Emergence of Quinolone-Resistant *Bordetella pertussis* in Japan[▽]

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Six *Bordetella pertussis* strains isolated from children in Japan from 2004 to 2006 showed high-level resistance to nalidixic acid (NAL; MIC, >256 µg/ml) and decreased susceptibilities to fluoroquinolones. All of the NAL-resistant strains had the same D87G mutation in *gyrA*.

Pertussis is an acute respiratory tract infection caused by *Bordetella pertussis* that is particularly serious for neonates and infants (2, 5, 19). Although the introduction of whole-cell and acellular vaccines caused a drastic decrease in the incidence of pertussis globally compared with that in the prevaccine era, developed countries have experienced a marked increase over the past 15 years (2). There has also been a recent shift in the age distribution of pertussis patients to adults and adolescents, an unrecognized but significant source of infection for neonates and infants (2, 5, 9, 19). Macrolides are widely used for antimicrobial treatment and postexposure prophylaxis of pertussis (2, 5). However, several erythromycin-resistant strains of *B. pertussis* have emerged in the United States (13, 14, 20) and alternative therapeutic agents are being sought to combat these strains. Several fluoroquinolones demonstrate excellent in vitro activity against *B. pertussis* (1, 3, 8, 11, 16, 22), and although contraindicated for children (1, 16), they might be candidate agents to treat adults with pertussis.

However, as we found six nalidixic acid (NAL)-resistant *B. pertussis* strains isolated from 2004 to 2006 in Japan by disk diffusion test (no inhibitory zone detected around a 30-µg NAL disk on Bordet-Gengou agar), the MICs of erythromycin, NAL, norfloxacin, ciprofloxacin, sparflaxacin, levofloxacin, gatifloxacin, and chloramphenicol were determined by Etest (AB Biodisk, Solna, Sweden) on Bordet-Gengou agar (7, 10). The quality control strains used were *B. pertussis* CCUG 30837^T (= ATCC 9797^T) and *Staphylococcus aureus* ATCC 29213 (10). Strains for which the MICs of erythromycin and NAL were less than or equal to 0.12 and 32 µg/ml were considered susceptible (4, 10). Whole sequences of *gyrA*, *gyrB*, *parC*, and *parE* of the NAL-resistant strains were determined by PCR and direct sequencing. To prepare template DNA, one loopful colony of each strain was resuspended in 100 µl of 20 mM Tris–2 mM EDTA buffer (pH 7.5), incubated at 100°C for 15 min, and then centrifuged for 5 min at 23,200 × g. The 50-µl reaction mixture contained 2 µl of the supernatant fluid, 25 pmol of each primer, a 0.4 mM concentration of each deoxynucleoside triphosphate, 2.5 U of TaKaRa LA-*Taq* poly-

merase (TaKaRa, Shiga, Japan), and LA-*Taq* GC buffer I (TaKaRa). After an initial denaturation at 95°C for 5 min, amplification proceeded for 30 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 2 min 30 s, with a final 10-min extension at 72°C. Following cleanup with a QIAquick PCR purification column (Qiagen, Valencia, CA), the PCR product was sequenced by using a Big Dye terminator (version 3.1; Applied

TABLE 1. Primers used in this study

Target and primer	Sequence (5' to 3')	Position ^a	Direction
<i>gyrA</i>			
gyrA1 ^b	CTCGGGTTCATCCTTACATA	–40 to –21	Forward
gyrA2	GCATGGCCACCAACATTC	530 to 547	Forward
gyrA3	ACTTCATCGCCATCATCA	1175 to 1192	Forward
gyrA4	TGTACTGGCTGAAGGTGT	1829 to 1846	Forward
gyrA5 ^b	CCGTGCCAGTCCAGCATTTTC	2776 to 2757	Reverse
gyrA6	ACACCTTCAGCCAGTACA	1846 to 1829	Reverse
gyrA7	TGATGATGGCGATGAAGT	1192 to 1175	Reverse
gyrA8	GAATGTTGGTGGCCATGC	547 to 530	Reverse
<i>gyrB</i>			
gyrB1 ^b	ATCTGATCCGCGACACAGAT	–100 to –80	Forward
gyrB2	ATCTTACCAACATCGAG	556 to 573	Forward
gyrB3	GCAAGAGCGTGCTGGAAG	1223 to 1240	Forward
gyrB4	CGAACGACCAAGGCATC	1878 to 1896	Forward
gyrB5 ^b	CACGCGTATCGGCCAACGTC	2568 to 2539	Reverse
gyrB6	GATGCCCTGGTGCGTTTCG	1896 to 1878	Reverse
gyrB7	CTTCCAGCACGCTCTTGC	1240 to 1223	Reverse
gyrB8	CGCGATGTTGGTGAAGAT	573 to 556	Reverse
<i>parC</i>			
parC1 ^b	GTGATCATTCCCAAGCGCG	–128 to –110	Forward
parC2	TGCCCGTGATGCTGCTCA	518 to 535	Forward
parC3	AAGAGTGGGTGGCGTTTC	1148 to 1165	Forward
parC4	CACCACCATGATCGATTC	1806 to 1823	Forward
parC5 ^b	GAACACACCGATGTTGACGA	2414 to 2395	Reverse
parC6	AGATCGATCATGGTGGTG	1823 to 1806	Reverse
parC7	GAAACGCCACCACTCTT	1165 to 1148	Reverse
parC8	TGAGCAGCATCAGGGCA	535 to 518	Reverse
<i>parE</i>			
parE1 ^b	ACTTGTCCGTAAAATGTGCGG	–47 to –28	Forward
parE2	GACGTGATCGAAGCACTG	445 to 462	Forward
parE3	CAAGGGCGTCAAGCTGCT	936 to 953	Forward
parE4	GATCCACGATATTTTCGGT	1410 to 1427	Forward
parE5 ^b	GTACGCATAGGCGGTGAATG	2051 to 2032	Reverse
parE6	ACCGAAATATCGTGGATG	1427 to 1410	Reverse
parE7	AGCAGCTTGACGCCCTTC	953 to 936	Reverse
parE8	CAGTGCTTCGATCAGCTC	462 to 445	Reverse

^a The position of each gene of *B. pertussis* Toham I (GenBank accession number BX640422) is shown.

^b The primers were used for PCR of each gene.

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TABLE 2. Quinolone susceptibilities and *gyrA* QRDR mutations of *B. pertussis* strains

Strain	Date isolated (day/mo/yr)	Patient		Location	MIC (μg/ml) ^a								NAL resistance ^d	Codon 87 ^e of <i>gyrA</i> (amino acid)
		Age (yr)	Sex		NAL	NAL with PAβN	NOR	CIP	SPX	LVX	GAT	CHL		
CCUG 30837 ^T					2	0.25	0.125	0.032	0.016	0.016	0.008	0.5	S	<u>G</u> AC(D); WT ^b
BP101	26/07/2005	6	Male	Oita	2	ND ^c	0.032	0.016	0.008	0.016	0.008	0.25	S	<u>G</u> AC(D); WT
BP106	13/10/2005	0	Male	Oita	1	ND	0.064	0.008	0.004	0.008	0.004	0.25	S	<u>G</u> AC(D); WT
BP109	12/12/2005	0	Unknown	Oita	2	ND	0.064	0.008	0.004	0.004	0.004	0.125	S	<u>G</u> AC(D); WT
BP111	06/01/2006	4	Unknown	Oita	2	ND	0.064	0.016	0.008	0.008	0.008	0.25	S	<u>G</u> AC(D); WT
BP112	22/01/2006	0	Male	Oita	1	ND	0.064	0.008	0.004	0.008	0.004	0.25	S	<u>G</u> AC(D); WT
BP113	08/02/2006	7	Male	Oita	2	0.25	0.064	0.008	0.004	0.008	0.004	0.5	S	<u>G</u> AC(D); WT
BP115	17/02/2006	8	Female	Oita	1	ND	0.064	0.016	0.004	0.008	0.008	0.5	S	<u>G</u> AC(D); WT
BP128	05/04/2007	0	Male	Osaka	1	ND	0.064	0.016	0.008	0.016	0.008	0.25	S	<u>G</u> AC(D); WT
BP58	24/04/2004	0	Male	Osaka	>256	64	1	0.125	0.125	0.064	0.032	0.5	R	<u>G</u> GC(G)
BP99	06/07/2005	0	Male	Oita	>256	32	1	0.125	0.032	0.064	0.032	0.25	R	<u>G</u> GC(G)
BP117	22/04/2006	0	Male	Osaka	>256	64	0.5	0.125	0.064	0.064	0.032	0.25	R	<u>G</u> GC(G)
BP118	04/05/2006	4	Male	Oita	>256	64	0.5	0.125	0.064	0.064	0.032	0.5	R	<u>G</u> GC(G)
BP121	16/05/2006	10	Female	Fukuoka	>256	32	1	0.125	0.064	0.064	0.032	0.25	R	<u>G</u> GC(G)
BP122	17/05/2006	5	Male	Fukuoka	>256	32	1	0.125	0.064	0.064	0.032	0.25	R	<u>G</u> GC(G)

^a NOR, norfloxacin; CIP, ciprofloxacin; SPX, sparfloxacin; LVX, levofloxacin; GAT, gatifloxacin; CHL, chloramphenicol.
^b WT, wild type.
^c ND, not done.
^d S, susceptible; R, resistant.
^e In each case, the amino acid changed is underlined and the amino acid substituted for it is in parentheses.

Biosystems, Foster City, CA). The primers used for PCR and direct sequencing are listed in Table 1.

The results are shown in Table 2. These six strains were erythromycin sensitive but showed high-level resistance to NAL (MIC, >256 μg/ml) and decreased susceptibilities to fluoroquinolones. All of the NAL-resistant strains showed the same mutation in the quinolone resistance-determining regions (QRDRs; e.g., in *Escherichia coli* at positions 67 to 122)

(18, 21) of *gyrA* from aspartic acid to glycine at position 87. *gyrB*, *parC*, and *parE* showed no mutations in the NAL-resistant strains (data not shown). Pulsed-field gel electrophoresis analysis (15) of XbaI-digested DNAs showed different patterns among the NAL-resistant strains tested (Fig. 1).

To our knowledge, this is the first report of quinolone resistance in *B. pertussis*. All of the strains were isolated from children and were genetically and epidemiologically unrelated.

FIG. 1. Pulsed-field gel electrophoresis patterns of XbaI-digested DNAs of NAL-resistant (*) and NAL-sensitive *B. pertussis* strains.

Although fluoroquinolones are not usually prescribed for children, they are widely used to treat respiratory tract infections in adults (3). In the vaccination era, an increasing proportion of pertussis cases occur in adults and adolescents who have lost immunity to *B. pertussis*, and adult pertussis is a likely source of infant pertussis outbreaks (2, 5, 9, 19). Treatment of unrecognized adult pertussis with fluoroquinolones might, therefore, be important for selecting quinolone-resistant *B. pertussis*.

There are three different mechanisms of quinolone resistance: mutations in drug targets such as DNA gyrase or topoisomerase IV, reduced accumulation of quinolones, and the existence of products that protect the microorganism from the lethal effects of quinolones, such as Qnr (17). The most common mutation observed in quinolone-resistant *E. coli* is at position 83 of *gyrA* (18, 21). In our studies, glutamine at position 83 and serine at position 84 are found in *B. pertussis* strains tested instead of serine at position 83 and alanine at position 84 in *E. coli* (data not shown). Since these two amino acids are consistent with the *gyrA* sequences in both the NAL-susceptible and NAL-resistant strains, their amino acids may be conservative and do not affect quinolone resistance in *B. pertussis*. Substitution of aspartic acid at position 87 is the second most commonly observed mutation in clinical isolates of quinolone-resistant gram-negative and gram-positive microorganisms (18). In this study, aspartic acid to glycine at position 87 of *gyrA* is the only substitution observed in all of the six NAL-resistant strains compared to NAL-sensitive strains.

A few studies have examined the two remaining mechanisms of quinolone resistance in *Bordetella* and related genera. Kadlec et al. (12) demonstrated that efflux-mediated resistance to NAL in *Bordetella bronchiseptica* strains was due to a FloR or CmlB1 exporter that could also export chloramphenicol and be inhibited by the efflux pump inhibitor Phe-Arg- β -naphthylamide (PA β N). This study found no differences in the MICs of chloramphenicol against both NAL-resistant and NAL-sensitive *B. pertussis* strains (Table 2). PA β N at 80 μ g/ml (one-fourth of the MIC) could change the MIC of NAL against NAL-resistant strains but similarly decreased the MIC of NAL against NAL-sensitive strains. The presence of Qnr increases the MICs of fluoroquinolones by 16- to 125-fold but affects the MIC of NAL only 2- to 8-fold (17). These quinolone resistance patterns are quite different from those of our NAL-resistant strains. Therefore, the single QRDR mutation in *gyrA* may constitute the main mechanism of quinolone resistance among the strains tested here.

Once a single mutation of *gyrA* occurs in gram-negative bacteria, additional mutations in *gyrA* or *parC* occur more frequently than in wild-type bacteria (6), and such a stepwise accumulation of multiple mutations increases resistance to fluoroquinolones. Continued surveillance of antimicrobial resistance among *B. pertussis* strains is clearly needed to control and eradicate *B. pertussis* transmission.

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