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Title: 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.

Key words: 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 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.

2.2. 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.

2.3. 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). A lyophilisate of 1 vial of Cefixime-Tellurite (CT) supplement (Merck) was dissolved in 1 ml of sterile distilled water and added to culture medium cooled to

50°C and finally poured into plates. 0.1 ml of 5-fold diluted raw sewage sample was cultured on CT-SMAC agar for 18-24 h at 37°C. Fifty non-sorbitol fermenting (NSF) (colorless) colonies were transferred individually to fresh Luria-Bertani (LB) broth and incubated at 37°C for 18 h. One microliter of culture from individual colony was used as template in a reaction volume of 25 μl for multiplex PCR.

2.5. Selective enrichment in mEC broth

For the preparation of mEC broth, the base medium was composed of Polypepton (Nihon Seiyaku) 2%; lactose (Wako) 0.5%; bile salts (Sigma) 0.112%; K_2HPO_4 (Wako) 0.4%; KH_2PO_4 (Wako) 0.15% and NaCl (Nacalai Tesque) 0.5%. Filter sterilized novobiocin (Wako), 20 mg ml^{-1}, was added to the autoclaved base medium after cooling to room temperature. Pellet from 50 ml sewage influent was inoculated in 15 ml of mEC broth and incubated for 18 h at 37°C. Six milliliter of enrichment broth was used to extract total DNA as described before. Serial 10-fold dilutions of enrichment broth were also cultured on CT-SMAC. Fifty NSF colonies were analyzed with multiplex PCR as described before.

2.6. Confirmation of PCR positive samples

Samples isolated from CT-SMAC and found positive for any one gene by PCR were confirmed as *E.coli* if they produced a dark blue colony by culturing on Chromocult coliform agar (Merck) [20], a widely used agar for the detection of *E.coli* and total coliforms. Isolates positive for O157 serotype by PCR were confirmed by using the O157-specific antibody (Kirkegaard & Perry Laboratories, Inc) against LPS antigen. Cells were lysed in 90% ethanol and 2 μl of sample was spotted on an Immobilin-P transfer membrane (Millipore). Peroxidase-labeled affinity-purified antibody to O157:H7 antigen and the ECL western blotting detection reagent (Amersham Pharmacia Biotech) were used for light emission. Appearance of black dots indicated the reaction of antibody to O157-LPS antigen and confirmed the presence of O157 serotype. Samples were further confirmed by checking the sensitivity against PP01 bacteriophage by spot test. PP01 phage is a T-even lytic phage specific to *E.coli* O157:H7 [21]. The receptor for this phage is *E.coli* O157:H7 specific outer membrane protein C (OmpC). Sixty microliter of bacterial culture in 0.5% agar was pipetted on the surface of 1.5% agar. Two microliter of phage lysate was spotted in the centre of the bacterial zone. The appearance of plaque in the centre after 7-8 h incubation at 37°C indicated cell lysis by phage and confirmed the presence of *E.coli* O157:H7.

3. Results and discussion

In our previous study, we reported the prevalence of *slt-Ⅱ* carrying bacteriophages infective to *E.coli* O157:H7 EDL 933 in the sewage influent [36]. Since phage can be used as an indicator for its host pathogenic bacteria, the presence of O157:H7 phages may suggest the coexistence of their host cell *E.coli* O157:H7 in the sewage. The results from that study [36], therefore, served as the basis for this investigation of the same wastewater treatment plant for the presence of virulence genes associated with EHEC O157:H7.

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

The total DNA from 50 ml of untreated sewage sample was examined for the presence of virulence genes (*slt-Ⅰ*, *slt-Ⅱ*, *eae*) and specific portions of *rfb* and *fliC* regions of *E.coli* O157:H7 serotypes by multiplex PCR. It is not possible to exactly quantify the amount of DNA by PCR amplification except real time PCR. However, to roughly estimate the level of prevalence of virulence genes in sewage, the detection limit of multiplex PCR was determined by spiking the sewage sample with known concentrations of DNA of pathogenic *E.coli* O157:H7. The detection limit of multiplex PCR was found to be 20 cells ml^{-1}. Thus sharp PCR amplification of the genes was observed when their concentration was above 20 cells ml^{-1} in the sewage. Slight amplification was also considered as a positive signal for a certain gene if it was identical to the target size and did not appear in negative control. In sewage

influent from October 2004 to September 2005, virulence genes associated with EHEC were detected every month (Fig.1). The most common virulence gene detected was *slt-Ⅰ* (11/12 samples [91%]) (Fig. 1, dotted box) and the most common virulence gene combination identified was *slt-Ⅰ*, *slt-Ⅱ*、*eae* (4 of 12 samples [33%]) samples. *slt-Ⅰ* with *eae* (3 of 12 [25%]) was also well represented. Of 12 samples carrying different virulence profiles, 8 (66%) samples showed amplification of O157 *rfb* region and H7 antigen region (Fig. 1, arrows) and 4 samples were positive only for H7 (Fig.1 Nov, Dec, Jun, Sep). One sample carrying *slt-Ⅰ* was found to be negative for both O157 and H7 (Fig.1 Jul). Of 8 samples positive for the O157 and H7, 5 samples carried all the virulence factors *eae*, *slt-Ⅰ*, *slt-Ⅱ* and the remaining 3 samples contained either one or two of the virulence genes. The data collected over one year, thus, revealed three types of gene profiles that represented STEC, STEC with intimin and EHEC O157.

3.2. Isolation of EHEC from CT-SMAC agar

For the isolation of EHEC particularly *E.coli* O157:H7, 100 µl inoculum of 5-fold diluted sewage sample was cultured onto CT-SMAC agar (detection limit 50 CFU ml^{-1}). Fifty NSF colonies per monthly sample were examined by multiplex PCR. Of 600 NSF isolates, one strain was detected as *slt-Ⅱ* carrying *E.coli* in March (Fig. 2 A; 1). Two strains carrying *eae*

along with *slt- I*, which were also positive for *rfb*E (O157) and *fliC* (H7) regions (Fig. 2 B; 2, 3), were detected in October 2004. PCR amplification using total DNA also revealed a similar profile of four genes corresponding to *slt- I*, *eae*, *rfb*E (O157) and *fliC* (H7) (Fig. 1, Oct) in October. These two strains were further confirmed as *E.coli* O157:H7 by Western blotting against O157-LPS antibody (Fig. 3 A) and by sensitivity against PP01 bacteriophage (Fig. 3 B). The formation of black dots because of reaction of LPS antigen with O157-specific antibody (Fig. 3 A; 2,3) and formation of plaques due to lysis by phage (Fig. 3 B; 2,3) confirmed that the two strains belonged to O157 serotype. *slt- II* carrying *E.coli* obtained in March was not found to be sensitive against O157-antibody reaction (Fig. 3 A;1) or PP01 phage (Fig. 3 B;1) which indicated that it was a non-O157 STEC strain. However, amplification band of *slt- I* using the pure culture of two strains of *E.coli* O157:H7 was found to be slighter (Fig. 2 B; 2, 3) relative to direct PCR using total DNA (Fig. 1, Oct). Frequent loss of SLTs upon subcultivation has been reported previously [7]. In the present study we subcultivated the individual colony from CT-SMAC to obtain pure culture for multiplex PCR, which may have resulted in a decrease in the concentration of *slt- I* in culture and subsequent low yield of amplification of *slt- I* (Fig. 2 B; 2,3).

The comparison of the results from multiplex PCR using the total DNA (detection limit 20 cells ml^{-1}) and that from colonies (detection limit of CT-SMAC= 50 CFU ml^{-1}) shows that the virulence genes associated with EHEC O157:H7 were present in sewage during most part of

the year (8 / 12 months) (Fig.1) at the density of 20-50 cells ml^{-1} except in October when two O157:H7 strains (Fig. 2 B; 2, 3) were recovered from CT-SMAC at the concentration of 10^2 CFU ml^{-1}. The origin of *slt* genes, whether from bacteria or bacteriophage, was not investigated in this study. However, the previous studies show that a majority of *slt* genes in *E.coli* are bacteriophage-borne [2, 16, 32], which may be important for the spread of STEC strains. Our previous study reported the existence of bacteriophage infective to pathogenic *E.coli* O157:H7 in sewage [36]. Similarly, other studies have reported significant numbers of free *slt*-bearing bacteriophages in the environment [22, 23], which may maintain the gene and infect new bacteria. Some studies have already shown that the transduction of *slt*-bearing bacteriophages to new bacterial hosts occurs in vivo [5] and in vitro [9] and plays an important role in evolutionary changes of EHEC/STEC [25]. Such studies together with the present report indicate that sewage samples should be recognized as a gene pool, from which EHEC or STEC can develop.

Most of the contamination in the sewage is of human origin. Since there was no animal farm in the vicinity of this sewage treatment plant, the origin of microbes, which carry the virulence genes, was probably from the inhabitants. However, during the period under observation, no outbreak resulting from EHEC or STEC was reported. The origin of microbes carrying the virulence genes might be healthy human inhabitants just as the isolation of EHEC/STEC has been reported not only from the feces of diarrheagenic [18] but from healthy

cattle [8] as well.

In the recent study performed with urban sewage in Spain [3], the data showed the high prevalence of non O157 STEC but EHEC O157:H7 was found to be relatively rare (1 out of STEC 55 strains). In our study, EHEC O157:H7 was commonly detected in sewage. These observations reinforce the findings that O157 STEC strains are of greater clinical significance as causes of human disease than non-O157 STEC strains in Japan but not in Spain [18].

Unlike what happens with cattle shedding of *E.coli* O157:H7, there were no significant seasonal differences in detection of EHEC in the monitored wastewater plant. These results are in agreement with those from Spain [3] and contaminated natural river waters in Japan [13], which found *E.coli* O157:H7 at densities between 10^2 to 10^4 cells ml^{-1} independently of the season.

3.3. Suitability of CT-SMAC agar for isolating EHEC from sewage

CT-SMAC has been proved to be an efficient and reliable screening aid for the detection of *E.coli* O157:H7 in stool specimens and cattle feces [29]. The frequency of NSF organisms in feces is 10% and on the average, no more than 10% of the samples need to be screened with specific tests to exclude non-target fecal NSF organisms [29]. However, for sewage sample in this study, the frequency of NSF was found to average of 43% (Table 2) and required

screening of 50 or more presumptive colonies for further tests. In the present study, the number of isolates screened was confined to 50, which sometimes did not represent all of the NSF colonies appearing on CT-SMAC. Omission of even a single colony may lead to false negative results particularly given the low prevalence of EHEC in the environment. Moreover, *slt-* I was detected in 92% of samples as depicted by multiplex PCR results using total DNA (Fig.1, dotted box). However, no *slt-* I-positive strain could be isolated from CT-SMAC, which further highlights the limitations of using CT-SMAC agar to detect non-O157 STEC strains, the majority of which appear to be sorbitol fermenters [3] and therefore remained undetected in this study. The multiplex PCR data using total DNA showed the prevalence of genes associated with EHEC (Fig.1) throughout the year. However, the number of O157 and *slt-* II-positive strains recovered from CT-SMAC agar was very little which can again be attributed to the inefficacy of CT-SMAC in enabling the recognition of *E.coli* O157:H7 in the presence of large numbers of NSF microbes. Out of 600 colonies screened only 3 positive isolates could be recovered, indicating the very low sensitivity (0.005 %.) of CT-SMAC.

3.4. Selective enrichment for improving the detection of EHEC from sewage

The mEC medium contains novobiocin and bile salts for inhibition of the growth of cohabitants and particularly Gram-positive bacteria while, lactose enhances the growth of

lactose-positive bacteria such as *E.coli* [1]. Considering the possibility of EHEC concentration lower than 5×10^{1} CFU ml^{-1} (below the detection limit of CT-SMAC) in sewage, the performance of selective enrichment in mEC broth relative to direct detection methods was evaluated from January to March 2006. After enrichment, the detection sensitivity of PCR increased such that total DNA from 6 ml broth in contrast to 50 ml without enrichment, revealed the clear amplification of five genes (Fig. 4 B). The result showed that enrichment in mEC selectively amplified the EHEC numbers and further suggested that EHEC were present in sewage in culturable and hence potentially infectious state. This contradicts the previous report from Japan in which failure in isolating *E.coli* O157:H7 strain was attributed to its possible existence in viable but nonculturable state[13].

The sub-culturing of enriched sample on CT-SMAC revealed the frequency of NSF colonies as 39% (Table 2) almost similar as obtained by direct culturing on CT-SMAC (43%). The concentration of NSF and SF increased simultaneously from 3 log units to 6 log units rendering the constant NSF/SF ratio after enrichment. This constant frequency indicated that most of the population appearing on CT-SMAC prior to enrichment was not sensitive to novobiocin and bile salts. Furthermore, the increase in number of NSF colonies made it further difficult to select the suspected colonies. Fifty NSF colonies were screened but no positive isolate was recovered unlike in bovine feces where enrichment in mEC broth

followed by CT-SMAC agar allowed the isolation of 32 STEC/EHEC strains [18].

4. Conclusion

Virulence genes associated with EHEC were detected throughout the year in the influent of a domestic wastewater treatment plant in Japan from October 2004 to September 2005. Sixty six percent of these samples also possessed the regions for O157 and H7 serotypes. One *slt-II* carrying *E.coli* (STEC) and two *slt-I* carrying *E.coli* O157:H7 were isolated from the influent, which confirmed the existence of virulence genes in sewage. CT-SMAC agar was found lacking in sensitivity and suitability for detection of EHEC in the sewage because of the high frequency (43%) of NSF strains and inability to identify sorbitol-fermenting STEC. Selective enrichment using mEC broth increased the sensitivity of PCR but did not enhance the selectivity of CT-SMAC. Use of CT-SMAC agar before and after enrichment cannot be the method of choice for the screening of EHEC/STEC in sewage. The present report suggests that human sewage in addition to cattle and other wild animals should be recognized as a gene pool, which can develop EHEC or STEC. Moreover, this study underscores the need for more appropriate screening techniques that can be routinely employed for the regular monitoring of sewage influent.

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Legends to figures

Figure 1: Multiplex PCR detection of virulence genes associated with enterohemorrhagic *E.coli* (EHEC) O157.

PCR reactions were performed by extracting total DNA from 50 ml of sewage influent on a

monthly basis over a period of 1 year from October 2004 to September 2005. 'M' is a 100 bp DNA ladder; 'P' is the positive control (purified DNA from EDL933); 'N' is the negative control (purified DNA from *E.coli* K12). The products correspond to (from top to bottom) H7, SLT-Ⅱ, Intimin, O157 and SLT-Ⅰ. The arrows indicate the presence of genes for O157:H7 with intimin and one or both SLTs. Among virulence genes, *slt-Ⅰ* (dotted box) was the most common (11/12 samples; 92%).

Figure 2: Isolation of EHEC from SMAC agar

100 μl of 5-fold diluted sewage sample was cultured on CT-SMAC agar. Fifty NSF colonies per monthly sample were examined by multiplex PCR. (A) shows the detection of *slt-Ⅱ* carrying *E.coli* in March 2005; (B) shows the detection of *slt-Ⅰ* carrying *E.coli* O157:H7 (2 and 3) in October 2004. 'M' is a DNA molecular weight marker; 'P' positive control (purified DNA from EDL933); 'N' negative control (purified DNA from *E.coli* K12).

Figure 3: Confirmation of serotype O157:H7

Isolates positive for O157 serotype by PCR were confirmed by using the O157-specific antibody against LPS antigen (A). Appearance of black dots indicated the reaction of antibody to O157-LPS antigen and confirmed the presence of O157 serotype. (B) Sensitivity against PP01 bacteriophage by spot test. The appearance of plaque in the centre after 7-8 h incubation

at 37°C indicated cell lysis by phage and confirmed the presence of *E.coli* O157:H7. P: positive control (*E.coli* O157:H7 ATCC43888), 2 and 3 are the *slt- I* carrying *E.coli* O157:H7 strains detected in October 2004; 1 is the *slt- II* carrying *E.coli* detected in March.

Figure 4: Comparison of direct versus enrichment culture procedures.

Municipal sewage was screened for an additional three months from January 2006 to March 2006 to compare the efficiency of both direct and enrichment culture procedure on the detection of EHEC. Multiplex PCR of the DNA extracted from sewage concentrate before (A) and after enrichment (B). 'M' is a DNA molecular weight marker; 'P' positive control (purified DNA from EDL933).