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PCR-Oligonucleotide Ligation Assay for Detection of Point Mutations Associated with Quinolone Resistance in *Streptococcus pneumoniae*

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We have developed a PCR-oligonucleotide ligation assay to rapidly identify base substitutions in topoisomerase genes that are associated with quinolone resistance in clinical isolates of *Streptococcus pneumoniae*. Thirty-seven strains for which the ciprofloxacin MICs were ≥4 μg/ml and 16 strains for which the MICs were ≤2 μg/ml were assayed. Compared with sequence data, the assay correctly identified the DNA bases that encoded amino acids at the four positions most commonly associated with quinolone resistance (Ser79 and Asp83 of ParC and Ser81 and Glu85 of GyrA). Therefore, this procedure can rapidly distinguish single base substitutions associated with quinolone-resistant topoisomerases in *S. pneumoniae*.

Streptococcus pneumoniae is a major pathogen causing acute bacterial exacerbations of chronic bronchitis, acute otitis media, meningitis, and community-acquired pneumonia. Newer quinolones such as levofloxacin, moxifloxacin, and gatifloxacin have enhanced activity against pneumococci compared with older quinolones and are used to treat infections caused by this organism. Studies show that the mechanism of action of quinolones is inhibition of the essential bacterial type II topoisomerases, DNA gyrase and topoisomerase IV (3, 10, 13). Amino acid substitutions in the quinolone resistance determining regions (QRDRs) of these enzymes are associated with resistance to quinolones in *S. pneumoniae* (2, 4, 7, 10). It has also been suggested that active efflux contributes to resistance against quinolones (1, 14).

Methods to identify mutations in topoisomerases are needed since the increased use of quinolones in the clinic may increase the prevalence of resistance. We developed a rapid assay to detect point mutations in the genes that code for two of the topoisomerase subunits, GyrA and ParC. This assay utilized PCR to amplify the DNA that codes for the QRDRs of the two subunits. The oligonucleotide ligation assay (OLA) was then used to identify specific base changes in the amplified product by colorimetric detection. In PCR-OLA, a 5′-biotinylated capture probe, in which the 3′ end is the base of interest, is annealed to the target sequence. A reporter probe labeled at the 3′ end with digoxigenin is annealed to the target sequence at the next base. Ligation of the two probes occurs when there is correct base pairing between the 3′ end of the capture probe and the target, resulting in a single oligonucleotide having a biotin group at the 5′ end and digoxigenin at the 3′ end, which is then captured on a solid surface. Ligation is reported by the colorimetric alkaline phosphatase assay after reaction of an antidigoxigenin antibody conjugated to alkaline phosphatase. When a base mismatch between the 3′ end of the capture probe and the target sequence exists, ligation does not occur; therefore, only the digoxigenin-free capture probe binds the solid surface. The PCR-OLA identified point mutations in DNA for a variety of diseases such as sickle cell anemia (9) and cystic fibrosis (9) as well as for macrolide resistance in *Helicobacter pylori* (12). OLA requires approximately 12 h to analyze two target sequences compared with automated DNA sequencing, which requires about 33 h to complete.

MICs were determined by the NCCLS broth microdilution method (8). Genomic DNA was extracted from *S. pneumoniae* as previously described (12). All oligonucleotides were constructed by Sigma-Genosys (The Woodlands, Tex.). Oligonucleotides used as PCR primers flanked the sequences coding for the QRDRs and were derived from the known sequences of the *gyrA* gene (GenBank accession no. NC_003028) and the *parC* gene (GenBank accession no. Z67739). The primers were 5′-TGGGTTGAAGCCGGTTCA and 5′-TGCTGGCAAGA CCGTTGG, which amplified bases 105 to 452 of the structural gene for GyrA, and 5′-CCGTCGCATTCTTTACG and 5′-AGTTGCTCCATTAACCA, which amplified bases 129 to 491 of the structural gene for ParC. Oligonucleotide capture probes (Table 1) were modified with a 5′ biotin group by incorporation of biotin-labeled phosphoramidite. Oligonucleotide reporter probes (Table 1) were modified by phosphorylation of the 5′ end and digoxigenin labeling of the 3′ end at Sigma-Genosys by proprietary methods.

PCR amplification was performed under the following conditions with SuperMix (Gibco-BRL, Gaithersburg, Md.). Thermocycling conditions for *parC* were 40 cycles of 95°C for 3 min, 94°C for 15 s, 52°C for 30 s, and 72°C for 45 s. Cycling conditions for *gyrA* were 40 cycles of 95°C for 3 min, 94°C for 15 s, 45°C for 30 s, and 72°C for 30 s. Amplified products were analyzed by electrophoresis in ethidium bromide-stained 2% agarose gels for confirmation that the products, 347 bp of *gyrA* and 362 bp of *parC*, were amplified. Ligation reactions (in 20-μl mixtures) and detection of ligated products were performed as described previously (12) except that reactions were conducted in a model 9600 DNA thermocycler (Perkin-Elmer, Foster City, Calif.) for 10 cycles of 94°C for 30 s and 52°C for 5 min. Amplified products were cycle sequenced with the Big Dye terminator cycle sequencing kit (Applied Biosystems, Fos-

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TABLE 1. Ligation probes for OLA (5′ to 3′)

Protein	Position	Target amino acid[b]	Codon	Ligation probe[a]	
				Capture	Reporter
ParC	79	Ser	TCT	TTCCACCCACACGGGGATTC	TTCTATCTATGATGCCATGGTTC
		Tyr	TAT	TTCCACCCACACGGGGATTA	TTCTATCTATGATGCCATGGTTC
		Phe	TTT	TTCCACCCACACGGGGATTT	TTCTATCTATGATGCCATGGTTC
		Ala	GCT	TTCCACCCACACGGGGATGC	TTCTATCTATGATGCCATGGTTC
	83	Asp	GAT	CGGGGATTCTTCTATCTATG	ATGCCATGGTTCGTATGTCT
		Tyr	TAT	CGGGGATTCTTCTATCTATT	ATGCCATGGTTCGTATGTCT
		Asn	AAT	CGGGGATTCTTCTATCTATA	ATGCCATGGTTCGTATGTCT
		His	CAT	CGGGGATTCTTCTATCTATC	ATGCCATGGTTCGTATGTCT
		Gly	GGT	CGGGGATTCTTCTATCTATG	GTGCCATGGTTCGTATGTCT
		Val	GTT	CGGGGATTCTTCTATCTATG	TTGCCATGGTTCGTATGTCT
GyrA	84	Ser	TCC	TATCACCCACACGGGGATTC	CTCTATTTATGAAGCCATGGTCC
		Phe	TTC	TATCACCCACACGGGGATTT	CTCTATTTATGAAGCCATGGTCC
		Ala	GCC	TATCACCCACACGGGGATGC	TCTATTTATGAAGCCATGGTCC
		Tyr	TAC	TATCACCCACACGGGGATTA	TCTATTTATGAAGCCATGGTCC
		Cys	TGC	TATCACCCACACGGGGATTG	TCTATTTATGAAGCCATGGTCC
	88	Glu	GAA	ACACGGGGATTACTCTATTTATG	AAGCCATGGTCCGTATGGCT
		Lys	AAA	ACACGGGGATTACTCTATTTATA	AAGCCATGGTCCGTATGGCT
		Gly	GGA	ACACGGGGATTACTCTATTTATG	GAGCCATGGTCCGTATGGCT

[a] Capture probes are labeled with biotin on the 5′ end. Reporter probes are phosphorylated on the 5′ end and labeled with digoxigenin on the 3′ end.
[b] For each position, the wild-type amino acid is given first.

ter City, Calif.) on an automated sequencer (model 373A; Applied Biosystems).

A total of 53 *S. pneumoniae* strains from recent Abbott-sponsored clinical trials were analyzed by PCR-OLA and DNA sequence analysis (Table 2). The ciprofloxacin MICs for 16 of the 53 were ≤2 μg/ml, and those for the remaining 37 were ≥4 μg/ml. Thirty of the 37 *S. pneumoniae* strains for which the ciprofloxacin MICs were ≥4 μg/ml had at least one amino acid substitution either in ParC at Ser79 or Asp83 or in GyrA at Ser81 or Glu85. A strain for which the ciprofloxacin MIC was 32 μg/ml had a single amino acid substitution, Ser81 to Phe (Ser81-Pro), in GyrA. All 16 strains for which the MICs were ≤2 μg/ml were wild type for both *parC* and *gyrA*. Four strains for which the ciprofloxacin MICs were 4 μg/ml, two strains for which the ciprofloxacin MICs were 8 μg/ml, and one strain for which the ciprofloxacin MIC was 16 μg/ml were wild type for both *parC* and *gyrA*.

Compared with sequence data from the same regions, PCR-OLA correctly identified DNA sequences that code for the amino acids at four QRDR positions most frequently associated with quinolone susceptibility or resistance in 53 strains (Table 2), indicating that the OLA is a valid method for detecting mutations associated with quinolone resistance. For the seven strains without QRDR mutations for which the ciprofloxacin MICs were 4 to 16 μg/ml the gatifloxacin and moxifloxacin MICs were less than 1 μg/ml. Therefore, efflux could be responsible for the higher MICs. Alternatively, mutations outside the amplified region could be present.

Other amino acid substitutions can also contribute to decreased susceptibility to quinolones. For instance, Ser80-Pro (5), Lys137-Asn, and Ala115-Pro (2) in ParC, Asp435-Asn (5) in ParE, and Glu474-Lys (11) in GyrB have been suggested. We did not have clinical isolates containing these mutations in this study. Therefore, we constructed oligonucleotides (approximately 40-mers) from the GenBank sequence that repre-

TABLE 2. Ciprofloxacin MICs and OLA results for 53 strains of *S. pneumoniae*

No. of strains	Ciprofloxacin MIC (μg/ml)	Amino acid substitution[b] at indicated position in:				Result[a]
		ParC		GyrA		
		79	83	81	85	
5	0.5	+	+	+	+	Yes
5	1	+	+	+	+	Yes
6	2	+	+	+	+	Yes
4	4	+	+	+	+	Yes
2	4	Phe	+	+	+	Yes
1	4	+	Asn	+	+	Yes
2	8	+	+	+	+	Yes
1	8	Phe	+	+	+	Yes
1	8	Phe	+	Phe	+	Yes
1	8	+	Asn	+	+	Yes
1	16	+	+	+	+	Yes
1	16	Phe	+	Phe	+	Yes
1	16	Phe	+	+	Lys	Yes
1	16	Tyr	+	Ala	+	Yes
1	16	+	Tyr	Phe	+	Yes
3	16	Tyr	+	Phe	+	Yes
1	16	Tyr	+	Tyr	+	Yes
1	16	Tyr	+	+	+	Yes
1	32	+	+	Phe	+	Yes
1	32	Tyr	+	Phe	+	Yes
1	32	Tyr	+	+	Lys	Yes
3	32	Phe	+	Phe	+	Yes
1	32	Tyr	+	+	Lys	Yes
1	32	+	Tyr	Phe	+	Yes
1	32	Tyr	+	Tyr	+	Yes
2	64	Phe	+	Phe	+	Yes
1	64	Phe	+	Tyr	+	Yes
1	64	Tyr	+	Phe	+	Yes
1	64	Tyr	Val	Phe	+	Yes
1	64	+	Asn	Tyr	+	Yes

[a] Yes, PCR-OLA in agreement with sequence results.
[b] +, wild-type amino acid.

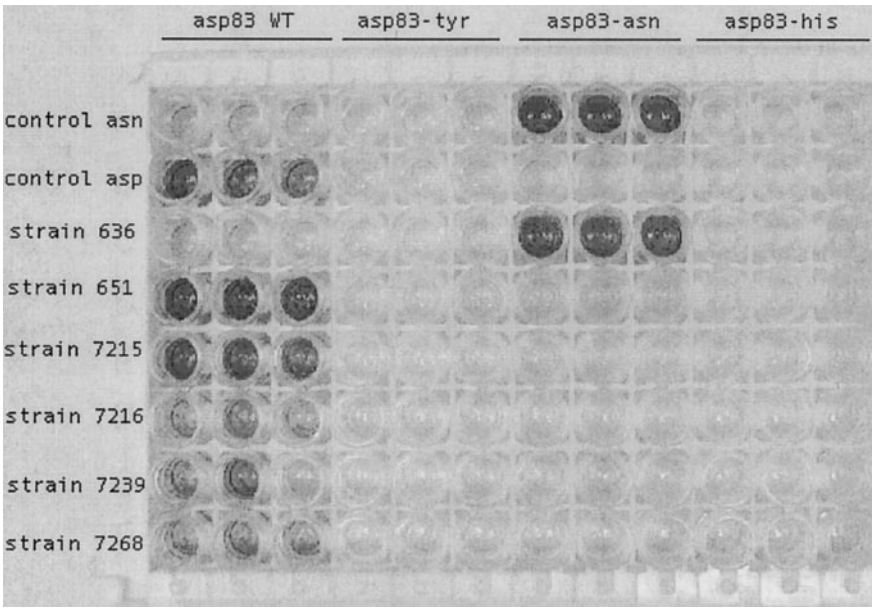

FIG. 1. PCR-OLA identification of quinolone resistance mutations in *parC* of *S. pneumoniae*. Shown is the 96-well colorimetric reaction for several strains of *S. pneumoniae*. Included were triplicate samples of strains with wild-type (WT) ParC (Asp83) and ParC with Asp83-Tyr, Asp83-Asn, and Asp83-His mutations. Top row, positive control for Asp83-Asn mutation; second row, positive control for WT Asp83; third to eighth rows, various strains of *S. pneumoniae*.

sented a wild-type sequence and a mutant sequence at these sites to serve as surrogate templates, and we synthesized the corresponding capture probes and reporter probes. The PCR-OLA identified the correct DNA sequence when used with the oligonucleotide templates that represented rarer mutations associated with quinolone resistance as well as more common mutations (data not shown). Thus, synthetic templates can be used to validate any mutation of interest prior to screening clinical isolates.

The OLA readily identified the bases at key QRDR sites in 51 of 53 strains. It gave a high signal-to-noise ratio for each targeted sequence (Fig. 1 and Table 3). Although PCR products were obtained from the remaining two isolates, they produced very weak signals at Ser81 and Glu85 for wild-type GyrA by PCR-OLA. Polymorphisms associated with the target sequence near the amino acid of interest likely prevented proper annealing and ligation of the probes. Based on the actual sequences of these strains, new capture and reporter probes that gave a higher positive signal in the OLA were constructed (data not shown). If limited polymorphisms at critical sites such as those seen with these two strains are prevalent in clinical isolates, pools containing equal molar amounts of modified probes specific for each polymorphic allele could be used to increase the likelihood of a strong positive signal. Alternatively, if polymorphisms resulting in failed or weak OLA reactions are rare, direct DNA sequencing could be used instead; in this study, sequencing would have been done on only 2 of 106 (2%) target sequences.

We used a single colorimetric detection system for all mutations of interest in separate reactions (Fig. 1). An alternative would be the development of a multiplex method using multicolor probes or fluorescently labeled probes with different excitation wavelengths to detect multiple mutations on a single PCR product and significantly increase the throughput of the assay. For example, multiplex OLA was used to genotype six different myostatin mutations in cattle (6). Automation can also be applied to screen multiple samples such as large collections of isolates from clinical trials or surveillance studies. In conclusion, the PCR-OLA is a rapid method that can discriminate single base pair mutations in DNA. In this study, we validated that PCR-OLA is an accurate method for identifying

TABLE 3. Ranges of optical densities for 53 isolates tested for *gyrA* and *parC* genes by PCR-OLA

Protein	Amino acid[a]	*n*[b]	OD_{492} range[c]
ParC	Negative (Ser79)	106	0.192–0.379
	Ser79 (wt)	29	0.746–1.896
	Phe79	12	0.676–1.650
	Tyr79	12	1.016–1.971
	Negative (Asp83)	159	0.052–0.120
	Asp83 (wt)	47	0.587–1.761
	Tyr83	2	1.287–1.420
	Val83	1	1.350–1.412
	Asn83	3	0.722–1.260
GyrA	Negative (Ser81)	159	0.073–0.493
	Ser81 (wt)	32	1.268–1.887
	Phe81	16	1.225–1.972
	Ala81	1	1.220–1.224
	Tyr81	4	1.235–1.986
	Negative (Glu85)	53	0.142–0.241
	Glu85 (wt)	50	0.486–1.882
	Lys85	3	0.715–0.902

[a] Negative, all negative reactions at indicated position; wt, wild type.
[b] *n*, number of target sequences.
[c] OD_{492}, optical density at 492 nm.

mutations associated with quinolone resistance in *S. pneumoniae*.

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