATTAACCCCAGG-3′
SLT-I	210	SLT-IF	5′-TGTAACTGGAAAGGTGGAGTATAC-3′
		SLT-IR	5′-GCTATTCTGAGTCAACGAAAAATAAC-3′
SLT-II	484	SLT-IIF	5′-GTTTTTCTTCGGTATCCTATTCCG-3′
		SLT-IIR	5′-GATGCATCTCTGGTCATTGTATTAC-3′

and Cefixime [27] to create CT-SMAC. O157 STEC strains are generally less susceptible to tellurite than are many other enteric organisms. To address the problem of low prevalence of pathogens in the environment, selective enrichment is carried out prior to subculture on selective agar (e.g. SMAC). Selective broths such as modified *E. coli* (mEC) broth [1] contain antibiotics (e.g. novobiocin) which suppress the growth of background microbial flora, particularly Gram-positive bacteria and allow the selective growth of bacteria including target bacteria. The advantages of enrichment are (i) amplification of cell numbers allowing for precise detection and (ii) detection of only culturable, hence potentially infectious cells.

EHEC appears to be transmitted primarily through the ingestion of fecally contaminated foods, particularly undercooked beef [26]. However, a large number of outbreaks of EHEC have also been associated with consumption of contaminated drinking water [4,14,30] or contact with recreational water [33]. *E. coli* comes from human and animal wastes. The high number of EHEC isolated from the feces of patients [35] raises the concern that these organisms could pose a significant health risk when sewage leaks into the water or is discharged into surface water, estuaries or coastal water. In a recent study from Spain about the distribution of the *slt-II* in sewage samples of different origin, the data revealed high levels of *slt-II* carrying bacteria in raw human sewage and suggested that human sewage in addition to cattle should be regarded as reservoirs of STEC [3].

Whether the sewage of human origin in Japan, where EHEC O157:H7 is considered to be of great clinical significance as a cause of human disease [24], constitutes the reservoir of EHEC, is a feature of the present study. For this purpose, we examined the presence of the virulence genes *slt-I*, *slt-II*, *eaeA* encoding SLT-I, SLT-II and intimin respectively, associated with EHEC, with particular emphasis on serovar O157:H7, in the raw municipal sewage of Japan. The potential application of SMAC to detect *E. coli* O157:H7 in stool cultures has been evidenced previously [29]. Culture following enrichment in mEC broth has also proved successful for isolation of serologically diverse STEC strains in bovine feces [18]. However, in view of the emerging importance of water-borne transmission of *E. coli* O157:H7, we examined the usefulness of CT-SMAC agar as a primary isolation medium, before and after selective enrichment in modified *E. coli* (mEC) broth, to aid in the detection of *E. coli* O157:H7 in sewage influent.

2. Materials and methods

2.1. Sewage collection

Sewage influent was collected monthly from a municipal wastewater treatment plant (Tokyo, Japan) over a period of one year from October 2004 to September 2005. The plant serves about 200,000 residents. Most of the contamination is of human origin. The plant does not treat any effluent from animal farms and industries. Samples were kept at 4 °C and examined within 24 h after collection.

2.2. Bacterial strains

DNA previously extracted [21] from *E. coli* O157:H7 EDL933, a STEC strain, was used as template for standard control in multiplex PCR. Where cells instead of DNA were required, non-pathogenic *E. coli* O157:H7 ATCC43888 was used as positive control as it does not produce SLTs because of the absence of genes for these toxins. DNA from *E. coli* K12 was used as negative control.

2.3. Detection of slt-I, slt-II, eae and H7 fliC and O157 rfb regions in raw sewage

Raw sewage influent (50 ml) was concentrated stepwise to a final volume of 1 ml. Total DNA was isolated by using DNA extraction kit (ISOIL, Nippon Gene Co., Ltd). The isolated DNA was resuspended in 50 μl of Tris-EDTA (TE) buffer at pH 8.0. Twenty microliter of elute was used as DNA template in the multiplex PCR assay. Multiplex PCR was performed by using five primer sets (Table 1) [34], that detect genes involved in biosynthesis of O157 and H7 antigens and the major known virulence traits of *E. coli* O157:H7, including SLTs (SLT-I and SLT-II) and the intimin. PCR amplification was performed as described previously [34], and amplified DNA fragments were resolved by gel electrophoresis using 2% agarose and stained with ethidium bromide.

2.4. Isolation of EHEC on CT- SMAC agar

SMAC agar (25.8 g, Merck) was suspended in 500 ml of deionized distilled water and autoclaved (15 min at 121 °C).