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An Intrinsic Pattern of Reduced Susceptibility to Fluoroquinolones in Pediatric Isolates of *Streptococcus pyogenes*

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Abstract

A total of 116 clinical isolates collected in 2003 from a tertiary pediatric hospital and a primary pediatric department in Chicago, Illinois were screened for reduced susceptibility to selected fluoroquinolones by disc diffusion. Correlation between reduced susceptibility and point mutations in the quinolone resistance-determining region of *parC* and *gyrA* genes were evaluated, and point mutations were compared with other reports of isolates derived from adult or mixed patient populations. 9% of isolates had reduced susceptibility to one or more of these fluoroquinolones by Etest: ciprofloxacin, levofloxacin, moxifloxacin. A single point mutation (Ser-79) in *parC* seemed responsible for the reduced susceptibility. Resistant *S. pyogenes* isolates were compared using M/*emm* type, RepPCR, and pulsed-field gel electrophoresis (PFGE). RepPCR provided no more separation of strains than M/*emm* typing and PFGE results with SgrA1 were more discriminatory than with SmaI. The majority of these isolates were M/*emm* type 6. PFGE analysis using SgrA1 demonstrated 2 different resistant strains among the M/*emm* type 6 isolates. The findings suggest that a population of *S. pyogenes* with an intrinsic reduced susceptibility to fluoroquinolones exists in pediatric clinical isolates. Monitoring of amino acid changes in both *parC* and *gyrA* will assist in the prediction of emergence of high level fluoroquinolone resistance.

Keywords

Streptococcus pyogenes; pediatric isolates; M/*emm* type; fluoroquinolones; resistance; point mutation

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Introduction

In contrast to *S. pneumoniae*, *S. pyogenes* remains uniformly susceptible to penicillin despite intensive exposure of the pathogen to the agent, and penicillin remains the drug of choice for the pathogen. For this reason, susceptibility testing of *S. pyogenes* isolates is not routinely performed. In the past six years, a few highly fluoroquinolone-resistant isolates of *S. pyogenes* have been reported (Yan et al., 2000b; Reinert et al., 2004; Richter et al., 2003). Analyses of the *gyrA* and *parC* gene sequences from these organisms indicate that point mutations along the quinolone resistance-determining regions (QRDRs) were the mechanisms for resistance (Yan et al., 2000b; Reinert et al., 2004; Orscheln et al., 2005). Additional studies demonstrated that isolates of *S. pyogenes* with moderate increase in MICs represent from 6–10% among surveyed clinical isolates and most of them were clustered in *M/emm* types 6 and 75 (Orscheln et al., 2005; Alonso et al., 2005; Malhotra-Kumar et al., 2005). However, the isolates of those studies were not exclusively derived from pediatric patients. Fluoroquinolones are not recommended for use in patients under 18 years old; therefore, susceptibility and sequence data from pediatric isolates collected from patients, particularly those of non cystic fibrosis pediatric patients, may provide a more reliable baseline of fluoroquinolone resistance. In this study, *M/emm* type, repPCR, and pulsed-field gel electrophoresis (PFGE) were performed on pediatric isolates collected from two hospitals in a typical metropolitan area in the United States concomitant with susceptibility testing and sequence analysis of *gyrA* and *parC*.

Materials and Methods

Bacterial strains and growth conditions

A total of 49 *S. pyogenes* isolates were collected from Children's Memorial Hospital (CMH), Chicago, IL and 67 isolates were collected from the University of Illinois Medical Center (UIC), Chicago, IL in 2003. Isolates were collected from patients who were 3 months to 16 years of age. None of the isolates were recovered from individuals with cystic fibrosis. All isolates were re-confirmed as *S. pyogenes* using a conventional identification scheme in the Clinical Microbiology Service at the National Institutes of Health.

Antimicrobial susceptibility testing

Screening for resistance to fluoroquinolones was performed by Kirby-Bauer disk diffusion testing on sheep blood Mueller-Hinton agar (Remel, Lenexa, KS) using disks for ciprofloxacin, norfloxacin, levofloxacin, enoxacin, lomefloxacin, gatifloxacin, and ofloxacin (Remel). Isolates with decreased inhibitory zone diameters to any of these antibiotics as compared to the ATCC12344 fluoroquinolone-sensitive control strain were tested for MICs to levofloxacin, ciprofloxacin, and moxifloxacin using Etest (AB Biodisk, Solna, Sweden). Isolates with reduced susceptibility to any of these three antibiotics (as compared to ATCC 12344) were considered to have reduced susceptibility to fluoroquinolones. These three antibiotics were chosen because they are commonly prescribed in the United States, and levofloxacin and moxifloxacin are both approved to treat infections with *S. pyogenes* and multi-drug resistant *S. pneumoniae*. Quality control conditions were based on recommendations of the Clinical and Laboratory Standards Institute (Formerly NCCLS).

DNA sequencing of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes

Genomic DNA preparation, primers for *gyrA* and *parC*, and mutation detection in the QRDRs of the fluoroquinolone-resistant isolates were investigated as described previously (Yan et al., 2000b). Primers for *gyrB* and *parE* were as follows: *gyrB* forward 5'GCGGCTCTTACTCGGGTCATCA (nucleotide location 790–811); *gyrB* reverse 5'TTCTGCGGCATCATCAACTGTAC (1722–1746), *parE* forward 5'-

GCTAGACCTATTGTAGAGAGC (970–991), and *parE* reverse 5'-TTATCCTCGATCCACTGACGG (1802–1823).

Determination of M/*emm* type

M-protein typing was performed at the World Health Organization Collaborating Center for Reference and Research on Streptococci at the University of Minnesota in Minneapolis. DNA sequencing of the M-protein gene (*emm*) was also performed, therefore, the serotype of isolates is referred to as “M/*emm* type”.

Rep-PCR and PFGE Analysis

Rep-PCR was performed according to the manufacturer's instructions using the DiversiLab Microbial Typing System (Spectral Genomics, Inc. Houston, TX). Briefly, DNA was extracted using the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA). Rep-PCR was set up using the DiversiLab Streptococcus DNA Fingerprinting Kit and amplified on an M-J thermacycler model PTC-200 (MJ Research, Inc., Watertown, MA). The DNA fragments were separated, detected and analyzed with the DiversiLab DNA Chip and the web-based Agilent 2100 Bioanalyzer version 2.1 (Agilent Technologies, Palo Alto, CA).

Genomic DNA for PFGE was extracted using a lysis solution that consisted of 1M NaCl, 100 mM EDTA (pH 7.5), 0.5% Brij-58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 2 mg/ml lysozyme, 25 U/ml mutanolysin, 6 mM Tris-HCl (pH 7.6), and 20 µg/ml RNAase. The proteolytic solution contained 0.5 M EDTA (pH 9.0–9.5), 1% sodium lauroyl sarcosine, and 150 µg/ml proteinase K. Plugs were then digested overnight with 10 U of SmaI (Invitrogen, Grand Island, NY) or 20 U of SgrAI (New England Biolabs, Beverly, MA). DNA fragments were separated in a 1% gel (Pulse Field Certified Agarose, Bio-Rad Laboratories, Richmond CA) on a Bio-Rad Chef-DR 111 System in 0.5 TBE (pH 8.0) at a 120° fixed angle, fixed voltage of 6 V/cm at 12° C for 24 hours with pulse time intervals of 5 to 35 sec. for the SmaI gel, and 5 to 40 sec. for the SgrAI gel. The gel image was captured and the PFGE patterns analyzed on the Kodak Image Station 2000R (Eastman Kodak Co., Rochester, NY).

Results

A total of 10 (9%) isolates of *S. pyogenes* had reduced susceptibility to one or more fluoroquinolone antibiotics. Table 1 summarizes the MICs and point mutations and compares the mutations in the QRDRs of the *gyrA* and *parC* identified in these isolates with previously reported *S. pyogenes* fluoroquinolone resistant isolates. The most common mutation detected was in the *parC* gene with a predicted amino acid change from serine to alanine at position 79, which is different from the mutation at this location in our previously reported highly-resistant isolate NIH-R01 (an M/*emm* type 2) where it carried a change from serine to tyrosine. Isolate CMH 47 (M/*emm* type 2) had reduced zones of inhibition by disc diffusion, but Etest confirmation indicated susceptibility to all three fluoroquinolones. Interestingly, this isolate did have a mutation in *gyrA* indicating a switch from methionine 99 to leucine, but had no mutation detected in *parC*. All 9 M/*emm* type 6 isolates had the serine-79 to alanine substitution in *parC* reported by Orescheln et al. (2005). The five isolates with Moxifloxacin at MIC of 0.5 µg/ml and NIH-R01 with moxifloxacin MIC of 4.0 µg/ml carried a mutation that translates to a switch from methionine-99 to leucine in *gyrA* in addition to the mutation in *parC*. Despite having an MIC of only 0.125 µg/ml, our previously reported isolate NIH-R02 (an M/*emm* 3) also had both of these mutations (Yan et al., 2000a). No mutations were detected in the QRDRs of *gyrB* and *parE* among the isolates studied.

We found that repPCR gave no more information than M/*emm* type data. Isolates with the same M/*emm* type had the same repPCR patterns. PFGE provided more data and was able to

distinguish unique strains among the same M/emm type. Although PFGE using SgrAI and SmaI grouped the isolates similarly, band patterns with SgrAI provided slightly more discriminatory power. Figure 1 shows the SgrAI band patterns of the fluoroquinolone resistant isolates. The nine M/emm type 6 isolates were separated into two distinct PFGE strain types. PFGE strain type A included 4 isolates from CMH and two from UIC. Four of the PFGE strain type A isolates (CMH21, CMH38, UIC62, UIC91) had mutations in both *parC* and *gyrA*.

The three PFGE strain type B isolates (M/emm type 6) were all from UIC and none of these had a mutation in *gyrA*. The M/emm type 2 isolate from Chicago, IL (CMH47) had a different PFGE pattern from the M/emm type 2 isolate from Bethesda, MD (NIH-R01).

Discussion

The 9 % prevalence of *S. pyogenes* with reduced susceptibility to fluoroquinolones from Chicago, IL in this report is similar to the 10.9% reported for isolates from St. Louis, MO (Orscheln et al., 2005) yet higher than the 3.4% incidence reported for isolates from Spain (Pérez-Trallero et al., 2001) and 5.4% reported from Belgium (Malhorta-Kumar et al., 2005), suggesting that the baseline of fluoroquinolone susceptibility in pediatric isolates of *S. pyogenes* has no major difference from adult or age mixed patient populations. Although M/emm type 6 is only responsible for 3–14 % of *S. pyogenes* infections (Alonso et al., 2005; Pérez-Trallero et al., 2001; Creti et al., 2007), it is the predominant isolate in surveys of fluoroquinolone resistance in *S. pyogenes* due to a polymorphism in *parC* (Orscheln et al., 2005). Our data from pediatric isolates of *S. pyogenes* confirmed this M/emm 6 predominance finding. However, it is interesting to note that although M/emm 6 isolates have intrinsic fluoroquinolone resistance, the highly resistant isolates reported in the literature have been M/emm types 2, 12, and 89 rather than M/emm type 6 (Yan et al., 2000b; Reinert et al., 2004; Richter et al., 2003).

Mutations in naturally arising resistant isolates of *S. pyogenes* occur in the QRDR of *gyrA* at Ser-81 with or without replacement at Met-99 and in the QRDR of *parC* at Ser-79 with or without change at Ala-121 (Yan et al., 2000b; Richter et al., 2003; Yan et al., 2000a; Alonso et al., 2002). The point mutation patterns among the pediatric isolates are similar to those previously documented in adult isolates and somewhat similar to those key residue replacements in *S. pneumoniae* but with much less variety of amino acid substitutions (Boos et al., 2001; Janoir et al., 1996). By comparison with published sequence data from naturally occurring and laboratory generated fluoroquinolone-resistant isolates, a significant increase in MIC appears to require mutations in both *parC* and *gyrA*, especially in residue serine-79 and serine-81, respectively, which are compatible to the development of fluoroquinolone resistance in *S. pneumoniae* (Boos et al., 2001; Janoir et al., 1996; Billal et al., 2007).

Previous surveys reporting the occurrence of the serine-79 to alanine substitution in *parC* of M/emm type 6 isolates found no mutations in *gyrA* leading to an amino acid substitution (Orscheln et al., 2005; Alonso et al., 2005; Malhotra-Kumar et al., 2005). We found 4 M/emm type 6 isolates that had both the inherent polymorphism in *parC* and a mutation in *gyrA* that results in a methionine-99 to leucine substitution. Three of these 4 isolates (CMH 21, CMH 38, and UIC 91) had an MIC to levofloxacin at 4.0 µg/ml (Table 1). Our data confirms the results reported by others that M/emm 6 isolates are not all from the same clone despite the consistent finding of the polymorphism in the QRDR of *parC* at Serine-79. Our isolates of M/emm 6 could be divided into two strains based on PFGE analysis with *SgrAI*, Alonso et al. reported 4 PFGE patterns with *SmaI*, and Albertí et al. reported 7 PFGE patterns with *SfiI* (Alonso et al., 2005; Alberti et al., 2003). We evaluated the utility of repPCR for strain typing of *S. pyogenes* and found that this method provided no more information than M/emm type data.

The findings from this work and others (Orscheln et al., 2005; Alonso et al., 2005; Malhotra-Kumar et al., 2005; Alberti et al., 2005) that *S. pyogenes* M/emm type 6 is the most reported serotype with reduced susceptibility to fluoroquinolones may warrant further studies. Although a polymorphism in the QRDR of the *parC* gene may explain the findings, concerns remain as to whether the intrinsic reduced susceptibility to fluoroquinolones in M/emm 6 concomitant with the pre-existing point mutation in *parC* would make these isolates more likely to become highly resistant clones following an increased use of fluoroquinolone agents with time (Orscheln et al., 2005). Malhotra-Kumar et al. (2005) concluded that the *parC* mutations in their fluoroquinolone-non-susceptible M/emm 6 strains do not seem to confer a significant fitness cost to the organism. We recently demonstrated that despite its intrinsic polymorphism in the QRDR of *parC*, *S. pyogenes* emm type 6 is not more likely to develop high-level resistance to fluoroquinolones after invitro induction and selection of fluoroquinolone-resistant mutants when compared to other emm types (Billal et al., 2007). Future studies are needed to explore other possible mechanisms that can confer fluoroquinolone-resistance to *S. pyogenes*, such as an activated efflux pump. In addition, it would be interesting to know the relative fitness of isolates with reduced susceptibility to fluoroquinolones compared to isolates that remain sensitive.

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Figure 1. Pulsed-Field Gel Electrophoresis (PFGE) band patterns using SgrAI performed on 13 *S. pyogenes* isolates with reduced susceptibility to fluoroquinolones and/or *gyrA*/*parC* mutations.

Table 1

Minimum inhibitory concentration of levofloxacin, ciprofloxacin, and moxifloxacin and mutations in *gyrA* and *parC* genes for *S. pyogenes* isolates from pediatric patients in comparison with previous reported isolates.

Isolate	M/ <i>emm</i> type	Levo	Susceptibility (µg/ml)			gyrA	Mutation	parC
			Cipro	Moxi				
This study								
ATCC 12344	1	0.25	0.25	0.125		none		none
CMH 21	6	4	4	0.5		Met99-Leu		Ser79-Ala
CMH 25	5/24/44	4	4	0.5		Met99-Leu		Ser79-Ala
CMH 38	6	4	4	0.5		Met99-Leu		Ser79-Ala
CMH 52	6	2	2	0.25		none		Ser79-Ala
CMH 47	2	0.25	0.25	0.125		Met99-Leu		none
CMH 60	6	2	2	0.25		none		Ser79-Ala
UIC 33	6	2	2	0.25		none		Ser79-Ala
UIC 62	6	2	2	0.5		Met99-Leu		Ser79-Ala
UIC 78	6	2	2	0.25		none		Ser79-Ala
UIC 79	6	2	2	0.25		none		Ser79-Ala
UIC 91	6	4	2	0.5		Met99-Leu		Ser79-Ala
Summary of previous reports								
References	# of isolates	Levo	Cipro	Moxi		gyrA		parC
NIH-R01, Yan, et al., 2000b	1	≥ 32	≥ 32	4		Ser81-Phe Met99-Leu		Ser79-Tyr
NIH-R02, Yan, et al., 2000a	3	4	4	0.125		Met99-Leu		Ser79-Ala
Richier, et al., 2003	1	16	≥ 32	2		Ser81-Tyr		Ser79-Phe, Ala121-Val
Reinert, et al., 2004	1	≥ 32	≥ 32	4		Ser81-Phe		Ser79-Phe, Asp91-Asn, Ser140-Pro
Alonso, et al., 2005	1	S	> 2	NA		None		Ser79-Ala
Alberti, et al., 2005	30	N/T	4	NA		None		Ser79-Ala or Ser79Phe; Asp91Asn
Orschem, et al., 2005	42	2-4	2-8	0.5		None		Ser79-Ala, Ser79-Phe, or Ser79-Tyr; Asp91-Asn, Ala121-Val, Gly128-Asp, or Ser140-Pro,
Malhotra-Kumar, et al., 2005	55	1-4	2-8	1.0		None		

Note: Levo: levofloxacin; cipro: ciprofloxacin; moxi: moxifloxacin; and NA: not available.