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Rapid detection of fluoroquinolone resistance by isothermal chimeric primer-initiated amplification of nucleic acids from clinical isolates of *Neisseria gonorrhoeae*

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Abstract

To ensure a complete response to fluoroquinolone therapy against *Neisseria gonorrhoeae* infections, rapid susceptibility determinations are required. We assessed a new approach, an isothermal chimeric primer-initiated amplification of nucleic acids (ICAN)/hybrid-chromatography method to detect rapidly fluoroquinolone resistance in *N. gonorrhoeae*. Comparison of the amplification results with fluoroquinolone minimum inhibitory concentrations (MICs), which were determined by an agar dilution method, showed that the new method accurately determined fluoroquinolone resistance in all ciprofloxacin- and/or gatifloxacin-resistant isolates, but agreed with results based on MICs in only 6 of 8 (75.0%) ciprofloxacin-susceptible and 7 of 12 (58.3%) gatifloxacin-susceptible isolates. Our results suggest that this method can rapidly and reliably detect point mutations in the *gyrA* gene as well as fluoroquinolone resistance in resistant isolates of *N. gonorrhoeae*.

Keywords: *Neisseria gonorrhoeae*; Fluoroquinolone; Susceptibility; Detection; *gyrA*

1. Introduction

In adolescents and adults, *Neisseria gonorrhoeae* is a common cause of sexually transmitted diseases (STD), specifically urethritis and pelvic inflammatory disease (Ison et al., 1998; Llanes et al., 2003). The 2002 STD treatment guidelines of the Centers for Disease Control and Prevention (CDC) recommended use of cephalosporins such as cefixime and ceftriaxone, or fluoroquinolones such as ciprofloxacin, ofloxacin and levofloxacin (plus azithromycin or doxycycline, if chlamydial infection is not ruled out) for treatment of uncomplicated gonococcal infections of the cervix, urethra and rectum (CDC, 2002). However, emergence and increasing prevalence of fluoroquinolone resistance in *N. gonorrhoeae* isolates have been reported worldwide, becoming a significant concern (Ison et al., 1998; Forsyth et al., 2000; Rahman et al., 2002; Tanaka et al., 2000). Clinical failure of treatment of gonococcal infections with fluoroquinolones has also been reported (Tapsell et al., 1995; Tanaka et al., 1998).

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Mechanisms of fluoroquinolone resistance that have been identified and characterized in *N. gonorrhoeae* include mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes, which encode subunits of DNA gyrase and topoisomerase IV, respectively (Deguchi et al., 1996, 1997). Alterations of amino acids at Ser91 and Asp95 in GyrA play major roles in development of fluoroquinolone resistance in *N. gonorrhoeae* (Deguchi et al., 1996). Alterations in ParC, identified only in isolates with GyrA alterations, contribute further to their decreased susceptibilities to fluoroquinolones (Deguchi et al., 1996; Belland et al., 1994).

Fluoroquinolone resistance generally is detected by conventional susceptibility testing, DNA sequencing or real-time polymerase chain reaction (PCR)-hybridization (Tanaka et al., 2000; Li et al., 2002). Recently, a new approach, the isothermal chimeric primer-initiated amplification of nucleic acids (ICAN) method, has been developed to rapidly and isothermally amplify nucleic acids from *N. gonorrhoeae* colonies. In using this procedure, a hybrid-chromatography method with gold-labeled anti-fluorescein isothiocyanate (FITC) antibody has been developed to detect point mutations in the QRDRs of the *gyrA* gene associated with amino acid mutations such as Ser91Phe and Ser91Tyr in GyrA. The Bed-side ICAN NG-QR detection kit (Takara Bio, Ohtsu, Japan) can be used for amplification of the QRDRs of the *gyrA* gene by the ICAN method to detect point mutations in these amplified regions by the hybrid chromatography.

In this study, we determined fluoroquinolone susceptibility in *N. gonorrhoeae* by means of both the new ICAN/hybrid-chromatography and the agar dilution methods, evaluating concordance between results obtained by these two techniques.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains used in this study were ATCC49226 and 76 clinical isolates of *N. gonorrhoeae* isolated from different patients in Japan between 2001 and 2003. Bacteria stored at −70 °C were inoculated on heart infusion agar (Nissui Pharmaceutical, Tokyo, Japan) plates supplemented with 5% heated horse blood (Nippon Bio-Test Laboratories, Tokyo, Japan) and incubated overnight at 37 °C in a 5% CO_2 atmosphere.

2.2. Antimicrobials

The antimicrobials used included ciprofloxacin (Bayer Yakuhin, Osaka, Japan), gatifloxacin (Kyorin Pharmaceutical, Tokyo, Japan), prulifloxacin (Meiji Seika Kaisha, Tokyo, Japan), sitafloxacin (Daiichi Pharmaceutical, Tokyo, Japan) and sparfloxacin (Dainippon Sumitome Pharma, Osaka, Japan).

2.3. Susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by an agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS, 2004). Susceptibility testing was performed on GC agar base supplemented with 1% defined growth supplement (GCHI enrichment and rehydrating solution; Cosmo Bio, Tokyo, Japan) according to the manufacturer's instructions. MIC interpretative criteria for ciprofloxacin and gatifloxacin followed those of the CLSI/NCCLS (NCCLS, 2004).

2.4. Detection of point mutations in the gyrA gene

DNA from 66 clinical isolates of *N. gonorrhoeae* and from ATCC49226 were purified with an ICAN NG-QR sample preparation kit (Takara Bio) as recommended by the manufacturer. A single colony of *N. gonorrhoeae* was incubated at 37 °C for 30 min in 200 μl of lysis solution provided by the manufacturer and denatured at 98 °C for 5 min. Detection of mutations at codon 91 in GyrA was performed with isothermal amplification for specific regions including the QRDR of the *gyrA* gene by using a Bed-side ICAN NG-QR detection kit (Takara Bio) according to the manufacturer's instructions. The DNA–RNA chimeric oligonucleotide primer sets provided by the manufacturer for ICAN were as follows: 5′-TCGGCGACGTCATCGGTAAA and 5′-biotin-CAGCACATAACGCATAGCGAA (ribonucleotides are underlined). The amplification was performed isothermally at 60 °C for 1 h in 50 μl of a reaction mixture containing 0.5 mM dNTPs, 2 μM of each chimeric primer, 1 U of exo$^-$Bca DNA polymerase and 4.4 U of RNase HII. Five microliters of the amplified products was mixed with 25 μl of hybridization buffer and then with 10 μl of denatured solution (Bed-side ICAN NG-QR detection kit). Twenty microliters of the denatured products was loaded on each of two chromatostrips immobilizing a streptavidin-line. Then, 0.75 pmol of an FITC-labeled probe to detect wild type Ser91 and that to detect mutations of both Ser91Phe and Ser91Tyr were, respectively, loaded on a chromatostrip, referred