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In Vitro Activity of Five Quinolones and Analysis of the Quinolone Resistance-Determining Regions of *gyrA*, *gyrB*, *parC*, and *parE* in *Ureaplasma parvum* and *Ureaplasma urealyticum* Clinical Isolates from Perinatal Patients in Japan

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***Ureaplasma* spp. cause several disorders, such as nongonococcal urethritis, miscarriage, and preterm delivery with lung infections in neonates, characterized by pathological chorioamnionitis in the placenta. Although reports on antibiotic resistance in *Ureaplasma* are on the rise, reports on quinolone-resistant *Ureaplasma* infections in Japan are limited. The purpose of this study was to determine susceptibilities to five quinolones of *Ureaplasma urealyticum* and *Ureaplasma parvum* isolated from perinatal samples in Japan and to characterize the quinolone resistance-determining regions in the *gyrA*, *gyrB*, *parC*, and *parE* genes. Out of 28 clinical *Ureaplasma* strains, we isolated 9 with high MICs of quinolones and found a single *parC* gene mutation, resulting in the change S83L. Among 158 samples, the ParC S83L mutation was found in 37 samples (23.4%), including 1 sample harboring a ParC S83L–GyrB P462S double mutant. Novel mutations of ureaplasmal ParC (S83W and S84P) were independently found in one of the samples. Homology modeling of the ParC S83W mutant suggested steric hindrance of the quinolone-binding pocket (QBP), and *de novo* prediction of peptide structures revealed that the ParC S84P may break/kink the formation of the α4 helix in the QBP. Further investigations are required to unravel the extent and mechanism of antibiotic resistance of *Ureaplasma* spp. in Japan.**

Ureaplasma spp. are among the smallest self-replicating organisms, in terms of both genome size and cellular dimensions. They lack cell walls and are thus resistant to penicillin and other β-lactams. These organisms exist in association with eukaryotic cells, mainly colonizing mucosal surfaces of the respiratory and urogenital tracts (1, 2). *Ureaplasma* spp. are common inhabitants of the lower genital tract and can be isolated from 40% to 80% of women of child-bearing age (1, 3). *Ureaplasma* spp. have been associated with a range of pathologies, including nongonococcal urethritis (NGU), miscarriage, preterm delivery, neonatal pneumonia, and chronic lung disease in preterm neonates (4, 5). Many reports have suggested that *Ureaplasma parvum* and/or *Ureaplasma urealyticum* may be associated with urogenital infections, infertility, and adverse pregnancy outcomes (6). Regarding the management of ureaplasmal infections, only a limited number of reports are available on the surveillance of antimicrobial resistance in clinical *Ureaplasma* strains, which is crucial for providing therapy empirically (6). The treatment of ureaplasmal infections is limited to tetracyclines, macrolides, and quinolones (5, 7). Resistance to all the three antibiotic classes in clinical *Ureaplasma* isolates has been documented, with unique nucleic acid substitutions identified as potential molecular mechanisms for each (5, 8–10).

Quinolones are used for treating urogenital infections and interact in bacteria with the type II topoisomerases DNA gyrase and topoisomerase IV, both of which are composed of two A and two B subunits; these subunits are encoded by the *gyrA* and *gyrB* genes for DNA gyrase and *parC* and *parE* genes for topoisomerase IV (9, 11–14). Beeton et al. determined the role of amino acid substitutions in GyrA, GyrB, ParC, and ParE proteins of *Ureaplasma* spp. in mediating quinolone resistance (5).

There is only one report of quinolone-resistant *Ureaplasma* in the field of urology in Japan (6). Quinolone resistance in *Ureaplasma* spp. may occur to some degree because of the widespread use of these drugs for the treatment of respiratory and urogenital infections. Data on antimicrobial resistance in *Ureaplasma* in perinatal patients are very limited, and there is no report in the field of perinatal medicine in Japan. We aimed to characterize quinolone

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susceptibility and identify the quinolone resistance-determining region (QRDR) in isolates obtained in the perinatal field.

MATERIALS AND METHODS

Patients. Isolates were obtained from patients admitted to four perinatal medical institutions located in two areas in Japan, Kyushu and Kinki, from January 2007 to December 2013. Vaginal and placental swabs and tracheal aspirates were obtained by obstetricians or neonatologists from the patients suspected of having *Ureaplasma* infections. Informed consent was obtained from the patients or their parents, and the study protocol was approved by the Ethics Committee at the Osaka Medical Center for Maternal and Child Health.

Microbiological laboratory tests for *Ureaplasma* spp. Clinical specimens were suspended in UMCHs medium (1) or urea arginine LYO2 medium (bioMérieux). After incubation at 37°C for 48 h, the color of the medium changed from yellow to red because of urea hydrolysis, indicating *Ureaplasma* positivity. We identified *Ureaplasma* spp. by colony formation and subsequent PCR-based assays using a modification of the method described by Kong et al. (15). For MIC determination in 28 clinical strains, single-colony cultivation was conducted. In total, 158 genomic DNA samples were extracted by the phenol-chloroform extraction method from 28 clinical strains (including one type strain) and 130 frozen ureaplasma-positive LYO2 culture medium samples. *U. parvum* and *U. urealyticum* were identified by the analysis of DNA sequences of the *ureB* gene and/or 16S rRNA gene (16). The serotypes of *U. parvum* were identified by PCR targeting the multiple-banded antigen (*mba*) gene (15).

Modified broth microdilution technique for MIC determination. The following agents were employed for MIC determination: sitafloxacin (STFX), levofloxacin (LVFX) (Daiichi Sankyo Co., Ltd., Tokyo, Japan), garenoxacin (GRNX), tosufloxacin (TFLX) (Toyama Chemical Co., Ltd., Tokyo, Japan), and ciprofloxacin (CPFX) (Wako Pure Chemical Industries., Ltd., Osaka, Japan). The quinolones STFX, LVFX, GRNX, and TFLX were dissolved appropriately in 0.1 M NaOH, 0.1 M HCl, double-distilled water (DDW), and 0.1 M NaOH, respectively, according to the manufacturer's recommendation. The pH of each UMCHs medium sample containing diluted agents was adjusted to 6.0. CPFX was dissolved in 0.1 M HCl, and the pH of UMCHs culture medium containing diluted agents was immediately adjusted to 6.0 to avoid decarboxylation of the agent. The potency of each quinolone was then assessed via serial dilution tests of susceptibility using McFarland standard number 1 of *Escherichia coli* K-12 JM109 (TaKaRa Bio) in Mueller-Hinton broth (data not shown).

The purity of each of these agents was above 98.0%. Quinolone susceptibility was determined by a modified breakpoint analysis in a 96-well broth microdilution format that enables a concurrent determination of bacterial load in a sample without prior knowledge of bacterial load. Antibiotic gradients were created for each agent. *Ureaplasma* organisms (20 μl) from the overnight culture with an unknown number of color-changing units (CCU) were added to each well in the columns (1:10 dilution). Plates were sealed and incubated at 37°C in a humidified cell culture incubator at an ambient CO_2 concentration for 48 h. The MIC was defined as the lowest concentration of antibiotic that prevented a color change after 48 h when read at 10^4 CCU (relative to the growth in the antibiotic-free medium) (9). Samples of UMCHs medium with and without antibiotics were also incubated with no *Ureaplasma* added to serve as negative color change controls. Although no official breakpoint values are available for *Ureaplasma*, reference values were based on the normal ranges of MICs reported for *Ureaplasma* (17).

Quinolone-resistant mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes. Known mutations of ureaplasmal genes associated with quinolone resistance were investigated (5). Using DNA from a single culture colony or extracted DNA, regions of *gyrA* (nucleotide positions 200 to 535), *gyrB* (nucleotide positions 1261 to 1570), *parC* (nucleotide positions 149 to 457), and *parE* (nucleotide positions 1210 to 1522) of *U. parvum* were amplified by PCR using previously described primers (9). Primers gyrA-1 and gyrA-2 were used for amplifying the *gyrA* gene, gyrB-3 and gyrB-4 for the *gyrB* gene, parC-5 and parC-6 for the *parC* gene, and parE-7 and parE-8 for the *parE* gene. The regions of *gyrA* (nucleotide positions 200 to 536), *gyrB* (nucleotide positions 1261 to 1570), *parC* (nucleotide positions 149 to 457), and *parE* (nucleotide positions 1159 to 1448) of *U. urealyticum* were amplified by PCR using the following primers: Uu-GyrA200F (5′-TTGCTGCTTTCGAAAATGG-3′) and Uu-GyrA536R (5′-ACCTGATGGCAAAACACTTGG-3′) for *gyrA*; Uu-GyrB 3F (5′-CCAGGTAAATTAGCTGATTG-3′) and Uu-GyrB 4R (5′-TTCGAATATGGCTACCATC-3′) for *gyrB*; Uu-ParC149F (5′-ATGCCATGAGCGAATTAGG) and parC-6 for *parC*; and Uu-ParE2F (5′-CGTGCTCGTGAAGAAACTAA-3′) and Uu-ParE2R 5′-AAATTCAGCACCAATTCCTGT-3′) for *parE*. PCR products were sequenced using the BigDye Terminator v3.1 cycle sequencing kit and analyzed on an ABI Prism 3130 genetic analyzer (Applied Biosystems), according to the manufacturer's instructions. The sequences of the *gyrA*, *gyrB*, *parC*, and *parE* genes of *U. parvum* and *U. urealyticum* were compared with those of the respective reference strains, *U. parvum* ATCC 700970 (GenBank AF222894) and *U. urealyticum* ATCC 33699 (GenBank CP001184.1) (6).

Homology modeling of ureaplasmal ParC S83L, S83W, and de novo prediction of peptide structure at around S84P. The molecular structure of ParC from *Ureaplasma* spp. remains unknown. We attempted to model *Ureaplasma parvum* ParC by homologous modeling of the quinolone-DNA cleavage complex of *Streptococcus pneumoniae* (PDB code 3RAE) using the Swiss-Model homology-modeling server (http://swissmodel.expasy.org/) (18). Data for the S83L and S83W mutants were calculated on the basis of our *U. parvum* ParC model structure using the software MolFeat (FiatLux), and the energy minimization was performed with the software DeepView/Swiss-PdbViewer (v4.1) (http://spdbv.vital-it.ch/) (19). Each *de novo* prediction of the peptide structure models was constructed via the PEP-FOLD server with the quality assessment by APOLLO (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) (20) using the wild-type ParC α3-α4 helices (SARTVGEVIGKYHPHGDSSIYEAMVRMSQDW) and S84P ParC (SARTVGEVIGKYHPHGDSPIYEAMVRMSQDW).

RESULTS

Samples from a total of 820 patients were sent to our laboratory between 2007 and 2013. Of these, 240 were positive for *Ureaplasma* spp. by culture color change and PCR, and from these, 28 strains could be analyzed for MIC and gene mutations. Patient characteristics are shown in Table 1. Twenty-four isolates were obtained from maternal vaginal swabs, two from placental swabs, and four from tracheal aspirates of premature infants who had respiratory complications with a diagnosis of severe neonatal chronic lung disease (new bronchopulmonary dysplasia). For all 28 strains, 19 were determined as *U. parvum* (68%) and nine as *U. urealyticum* (32%). Amniotic fluid samples of the patients with vaginal swabs positive for strains UP12, UU4, and UU6 were also screened for *Ureaplasma* spp. Among these three amniotic fluid samples, only one, that from the woman who was swab positive for strain UP12, was culture positive for *Ureaplasma*. Comparative *in vitro* activities of quinolones (CPFX, LVFX, TFLX, GRNX, and STFX) against *Ureaplasma* spp. are shown in Table 2. All isolates had high-level resistance to the older quinolones, such as CPFX, with MICs ranging from 4 to 128 μg/ml. Susceptibility to the newer quinolones, such as LVFX and TFLX, depended strongly on the individual isolate, with MICs ranging from 1 to 16 μg/ml and 2 to >16 μg/ml, respectively. The newer quinolones GRNX and STFX exhibited MICs ranging from 0.5 to 4 μg/ml in the wild-type bacterium, whereas their MICs in the S83L strains ranged from 1 to 4 μg/ml, illustrating that the mutation slightly affected MICs. The increase of MICs against GRNX and STFX for strains UP3,

TABLE 1 Sources of the 28 clinical *Ureaplasma* isolates from perinatal patients in Japan[a]

Isolate	Patient sex and age[b]	Species (serovar)	Specimen source	Past pregnancies	Pregnancy outcome[c]	Gestational age (wks)	Presence of *Lactobacillus* spp.
UP1	F, 31	*U. parvum* (SV3)	Placental	G1P1		26	ND
UP2	F, 30	*U. parvum* (SV3)	Vaginal	G1P0	M	13	+
UP3	F, 30	*U. parvum* (SV6)	Placental	G1P1		39	ND
UP4	F, 33	*U. parvum* (SV3)	Vaginal	G4P1	M, P	Not pregnant	−
UP5	F, 36	*U. parvum* (SV6)	Vaginal	G5P2	M	Not pregnant	ND
UP6	M, 12 days	*U. parvum* (SV6)	Tracheal	ND	P	ND	ND
UP7	M, 91 days	*U. parvum* (SV6)	Tracheal	G2P1	P	ND	ND
UP8	F, 26	*U. parvum* (SV3)	Vaginal	G3P1	M	32	−
UP9	F, 25	*U. parvum* (SV6)	Vaginal	G1P1		20	+
UP10	F, 31	*U. parvum* (SV6)	Vaginal	G7P1	M, P	33	−
UP11	F, 35	*U. parvum* (SV6)	Vaginal	G3P0	M	18	+
UP12	F, 44	*U. parvum* (SV3)	Vaginal	G1P0	M	8	+
UP13	M, 3 days	*U. parvum* (SV3)	Tracheal	G5P4	P	ND	ND
UP14	F, 42	*U. parvum* (SV6)	Vaginal	G3P0	M	ND	−
UP15	F, 37	*U. parvum* (SV6)	Vaginal	G2P1	M	Not pregnant	+
UP16	F, 37	*U. parvum* (SV6)	Vaginal	G3P1	M	Not pregnant	−
UP17	F, 33	*U. parvum* (SV6)	Vaginal	G2P1	M, P	Not pregnant	−
UP18	F, 34	*U. parvum* (ND)	Vaginal	G4P1	M	Not pregnant	+
UP19	F, 30	*U. parvum* (SV6)	Vaginal	G2P0	M	18	−
UU1	F, 43	*U. urealyticum*	Vaginal	G5P0	M	ND	−
UU2	F, 1day	*U. urealyticum*	Tracheal	G1P1	P	ND	ND
UU3	F, 36	*U. urealyticum*	Vaginal	G3P0	M	33	+
UU4	F, 26	*U. urealyticum*	Vaginal	G2P2	P	9	+
UU5	F, 27	*U. urealyticum*	Vaginal	G4P2	P	10	+
UU6	F, 20	*U. urealyticum*	Vaginal	G0P0		25	−
UU7	F, 28	*U. urealyticum*	Vaginal	G1P1		11	+
UU8	F, 22	*U. urealyticum*	Vaginal	G5P2	P	34	+
UU9	F, 29	*U. urealyticum*	Vaginal	G1P0	P	6	−

[a] Age is given in years unless otherwise specified.
[b] ND, not determined.
[c] M, miscarriage; P, preterm delivery.

UP4, UP10, UP12, UP18, and UU8 were less pronounced than that for strains UP9, UP15, and UU6.

For *Ureaplasma* spp., the positions of mutations associated with quinolone resistance include GyrA 100 and 104 (84 and 88 for *Escherichia coli*), GyrB 462 (445 for *E. coli*) (13), ParC 82, 83, 87, and 88 (79, 80, 84, and 85 for *E. coli*), and ParE 457 (447 for *E. coli*) (9, 12, 13, 21). Nine strains (UP3, UP4, UP9, UP10, UP12, UP15, UP18, UU6, and UU8) out of the 28 (32.1%) showed a highly increased MICs of quinolones, including seven strains of *U. parvum* and two strains of *U. urealyticum*. The lowest MICs (≦4 μg/ml) in our clinical *Ureaplasma* strains were observed for GRFX and STFX, in contrast to those of the three other quinolones, which were characterized by increased MICs compared with those for the reference strain ATCC 700970. Among the strains showing high-level MICs of quinolones, we found identical mutations in the *parC* gene, corresponding to a Ser83-to-Leu (S83L) amino acid substitution (Table 2). No mutations associated with the quinolone-resistant alterations of the hot spots in *gyrA*, *gyrB*, and *parE* genes were detected in our strains. The ParC S83L substitution resulted in an increase in the MIC of up to 32-fold against tested quinolones.

We found the following amino acid substitutions in the QRDRs of 130 *Ureaplasma* culture-positive samples based on the DNA sequence: ParC S83L, serine (TCA) to leucine (TTA) (28 samples); ParC S83W, serine (TCA) to tryptophan (TGA) (TGA codes for tryptophan instead of being a termination codon in the *Mycoplasmataceae* [22]) (1 sample); ParC S84P, serine (TCA) to proline (CCA) (1 sample); GyrA D112H, aspartic acid (GAC) to histidine (CAC) (1 sample); and GyrB P462S, proline (CCA) to serine (TCA) (1 sample) (Table 3). The ParC mutation S83L was also detected in a DNA sample harboring GyrB P462S in the QRDR. Amino acid sequence alignment of QRDRs from type II topoisomerases is shown in Fig. 1A. Serines 83 and 84 are located at the second and third positions of the α4 helix, respectively. To evaluate the structural importance of the substituted amino acids of ParC S83L and S83W, homology modeling of *U. parvum* ParC was performed. The C-3 carboxylic acid and C-4 carbonyl of the quinolone together with a divalent magnesium cation are important for the binding interaction with the DNA-topoisomerase complex (Fig. 1B). From the model, the side chains of S83L (Fig. 1C) and S83W (Fig. 1D) occupied the quinolone binding pocket of the levofloxacin C-3 carboxylic acid. These S83L and S83W mutations in the side chains would interfere with the proper binding of quinolones via steric hindrance.

ParC serine 84 is located at the third position of the α4 helix based on the sequence alignment (Fig. 1A). Secondary structures of the predicted top 5 models are shown in Fig. 2A. Four of five models (80%) of wild-type QRDR formed α-helices (Fig. 2B), whereas the S84P mutant was predicted to form an α-helix in only one of five models (20%) (Fig. 2C). Our model suggested that the S84P mutant was less likely to form an α4 helix structure in QRDR than wild-type ParC.

TABLE 2 Characteristics of 28 *Ureaplasma* clinical isolates from perinatal patients in Japan

Strain	MIC (μg/ml)					Amino acid change at the indicated position in[a]:							
						GyrA			ParC				
	CPFX	LVFX	TFLX	GRNX	STFX	100	104	GyrB, 462	82	83	87	88	ParE, 457
ATCC 700970	4	2	2	0.5	1								
UP1	8	2	4	1	1								
UP2	8	2	4	1	2								
UP5	8	2	4	0.5	0.5								
UP6	16	4	16	4	1								
UP7	4	2	4	0.5	0.5								
UP8	8	2	4	1	1								
UP11	16	2	4	0.5	0.5								
UP13	4	1	2	0.5	0.5								
UP14	32	8	16	2	2								
UP16	8	1	2	0.5	0.5								
UP17	8	2	4	1	1								
UP19	8	2	4	1	0.5								
UU1	8	1	8	0.5	1								
UU2	16	2	8	0.5	1								
UU3	16	4	8	0.5	1								
UU4	16	4	8	0.5	1								
UU5	16	4	8	0.5	1								
UU7	16	4	16	0.5	2								
UU9	16	4	8	1	1								
S83L strains													
UP3	128	16	>128	2	1					Leu			
UP4	64	16	32	2	1					Leu			
UP9	64	16	64	4	2					Leu			
UP10	32	8	16	2	1					Leu			
UP12	64	8	32	2	1					Leu			
UP15	64	16	64	4	2					Leu			
UP18	32	8	32	1	1					Leu			
UU6	64	8	>128	2	4					Leu			
UU8	64	8	>128	1	2					Leu			

[a] Amino acid position where an amino acid change is reported in quinolone-resistant mutants of *U. parvum* and/or *U. urealyticum*.

DISCUSSION

Ureaplasma spp. are commonly found in the lower urogenital tracts of healthy women and have been implicated as the cause of acute and chronic urinary tract infections (21, 23–26). We recently reported an epidemiological relationship between *Ureaplasma* infection and pathological chorioamnionitis (1) and confirmed the lipoprotein multiple-banded antigen of *Ureaplasma* spp. as a virulence factor for preterm and intrauterine fetal death

TABLE 3 Amino acid substitutions and DNA mutations of QRDRs of *Ureaplasma* spp. among 130 DNA samples

Protein	Position	Amino acid	DNA sequence	*U. urealyticum* ($n = 24$)			*U. parvum* ($n = 106$)		
				Amino acid substitution	DNA mutation	No. of mutant strains	Amino acid substitution	DNA mutation	No. of mutant strains
GyrA	100	Gln				0			0
	104	Gln				0			0
	112	Glu/Asp	GAA/GAC			0	His	CAC	1
GyrB	462	Pro	CCA			0	Ser	TCA	1
ParC	82	Asp				0			0
	83	Ser	TCA	Leu	TTA	3	Leu	TTA	25
							Trp	TGA[a]	1
	84	Ser	TCA			0	Pro	CCA	1
	87	Glu				0			0
	88	Ala				0			0
ParE	457	Ala				0			0

[a] The TGA codon encodes tryptophan in the *Mycoplasmataceae* (22).

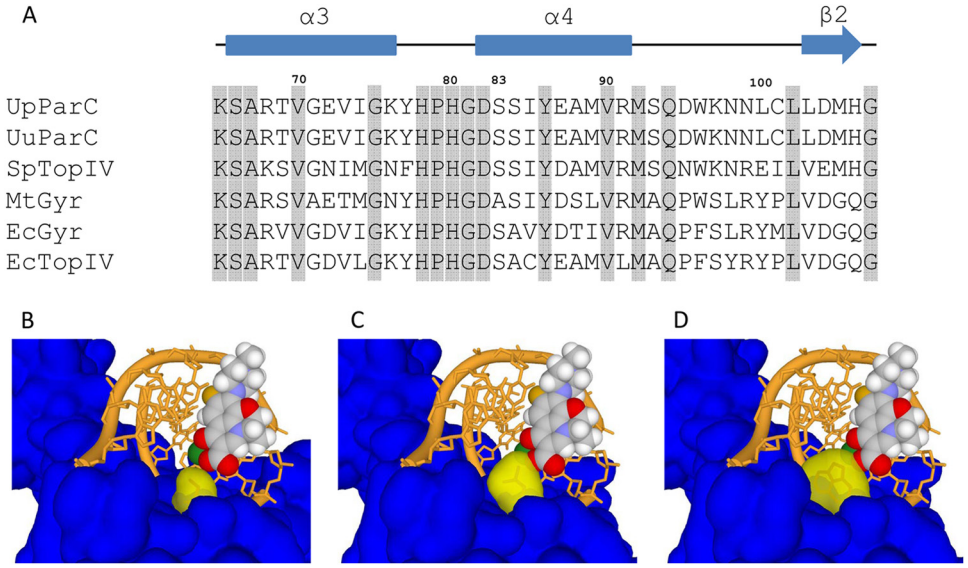

FIG 1 The QRDR model structures of *U. parvum* wild-type ParC and S83L and S83W mutants. (A) Sequence alignment of the QRDR domain from type II topoisomerases. The sequences are labeled as follows: UpParC, *U. parvum* ATCC 700970 ParC (GenBank no. AAF30879); UuParC, *U. urealyticum* ATCC 33699 ParC (GenBank no. ACI60320); SpTopIV, *Streptococcus pneumoniae* topoisomerase IV (PDB code 3RAE); MtGyr, *Mycobacterium tuberculosis* DNA gyrase (PDB code 3IFZ); EcGyr, *Escherichia coli* DNA gyrase (PDB code 1AB4); EcTopIV, *E. coli* topoisomerase IV (PDB code 1ZAU). Numbers above the sequences denote the amino acid positions according to *U. parvum*. The α3 and α4 helices (blue rectangles), the β2 strand (blue arrow), and identical amino acids (gray shading) are indicated. (B to D) Surface models of *U. parvum* wild-type ParC (by SWISS-MODEL server) (B) and the S83L (C) and S83W (D) mutants. Amino acid position 83 is in yellow; others are in blue. LVFX (ball), DNA (orange), and Mg^{2+} (green) are superimposed from PDB code 3RAE.

in pregnant mice (27). In Japan, Kamiya et al. detected a gene mutation associated with quinolone resistance in *U. parvum* and *U. urealyticum* by DNA sequencing of isolates from urine specimens from males with NGU (6). The eradication of ureaplasmas from the female genital tract is difficult, sometimes requiring prolonged courses of antimicrobial therapy (21, 28). Our data show that *Ureaplasma* spp. with a high MIC against quinolones are already widely distributed among females of reproductive age in Japan, which may complicate the eradication of ureaplasmas from the urogenital tract even in nonpregnant reproductive women, underscoring the importance of keeping quinolone treatment of adult NGU and respiratory infections under surveillance.

The S83L and S83W mutations were detected in the *gyrA* gene of *E. coli* and *Staphylococcus pseudintermedius* (S84W) (29, 30). A

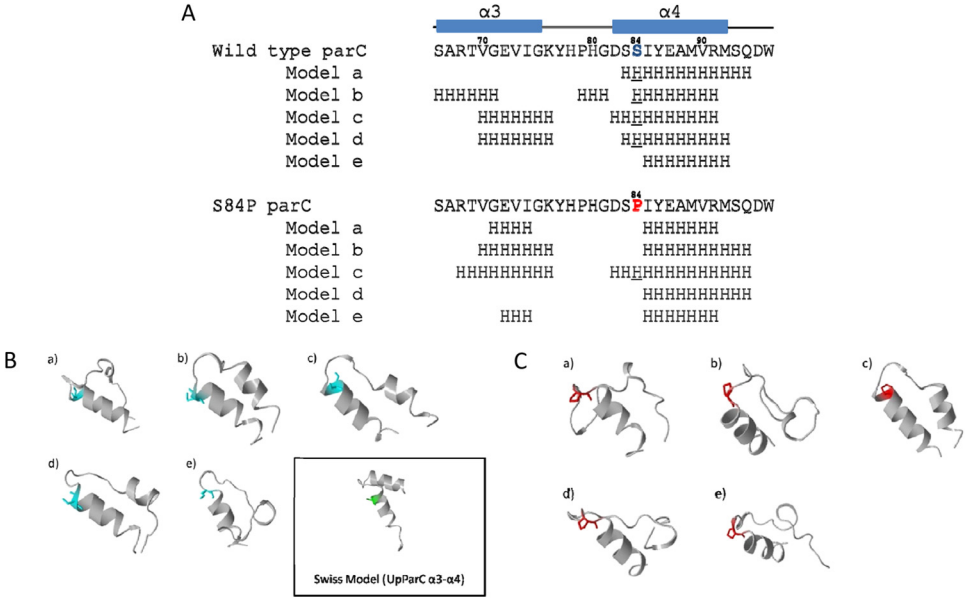

FIG 2 *De novo* prediction of *U. parvum* ParC peptide structures (amino acid positions 66 to 96) by the PEP-FOLD server. (A) The secondary structures of the top 5 predicted models are shown. Numbers above the sequences denote the amino acid positions according to *U. parvum*. Serine 84 is in blue, and proline 84 is in red. H refers to the α-helical structure, and underlining indicates helical serine 84 and helical proline 84. (B and C) Tertiary structures of the top 5 predicted peptide models of wild-type ParC (B) and ParC S84P (C). The outlined panel shows *U. parvum* ParC α3-α4 helix structures obtained by homology modeling.

GyrA A87V/S88P (corresponding to positions 83 and 84, respectively, in *Escherichia coli*) double mutant was recently reported in multidrug-resistant *Ochrobactrum intermedium* (31). In *Ureaplasma* spp., the mutation corresponding to S83L is located in the *parC* gene. At present, a functional analysis of the ureaplasmal *gyrA* and/or *parC* gene product has not been provided. These two genes were assigned by *in silico* homology search against bacterial databases. Therefore, in accordance with previous reports, we report that the mutations corresponding to S83L, S83W, and also S84P were detected in the (putative) *parC* gene.

We found 9 strains from placental or vaginal swabs harboring the S83L mutation (32.1%; 9/28 strains) and showing high MICs of quinolones (Table 2). The S83L mutation in ParC was also detected in 28 of 130 ureaplasma-positive samples (21.5%) (Table 3). A few quinolone-resistant clinical *Ureaplasma* isolates have been described in China (32, 33), France (5, 10, 12), the United States (13, 21), Italy, South Korea (34), and Germany (35). Overall, the S83L mutant was identified in 37 of 158 samples (23.4%), indicating that quinolone-resistant *Ureaplasma* spp. are already widespread in the perinatal field in Japan. GRNX is one of the newer quinolones with strong activity against ureaplasmas (36). However, unfortunately, GRNX cannot be prescribed for patients with urogenital infections because it is permitted only for patients with respiratory infections in Japan. STFX is also one of the newer quinolones whose *in vitro* activity is higher than that of the older quinolones (37) or other antimicrobial agents (38). In our study, GRNX and STFX showed relatively high activity against ureaplasmas compared with CPFX, LVFX, and TFLX. Furthermore, GRNX and STFX had better activity against the S83L strains. UP6, UP14, and some other strains exhibited relatively high MICs of quinolones (CPFX, LVFX, and TFLX); however, we could not detect any known hot spot mutations associated with quinolone resistance. Resistance in these isolates could be linked to mutations outside the sequenced regions or alternative mechanisms, such as altered membrane permeability (5, 39).

A plasmid-mediated quinolone resistance gene was reported in *Klebsiella pneumoniae* (40) and *Proteus mirabilis* (41). To date, 20 human *Ureaplasma* sp. genomes have been fully sequenced. However, there is no evidence for the existence of a plasmid in 19 human *Ureaplasma* genomes (42), nor was any evidence uncovered in our previous study (43). Codon usage by the family *Mycoplasmataceae*, including ureaplasmas, differs from that of other bacteria, and the GC content of these species is lower than that of other bacteria (43). The plasmid gene transfer system does not appear to be a general evolution mechanism of ureaplasmas.

We found ParC S83W and S84P mutations in the QRDRs of *Ureaplasma* spp. Unfortunately, living bacteria harboring ParC S83W and S84P could not be recovered from the frozen LYO2 culture medium for MIC tests. To date, an efficient gene manipulation technology for *Ureaplasma* spp. has not yet been established. For these reasons, we performed *in silico* homology modeling of S83W and *de novo* predictions for S84P helix formation. In the analyses, S83W exhibited the potential to cause quinolone resistance via steric hindrance (Fig. 1D).

There is no available determined structure of S84P for homology modeling. Proline can be found at the N termini, but not in the middle, of the α-helices (44). Among all amino acids, proline has the lowest helix propensity (45) and the highest disorder propensity (46). For these reasons, proline is known to break or kink helical structures. This unique characteristic is associated with the backbone of proline, which cannot form hydrogen bonds and which exhibits rigid N-C^{α} rotation. Consequently, we performed *de novo* prediction using the S84P peptide structure instead of homology modeling using the wild-type serine 84 geometry (Fig. 2). As expected, the S84P protein exhibited a lower propensity for α4 helix formation, suggesting that this mutation may be an obstacle to the formation of the proper α3-α4 structure of QRDR. However, further research is needed, especially MIC tests for S83W and S84P. We also found a GyrA D112H mutation/polymorphism in one sample; however, we could not detect any evidence that this mutation is associated with quinolone resistance from the structural view (data not shown).

We report the first *in vitro* quinolone-resistant clinical strains of *Ureaplasma* spp. associated with an S83L mutation, and we also identified S83W and S84P mutations in the *parC* gene and a P462S mutation in the *gyrB* gene in samples obtained in the field of perinatal medicine in Japan. However, this study has some limitations. The total number of patients included in this study was relatively small. In addition, continuous surveillance of *Ureaplasma* antimicrobial resistance and appropriate treatments are required to prevent perinatal complications through *Ureaplasma* infections.

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