Molecular characterisation of the quinolone resistance-determining region (QRDR) of the *parC* gene locus in viridans-group streptococci

M. MURAYAMA[*†], Y. MAEDA[*‡], C. E. GOLDSMITH[*], W. A. COULTER[§], C. MASON[§], B. C. MILLAR[*], M. MATSUDA[†] and J. E. MOORE[*‡]

[*]*Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast;* [†]*Laboratory for Molecular Biology, School of Environmental Health Sciences, Azabu University, Sagamihara, Japan;* [‡]*School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine; and* [§]*School of Dentistry, Queen's University of Belfast, Royal Group of Hospitals, Grosvenor Road, Belfast, Northern Ireland, UK*

Accepted: 24 December 2010

ABSTRACT

Forty-eight isolates of viridans-group streptococci (VGS) from adults and children in the community are examined for their resistance to ciprofloxacin phenotypically by determination of the minimum inhibitory concentration (MIC). In addition, the *parC* gene locus is amplified and sequenced in all isolates and mutations noted. Overall, 44 VGS organisms were found to be susceptible to ciprofloxacin by the broth microdilution method, and the remaining four strains had intermediate susceptibility. Reduced MICs were observed with intermediate strains when reserpine was added to the broth, inhibiting any efflux activity. Overall, the effect of adding reserpine to the broth medium was to add one doubling dilution to the MIC in the case of *Streptococcus mitis, S. oralis* and *S. salivarius*, as well as to increase the MIC by two doubling dilutions in two of the three *S. parasanguinis* isolates. Amino acid sequence analysis of the quinolone resistance-determining region (QRDR) of the *parC* gene locus showed good correlation to the phenotypic resistance to ciprofloxacin, where no confirmed mutation conferring quinolone resistance was found. Eleven amino acid positions showed discordance with *S. pneumoniae* R6 and eight (S52, F55, S58, N91, E135, K137, F141 and S167) were common in the VGS species examined. In addition, minor substitutions were found at three positions (D51, T54 and V86). In conclusion, this study demonstrates the low occurrence of ciprofloxacin resistance in a population of VGS isolated from the community. In addition, several silent mutations were noted in VGS organisms without any increase in MIC against ciprofloxacin.

KEY WORDS: Ciprofloxacin.
Drug resistance, microbial.
Fluoroquinolones.
parC gene product.
Streptococcus pneumoniae.

Introduction

The gene locus *parC* is a subunit consisting of DNA topoisomerase IV and plays an important role in chromosome segregation during DNA replication.[1] It is considered to be the primary target of ciprofloxacin activity, whereas the target is DNA gyrase A (GyrA) in Gram-negative bacteria.[2] The mechanism of quinolone resistance involves mutations at the quinolone resistance-determining region (QRDR) of the *parC* and *gyrA* genes, as well as in *parE* and *gyrB* genes.[3] There appears to be a tendency for mutations in *parC* to occur first, followed by mutations in *gyrA*, leading to double mutations which in turn leads to high-level resistance to quinolones.[4] A previous report describes the importance of this first mutation at the *parC* locus. In this report, *S. pneumoniae* isolated from a bacteraemic patient showed a levofloxacin minimum inhibitory concentration (MIC) of 1 µg/mL and a *parC* mutation. After failure of levofloxacin treatment, these workers isolated the same clonal lineage of *S. pneumoniae* with mutations in *parC* and *gyrA*.[5] The message from this study is that phenotypic testing of susceptible strains may disguise the existence of important *parC* mutations.

Increasing rates of ciprofloxacin-resistant *S. pneumoniae* infection have been reported in Northern Ireland.[6] The spread of quinolone resistance may be due to intraspecies and interspecies horizontal gene transfer (HGT) of quinolone resistance genes.[7,8] In particular, HGT from viridans-group streptococci (VGS) to *S. pneumoniae* is likely to happen naturally, as VGS are commensal bacteria and are often exposed to antimicrobial selective pressure, thereby becoming a potential reservoir of antibiotic resistance genes.[9,10] Such transmissibility has been described under laboratory conditions, with a rate of 10^{-3} to 10^{-6}, while the mutation frequency of ciprofloxacin-resistant *S. pneumoniae* is 10^{-8} to 10^{-9}.[11] Therefore, the aim of this study is to characterise the *parC* gene in VGS isolated in Northern Ireland.

Correspondence to: Professor John E. Moore
Northern Ireland Public Health Laboratory, Department of Bacteriology
Belfast City Hospital, Belfast BT9 7AD, Northern Ireland, UK
Email: jemoore@niphl.dnet.co.uk

Materials and methods

Bacterial isolates

Forty-eight isolates of VGS isolated from nasal swabs of patients attending local general practices were used in this

study. Briefly, streptococcal isolates were isolated using Mitis-Salivarius agar (Becton Dickinson, Oxford, UK) and were incubated for 48 h at 37°C under microaerophilic conditions. Following incubation, visually distinct morphological variants were subcultured on Columbia blood agar (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37°C under microaerophilic conditions. The isolates were identified to species level by *rnpB* gene sequencing, as described by Maeda *et al*. (personal communication). The resulting population of VGS comprised *S. salivarius* (*n*=42), *S. parasanguinis* (*n*=3), *S. mitis* (*n*=2) and *S. oralis* (*n*=1).

Table 1. Minimum inhibitory concentration range, geometric MIC mean, MIC_{50} and MIC_{90} for each VGS species identified against ciprofloxacin.

Species	MIC range (µg/mL)	Mean	MIC_{50}	MIC_{90}
S. mitis (*n*=2)	1	1	1	1
S. oralis (*n*=1)	2	2	2	2
S. parasanguinis (*n*=3)	1–4	3	4	4
S. salivarius (*n*=42)	1–4	1.58	2	2

Determination of minimum inhibition concentration

The MIC of ciprofloxacin was determined by broth microdilution using the CLSI standard method.[12] In order to estimate ciprofloxacin resistance, ofloxacin criteria for "*Streptococcus* spp. other than *Streptococcus pneumoniae*" was used, where resistance was an MIC ≥8 µg/mL, intermediate resistance MIC = 4 µg/mL and susceptible MIC ≤2 µg/mL. The effect of active efflux pumps was examined by supplementation with 7.5 µg/mL reserpine. Lyophilised antimicrobial tablets were obtained from Mast (Merseyside, UK).

DNA extraction

Bacterial strains were subcultured on Columbia blood agar supplemented with 5% (v/v) horse blood (Oxoid, Basingstoke, UK) for 24 h at 37°C under microaerophilic conditions. All DNA isolation procedures were carried out in a Class II Biological Safety Cabinet (MicroFlow, UK) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room in accordance with the Good Molecular Diagnostic Procedure (GMDP) guidelines of Millar *et al.*,[13] in order to minimise contamination. Bacterial genomic DNA was extracted from a few colonies of each strain using the Roche High Purity PCR Template Preparation Kit (Roche Diagnostics, Sussex, UK), in accordance with the manufacturer's instructions. Extracted DNA was stored at –20°C prior to PCR amplification.

PCR amplification of QRDRs

The QRDR of the *parC* gene was amplified using a degenerate primer set, as described by Maeda *et al.* (personal communication). The PCR reaction mixes (25 µL) contained 1 µL DNA template (approximately 25 ng DNA), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 200 µmol each dNTP, 0.6 units *Thermophilus aquaticus* (*Taq*) DNA polymerase (New England Biolabs, Hertfordshire, UK) and 10 µmol each primer. Following a 'hot start', the reaction mixtures were subjected to the following thermal cycling parameters in a GeneAmp PCR 9700 thermocycler system (Applied Biosystems, Warrington, UK): 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec, followed by a final extension at 72°C for 7 min. Following amplification, the PCR products were visualised on 1.5% (w/v) agarose gels in 0.5xTBE buffer, using ethidium bromide (0.5 µg/mL) staining and ultraviolet (UV) illumination from a gel image analysis system (UVP Products, Cambridge, UK).

DNA sequencing and amino acid analysis

Amplicons for sequencing were purified using a QIAquick PCR purification kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. The QRDR of the *parC* gene sequences was determined using the BigDye Terminator cycle sequencing kit and an ABI 3100 genetic analyser (Applied Biosystems). Sequencing analysis was carried out using the Genetyx system (Genetyx, Tokyo, Japan).

Results

Forty-four isolates examined in this study were found to be susceptible to ciprofloxacin by broth microdilution and the remaining four strains had intermediate susceptibility

Table 2. Amino acid positions which differed from the *S. pneumoniae* R6 strain.

	Amino acid substitution at the QRDRs of *parC*										
Species	51[a]	**52**[b]	54	**55**	**58**	86	**91**	**135**	**137**	**141**	**167**
S. pneumoniae R6	D	**S**	T	**F**	**S**	V	**N**	**E**	**K**	**F**	**S**
S. mitis	–	G	–	H/Y	–	–	D	–	–	–	–
S. oralis	–	G	–	–	–	–	D	–	–	–	–
S. parasanguinis	Y	G	–	H	G	–	D	–	N	–	A
S. salivarius		G	A	G	G	A	D	D	N	L/W	A

[a] Amino acid position of *S. pneumoniae* R6

[b] Bold characters indicate amino acid positions commonly different from *S. pneumoniae* R6.

(Table 1). Reduced MICs were observed with intermediate strains when reserpine was added to the broth, inhibiting any efflux activity. Overall, the effect of adding reserpine to the broth medium was to add one doubling dilution to the MIC in the case of *S. mitis, S. oralis* and *S. salivarius*, as well as to increase the MIC by two doubling dilutions in two of the three *S. parasanguinis* isolates. Amino acid sequence analysis of the QRDR of *parC* showed good correlation to the phenotypic resistance to ciprofloxacin, where no confirmed mutation-conferring quinolone resistance was found. Some amino acid positions showed discordance with *S. pneumoniae* R6 at positions S52, F55, S58, N91 E135, K137, F141 and S167 (Table 2). In addition, minor substitutions were found at three positions (D51, T54 and V86). All detected mutations in the *parC* gene locus have been deposited in GenBank with the accession numbers GQ999579–GQ999589.

Discussion

Emergence of antibiotic-resistant *S. pneumoniae* is of growing concern worldwide. Although the prevalence of antibiotic-resistant pneumococci remains low (<5%), this resistance rate is expected to increase.[14] Such a rise in resistance may be due to several factors including the increased prescription of fluoroquinolones for community-acquired pneumococcal infection. One American study has shown fluoroquinolones to be the most prescribed antibiotic, having overtaken other antibiotics such as the penicillins and the macrolides. Recent studies have shown that VGS may be a reservoir of antibiotic resistance determinant genes,[9] which are seen more frequently than mutations conferring quinolone resistance.[11] Therefore, the present study characterised QRDR of the *parC* gene (which primarily produces fluoroquinolone resistance[4]) in VGS species isolated from patients attending local general practices in Northern Ireland.

No fluoroquinolone-resistant VGS were found in this patient population. Eleven amino acid positions showed discordance with *S. pneumoniae* R6 and eight were common in the VGS species examined, although none contributed to ciprofloxacin resistance (Maeda *et al*., personal communication). Three spontaneous mutations were observed and these appeared to have no effect on ciprofloxacin resistance, as they were all found in ciprofloxacin-susceptible isolates.

Overall, a low prevalence of ciprofloxacin-resistant VGS and no confirmed amino acid mutations were found in this study. First-step mutations at the QRDR of *parC* are key in the development of fluoroquinolone resistance in VGS and *S. pneumoniae*. However, the first-step *parC* mutation can be missed if only antibiotic susceptibility tests are carried out, resulting in subsequent double mutations in *gyrA* and other QRDR gene loci.

The emergence of ciprofloxacin-resistant *S. pneumoniae* has been reported and one event that may contribute to this is the potential horizontal gene transfer of the quinolone resistance determinant genes from VGS to pneumococci.

In conclusion, this study investigated the prevalence of ciprofloxacin-resistant VGS and characterised these data in terms of the organisms' *parC* gene mutations. No resistance was found in 44 VGS isolates but it was intermediate in four isolates examined. Amino acid sequences showed no confirmed mutation that would confer quinolone resistance. □

This work was supported financially through HSC R&D Office commissioned grant: Antimicrobial Resistance Action Plan (AMRAP) (COM/2730/04).

References

1 Hooper DC. Fluoroquinolone resistance among Gram-positive cocci. *Lancet Infect Dis* 2002; **2** (9): 530–8.

2 González I, Georgiou M, Alcaide F, Balas D, Liñares J, de la Campa AG. Fluoroquinolone resistance mutations in the *parC*, *parE*, and *gyrA* genes of clinical isolates of viridans-group streptococci. *Antimicrob Agents Chemother* 1998; **42** (11): 2792–8.

3 Ip M, Chau SS, Chi F, Qi A, Lai RW. Rapid screening of fluoroquinolone resistance determinants in *Streptococcus pneumoniae* by PCR-restriction fragment length polymorphism and single-strand conformational polymorphism. *J Clin Microbiol* 2006; **44** (3): 970–5.

4 Pan XS., Ambler J, Mehtar S, Fisher LM. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae. Antimicrob Agents Chemother* 1996; **40** (10): 2321–6.

5 de Cueto M, Rodríguez JM, Soriano MJ, López-Cerero L, Venero J, Pascual A. Fatal levofloxacin failure in treatment of a bacteremic patient infected with *Streptococcus pneumoniae* with a preexisting *parC* mutation. *J Clin Microbiol* 2008; **46** (4): 1558–60.

6 Goldsmith CE, Moore JE, Murphy PG, Ambler JE. Increased incidence of ciprofloxacin resistance in penicillin-resistant pneumococci in Northern Ireland. *J Antimicrob Chemother* 1998; **41** (3): 420–1.

7 Stanhope MJ, Walsh SL, Becker JA *et al*. Molecular evolution perspectives on intraspecific lateral DNA transfer of topoisomerase and gyrase loci in *Streptococcus pneumoniae*, with implications for fluoroquinolone resistance development and spread. *Antimicrob Agents Chemother* 2005; **49** (10): 4315–26.

8 Ip M, Chau SS, Chi F, Tang J, Chan PK. Fluoroquinolone resistance in atypical pneumococci and oral streptococci: evidence of horizontal gene transfer of fluoroquinolone resistance determinants from *Streptococcus pneumoniae. Antimicrob Agents Chemother* 2007; **51** (8): 2690–700.

9 Balsalobre L, Ferrándiz MJ, Liñares J, Tubau F, de la Campa AG. Viridans group streptococci are donors in horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae. Antimicrob Agents Chemother* 2003; **47** (7): 2072–81.

10 Fantin B, Duval X, Massias L *et al*. Ciprofloxacin dosage and emergence of resistance in human commensal bacteria. *J Infect Dis* 2009; **200** (3): 390–8.

11 Ferrándiz MJ, Fenoll A, Liñares J, de la Campa AG. Horizontal transfer of *parC* and *gyrA* in fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae. Antimicrob Agents Chemother* 2000; **44** (4): 840–7.

12 Clinical and Laboratory Standards Institute (CLSI): *Performance standards for antimicrobial susceptibility testing*. Document M7-A6. Wayne, Pennsylvania: CLSI, 2005.

13 Millar BC, Xu J, Moore JE. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *J Clin Microbiol* 2002; **40** (5): 1575–80.

14 de la Campa AG, Ardanuy C, Balsalobre L *et al*. Changes in fluoroquinolone-resistant *Streptococcus pneumoniae* after 7-valent conjugate vaccination, Spain. *Emerg Infect Dis* 2009; **15** (6): 905–11.