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Analysis of mutations within multiple genes associated with resistance in a clinical isolate of *Neisseria gonorrhoeae* with reduced ceftriaxone susceptibility that shows a multidrug-resistant phenotype

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Abstract

A *Neisseria gonorrhoeae* strain with a reduced susceptibility to ceftriaxone (minimum inhibitory concentration (MIC) = 0.5 μg/mL) was isolated among 398 clinical isolates obtained from 2000–2001 in Fukuoka City, Japan. The *N. gonorrhoeae* strain was negative for penicillinase production but it showed multidrug resistance against penicillin (MIC = 8 μg/mL), tetracycline (MIC = 4 μg/mL), azithromycin (MIC = 0.5 μg/mL) and ciprofloxacin (MIC = 16 μg/mL). The molecular mechanisms of the multidrug-resistant phenotype in this strain were analysed. Polymerase chain reaction and direct DNA sequencing were performed to identify mutations within the *penA, ponA, mtrR, penB, gyrA* and *parC* genes of the gonococcal strain, which thus explain the multidrug-resistant phenotype. The *N. gonorrhoeae* strain contained a significantly different sequence of the *penA* gene from that of the ceftriaxone-susceptible strains. Some regions of the transpeptidase domain within this *penA* gene were closely similar to those found in other *Neisseria* species such as *Neisseria subflava*, *Neisseria flavescens* or *Neisseria perflava*/*sicca*. This strain also included a *ponA* mutation that is associated with high-level resistance to penicillin, *mtrR* mutations that mediate overexpression of the MtrCDE efflux pump responsible for resistance to hydrophobic agents such as azithromycin, and *penB* mutations that reduce porin permeability to hydrophilic agents such as tetracycline. Moreover, this strain contained *gyrA* and *parC* mutations that confer high-level resistance to ciprofloxacin. These results indicate the emergence of a *N. gonorrhoeae* strain with reduced susceptibility to ceftriaxone, which also showed a multidrug-resistant phenotype that can be explained by the presence of multiple loci mutations associated with antibiotic resistance.

Keywords: *Neisseria gonorrhoeae*; Ceftriaxone; Resistance; Susceptibility; Mutation

1. Introduction

The evolution of resistance to antimicrobial agents in *Neisseria gonorrhoeae* isolates is a global problem in the treatment of gonococcal infections. The gonococcal resistance level to penicillins, tetracyclines, fluoroquinolones and oral cephalosporins has recently begun to increase in Japan [1,2]. Therefore, a regimen of parenteral cephalosporin such as ceftriaxone or spectinomycin is now generally recommended as a first-line treatment for uncomplicated gonococcal infections, and this regimen has also been proven to show an excellent clinical efficacy in Japan. However, we have recently isolated a *N. gonorrhoeae* strain with a reduced susceptibility to ceftriaxone in Fukuoka City, Japan. This gonococcal strain was also found to demonstrate a chromosomally-mediated multidrug-resistant phenotype. The treatment of gonorrhoea may therefore become increasingly more complicated owing to its resistance to a variety of antimicrobial agents, including parenteral cephalosporins.

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The genetic mechanisms of chromosomally-mediated penicillin and tetracycline resistance in *N. gonorrhoeae* have been investigated in laboratory mutants and clinical isolates, and the mechanisms are thought to be due to mutations in the three loci of *penA*, *mtrR* and *penB* genes [3]. Mutations in the *penA* gene are associated with a reduced binding of penicillin by penicillin-binding protein (PBP)2, which is a very important target for penicillin [4,5]. Mutations in the *mtrR* gene confer non-specific resistance to erythromycin, azithromycin, rifampicin and hydrophobic agents owing to increased expression of the MtrCDE efflux pump system [6]. The *penB* mutations reduce porin permeability of the outer membrane to hydrophilic antibiotics such as penicillin and tetracycline [7,8]. The *penB* phenotype is apparent only in strains with the MtrR phenotype. In addition to affecting resistance to penicillin, the *penA*, *mtrR* and *penB* loci appear to increase resistance to cephalosporins in *N. gonorrhoeae*. Moreover, recent studies have indicated that alterations in PBP1, encoded by the *ponA* gene, are involved in high-level penicillin resistance in chromosomally-mediated resistant *N. gonorrhoeae* [9,10].

However, little is known about the molecular mechanisms for cephalosporin resistance in *N. gonorrhoeae*. The aim of this study was to determine the molecular basis for ceftriaxone resistance with a multidrug-resistant phenotype in a strain of *N. gonorrhoeae* isolated from our patient population.

2. Materials and methods

2.1. Neisseria gonorrhoeae strains

Antimicrobial susceptibility testing was performed on a total of 398 clinical isolates of *N. gonorrhoeae* that had been obtained from January 2000 to December 2001. All isolates of *N. gonorrhoeae* were collected from male patients with urethritis attending a sexually transmitted disease clinic in Fukuoka City, Japan. None of the isolates were either post-treatment isolates or repeat isolates from the same patient. The clinical efficacy of ceftriaxone or other antibiotics against *N. gonorrhoeae* isolates was not known. The organisms were identified by Gram staining, oxidase activity and reaction using the Gonochek II test (EY Laboratories, San Mateo, CA). The isolates were suspended in a cryoprotective medium [11] and stored at −80 °C until they were tested. After antimicrobial susceptibility testing, the identities of five isolates (GP853, GP984, GP986, GP998 and A69W) were further confirmed using the biochemical test Vitek NHI (bioMerieux, Tokyo, Japan).

2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) for all isolates was determined by an agar dilution technique with a GC agar base (Becton Dickinson, Sparks, MD) containing 1% Iso VitaleX (Becton Dickinson) and two-fold dilutions of antibiotics as specified in the National Committee for Clinical Laboratory Standards (NCCLS) protocol [12]. Briefly, the plates were inoculated with ca. 10^4 colony-forming units/spot of each isolate with a multipoint inoculator. The World Health Organization reference *N. gonorrhoeae* strains A, B, C, D and E, and *N. gonorrhoeae* ATCC 49226 strain were included as quality controls. The plates were incubated for 24 h at 35 °C in a 5% CO_2 atmosphere. MICs were defined as the lowest antibiotic concentration observed to inhibit bacterial growth. The antimicrobial agents tested were penicillin G (Sigma Chemical Co., St Louis, MO), tetracycline (Wyeth Ledere Japan, Tokyo, Japan), ceftriaxone (Nippon Roche, Tokyo, Japan), cefixime (Astellas Pharma, Tokyo, Japan), ciprofloxacin (Bayer Yakuhin, Osako, Japan), azithromycin (Pfizer Pharmaceuticals, Tokyo, Japan) and spectinomycin (Sigma Chemical Co.). All of the antibiotics were obtained in powder form at the stated potencies determined by their manufacturers. The antimicrobial susceptibility was determined according to the breakpoint criteria defined by the NCCLS [12].

2.3. Polymerase chain reaction (PCR) amplification and DNA sequencing

For the PCR amplification of the *penA* gene, three sets of oligonucleotide primers (1S 5′-CGAATATAAGCCCCGGAT-3′, PA2 5′-ACAATCTCGTTGATACTCG-3′ [13]; B1 5′-TGCCGGAATCGGATTCCT-3′, B2 5′-CGATGACGTGTGCAAAGA-3′; and C1 5′-TTACGGCCTGCAATTGAG-3′, C2 5′-GGTCGGGATGCCGGTTTC-3′) were used. The *ponA* gene was amplified with the following primers: 5′-CGCGGTGCGGAAAACTGATATCGAT-3′ (nucleotides 955–978 of the *ponA* open reading frame) and 5′-AGCCCGGATCGGTTACCATACGTT-3′ (nucleotides 2218–2195 of the *ponA* open reading frame) [10]. To amplify the promoter and coding regions of the *mtrR* gene (nucleotides 860–1175; GenBank accession no. Z25796), primers MTR1 (5′-AACAGGCATTCTTATTTCAG-3′) and MTR2 (5′-TTAGAAGAATGCTTTGTGTC-3′) published by Mavroidi et al. [13] were used. Primers PorB1 (5′-AAAGGCCAAGAAGACCTCGGC-3′) and PorB2 (5′-GAGAAGTCGTATTCCGCACCG-3′) were used for amplification of a part of the *por* gene (nucleotides 160–917; EMBL accession no. AJ004943) [13].

PCR amplification was performed in a reaction mixture containing 5.0 μL of 10× *Taq* polymerase buffer (500 mM KCl, 100 mM Tris–HCl (pH 8.3), 15 mM $MgCl_2$, 0.1% gelatin), 2.0 μL of each of the two primers (25 pmol/μL), 1.0 μL of each of the four deoxynucleotide triphosphates (10 mM), 0.25 μL of *Taq* DNA polymerase (5 U/μL) (Takara Biomedicals, Otsu, Shiga, Japan) and 1.0 μL of template DNA (100 ng/μL). Thirty-five cycles were performed for each reaction. Each cycle consisted of denaturation at 96 °C for 0.5 min, annealing at 56 °C for 0.5 min and extension at 74 °C for 0.5 min. The PCR amplification products were directly sequenced using a DYEnamic ET Terminator Cycle