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Mutant Selection Window and Characterization of Allelic Diversity for Ciprofloxacin-Resistant Mutants of *Rhodococcus equi*[▽]

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The mutant prevention concentration (MPC) for ciprofloxacin was determined for two *Rhodococcus equi* strains. The MPC for both strains was 32 μg/ml, which is above the peak serum concentration of ciprofloxacin obtainable by oral administration in humans. Nine single nucleotide changes corresponding to eight amino acid substitutions in the quinolone resistance-determining regions of DNA gyrase subunits A and B were characterized. Only mutants with amino acid changes in Ser-83 of GyrA were highly resistant (≥64 μg/ml). Our results suggest that ciprofloxacin monotherapy against *R. equi* infection may result in the emergence of ciprofloxacin-resistant mutants.

Rhodococcus equi, a facultative intracellular Gram-positive coccobacillus phylogenetically related to the genus *Mycobacterium*, is a known cause of suppurative pneumonia and ulcerative enteritis in 1- to 3-month-old foals (24). *R. equi* is also known as an important pathogen of immunosuppressed human patients (27). Recently, fluoroquinolones have been used in humans and have also been used on an experimental basis to treat horses for *R. equi* infection (8, 17, 25). Although the majority of clinical isolates of *R. equi* from previous surveillance studies were reported to be susceptible to fluoroquinolones (1, 16, 21), treatment failures and fluoroquinolone-resistant isolates have been reported (12, 19, 22).

The development of bacterial resistance has been hypothesized to be due in part to the use of antibiotics at therapeutic doses that usually block only the growth of susceptible wild-type cells (5, 6). However, under these conditions, the concentration may be too low to eliminate the small numbers of spontaneous drug-resistant mutants in the population of infecting bacteria. Drug-resistant mutants in the infecting population were hypothesized to be enriched during growth within a drug concentration range known as the mutant selection window (MSW) (5, 6). The lower boundary of the MSW is the MIC_{99} of the drug. The upper boundary consists of the mutant prevention concentration (MPC), defined as the drug concentration that inhibits the growth of the subpopulation of cells that are the least susceptible due to spontaneous single-step mutants in the population. Experimentally, MPC is the lowest drug concentration that prevents mutant bacterial colony formation from $\geq 10^{10}$ CFU (4). This suggests that maintaining antibiotic concentrations above the MPC throughout therapy may reduce the chance for enrichment of single-step resistance mutants and selection of high-level resistance. A higher level of resistance would be a rare event since it would require the acquisition of two or more spontaneous mutations by bacterial cells.

Niwa et al. (20) previously reported on the association between fluoroquinolone resistance and a single amino acid substitution in the quinolone resistance-determining region (QRDR) of DNA gyrase subunit A gene (*gyrA*) of *R. equi.* However, susceptibility was determined only at a single drug concentration, and the diversity of the mutant population was not examined over a variable range of ciprofloxacin concentrations using a large number of cells. Therefore, in order to determine the risk of selecting for ciprofloxacin resistance and to correlate mutations with a resistance phenotype in *R. equi*, we determined the MPC and characterized the diversity of ciprofloxacin-resistant mutants by DNA sequence analysis of the QRDR in the target genes encoding DNA gyrase subunit A (*gyrA*) and subunit B (*gyrB*).

R. equi strain W5234 was isolated from an HIV-positive patient, and *R. equi* strain ATCC 6939^T was isolated from a lung abscess of a foal (14). *R. equi* cells were grown in brain heart infusion broth (Becton Dickinson and Co., LePont de Claix, France) for 48 h at 35°C with vigorous shaking, harvested by centrifugation, and then resuspended in 20 ml of fresh brain heart infusion broth. The numbers of CFU/ml were determined by plating serial 10-fold dilutions on drug-free Columbia blood agar base plates (Becton Dickenson and Co.). At least 1×10^{10} cells were applied onto five Columbia blood agar base plates containing various concentrations of ciprofloxacin (USP, Rockville, MD). The agar plates were examined for the emergence of ciprofloxacin-resistant colonies after incubation at 35°C for 4 days. The MIC_{99} was determined after individual ciprofloxacin-resistant colonies were counted. The fraction of resistant colonies recovered from the agar plates was plotted against the drug concentration, and the MPC was determined. The MICs of ciprofloxacin for parent strains and mutants were determined by the broth microdilution method using cation-supplemented Mueller-Hinton broth (Difco, Detroit, MI) according to the guidelines of the current NCCLS/CLSI M24-A standard (2). At least two ciprofloxacin-resistant mutants were selected at each concentration. Mutants were confirmed by a combination of two passages on an agar plate

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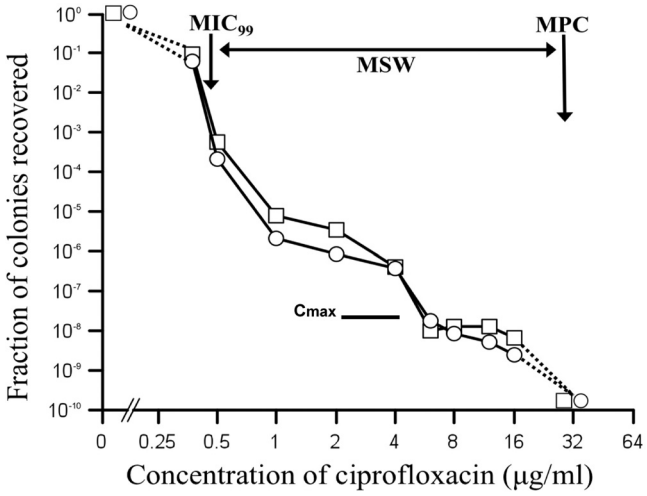

FIG. 1. Effect of ciprofloxacin concentration on recovery of single-step ciprofloxacin-resistant mutants. *R. equi* ATCC strain W6939^T (open squares) and *R. equi* strain W5234 (open circles) were applied to Columbia blood agar base agar plates supplemented with ciprofloxacin and then incubated for 4 days. Cell survival is shown as the fraction of ciprofloxacin-resistant colonies relative to the number of CFU applied to ciprofloxacin-free plates. The small downward arrow indicates the MIC_{99}, whereas the larger downward arrow indicates the mutant prevention concentration (MPC) for ciprofloxacin. The horizontal double arrow shows the mutant selection window. C_{max} is denoted by the thick horizontal line.

whose drug concentration was the same as that upon which the mutant was selected and one passage on a drug-free agar plate. Each experiment was carried out twice.

Figure 1 shows the MSW of ciprofloxacin for two *R. equi* strains (double arrow). The number of colonies recovered dropped gradually until reaching a plateau at approximately 1 to 4 μg/ml and then dropped a second time at higher drug concentrations. The MIC, MIC_{99}, and MPC (Fig. 1, vertical arrows) of both W5234 and ATCC 6939^T were 0.5, 0.42, and 32 μg/ml, respectively. In comparison, the C_{max} for ciprofloxacin, the maximum concentration that can be achieved in serum, is 2.5 to 4.4 μg/ml (4, 13). The AUC_{24}, the area under the concentration-time curve from 0 h to 24 h, is 24 μg · h/ml (26). The AUC_{24}/MPC and AUC_{24}/MIC ratios for both strains of *R. equi* were 0.75 h and 48 h, respectively. In comparison, an increase in resistance was observed when the AUC_{24}/MIC ratio ranged between 20 and 150 h in a rabbit model of infection by *Staphylococcus aureus* (3), and the greatest increase in fluoroquinolone resistance was observed at an AUC_{24}/MIC ratio of 24 to 62 h (7). With fluoroquinolones, an AUC_{24}/MPC ratio ranging between 20 and 70 restricts the emergence of resistance mutants (6). The low AUC_{24}/MPC ratio of 0.75 obtained for both isolates of *R. equi* again indicates the potential to enrich the mutant population. Together, our results suggest that monotherapy using ciprofloxacin against *R. equi* infection may be of high risk for the emergence of ciprofloxacin-resistant mutants since the obtainable therapeutic doses clearly remain within the MSW. Similarly, resistance of *Mycobacterium tuberculosis* during levofloxacin monotherapy has been reported (9, 23). These results indicate that careful use of fluoroquinolones for the treatment of infection with *R. equi* and monitoring of MICs during the administration of ciprofloxacin should be considered.

To determine the correlation between ciprofloxacin susceptibility and genetic variation, the parent strains and ciprofloxacin-resistant mutants were characterized by sequence analysis of the QRDR of *gyrA* and *gyrB* as described previously (20). Totals of 36 and 38 ciprofloxacin-resistant colonies were obtained from *R. equi* strains W5234 and ATCC 6939^T, respectively, at the ciprofloxacin concentrations shown in Fig. 2. The correlation between each amino acid substitution and the ciprofloxacin concentration is presented in Table 1. Nine nucleotide mutants corresponding to seven different amino acid substitutions denoted as mutant types I to VII in *gyrA* were observed in 56 of 74 mutants. Importantly, high-level resistance (MIC of ≥64 μg/ml), was observed only for mutants harboring amino acid substitutions at Ser-83. These mutants were isolated from plates containing a relatively high level of ciprofloxacin (≥12 μg/ml) but not at a lower drug concentration. The high level of ciprofloxacin resistance in mutants with

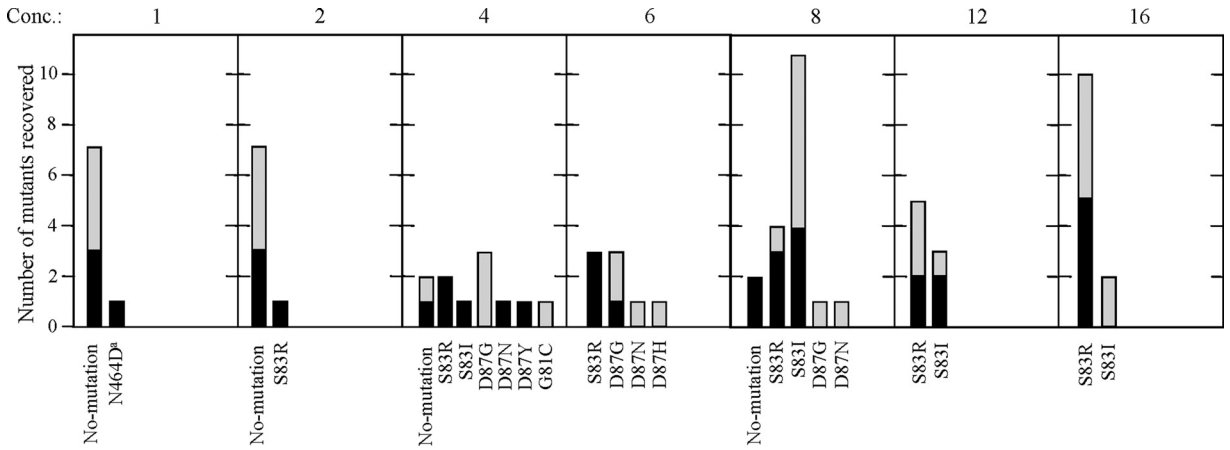

FIG. 2. Effect of ciprofloxacin concentration on the diversity of ciprofloxacin-resistant *R. equi* mutations. Each section shows the resistance mutation alleles recovered from agar plates supplemented with the indicated concentration of ciprofloxacin (μg/ml). Vertical bars indicate the number of mutants analyzed; black bars represent mutants derived from *R. equi* W5234, and gray bars represent mutants derived from *R. equi* ATCC 6939^T. All amino acid substitutions were in GyrA except N464D[a], derived from an amino acid substitution in GyrB.

TABLE 1. Correlation among amino acid substitutions in GyrA and GyrB, concentrations of ciprofloxacin on which mutants were selected, and MICs

Mutation type	Amino acid substitution[a] in:		Concn of drug	Result[b] using strain:			
				W5234 (MIC, 0.5 μg/ml)		ATCC 6939^T (MIC, 0.5 μg/ml)	
	GyrA	GyrB		No. of mutants isolated	MIC (μg/ml) or range	No. of mutants isolated	MIC (μg/ml) or range
I	Ser-83 to Arg	ND	16	5	64	5	32–64
			12	2	64	3	128
			8	3	32–64	1	64
			6	3	32–64	0	NA
			4	2	32	0	NA
			2	1	8	0	NA
II	Ser-83 to Ile	ND	16	0	NA	2	64
			12	2	64	1	128
			8	4	32–64	7	32–128
			4	1	32	0	NA
III	Asp-87 to Gly	ND	8	0	NA	1	16
			6	1	16	2	16
			4	0	NA	3	8
IV	Asp-87 to Asn	ND	8	0	NA	1	32
			6	0	NA	1	16
			4	1	16	0	NA
V	Asp-87 to His	ND	6	0	16	1	16
VI	Asp-87 to Tyr	ND	4	1	8	0	NA
VII	Gly-81 to Cys	ND	4	0	NA	1	8
VIII	ND	Asn-464 to Asp	1	1	4	0	NA
IX	ND	ND	8	2	16–32	0	NA
			4	1	8	1	4
			2	3	2	4	2–4
			1	3	2–4	4	1–4

[a] The first amino acid represents the wild-type amino acid, and the second amino acid represents the mutant amino acid occurring at that position. Numbering of the amino acid residues in GyrA and GyrB for the mutants analyzed in this study was according to those of *E. coli* GyrA (GenBank accession no. CAA68611) and GyrB (GenBank accession no. BAA20341). ND, not detected.

[b] NA, not applicable.

amino acid substitutions at Ser-83 suggests that this amino acid may play an important function in reducing the affinity of the active site of DNA gyrase to quinolones (11). Mutants with an intermediate level of resistance (8 μg/ml to 32 μg/ml) were also identified (Table 1). These mutants were obtained from plates with lower ciprofloxacin concentrations of 8 to 16 μg/ml. A single amino acid substitution, Asn-464 to Asp, was detected in the QRDR of *gyrB*. The Asn-464 to Asp mutation in the QRDR of *gyrB* has been previously observed in *Pseudomonas aeruginosa* (18). In 18 of 74 resistant colonies, nucleotide changes were not observed in the QRDR of either the *gyrA* or *gyrB* gene of W5234 and ATCC 6939^T (Table 1). Two of 18 non-gyrase mutants had an intermediate MIC of 16 to 32 μg/ml, whereas 16 of 18 mutants showed low levels of resistance. Resistance in non-gyrase mutants may be due to an alteration of membrane permeability or the activation of efflux pumps, whereas high levels of resistance were only associated with substitutions in the target enzyme (21, 29). Alternatively, resistance to ciprofloxacin in the non-gyrase mutants may be due to mutations in the genes for topoisomerase IV (*parC* and *parE*) that were previously identified as a target for fluoroquinolones (6, 11, 18, 26); however, *parC* and *parE* have not been identified in other mycolic acid-containing bacteria phylogenetically related to *R. equi* (10, 28). Overall, resistant mutants detected in the wild-type population were diverse and may be a clinically relevant reservoir following enrichment within the MSW.

In this study, we investigated the potential risk for the emergence of ciprofloxacin resistance in *R. equi* by determining its MSW. Molecular characterization of the mutations associated with ciprofloxacin phenotypes may provide an important adjunct diagnostic tool for patient management to reduce the emergence of resistance. Although fluoroquinolones are still effective and requisite agents against most clinical strains of *R. equi* infection of humans and horses, further study will be needed for better dosing regimens of ciprofloxacin, or a combination of agents, to treat *R. equi* infection while preventing the emergence of resistant mutants.

Nucleotide sequence accession numbers. The GenBank sequence accession numbers for the partial nucleotide sequences of the *gyrA* and *gyrB* ciprofloxacin-resistant mutants isolated in this study are GQ468775 to GQ468790 and GQ369761 to GQ369763, respectively.

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