Resistance to Fluoroquinolone by a Combination of Efflux and Target Site Mutations in Enteroaggregative Escherichia coli Isolated in Korea

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Abstract

Objectives: Enteroaggregative Escherichia coli (EAEC) was recently reported as a major diarrheagenic pathogen in infant and adult travelers, both in developing and developed countries. EAEC strains are known to be highly resistant to antibiotics including quinolones. Therefore in this study we have determined the various mechanisms of quinolone resistance in EAEC strains isolated in Korea.

Methods: For 26 EAEC strains highly resistant to fluoroquinolone, minimal inhibitory concentrations for fluoroquinolones were determined, mutations in the quinolone target genes were identified by PCR and sequencing, the presence of transferable quinolone resistance mechanism were identified by PCR, and the contribution of the efflux pump was determined by synergy tests using a proton pump inhibitor. The expression levels of efflux pump-related genes were identified by relative quantification using real-time PCR.

Results: Apart from two, all tested isolates had common mutations on GyrA (Ser83Leu and Ser87Gly) and ParC (Ser80Gln). Isolates EACR24 and EACR39 had mutations that have not been reported previously: Ala81Pro in ParC and Arg157Gly in GyrA, respectively. Increased susceptibility of all the tested isolates to ciprofloxacin and norfloxacin in the presence of the pump inhibitor implies that efflux pumps contributed to the resistance against fluoroquinolones. The expression levels of efflux pump-related genes were identified by relative quantification using real-time PCR.

Conclusion: These results indicate that quinolone resistance of EAEC strains mainly results from the combination of mutations in the target enzyme and an increased expression of efflux pump-related genes. The mutations Ala81Pro in ParC and Arg157Gly in GyrA have not been reported previously; the exact influence of these mutations should be investigated further.
1. Introduction

Enteroaggregative *Escherichia coli* (EAEC) is one of the diarrheagenic *E. coli* strains, and its importance in public health is increasing around the world [1]. EAEC has been implicated as major etiologic pathogens of infant gastroenteritis in developing countries, and associated with persistent diarrhea longer than 14 days, which is related to high mortality in these regions [2,3]. It was recently also reported to be one of the major diarrheagenic pathogens in young children and adults traveling in developed countries [4].

EAEC is defined by "stacked-brick" type aggregative adherence to HEp-2 cells. The pathogenesis of EAEC infection is not fully understood but it is presumed to result from the characteristic that it adheres to the intestinal mucosa using adherence fimbriae, and causes damage to the mucosal epithelium [2,5,6]. The putative virulence factors are mostly in the 60–65 MDa aggregative adherence plasmids [1,7].

The ratio of antibiotic resistant strains in EAEC is higher than in other diarrheagenic *E. coli* strains. In Peru, the antibiotic resistance ratio of diffuse adhering *E. coli* and EAEC was higher than that of other *E. coli* strains, and, in Iran, the EAEC isolates showed 100% resistance to ampicillin, and 64.3%, 57.1%, and 42.9% to tetracyclin, nalidixic acid and ciprofloxacin (CIP), respectively [8,9].

Generally, EAEC strains are highly resistant to ampicillin, nalidixic acid, cotrimoxazol, and others. It is presumed that persistent diarrhea increases the chance of EAEC being exposed to the various antibiotics causing the highly antibiotic resistant nature of EAEC [9].

Quinolone resistance in enteric bacteria is mainly a result of mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA, gyrB, parC*, and *parE* genes have been previously described [15]. The PCR was performed in a final volume of 25 μL containing 2 μL of template DNA, 1× reaction buffer, 0.2 mM of each deoxynucleoside triphosphate, 20 pM of each primer, and 3.5 units of Taq polymerase. The assays were performed in a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) under the following conditions: one cycle of 94 °C for 5 minutes followed by 30 cycles of 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute and a final extension of 72 °C for 5 minutes.

2. Materials and Methods

2.1. Bacterial isolates

We received 227 clinical EAEC isolates from the national public health network of Korea in 2008–2009. The antibiotic susceptibility of these isolates was performed by the disk diffusion test, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. We selected 26 EAEC isolates showing high resistance to CIP (inhibitory zone was <6 mm).

2.2. Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of CIP and norfloxacin (NOR) were determined by E-test (AB Biodisk, Solna, Sweden) according to the manufacturer’s instruction. *E. coli* ATCC 25922 was used as a quality assurance strain [16,17]. CLSI criteria were used to categorize strains as susceptible, intermediate, or resistant [16].

2.3. DNA sequence analysis of the quinolone resistance-determining regions

The quinolone resistance-determining regions (QRDRs) of *gyrA, gyrB, parC*, and *parE* genes were amplified by PCR [15] and sequenced using an ABI 3700 sequencer (Applied Biosystem, Foster, CA, USA). These portions of the QRDRs corresponded to amino acid residues 54–171 of *GyrA*, 397–520 of *GyrB*, 12–130 of *ParC*, and 421–524 of ParE.

2.4. Amplification of qnrA, qnrB, qnrS, and aac(6′)-Ib-cr genes

Protocols for the amplification of *qnrA, qnrB, qnrS*, and *aac(6′)-Ib-cr* genes have been previously described [15].

2.5. Synergy tests

Synergy experiments were performed using the fluoroquinolones CIP and NOR, and the efflux pump inhibitor, reserpine. Reserpine was added in Muller-Hinton agar at the concentration of 100s added in Muller-Hin testing to CIP and NOR by E-test was performed in agar plates with or without reserpine [17,18].

2.6. Expression of mdfA, tolC, and ydhE

The expression of each gene was investigated using real-time PCR as described previously [15]. The cycling conditions were 40 cycles of 95 °C for 15 seconds followed by 60 °C for 1 minute. Quantification was based on the threshold cycle value, which was determined with the aid of the SDS 2.1 software system (Applied Biosystems). Relative gene expression level was calculated.
using threshold cycle (CT) as \(2^{-\Delta\Delta CT}\), where \(\Delta\Delta CT = \Delta CT\) (sample) – \(\Delta CT\) (control), and \(\Delta CT\) is the CT of the target gene subtracted from the CT of the housekeeping gene [19]. All tests were repeated five times and the relative gene expression value is indicated average of five tests. Relative gene expression level of efflux pump related genes were compared with those of the quinolone susceptible strain *E. coli* ATCC 25922.

3. Results

3.1. Susceptibility of *Shigella* isolates to fluoroquinolones

All of the 26 EAEC isolates were found to be highly resistant to the fluoroquinolones tested: CIP (MIC, 32–256 μg/mL), and NOR (MIC ranges of 32–256 μg/mL).

3.2. Mutations in the genes gyrA and parC

From the sequence analysis of fluoroquinolone resistance-related genes, there were no alterations of amino acid sequence in GyrB and ParE, while several mutations were detected in the GyrA and ParC (Table 1). Seventeen isolates among the tested fluoroquinolone resistance isolates had multiple mutations in GyrA (Ser83Leu and Ser87Gly) and ParC (Ser80Gln and Glu84Val or Gly). Seven isolates were observed to have double mutations in GyrA (Ser83Leu and Ser87Gly) and one in ParC (Ser80Gln). The combination of GyrA gene (Ser83 and/or Asp87) and ParC gene (Ser80 and/or Glu84) were frequently found in fluoroquinolone highly resistant isolates. However, fluoroquinolone resistant isolates EACR 24 and 39 were found to have novel mutations in ParC and GyrA, respectively. EACR 24 contained two substitutions, one in GyrA (Ser83Leu) and one in ParC (Ala81Pro; GenBank No. EU512995). EACR 39 exhibited four substitutions, three in GyrA (Ser83Leu, Asp87Gly, Arg157Gly; GenBank No. HM753589) and ParC (Ser80Gln). The mutations Arg157Gly in GyrA and Ala81Pro in ParC have not been reported previously. There was no notable difference in MIC for CIP and NOR between the triple and quadruple mutants.

3.3. Presence of *qnrA, qnrB, qnrS,* and *aac(6’)-Ib-cr* genes

Among the 26 fluoroquinolone resistant isolates, *qnrA* was only detected in EACR 33. Three isolates (EACR 25, 29, and 32) were PCR positive for *aac(6’)-Ib-cr*. However, *qnrB* and *qnrS* were not identified in any of the tested fluoroquinolone resistant isolates.

3.4. Involvement of putative efflux pumps for fluoroquinolone resistance

To elucidate the involvement of the efflux pump in fluoroquinolone resistance in EAEC isolates, synergy experiments were performed using the efflux pump inhibitor, reserpine and the fluoroquinolones CIP and NOR. The MICs for 26 isolates of fluoroquinolones were decreased 32- to 256-fold in agar plate containing reserpine in comparison with those observed in reserpine-free medium (Table 1).

3.5. Expression of genes, *mdfA, tolC, and ydhE,* encoding efflux pump

EACR07, EACR29, and EACR33 were selected to investigate the extent of intervention of efflux pump in fluoroquinolone resistance, because EACR07 and EACR29 showed different MIC for fluoroquinolones but had same mutation profile on quinolone target enzyme, and EACR29 and EACR31 showed a similar MIC but have different mutation profiles. Real-time PCR showed that isolates EACR07, EACR29, and EACR33 exhibited approximately 9 times higher expression of *tolC, mdfA,* and *ydhE,* respectively, than the *E. coli* ATCC 25922 control strain (Figure 1). When CIP was added to medium, the expression of the three genes were greatly increased in all three isolates. The EACR07 isolate expressed 57.33 times more *tolC,* 2.9 times more *mdfA,* and 5.8 times more *ydhE* than the control strain. EACR29 exhibited 143.2 times more *tolC,* 9.7 times more *mdfA,* and 13.6 times more *ydhE* than the control strain. EACR33 exhibited 180.8 times more *tolC,* 6.3 times more *mdfA,* and 12.9 times more *ydhE* than the control strain.

4. Discussion

EAEC is a main cause of persistent diarrhea in children in developing regions and a prevalent cause of traveler’s diarrhea. Recently it was reported that EAEC also can cause acute diarrhea in children in both developing and industrialized countries [4]. Because of this, even though the EAEC was most recently categorized as diarrheagenic *E. coli,* it became an emerging enteric pathogen.

EAEC is also known to have relatively high resistance to β-lactams and quinolones compared with other *E. coli* because it has repeated exposure to antimicrobial agent or may interact with other resistant flora that cause chronic diarrhea [8].

In this study, 26 EAEC clinical isolates with high resistance to fluoroquinolones were examined to identify their quinolone resistance mechanism. Resistance to quinolones is usually due to mutations of the bacterial targets, DNA gyrase and topoisomerase IV [10]. In this study, all tested isolates had multiple mutations on GyrA and ParC at the QRDR of each.

Apart from EACR24 and EACR 39, all tested isolates had the common mutations on GyrA (Ser83Leu and Ser87Gly) and ParC (Ser80Gln). Seventeen isolates had double mutations on both GyrA and ParC, seven isolates...
Table 1. Characteristics of EAEC isolates with resistance to fluoroquinolones

| Strain no. | CIP + Reserpine | Nor + Reserpine | gyrA amino acid at codon 83 GAC (Asp) | gyrA amino acid at codon 87 GAC (Asp) | gyrA amino acid at codon 157 CGT (Arg) | parC amino acid at codon 80 AGC (Ser) | parC amino acid at codon 81 GCC (Ala) | parC amino acid at codon 84 GAA (Glu) | qnr, aac(6')Ib-cr, qepA type |
|------------|----------------|----------------|--------------------------------------|--------------------------------------|--------------------------------------|----------------------------------|---------------------------------|----------------------------------|------------------------------------|
| EACR01     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR02     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR03     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR04     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR05     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR06     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR07     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR09     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR10     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR11     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR12     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR15     | 32 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR17     | 32 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR18     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR21     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR22     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR23     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR24     | 32 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR25     | 128 <2         | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR29     | 256 <2         | 64 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR31     | 256 <2         | 64 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR32     | 128 <2         | 64 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR33     | <256 <2        | 256 <2         | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR38     | 128 <2         | 64 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR39     | 128 <2         | 64 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |
| EACR41     | 64 <2          | 32 <2          | -T-(Leu)                             | -G-(Gly)                             | -G-(Gly)                             | -G-(Gly)                        | -G-(Gly)                        | -G-(Gly)                        |                                   |

Each value is the average of five replicates.

CIP = ciprofloxacin; NOR = norfloxacin.

had double mutations on GyrA and a single mutation on ParC. In EACR 39, we found a substitution of arginine to glycine at Codon 157 in GyrA, which has not been reported previously. This mutation, however, is presumed to contribute insignificantly to the resistance, because the MIC for fluoroquinolone is similar to that of other resistant strains that have the same mutations apart from Arg157Gly in GyrA. In EACR24, we also found a mutation that has not been reported before: Ala81Pro in ParC. The MIC value for CIP of EARC24 was 32 μg/mL, and it had two mutations in the target genes GyrA and ParC; therefore, this newly reported mutation seems to have a great impact on fluoroquinolone resistance. The exact influence of these two newly reported mutations should be investigated further.

In general, reduced susceptibility due to a change of Ser83 in GyrA leads to an increase in resistance with the addition of one or two mutations in GyrA or ParC [20]. However, there was no comparable difference in MICs for CIP and NOR. For example, EACR07 and EACR29 have the same mutation profile, two in GyrA and one in ParC, but the MIC of CIP was 64 μg/mL for EACR04.

Figure 1. Expression of tolC, mdfA, and ydhE measured by RT-PCR in the absence and presence of ciprofloxacin. Each value is the average of five culture replicates, each of which was evaluated twice. Relative gene expression levels were calculated as $2^{-\Delta \Delta CT}$, where $\Delta \Delta CT = \Delta CT$ (sample) $- \Delta CT$ (control) [3]. CT = threshold cycle.
and 256 μg/mL for EACR33. Therefore, we can deduce that other resistance mechanisms such as efflux are concerned with these isolates.

Efflux pumps utilize the energy of the proton motive force to transport out the antibiotics out of cells [21]. In all the 26 isolates, the contribution of efflux pump to fluoroquinolone resistance was determined, because increased susceptibility of all the tested isolates to CIP and NOR in the presence of a proton pump inhibitor implied the presence of energy-dependent active efflux pumps for fluoroquinolones. The loss of multidrug efflux could neutralize the resistance contributed by target site alteration as has been shown in E. coli, Shigella, and Salmonella typhimurium [12,14,15].

Expression of tolC for resistance-nodulation-cell division superfamily transporter, mdfA for major facilitator superfamily transporter, and ydhE for multidrug extrusion protein family transporter was tested in EACR07, EACR29, and EACR33. There was a clear relationship between increased expression of genes for efflux pumps and resistance to fluoroquinolones in all three isolates. Expression of tolC, mdfA, and ydhE was greatly induced in the presence of ciprofloxacin, but the extent was different from the tested isolates. EACR33 produced the largest amount of tolC in the presence of ciprofloxacin, and it has highest MIC value for fluoroquinolones among the tested isolates. However, the expression level of mdfA and ydhE was less than that of EACR24. This can be explained by previous reports that the fluoroquinolone efflux pump of E. coli is mainly encoded by acrAB and tolC genes [22,23]. EACR29 produced 2.3–3.3 times more transporters than EACR07 in the presence of ciprofloxacin. This may explain the difference in MICs between the isolates that have the same mutation profile on the target enzyme. In conclusion, these results indicate that resistance to fluoroquinolones resulted from both the presence of target site mutations and the expression of genes for efflux pumps.

In recent studies, the qnrA, B, S and aac(6')Ib-cr genes were shown to be related to quinolone resistance [11]. We detected aac(6')Ib-cr genes in EACR29, and in qnrA, EACR33. Therefore, further studies are needed to determine whether the expression of these genes contribute to fluoroquinolone resistance. To our knowledge, this is the first report of the mutations Ala81Pro in ParC and Arg157Gly in GyrA.

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