Molecular characterisation of the quinolone resistance-determining regions (QRDR) including *gyrA*, *gyrB*, *parC* and *parE* genes in *Streptococcus pneumoniae*

Y. KAKINUMA[*†], Y. MAEDA[*‡], C. MASON[§], C. E. GOLDSMITH[*], W. A. COULTER[§], M. MATSUDA[†], J. S. G. DOOLEY[†], C. J. LOWERY[†] and J. E. MOORE[*‡]

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

Accepted: 25 May 2012

ABSTRACT

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia (CAP). Currently, empirical treatment with quinolones is being used due to the emergence of β-lactam and macrolide resistance in *S. pneumonaie*. Although the prevalence of quinolone-resistant *S. pneumoniae* remains low, increasing numbers of resistant isolates are being seen. Genetic mechanisms leading to fluoroquinolone resistance in pneumococci are complex. This study aims to use molecular methods to characterise all isolates through sequence analysis of their QRDR regions. Thirty-two *S. pneumoniae* isolates were obtained from nasal swabs from adult and paediatric patients attending local general practices in Northern Ireland. Phenotypic minimum inhibitory concentration (MIC) was determined for Clinical and Laboratory Standards Institute (CLSI) broth microdilution against ciprofloxacin, levofloxacin and norfloxacin. Simultaneously, the QRDR regions of *gyrA*, *gyrB*, *parC* and *parE* were analysed by sequence typing for all pneumococci obtained. Only one isolate (3.1%) showed reduced susceptibility to ciprofloxacin and levofloxacin. Two amino acid positions were discordant in the *S. pneumoniae* R6 strain and eight (25%) and 23 (71.9%) isolates contained the mutations Ile460Val in *gyrA* and Lys137Asn in *parC* (deposited in GenBank, accession numbers GQ999587–GQ999589), respectively. No mutations were found in either the *gyrB* or *parE* loci. In conclusion, the study demonstrated increased fluoroquinolone resistance which could not be accounted for simply through QRDR mutations, and, reciprocally, that mutations in the QRDR region do not necessarily result in overt phenotypic resistance.

KEY WORDS: Drug resistance, microbial.
Mutation.
Polymerase chain reaction.
Quinolones.
Streptococcus pneumoniae.

Introduction

The incidence of community-acquired pneumonia (CAP) in the elderly population in the USA is estimated to be 915,900 cases, where *Streptococcus pneumoniae* is the leading cause of this infection.[1] Recommendations for empirical treatment of CAP is use of quinolones, as emergence of β-lactam- and macrolide-resistant *S. pneumoniae* has been reported.[2] Although the rate of quinolone-resistant *S. pneumoniae* remains low, several studies have described increasing quinolone resistance in *S. pneumoniae* worldwide.[3–5]

The mechanism of quinolone resistance in this organism has been studied extensively. Point mutations in DNA gyrase subunits *gyrA* and *gyrB*, and in DNA topoisomerase IV subunits *parC* and *parE*, are largely responsible for such quinolone resistance.[6]

Prevalence of ciprofloxacin resistance in *S. pneumoniae* isolated from community-acquired lower respiratory tract infection in the UK during the year 1994 to 1995 has been reported as 14.8%, ranging from 4% in Liverpool to 42.9% in Belfast.[7] Following the survey, Smith and colleague have shown that prescription of antimicrobial agents in the community in the UK reduced by approximately 22% between 1995 and 2000.[8]

It remains unclear to what extent asymptomatically colonising *S. pneumoniae* exhibits resistance to quinolones after this reduction in antibiotic prescription. Therefore, the aim of this study is to investigate the occurrence and genetic mechanisms of quinolone resistance in community isolates of *S. pneumoniae*.

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

Thirty-two *S. pneumoniae* isolates were obtained from nasal swabs from adult and paediatric patients attending local general practices in Northern Ireland. These isolates were obtained with informed patient consent as part of a study with approval from the Office of Research Ethics Committees, Northern Ireland (ORECNI). All samples were plated on selective Mitis-Salivarius agar (Cat No: 229810, Becton Dickinson, Oxford, UK) containing 1% (w/v) tellurite solution, and were incubated for 48 h at 37°C under microaerophilic conditions in a CO_2 incubator regulated at 5% (v/v) CO_2. Following incubation, presumptive *S. pneumoniae* colonies were subcultured on Columbia blood agar (CM0331, Oxoid, Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37°C under microaerophilic conditions, as detailed above.

The species of all presumptive pneumococcal isolates were confirmed phenotypically by optochin susceptibility testing, as well as bile solubility testing. In addition, all isolates were confirmed by molecular methods using sequence analysis of the *rpnB* and 16S rRNA gene loci. In addition, three reference strains (G7, J5 and 27) with known fluoroquinolone resistance were employed as positive controls.

Minimum inhibition concentration

The minimum inhibitory concentration (MIC) for all isolates against ciprofloxacin, levofloxacin and norfloxacin was determined by a broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) standard method. The MICs for ciprofloxacin ≥4.0 µg/mL and norfloxacin >16 µg/mL were considered to demonstrate reduced susceptibility, as proposed previously.[9] Lyophilised antimicrobial tablets were purchased from Mast (Merseyside, UK).

DNA extraction

Bacterial isolates were subcultured on Columbia blood agar supplemented with 5% (v/v) horse blood (Oxoid), for 24 h at 37°C under microaerophilic conditions. Bacterial genomic DNA was extracted from a few colonies of each isolate, using the Roche High Purity PCR Template Preparation Kit (Roche Diagnostics, Sussex, UK), following the manufacturer's instructions. Extracted DNA was stored at –20°C prior to polymerase chain reaction (PCR) amplification.

Amplification of quinolone resistance-determining regions

The quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* gene loci in each isolate was amplified using a previously described method.[6] Following amplification, PCR products were visualised on 1.5% (w/v) agarose gels in 0.5×TBE buffer, followed by staining with ethidium bromide (0.5 µg/mL) and ultraviolet (UV) illumination with a gel image analysis system (UVP Products, Cambridge, UK).

Table 1. Minimum inhibitory concentration (MIC) of *Streptococcus pneumoniae* (n=32 isolates) to three quinolone antibiotic agents.

	MIC (µg/mL)			
	Range	Mean	MIC_{50}	MIC_{90}
Ciprofloxacin	<0.125–4	1.49	1	2
Levofloxacin	0.25–4	1.32	1	2
Norfloxacin	2–16	9.56	8	8

DNA sequencing and analysis

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

Results and discussion

Only one isolate (3.1%) showed reduced susceptibility to ciprofloxacin and levofloxacin. Table 1 details the range of MIC, geometric MIC mean, MIC_{50} and MIC_{90} against the three quinolones tested. Two amino acid positions were discordant in the *S. pneumoniae* R6 strain. Eight (25%) and 23 (71.9%) of the isolates showed the mutation Ile460Val in *gyrA* and Lys137Asn in *parC* (deposited in GenBank, accession numbers: GQ999587–GQ999589), respectively. No mutations were found in either the *gyrB* or *parE* loci.

Nucleic acid and amino acid analyses of the QRDR loci showed good correlation to the phenotypic susceptibility results obtained. Of interest, this study showed that one isolate (isolate 25; 104250) had reduced susceptibility to ciprofloxacin and levofloxacin, but did not contain any mutations or amino acid substitutions in any of the four QRDR gene loci examined. Hence, this reduced susceptibility may be attributed to the activity of an active efflux pump, expelling the fluoroquinolone agent from bacterial cells, or, alternatively, is under regulatory genetic control elsewhere in the genome.

Efflux pump-mediated resistance is considered to play an important role in the development of quinolone resistance. Although it usually results only in low-level resistance in *S. pneumoniae*, this is important in conditioning the organism to survive in low concentrations of quinolones, where *S. pneumoniae* will have an extended opportunity to develop further resistance by point mutations within its QRDR genetic machinery.[10]

Two mutations at the QRDRs in *gyrA* and *parC* were identified in this study and these have been reported previously as common mutations in *S. pneumoniae*, neither of which has any effect on quinolone resistance.[11]

Overall, this study demonstrates that the prevalence of quinolone-resistant *S. pneumoniae* is rare in the community in Northern Ireland, both by standard phenotypic broth microdilution techniques and by genotypic sequence typing. Reports from elsewhere (e.g., Hong Kong and Turkey) reveal a range of quinolone-resistant *S. pneumoniae* isolated from healthy children, showing rates of 57% and 3.6%, respectively.[12,13]

In conclusion, mechanisms leading to fluoroquinolone resistance in pneumococci are complex. This study has demonstrated increased fluoroquinolone resistance that could not explained simply through QRDR mutations, and, reciprocally, that mutations in QRDR regions do not necessarily result in overt phenotypic resistance.

Fluoroquinolone reference strains (G7, J5 and 27) were kindly donated by Dr Cassie Pope and Professor Stephen Gillespie, UCL, Royal Free Hospital, London. This work was supported financially through HSC R&D Office-commissioned grant Antimicrobial Resistance Action Plan (AMRAP) (COM/2730/04). YK and YM contributed equally to this study and hence should be considered equal first authors.

References

1 Jackson ML, Neuzil KM, Thompson WW *et al*. The burden of community-acquired pneumonia in seniors: results of a population-based study. *Clin Infect Dis* 2004; **39** (11): 1642–50.

2 Mandell LA, Wunderink RG, Anzueto A *et al.*; Infectious Diseases Society of America; American Thoracic Society. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin Infect Dis* 2007; **44** (Suppl 2): S27–72.

3 Jones RN, Fritsche TR, Sader HS, Stilwell MG. Activity of garenoxacin, an investigational des-F(6)-quinolone, tested against pathogens from community-acquired respiratory tract infections, including those with elevated or resistant-level fluoroquinolone MIC values. *Diagn Microbiol Infect Dis* 2007; **58** (1): 9–17.

4 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.

5 Osawa M, Ito Y, Ishida T, Imai S, Ichiyama S, Mishima M; Kansai Community Acquired Pneumococcal Pneumonia Study Group. Molecular characterization of quinolone resistance-determining regions and their correlation with serotypes and genotypes among *Streptococcus pneumoniae* isolates in Japan. *Eur J Clin Microbiol Infect Dis* 2010; **29** (2): 245–8.

6 Heaton VJ, Goldsmith CE, Ambler JE, Fisher LM. Activity of gemifloxacin against penicillin- and ciprofloxacin-resistant *Streptococcus pneumoniae* displaying topoisomerase- and efflux-mediated resistance mechanisms. *Antimicrob Agents Chemother* 1999; **43** (12): 2998–3000.

7 Felmingham D, Robbins MJ, Dencer C, Nathwani A, Grüneberg RN. Antimicrobial susceptibility of community-acquired bacterial lower respiratory tract pathogens. *J Antimicrob Chemother* 1996; **38** (4): 747–51.

8 Smith GE, Smith S, Heatlie H *et al.* What has happened to antimicrobial usage in primary care in the United Kingdom since the SMAC report? – description of trends in antimicrobial usage using the General Practice Research Database. *J Public Health (Oxf)* 2004; **26** (4): 359–64.

9 Chen DK, McGeer A, de Azavedo JC, Low DE. Decreased susceptibility of *Streptococcus pneumoniae* to fluoroquinolones in Canada. Canadian Bacterial Surveillance Network. *N Engl J Med* 1999; **341** (4): 233–9.

10 Jumbe NL, Louie A, Miller MH *et al*. Quinolone efflux pumps play a central role in emergence of fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2006; **50** (1): 310–7.

11 Touyama M, Higa F, Nakasone C *et al. In vitro* activity of sitafloxacin against clinical strains of *Streptococcus pneumoniae* with defined amino acid substitutions in QRDRs of gyrase A and topoisomerase IV. *J Antimicrob Chemother* 2006; **58** (6): 1279–82.

12 Boost MV, O'Donoghue MM, Dooley JS. Prevalence of carriage of antimicrobial resistant strains of *Streptococcus pneumoniae* in primary school children in Hong Kong. *Epidemiol Infect* 2001; **127** (1): 49–55.

13 Uzuner A, Ilki A, Akman M *et al.* Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* in healthy children. *Turk J Pediatr* 2007; **49** (4): 370–8.