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BRIEF REPORT

Multiplex Real-Time Polymerase Chain Reaction-Based Method for the Rapid Detection of *gyrA* and *parC* Mutations in Quinolone-Resistant *Escherichia coli* and *Shigella* spp.

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Abstract
Two real-time polymerase chain reaction assays were developed to detect mutations in codons 83 and 87 in *gyrA* and in codons 80 and 91 in *parC*, the main sites that causes quinolone resistance in pathogenic *Escherichia coli* and *Shigella* spp. isolates. These assays can be employed as a useful method for controlling infections caused by quinolone-resistant *E coli* and *Shigella* isolates.

1. Introduction

Fluoroquinolone is highly effective against enteric Gram-negative bacteria [1]. Within a short period of time, however, the widespread use of this drug has resulted in the emergence of fluoroquinolone-resistant, Gram-negative bacteria [2–4].

Resistance to fluoroquinolones is mainly caused by mutations in the quinolone-resistance determining regions (QRDRs) of *gyrA* and *parC*, which encode the GyrA and ParC proteins, respectively [5]. Most quinolone-resistant isolates have at least one *gyrA* mutation, and many have additional *gyrA* and/or *parC* mutations [2,3]. Mutations in the *gyrA* amino acid codons for Ser83 and/or Asp87 are commonly found in

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fluoroquinolone-resistant, Gram-negative bacteria [6]. Targeted mutations are also frequently present in *parC* in quinolone-resistant Gram-negative bacteria, including mutations in the codons for Ser80, Glu84, and/or Arg91 [7,8]. Mutations in *gyrA* or *parC* have less of an effect on resistance in Gram-negative bacteria by themselves than they do when found in combination with other mutations in these genes [9].

Currently, nucleotide sequencing is a common method for the identification of mutations in the QRDRs of *gyrA* and *parC*. Despite the widespread use of sequencing, it is slow and expensive [10]. Various alternative methods to replace sequencing have been proposed, including polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) [11], real-time (RT)-based detection [12], and single-stranded conformation polymorphism (SSCP) [13].

Our objective was to develop a quicker method for the detection of quinolone resistance-related mutations at the *gyrA* codons of Ser83 and Asp87 and the *parC* codons of Ser80 and Arg91 in quinolone-resistant *Escherichia coli* and *Shigella* spp. isolates. This technique is based on the detection of fluorescent emissions from the PCR products of wild-type *gyrA* and *parC*; isolates with mutations at specific sites in *gyrA* and *parC* do not emit detectable fluorescence.

2. Materials and Methods

The isolates used in this study are listed in Table 1. Quinolone-resistant pathogenic *E coli* and *Shigella* spp. isolates were obtained from the National Public Health Network of Korea. Each isolate was identified according to the Giraud method with some modifications [14].

Two multiplex PCR assays were developed to detect mutations in the QRDRs of *gyrA* and *parC*. In the first assay (named Gyr), Taqman MGB probes were designed to detect wild-type nucleotide sequences corresponding to the codons of Ser83 and Asp87 in *gyrA*; in the second assay (named Par), probes were designed to detect wild-type nucleotide sequences corresponding to the codons of Ser80 and Agr87 in *parC*. Selective probes for both assays were designed using Primer Express (version 3.0; Applied Biosystems, Foster City, CA, USA) to detect mutations in the second base of the each targeted codon.

Template DNA was prepared from each isolate by boiling in 100 μL of 10 mM Tris-HCl (pH 8.0) for 10 minutes. The primers were synthesized by Bioneer (Seoul, Korea), and the probes were synthesized by Applied Biosystems. For each assay set, the probe for one allele was labeled with FAM (6-carboxy-fluorescein) and the other was labeled with VIC (4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein). The primers and probes used in this study are shown in Table 2.

All of the PCR assays were used in a final volume of 20 μL that contained 5 μL of template DNA with 0.2 μM of each primer, 0.5 μM of each probe, and 10 μL of RT-PCR master mix (Kogen Biotech, Seoul, Korea). All of the assays used the same thermocycling parameters. The reactions were performed in a LightCycler 480 system (Roche Diagnostics, Basel, Switzerland) under the following conditions: one cycle of 50°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

3. Results

After optimizing each assay, 14 *Shigella* spp. strains with known mutations in *gyrA* and/or *parC* were tested using the Gyr and Par assays. All of the known mutations at Ser83 and Asp87 were successfully detected in the *gyrA* mutants using the Gyr assay: 10 strains had mutations at Ser83 and one strain had a mutation at both Ser83 and Asp87. Additionally, all of the known mutations in Ser80 and Arg87 were successfully detected in the *parC* mutants using the Par assays. We assessed PCR amplification in all of the mutant isolates (i.e., those that did not emit fluorescence). Amplification of both the 78-base pair (bp) Gyr assay PCR product and the 98-bp Par assay PCR product were confirmed in all of the tested isolates using electrophoresis (data not shown).

To determine the accuracies of the RT-PCR assays, 48 strains of quinolone-resistant pathogenic *E coli* and 14 *S flexneri* isolates were tested using both the Gyr and Par assays. The fluorescent signals generated from the RT-PCR Gyr and Par assays were analyzed to determine the presence of mutations in *gyr*A and/or *par*C. The PCR results were confirmed by sequencing. All of the PCR results corresponded 100% with the sequencing results (Table 1). Notably, in strain QR24, a fluorescent signal was detected with the Par assay, but sequencing revealed a mutation in *parC* codon Ala81; however, this mutation at codon Ala81 did not affect the PCR results.

4. Discussion

Resistance to quinolone is caused by a variety of mechanisms, such as a mutation in the targeted enzyme's coding sequence, participation of the efflux system, and the actions of *qnr* [15]. Compared with other quinolone-resistance mechanisms, the largest contributing factor to resistance is alteration of the target sequence. Thus, the fast, precise detection of mutations in the QRDRs of the target enzymes is very important for controlling infections caused by quinolone-resistant strains [9].

The RT-PCR assay developed for this study can simultaneously identify mutations in specific regions of the *gyrA* and *parC* genes. Compared with similar PCR