Introduction

Escherichia coli is a common gut bacteria that can cause a variety of infections, including diarrhea, in humans. Some strains of E. coli, known as pathogenic E. coli (PEC), can cause severe illness due to the expression of virulence factors. These factors enable the bacteria to adhere to and damage the intestinal lining, leading to symptoms such as diarrhea. The study aimed to characterize the genetic and serological diversity of PEC isolates from diarrheal patients in Korea between 2003 and 2006.}

Materials and Methods

The study used 111 E. coli strains isolated from diarrheal patients in Korea. The strains were tested through polymerase chain reaction (PCR) and slide agglutination method for the detection of virulence genes and serotypes, respectively. To compare the expression of Shiga toxin (stx)-1 and stx2 genes, real-time quantitative reverse-transcriptase PCR and rapid expression assay, reversed-passive latex agglutination, were performed.

Results

Forty-nine Shiga toxin-producing E. coli (STEC) strains and 62 non-STEC strains, including 20 enteropathogenic E. coli, 20 enterotoxigenic E. coli, 20 enteroaggregative E. coli, and 2 enteroinvasive E. coli were randomly chosen from the strains isolated from diarrheal patients in Korea between 2003 and 2006. PCR analysis indicated that locus of enterocyte effacement pathogenicity island, that is, eaeA, espADB, and tir genes were present in STEC, enteropathogenic E. coli, and enteroinvasive E. coli. Quorum sensing-related gene luxS was detected in most of pathogenic E. coli strains. Major serotypes of the STEC strains were O157 (26%) and O26 (20%), whereas the non-STE strains possessed various serotypes. Especially, all the strains with serotype O157 carried stx2 and the tested virulence factors. Of the STEC strains, the data of real-time quantitative reverse-transcriptase PCR and reversed-passive latex agglutination tests showed that messenger RNA- and protein expression of stx2 gene were higher than those of stx1 gene.
1. Introduction

Diarrhoea is an extraordinarily common disease with worldwide distribution, and diarrhoeagenic *Escherichia coli* is an important bacterium to cause diarrhoeal disease.\(^1,2\) In a surveillance of bacterial pathogens associated with acute diarrhoeal disease in the Republic of Korea, it has been found that pathogenic *E. coli* are frequently isolated from diarrhoeal patients (around 20%), and enterohaemorrhagic *E. coli* (EHEC) accounts for ca. 2% among the isolated pathogenic *E. coli*.\(^3\) The pathogenic strategies of the diarrhoeagenic *E. coli* strains exhibit remarkable variety. Three general paradigms have been described by which *E. coli* may cause diarrhoea;\(^2\) each is described in detail in the appropriate section below: enterotoxin production [enterotoxigenic *E. coli* (ETEC) and enteraggregative *E. coli*, (EAEC)], invasion [enteroinvasive *E. coli* (EIEC)], and intimate adherence with membrane signalling [enteropathogenic *E. coli* (EPEC) and EHEC).\(^4\)–\(^7\)

The major virulence factor, which is a defining characteristic of EHEC, is Shiga toxin (Stx). Shiga toxin-producing *E. coli* (STEC) strains produce one or both of two major types of shiga toxin, designated Stx1 and Stx2, and the production of the latter is associated with an increased risk of developing haemolytic-uremic syndrome.\(^1,8\) The prototypical Stx1 and Stx2 toxins have 55% and 57% sequence homology in the A and B subunits, respectively.\(^2,6\) ETEC strains are identified by 55% and 57% sequence homology in the A and B subunits, respectively.\(^2,6\) ETEC strains are identified by

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**Conclusion**

Our results provide the epidemiological information regarding the trend of STEC and non-STEC infections in the general population and show the fundamental data in association of serotypes with virulence genes in diarrhoeagenic *E. coli* strains from Korea.
the following conditions: initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 72°C for 1 minute, and final cycle 72°C for 5 minutes. Amplified PCR products were analysed by gel electrophoresis in 2% agarose gels stained with ethidium bromide, visualized with ultraviolet illumination, and imaged with the Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA, USA).

2.3. Total RNA isolation

RNA extracts of the strains were prepared using the Qiagen RNeasy midi-prep kit and RNA BacteriaProtect (QIAGEN Co. Ltd, Germany) according to the manufacturer’s instructions for gram-negative bacteria.

2.3.1. Amplification of target genes by real-time reverse-transcriptase-PCR and analysis

For quantitative real-time reverse-transcriptase-PCR (qPCR), the 20 μL reaction mixture was prepared by 2 μL of total RNA, 0.6 μM of each primer, and reference dye Synergy Brands Inc. Green. The number of copies was calculated, and dilutions ranging from 100 pg to 100 ng copies of this standard were prepared in a Tris-

| Target gene | Primer sequence (5' to 3') | Size of the PCR product (bp) |
|-------------|-----------------------------|-----------------------------|
| Shiga toxin 1 (stx1) | CGTACGGGGATGCAGATAATCGC | 210 |
| Shiga toxin 2 (stx2) | GTTCGCGTTTGTGCAGTGCAC | 326 |
| Heat-stable enterotoxin (esta1) | ATGCCCTACACAGATATAGCC | 119 |
| Attaching and effacing (eaeA) | ATGCCTCGGATGTACACTGG | 233 |
| Heat-labile toxin (lt) | GATCAGCGAGGAAACCAAAACC | 366 |
| Heat-stable toxin (st) | CTTTCCCCCTTGTAGTACT | 302 |
| Invasion-associated locus (ial) | GTTGGCGCTTGGTTGGGATATC | 356 |
| Hemolysin (hlyA) | GCATCATCAAGGTCAGTCC | 519 |
| Type III secretion protein (espA) | GTTTTTCAGGCTGATTTC | 187 |
| Type III secretion protein (espD) | AAAAGACGTGCCAAGACA | 145 |
| Translocated intimin receptor (iir) | GCCTGCACTCCCATGATCT | 107 |
| Autoinducer-2 synthetase (luxS) | GTGCAGCGAGGCTGATTACA | 792 |

PCR = polymerase chain reaction; bp = base pair.

Table 1 Primers used in this study
Quantitative (expression) ratio = \frac{2^{\Delta C/tar} - test}{2^{\Delta C/ end}}.

performed in triplicate. The threshold cycle (Ct) values of the known standards were plotted versus the logarithm of the concentration of each standard creating a standard curve. Samples of unknown concentration were plotted onto the standard curve to calculate their concentration. Normalization of the quantification results from stx1 and stx2 was performed by the incorporation of the quantification results of gapA mRNA into the following equation:

Here, \Delta Ct endogenous gene (control – test) = Ct value of the endogenous gene (gapA) with the control RNA – Ct value of the endogenous gene (gapA) from the sample RNA. Also, \Delta Ct target gene (control – test) = Ct value of the target gene (stx1 or stx2) with the control RNA – Ct value of the target gene (stx1 or stx2) with the test RNA.

2.4. Reversed-passive latex agglutination test for the detection of Shiga toxin

The production of Stx1 and Stx2 by the isolates was determined by using a reversed-passive latex agglutination (RPLA) kit (VTEC-RPLA; Denka Seiken Co., Ltd., Tokyo, Japan) after having been grown and shaken in 5 mL of Tryptone Soya Broth overnight at 37°C. Of this suspension, 1 mL was centrifuged for 20 minutes at 13,000 rpm. The titre of the supernatant was determined by the titration method with the method of Guine´ e et al.18 using all available O antisera (O1–O181). All antisera were absorbed with the corresponding cross-reacting antigens to remove the non-specific agglutinins.

2.5. Serotyping of O antigen

The presence of O antigens was determined by slide agglutination with the method of Guine´ e et al.18 using the available O antisera. All antisera were absorbed with the corresponding cross-reacting antigens to remove the non-specific agglutinins.

3. Results

3.1. Diversity of virulence genes in pathogenic E coli isolates from diarrhoeal patients

To characterize genetic diversity of virulence factors, 111 pathogenic E coli strains, 49 STEC strains, and 62 non-STEC strains, were chosen from the strains isolated from diarrhoeal patients in Korea between 2003 and 2006. All the strains were tested through PCR with the primers as shown in Table 1. As shown in Table 2, in the genomic DNA of the 49 STEC strains, 22 strains were stx1 positive and 17 were stx2 positive. Ten possessed both stx1 and stx2 genes.

To detect major virulence genes for pathogenic E coli, eaeA gene, major virulence factor of EPEC, lt and st genes for ETEC, eae1 gene for EAEC, and invasion-associated locus gene for EIEC were analysed in the STEC and non-STEC strains by PCR with the specific primers for these genes. Among the non-STEC strains, 20 EPEC, 20 ETEC, 20 EAEC, and 2 EIEC strains were randomly chosen among the pathogenic E coli strains isolated from diarrhoeal patients in Korea. The eaeA gene was present in EPEC, STEC, and EIEC strains.

Table 2 Distribution of virulence genes in pathogenic Escherichia coli from diarrhoeal patients

| Pathogen (n) Major genes (n) | eaeA | lt | st | east1 | ial | hlyA | espA | espD | espB | tir | luxS |
|-----------------------------|------|----|----|-------|----|------|------|------|------|-----|------|
| STEC (49)                   |      |    |    |       |    |      |      |      |      |     |      |
| stx1 only (22)              | 12 (55) | 0 (0) | 0 (0) | 3 (14) | 0 (0) | 14 (64) | 14 (64) | 13 (59) | 14 (64) | 13 (59) | 22 (100) |
| stx2 only (17)              | 13 (77) | 1 (6) | 0 (0) | 2 (12) | 0 (0) | 13 (77) | 11 (65) | 13 (77) | 11 (65) | 12 (71) | 16 (94) |
| stx1 and 2 (10)             | 7 (70) | 1 (10) | 0 (0) | 3 (30) | 0 (0) | 8 (80) | 6 (60) | 7 (70) | 5 (50) | 5 (50) | 10 (100) |
| EPEC (20)                   |      |    |    |       |    |      |      |      |      |     |      |
| eaeA (20)                   | 20 (100) | 2 (10) | 0 (0) | 5 (25) | 0 (0) | 2 (10) | 11 (55) | 20 (100) | 20 (100) | 11 (55) | 11 (55) |
| ETEC (20)                   |      |    |    |       |    |      |      |      |      |     |      |
| lt (6)                      | 0 (0) | 6 (100) | 0 (0) | 3 (50) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 6 (100) |
| st (1)                      | 0 (0) | 0 (0) | 1 (100) | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (100) |
| lt and st (13)              | 0 (0) | 13 (100) | 13 (100) | 13 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 13 (100) |
| EAEC (20)                   |      |    |    |       |    |      |      |      |      |     |      |
| east1 (20)                  | 0 (0) | 0 (0) | 0 (0) | 20 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 14 (70) |
| EIEC (2)                    |      |    |    |       |    |      |      |      |      |     |      |
| ial (2)                     | 1 (50) | 2 (100) | 0 (0) | 0 (0) | 2 (100) | 0 (0) | 1 (50) | 1 (50) | 1 (50) | 1 (50) | 1 (50) |

STEC = Shiga toxin-producing E coli; EPEC = enteropathogenic E coli; EAEC = enteroaggregative E coli; ETEC = enterotoxigenic E coli; EIEC = enteroinvasive E coli.
The *lt* and *st* genes showed different prevalence in the pathogenic *E. coli* strains. The *lt* gene was found in various pathogenic *E. coli* groups: ETEC, STEC, EPEC, and EIEC; whereas the *st* gene was detected only in ETEC strains. Interestingly, the *eas*1 gene was also distributed in various pathogenic *E. coli* groups except EIEC strains. Eighty-five percent (17 of 20 strains) of the STEC strains harboured the *eae* gene. This gene was found in the one of the two EIEC strains. Most of the pathogenic *E. coli* strains carried *luxS* gene. This gene was present in all ETEC strains and 97% of the STEC strains. The presence of this gene was similar in EPEC (55%), EAEC (70%), and EIEC (50%) strains (Table 2).

### 3.2. Serotyping of pathogenic *E. coli* isolates from diarrhoeal patients

As shown in Table 3, the O antigen was typeable for 95 strains, which could be classified into 38 different serotypes, and 15 strains were non-typeable. Among the STEC strains, 12 serotypes were identified. Major serotypes of the STEC strains were O157 (26%) and O26 (20%). Under the non-STEC strains, the EPEC, ETEC, and EAEC strains showed diversity of serotypes. However, the serotype of two EIEC strains was non-typeable.

### 3.3. Association of serotypes of pathogenic *E. coli* isolates with virulence genes

The serotypes of STEC strains were examined in association with virulence genes. The data of serotypes in association with *stx* genes were described in Table 4. The *stx*1 gene was mostly distributed between the O26 (80%) and O103 (100%) serotypes. The *stx*2 gene was present in the great majority of the O157 (100%) and O121 (100%) serotypes. As shown in Figure 1, all O157 strains harboured all the tested genes, that is, *eaeA*, *hlyA*, *espADB*, and *tir* genes. The serotypes that possessed all the genes described above were O26 (6 of 10 strains), O103 (three of six strains), and O121 (two of four strains), except for serotype O157 (Group A). Among the four O26 strains, *eaeA*-negative strain (Group B), *hlyA*-negative strain (Group B), *espD*-negative strain (Group C), and *eaeA*, *hlyA*, *espADB*, *tir*-negative strain (Group H) were found.
3.4. Expression of stx genes in STEC O157 strains

To compare mRNA and protein expression of stx1 and stx2 genes, qPCR and rapid expression assay, RPLA were performed in the STEC O157 strains (n = 13). Seven strains possessed stx2 gene only and six strains carried both stx1 and stx2 genes. For each isolate, analysis of gene expression of gapA, stx1, and stx2 was performed in the same PCR run. Application of qPCR assays indicated that the mRNA expressions of stx2 gene were higher than those of stx1 gene. Using dilution gradients of culture supernatant fluids by RPLA, expression titres of stx2 genes were given. The expression titres of Stx2 was also higher than those of Stx1 (Table 5). However, in some strains, low expressions of stx2 gene were found; for example, mRNA expression of Strain 2 and protein expressions of Strains 3 and 4.

4. Discussion

The present study employed a range of several E. coli organisms in the aetiology of diarrhoeal patients in Korea providing to the genetic characterization with regard to their harbouring of potential virulence genes.

To obtain the genetic and serological diversity of pathogenic E. coli, the association between the virulence factors and serotypes of isolates found in human was examined in this study. We showed that the genotypes of O157 serotype and non-O157 serotypes in STEC strains were different. The results indicated that all O157 serotypes of the STEC strains carried all the tested virulence genes, whereas these genes were detected in a lesser percent of the non-O157 STEC strains. Moreover, the stx2 gene was present in the great majority of the O157. These crude data suggest an association of stx2 with isolates of serotypes found in humans with severity of disease.

O’Brien et al. 19 reported that 67 EHEC O157 strains tested possessed the EHEC hlyA gene. Boerlin et al. 20 reported that eaeA and stx2 were significantly more frequent in isolates from serotypes found in humans with severe disease. It has been shown that the chromosomal virulence genes of EHEC and EPEC are organized as a cluster referred to as a pathogenicity island. 5 Our data showed that the genotype in STEC and EPEC was similar (Table 2). Genes for both classes are found predominantly on plasmids, and some ST-encoding genes have been found on transposon. 2 STa has about 50% identity to east 1 of EAEC. It has recently been reported that some strains of ETEC may also express east1 in addition to STa. 2,12 STb...
has been found only in ETEC. The main conclusion of these previous investigations is that no single factor is responsible for the virulence of E. coli strains.

The second part of our study showed differences of stx genes in O157 strains that are known as a pathogen in association with severe disease. Previous studies have shown that the virulence of STEC for humans may be related to the type of stx, which is produced by the bacteria and serotype. In a study concerning Stx association with severe disease, previous studies have indicated that the expression of stx genes was different. Although low expressions of stx2 gene were found in some strains (Table 5), we suggest that most O157 strains show more expression of stx2 mRNA and protein than stx1. The low expression of stx2 gene implies that the stx expression may be influenced by environmental conditions of each strain.

In conclusion, the present study demonstrates the diversity of virulence genes and serotypes in pathogenic E. coli isolated from diarrhoea patients and the importance of stx2 gene in the infection of STEC O157. Thus, it can provide the epidemiological information regarding the trend of STEC and non-STEC infections in the general population and show the fundamental data in association of serotypes with virulence genes in diarrhoeagenic E. coli strains from Korea.

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