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Cultivation of *Neisseria gonorrhoeae* in Liquid Media and Determination of Its In Vitro Susceptibilities to Quinolones

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The cultivation of *Neisseria gonorrhoeae* by use of fastidious broth (FB) was evaluated. FB was found to be able to support the growth of all *N. gonorrhoeae* strains tested in this study without a rapid decrease in the viable count after exponential growth. After 24 h of incubation at 35°C with 5% CO_2, viable counts of all strains reached over 10^8 CFU/ml in FB. Similar growth of the wild-type strain and its target-altered quinolone-resistant derivatives was observed. The susceptibilities of laboratory-adapted strains and clinical isolates to quinolones were tested by the microdilution method using FB. The MICs determined by microdilution were not significantly different from those determined by the agar dilution method recommended by the CLSI (formerly National Committee for Clinical Laboratory Standards). Moreover, the concentration-dependent time-kill of quinolones such as gatifloxacin and ciprofloxacin was observed in FB. At 2 to 4 times the MIC, gatifloxacin and ciprofloxacin were predominantly bactericidal against *N. gonorrhoeae* WHO A. At the MIC, the activities of both quinolones ranged from bactericidal to bacteriostatic. At 0.25 to 0.5 times the MIC, gonococcal growth was comparable to that of the growth control. These results suggest that the cultivation of *N. gonorrhoeae* by use of FB may be useful for evaluation of the antibacterial effects of quinolones.

Neisseria gonorrhoeae is one of the causatives of sexually transmitted diseases, and it is a fastidious organism. This organism is typically cultured using an agar medium such as chocolate agar plate (GCII agar base with 1% IsoVitaleX [BBL] and purified hemoglobin). The CLSI (formerly the National Committee for Clinical Laboratory Standards) recommends only the agar dilution procedure for antibacterial susceptibility testing of *N. gonorrhoeae* (20). Before the 1980s, a number of studies were published regarding the liquid culture methodology for *N. gonorrhoeae* (9, 13, 21, 23, 25). However, these reports demonstrated that the number of bacteria decreased rapidly after bacterial exponential growth, and broth microdilution methods for susceptibility testing of *N. gonorrhoeae* were reported to give higher MICs of β-lactam antibiotics against penicillinase-producing *N. gonorrhoeae* than agar dilution methods (7, 22).

Recently, the antibacterial activity of antigonococcal agents against clinical isolates of *N. gonorrhoeae* has been declining (1, 11, 24). Quinolones initially appeared to be promising agents for the treatment of *N. gonorrhoeae* infections; however, the extensive clinical use of quinolones carries the risk of the development of resistance, and indeed, there have been increasing numbers of recent isolates of *N. gonorrhoeae* that are highly resistant to quinolones, especially in Asia (11, 24, 27). Therefore, the evaluation of the antibacterial susceptibility of *N. gonorrhoeae* is important in the clinical setting. According to the CLSI (formerly NCCLS) procedure, the drug susceptibility of *N. gonorrhoeae* should be measured by the agar dilution method, and it is recommended that direct colony suspension be used for the inoculum preparation for the susceptibility test of *N. gonorrhoeae* (20). However, these approaches are tedious, time-consuming, and inconvenient, especially in the investigation of the antimicrobial susceptibility of large numbers of isolates and of the activities of many antimicrobial agents. The broth microdilution method and the growth method using liquid culture for the inoculum preparation are both simple methods to test the susceptibilities of clinical isolates; however, they have not yet been established for testing the susceptibility of *N. gonorrhoeae*. To date, few studies have been reported regarding the susceptibilities of antibacterial-resistant *N. gonorrhoeae* to antigonococcal agents by use of the microdilution method (7, 22). Therefore, a more convenient procedure than the agar dilution procedure recommended by the CLSI (formerly NCCLS) is still needed.

In addition to its potential usefulness for susceptibility testing, a modified liquid culture methodology is necessary for conducting bactericidal studies of antibiotics, such as in vitro pharmacokinetic/pharmacodynamic (PK/PD) analysis. In vitro PK/PD analysis of antimicrobial agents offers an alternative method for the determination of an adequate clinical regimen and is useful for predicting the clinical efficacy of a given antibiotic against various bacteria (2, 6, 8, 14–16). Therefore, the in vitro PK model, which can simulate human PK, is rapidly becoming one of the most important examinations in the development of new antimicrobial agents. In order to simulate human PK in vitro, it is essential to use a liquid culture. In vitro PK/PD analysis for *N. gonorrhoeae* has not yet been performed,

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TABLE 1. Strains used in this study

Strain	Mutation(s)	Source or reference
WHO A	Wild-type (parent)	26
R-4/5	*gyrA* (Ser91Phe)	26
R-8/1	*gyrA* (Asp95Tyr)	26
R-8/4	*gyrA* (Ser91Tyr)	26
R-8/5	*gyrA* (Asp95Asn)	26
R-4/5/1	*gyrA* (Ser91Phe), *parC* (Glu91Lys)	26
R-4/5/2	*gyrA* (Ser91Phe), *parC* (Asp86Asn)	26
R-4/5/3	*gyrA* (Ser91Phe)	26
ATCC49226	Wild-type (parent)	American Type Culture Collection
N-4-2	*gyrA* (Ser91Phe)	This study
N-4-2-4-2	*gyrA* (Ser91Phe), *parC* (Glu91Lys)	This study
TK106	*gyrA* (Ser91Phe), *parC* (Ser87Ile)	5
TK109	*gyrA* (Ser91Phe), *parC* (Ser87Ile)	5

because no liquid culture methodology has yet been established. Therefore, the development of such an approach remains necessary for in vitro PK/PD studies, as well as for testing the susceptibility of *N. gonorrhoeae*.

Cartwright et al. reported developing a fastidious broth (FB) that is capable of growing fastidious organisms (3). FB is used primarily for the recovery of clinically significant organisms from specimens. It is known that the viability of fastidious organisms, including *N. gonorrhoeae*, does not decline for several days in FB (3). However, thus far there has been no detailed report on the growth of *N. gonorrhoeae* in FB. In the present study, we examined the growth of *N. gonorrhoeae* in FB and considered whether or not FB could be used for the biological evaluation of *N. gonorrhoeae*, such as in vitro susceptibility testing and bactericidal activity of quinolones.

MATERIALS AND METHODS

Quinolones, bacterial strains, and media. The quinolones tested here were synthesized at Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan) or were purchased from commercial sources. Data on the bacterial strains used in this study are shown in Table 1. *N. gonorrhoeae* WHO A and its quinolone-resistant derivatives have been described previously (26). *N. gonorrhoeae* ATCC49226 was a quality control strain of the CLSI (formerly NCCLS), and its *gyrA* mutant (N4-2) and *gyrA parC* double mutant (N4-2-4-2) were obtained by selection with norfloxacin in the present study. Mutations of the quinolone resistance-determining region of *gyrA* and *parC* were determined following a previously described method (4). *N. gonorrhoeae* TK106 and TK109 are known to be quinolone-resistant clinical isolates, as reported previously (5). The clinical isolates used in this study were isolated from patients with gonococcal urethritis in Japan.

GCII agar base medium (Becton Dickinson, Cockeysville, MD) supplemented with 1% IsoVitaleX (Becton Dickinson) and chocolate agar were used to prepare the inocula, determine the visible colony count, and confirm the presence of *N. gonorrhoeae* by the oxidase test. FB was prepared as described previously by Cartwright et al. (3), and consisted of 35 g of Columbia broth base, 5 g of glucose, 5 g of yeast extract, 2 g of neopeptone, and 0.75 g of agarose dissolved in 960 ml of distilled water. A total of 30 ml of hematin solution (0.05% [wt/vol] in 0.1 M NaOH) and 5 ml of Tween 80 (10% [vol/vol]) was then added, and the resultant broth was sterilized by autoclaving, after which 6 ml of pyridoxal solution (0.1% [wt/vol]) and 1.5 ml of NAD solution (1% [wt/vol[) were added.

Growth studies using FB. Colonies were removed from GCII agar after 24 h of incubation at 35°C in a moist atmosphere containing 5% CO_2. The colonies were dispersed in sterile saline, and the inoculum turbidity was adjusted to approximately 1.0 at an optical density of 520 nm (ca. 10^8 to 10^9 CFU/ml). A total of 100 μl of this suspension was transferred to test tubes containing 10 ml of FB. The final inoculum was approximately 10^6 CFU/ml. Test tubes were incubated at 37°C in a water bath in the absence of CO_2 and were shaken at 100 rpm. Growth was monitored by determination of the viable count. Portions (0.1 ml) of the cultures were removed at the indicated time points and were plated onto drug-free GCII agar supplemented with 1% IsoVitaleX, after dilution as necessary. The numbers of colonies were counted after 24 h of incubation at 35°C in a moist atmosphere containing 5% CO_2.

Determination of MICs. The MICs were determined by agar dilution with GCII agar supplemented with 1% IsoVitaleX and by microdilution with FB.

The agar dilution procedure was identical to that recommended by the CLSI (formerly NCCLS) (20). The MIC was defined as the lowest concentration of an antibacterial agent that inhibited visible growth after incubation for 24 h at 35°C in a moist atmosphere containing 5% CO_2.

Microdilution plates were prepared using serial twofold dilutions of antimicrobial agents in FB. To prepare the inoculum, the turbidity of the actively growing FB culture was adjusted with sterile saline to obtain turbidity optically comparable to that of the 0.5 McFarland standard. The suspension was diluted 10-fold in sterile saline, and 5 μl of diluent was inoculated into 100 μl of FB. The final inoculum was approximately 5×10^5 CFU/ml (ca. 5×10^4 CFU/well). The plates were incubated for 24 h at 35°C in a moist atmosphere containing 5% CO_2. The MIC was defined as the lowest concentration of an antibacterial agent that prevented macroscopically visible growth under the test conditions.

Killing curve study. The bactericidal activities of gatifloxacin and ciprofloxacin were measured by a previously described method (10, 19) with minor modifications. In brief, representative strains incubated in FB for 24 h at 35°C in a moist atmosphere containing 5% CO_2 were diluted with fresh broth to approximately 10^6 CFU/ml, and the diluted cultures were incubated for 2 h at 37°C in the absence of CO_2 with shaking. After 2 h of preincubation, gatifloxacin and ciprofloxacin were added to the culture at various concentrations around the MIC. Portions (0.1 ml) of the cultures were removed at the indicated time points and were plated onto drug-free GCII agar supplemented with 1% IsoVitaleX, after dilution as necessary. The numbers of colonies was counted after 24 h of incubation at 35°C in a moist atmosphere containing 5% CO_2.

In vitro PK model. The in vitro PK model has been described previously (6). A dilutional in vitro PK model (PASS-400; Dainipponseiki, Kyoto, Japan) was used to simulate serum concentrations of ciprofloxacin on the basis of the PK parameters reported previously (17). *N. gonorrhoeae* R-4/5/1, which possessed single-point mutations in the quinolone resistance-determining region of both GyrA (Ser91→Phe) and ParC (Glu91→Lys), were used. Representative strains incubated in FB for 24 h at 37°C with shaking were diluted with fresh broth to approximately 10^6 CFU/ml, and the diluted cultures were incubated for 2 h at 37°C in the absence of CO_2 with shaking. After 2 h of preincubation, ciprofloxacin was then added to the culture chamber according to the dosing regimen. Aliquots of the cultures were collected via an outflow tube at the indicated time points and were plated onto drug-free GCII agar supplemented with 1% IsoVitaleX, after dilution as necessary. The number of colonies was counted after 24 h of incubation at 35°C in a moist atmosphere containing 5% CO_2.

RESULTS

Culture of *N. gonorrhoeae* by FB. The cultivation of *N. gonorrhoeae* was examined using FB. All of the tested strains of *N. gonorrhoeae* were found to be able to grow in FB (Fig. 1). *N. gonorrhoeae* WHO A (wild type) and its quinolone-resistant derivatives grew well in FB without any rapid decrease in the viable count after exponential growth. No significant changes in the growth rate were observed for the wild-type strain and its target-altered quinolone-resistant derivatives. The generation times for WHO A (wild type), R-4/5 (*gyrA*, Ser91→Phe), R-4/5/1 (*gyrA*, Ser91→Phe; *parC*, Glu91→Lys), R-4/5/2 (*gyrA*, Ser91→Phe; *parC*, Asp86→Asn), and R-4/5/3 (*gyrA*, Ser91→Phe; *parC*, unknown) were 83, 105, 94, 90, and 94 min, respectively. At 24 h after inoculation, the viable counts of the bacteria in culture had reached their maximum numbers, which ranged from 6×10^8 to 9×10^8 CFU/ml.

For *N. gonorrhoeae* ATCC49226 (wild type), which was the quality control strain recommended by the CLSI (formerly

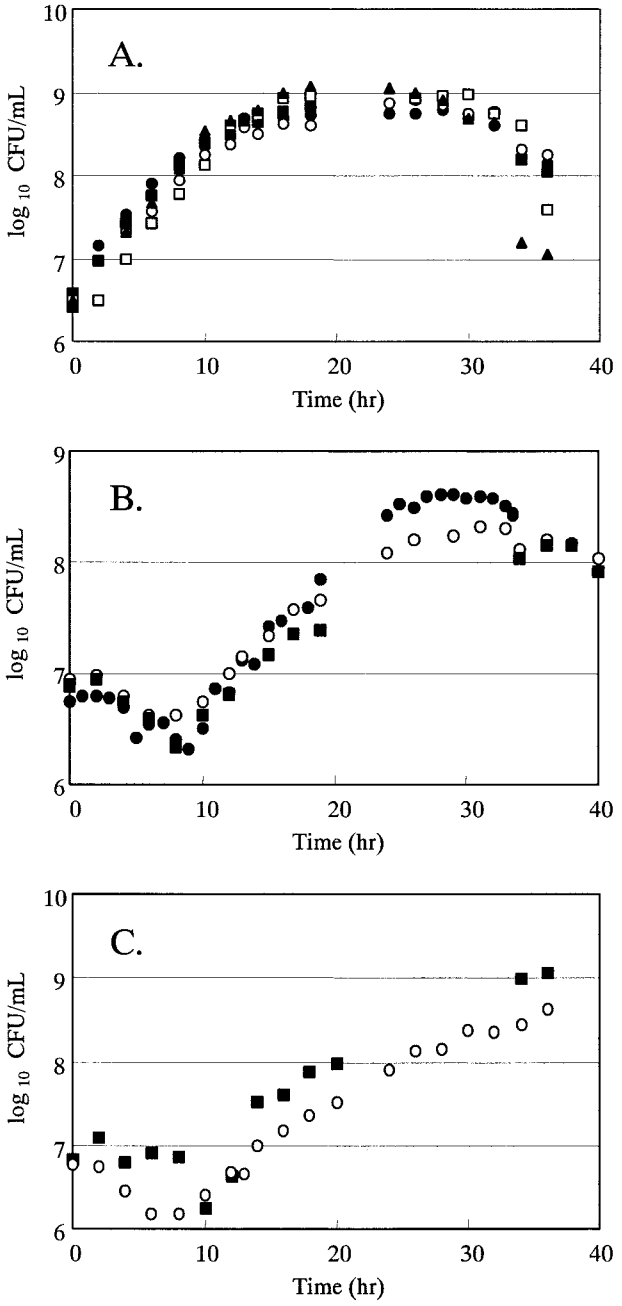

FIG. 1. Growth of *N. gonorrhoeae* strains, including quinolone-resistant strains, in FB. Symbols: A. ●, WHO A (wild type); ○, R-4/5 (*gyrA*); ■, R-4/5/1 (*gyrA parC*); □, R-4/5/2 (*gyrA parC*); ▲, R-4/5/3 (*gyrA*); B. ●, ATCC49226 (wild type); ○, N4-2 (*gyrA*); ■, N4-2-4-2 (*gyrA parC*); C. ■, TK106; ○, TK109 (quinolone-resistant clinical isolate).

NCCLS), and its target-altered quinolone-resistant derivatives, the exponential growth phase was observed for 15 h after a lag phase of 10 h. Maximal growth was observed 24 h after incubation with viable counts of approximately 4×10^8 CFU/ml. The generation time of the exponential phase of ATCC49226 (wild type), N4-2 (*gyrA*, Ser91→Phe), and N4-2-4-2 (*gyrA*, Ser91→Phe; *parC*, Asp86→Asn) were 127, 173, and 197 min, respectively.

Viable cells could be counted at 10 h after incubation for the clinical isolates of *N. gonorrhoeae* TK106 and TK109. The growth rates of these strains were the lowest among the strains tested, since their generation times were 222 min (TK106) and 215 min (TK109). The viable counts under the present culture conditions ranged from 4×10^8 (TK109) to 1×10^9 CFU/ml (TK106) at 36 h after inoculation.

Bacterial growth in FB was evaluated for the remaining 20 strains of *N. gonorrhoeae*, including laboratory-adapted strains and clinical isolates. One colony of each strain was transferred into a tube containing 0.5 ml of FB and was then cultivated at 35°C in a moist atmosphere containing 5% CO_2. All tested strains were found to have grown to over 10^8 CFU/ml at 24 h after inoculation. Additionally, *N. gonorrhoeae* ATCC49226 was found to have grown to over 10^8 CFU/ml at a stationary phase after 10^4, 10^5, and 10^6 CFU/ml inoculation (data not shown).

Comparison of the MICs of quinolones determined by microdilution and agar dilution procedures. No difference in the MICs of quinolones was observed by use of an agar dilution procedure using both direct colony suspension as recommended by the CLSI (formerly NCCLS) and FB culture for inoculum preparation (data not shown).

The MICs of quinolones used in this study are summarized in Table 2 to Table 4. The MICs of quinolones, as measured by the microdilution procedure using FB, were within a fourfold deviation, compared with those obtained by the agar dilution procedure. Additionally, the MICs of quinolones measured by the microdilution procedure for *N. gonorrhoeae* ATCC49226 were within the ranges permitted by the CLSI (formerly NCCLS) using the agar dilution procedure. Furthermore, the susceptibility to quinolones of target-altered quinolone-resistant strains was found to have decreased compared with that of the parent strains. Against clinical isolates, the MIC_{50}s and the MIC_{90}s obtained by the microdilution procedure were comparable to those observed with the agar dilution procedure. The percentage of agreement between the agar dilution procedure and microdilution procedure within a single doubling dilution exceeded 90% for all quinolones tested (Table 5).

Bactericidal activity of quinolones against *N. gonorrhoeae*. Killing curve studies were performed with gatifloxacin and ciprofloxacin against *N. gonorrhoeae* WHO A, and the bactericidal activities were found to increase with the concentrations of both gatifloxacin and ciprofloxacin (Fig. 2). More than 99% of the initial viable *N. gonorrhoeae* were killed within 2 to 4 h of incubation with quinolones at concentrations in excess of 2 × MIC. Bacteriostatic activity was observed at a concentration equal to the MIC.

Bacterial time-kill study using an in vitro simulation model. The time-kill curve for ciprofloxacin at the simulated serum concentration after oral dosing of 200 mg three times a day (t.i.d.), which is the Japanese clinical regimen, is shown in Fig. 3. The growth control curve demonstrated logarithmic growth to approximately 10^9 CFU/ml. At a dose of 200 mg t.i.d., ciprofloxacin reduced the number of quinolone-resistant *N. gonorrhoeae* R-4/5/1 bacteria to below the limit of detection ($<10^2$ CFU/ml) within 8 h following the initial exposure. No regrowth was observed until 24 h after exposure.

TABLE 2. Comparison of MICs by microdilution versus agar dilution for type strains of *N. gonorrhoeae*

N. gonorrhoeae strain	MIC (μg/ml) of[a]:									
	Norfloxacin		Ciprofloxacin		Ofloxacin		Levofloxacin		Gatifloxacin	
	AD	MD	AD	MD	AD	MD	AD	MD	AD	MD
ATCC49226	0.016	0.031	0.002	0.004	0.008	0.016	0.004	0.008	0.002	0.004
WHO A	0.008	0.031	0.002	0.004	0.008	0.016	0.004	0.008	0.002	0.004
Type	0.008	0.016	0.002	0.004	0.008	0.008	0.004	0.004	0.002	0.004
IID835	0.008	0.016	0.002	0.004	0.008	0.016	0.008	0.008	0.008	0.004
	Moxifloxacin		Sparfloxacin		Trovafloxacin		Garenoxacin		Gemifloxacin	
	AD	MD	AD	MD	AD	MD	AD	MD	AD	MD
ATCC49226	0.008	0.008	0.004	0.004	0.008	0.008	0.008	0.004	0.002	0.002
WHO A	0.004	0.008	0.001	0.002	0.002	0.002	0.001	0.002	0.001	0.002
Type	0.004	0.004	0.001	0.002	0.002	0.004	0.002	0.002	0.001	0.002
IID835	0.008	0.008	0.002	0.004	0.004	0.008	0.008	0.008	0.004	0.004

[a] AD, agar dilution; MD, microdilution.

DISCUSSION

It was observed that FB was capable of supporting the growth of all of the *N. gonorrhoeae* strains tested in the present study. In some strains, a lag growth phase atypical for *N. gonorrhoeae* was observed prior to the log growth phase. The duration of the lag phase differed according to the strain tested. A long lag phase was observed for the growth of *N. gonorrhoeae* ATCC49226, N4-2, N4-2-4-2, TK106, and TK109 (10 h). However, after 24 h of incubation, the viable counts of

TABLE 3. Comparison of MICs by microdilution versus agar dilution for quinolone-resistant strains of *N. gonorrhoeae*

N. gonorrhoeae strain	MIC (μg/ml) of[a]:									
	Norfloxacin		Ciprofloxacin		Ofloxacin		Levofloxacin		Gatifloxacin	
	AD	MD	AD	MD	AD	MD	AD	MD	AD	MD
WHO A	0.008	0.031	0.002	0.004	0.008	0.016	0.004	0.008	0.002	0.004
R-4/5	0.25	0.5	0.031	0.063	0.125	0.25	0.063	0.125	0.016	0.016
R-8/1	0.125	0.5	0.031	0.063	0.063	0.125	0.031	0.063	0.008	0.016
R-8/4	0.25	0.5	0.031	0.063	0.125	0.25	0.063	0.125	0.016	0.031
R-8/5	0.25	0.5	0.016	0.063	0.063	0.125	0.031	0.063	0.016	0.016
R-4/5/1	0.5	2	0.063	0.125	0.125	0.25	0.063	0.25	0.016	0.031
R-4/5/2	0.5	2	0.063	0.125	0.125	0.5	0.063	0.25	0.016	0.031
R-4/5/3	0.5	2	0.063	0.125	0.125	0.25	0.063	0.125	0.016	0.031
ATCC49226	0.016	0.031	0.002	0.004	0.008	0.016	0.004	0.008	0.002	0.004
N-4-2	0.25	0.5	0.063	0.125	0.125	0.25	0.063	0.125	0.016	0.031
N-4-2-4-2	1	2	0.125	0.25	0.25	0.25	0.125	0.125	0.031	0.031
TK106	4	2	0.5	0.5	1	1	0.5	0.5	0.125	0.125
TK109	16	16	2	2	4	8	4	4	0.5	0.5
	Moxifloxacin		Sparfloxacin		Trovafloxacin		Garenoxacin		Gemifloxacin	
	AD	MD	AD	MD	AD	MD	AD	MD	AD	MD
WHO A	0.004	0.008	0.001	0.002	0.002	0.002	0.001	0.002	0.001	0.002
R-4/5	0.016	0.031	0.008	0.031	0.008	0.016	0.008	0.016	0.008	0.008
R-8/1	0.016	0.031	0.008	0.016	0.008	0.016	0.008	0.008	0.008	0.008
R-8/4	0.016	0.031	0.016	0.031	0.008	0.016	0.008	0.008	0.008	0.008
R-8/5	0.016	0.031	0.008	0.016	0.008	0.016	0.008	0.008	0.008	0.008
R-4/5/1	0.031	0.063	0.031	0.063	0.063	0.25	0.063	0.063	0.031	0.063
R-4/5/2	0.031	0.063	0.031	0.063	0.063	0.125	0.031	0.063	0.031	0.063
R-4/5/3	0.031	0.063	0.016	0.063	0.031	0.063	0.016	0.031	0.008	0.016
ATCC49226	0.008	0.008	0.004	0.004	0.008	0.008	0.008	0.004	0.002	0.002
N-4-2	0.031	0.031	0.063	0.063	0.031	0.063	0.031	0.031	0.016	0.016
N-4-2-4-2	0.063	0.063	0.125	0.25	0.5	1	0.25	0.25	0.125	0.125
TK106	0.125	0.125	0.25	0.25	0.5	1	0.25	0.25	0.25	0.5
TK109	0.5	1	0.5	1	0.5	1	0.25	0.5	0.5	1

[a] AD, agar dilution; MD, microdilution.

TABLE 4. Comparative antimicrobial susceptibilities of *N. gonorrhoeae* clinical isolates (71 strains)

Antimicrobial agent	MIC (μg/ml)					
	Agar dilution			Microdilution		
	50%	90%	Range	50%	90%	Range
Norfloxacin	8	32	0.008–64	8	32	0.016–64
Ciprofloxacin	2	16	0.001–32	4	16	0.002–32
Ofloxacin	4	16	0.008–32	4	16	0.008–32
Levofloxacin	2	8	0.004–8	2	8	0.004–16
Gatifloxacin	0.5	2	0.002–2	0.5	2	0.002–2
Moxifloxacin	1	4	0.004–4	2	4	0.004–4
Sparfloxacin	1	4	0.001–8	2	8	0.001–16
Trovafloxacin	0.5	4	0.001–8	1	8	0.002–16
Garenoxacin	0.5	2	0.001–4	0.5	2	0.001–4
Gemifloxacin	0.5	2	0.001–4	0.5	4	0.001–8

TABLE 5. Agreement of microdilution with agar dilution for susceptibility testing of *N. gonorrhoeae*

Antimicrobial agent	% Deviation between MICs at dilution step:					
	−2	−1	0	+1	+2	+3
Norfloxacin		5.6	53.5	40.8		
Ciprofloxacin		2.8	54.9	40.8		1.4
Ofloxacin		4.2	53.5	42.3		
Levofloxacin		7.0	47.9	43.7	1.4	
Gatifloxacin		14.1	54.9	31.0		
Moxifloxacin		9.9	50.7	35.2	4.2	
Sparfloxacin		1.4	28.2	60.6	9.9	
Trovafloxacin	1.4	9.9	29.6	54.9	4.2	
Garenoxacin	5.6	16.9	42.3	35.2		
Gemifloxacin	1.4	9.9	28.2	57.7	2.8	

all strains reached levels of more than 10^8 CFU/ml in FB. Therefore, it is possible that FB can be used to culture *N. gonorrhoeae* for various types of biological evaluation.

The evaluation of antibacterial agents, e.g., by in vitro PK/PD analysis, is performed based on the MICs. Therefore, the susceptibility of *N. gonorrhoeae* in FB would not be expected to differ substantially from that generally reported in the literature. Thus, the antibacterial susceptibilities determined by these microdilution procedures were compared with those determined by the agar dilution procedures recommended by the CLSI (formerly NCCLS). As regards the quality control strain of the CLSI (formerly NCCLS) (ATCC49226) and the laboratory-adapted strains used in this study, no significant differences were observed between the MICs obtained by either of the procedures. Additionally, the MIC_{90}s of quinolones against clinical isolates determined by microdilution were almost equal to or even twofold higher than those determined by agar dilution; moreover, the MICs associated with more than 90% of the clinical isolates tested were observed to be within only 1 doubling difference when the agar dilution and microdilution procedures were compared. As regards various types of bacteria, including both laboratory-adapted strains and clinical isolates, the MICs of quinolones determined by both procedures were found differ slightly. It appears that such slight differences between the MICs are reflective of the standard deviation in this study. Therefore, the microdilution method may prove to be useful for the determination of the antibacterial activities of quinolones against *N. gonorrhoeae*, and the present culture method using FB could be applied for various types of evaluation of quinolone.

In Asia, the number of gonococcal infections caused by antibacterial-resistant strains has gradually increased since the mid-1990s. The rapid emergence of clinical isolates of *N. gonorrhoeae* with decreased susceptibility to quinolones has been reported previously (11, 24). In Hawaii, it was reported that a total of 10.4% of gonococcal isolates in 2000 were ciprofloxacin resistant, compared with <1.5% per year from 1990 to 1997 (12). In order to investigate this emerging quinolone resistance among clinical isolates of *N. gonorrhoeae*, it will be necessary to develop more expedient studies such as sequential resistance development and in vitro PK/PD analysis as well as time-kill studies, etc. Growth methods using a liquid medium offer several advantages over the more commonly used agar methods.

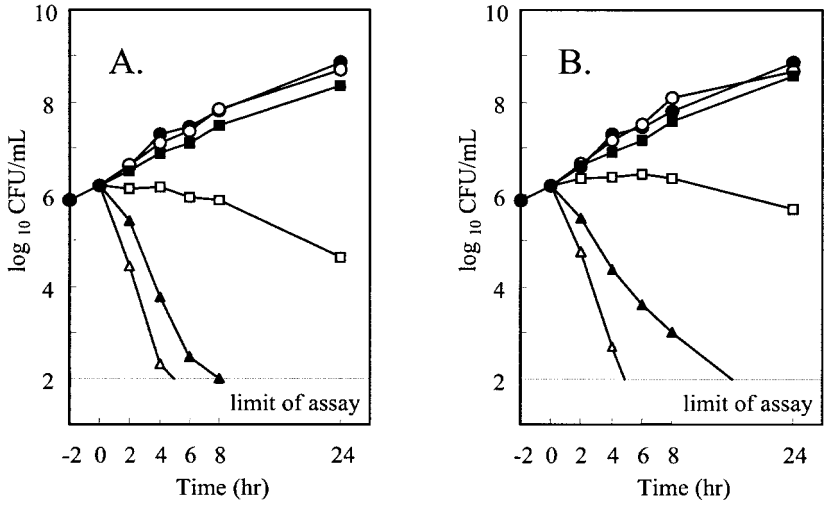

FIG. 2. Time-kill curves of gatifloxacin (panel A; MIC, 0.004 μg/ml) and ciprofloxacin (panel B; MIC, 0.004 μg/ml) against *N. gonorrhoeae* WHO A in FB. Symbols: ●, control; ○, 0.25 × MIC; ■, 0.5 × MIC; □, 1 × MIC; ▲, 2 × MIC; Δ, 4 × MIC.

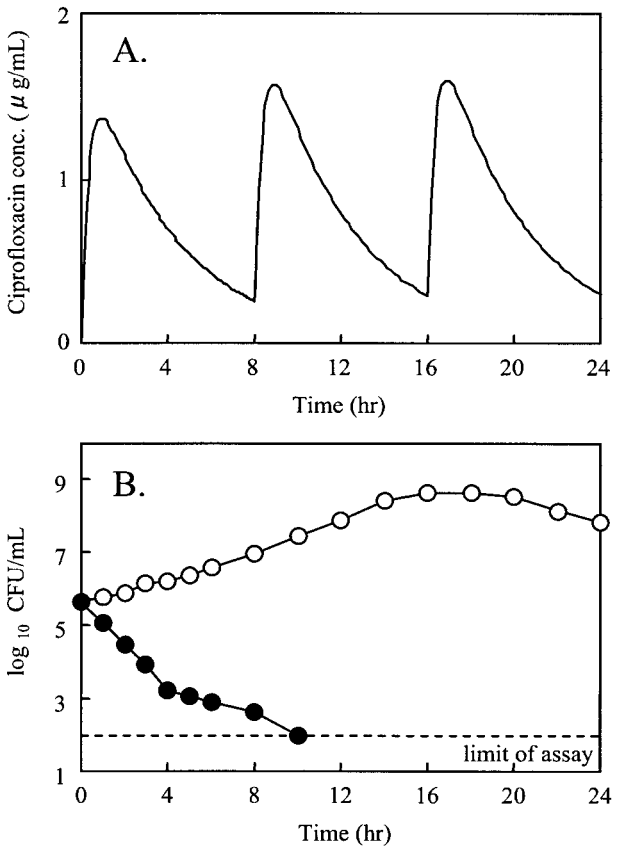

FIG. 3. Bactericidal effect of ciprofloxacin with a simulation concentration in serum at a dose of 200 mg t.i.d. on quinolone-resistant *N. gonorrhoeae* R-4/5/1 (A); MIC, 0.125 μg/ml (B). Symbols: ○, control; ●, 200 mg t.i.d. simulation.

Here, we performed time-kill kinetic studies of gatifloxacin and ciprofloxacin. These results indicated that both quinolones produced concentration-dependent killing effects over a 24-h period. At 2 to 4 × MIC, both compounds were predominantly bactericidal, and at the MIC, the activity ranged from bactericidal to bacteriostatic. At 0.25 to 0.5 × MIC, gonococcal growth was comparable to that of the growth control. Concentration-dependent time-kill was observed among the quinolones. These results suggest that the cultivation of *N. gonorrhoeae* in FB is useful for PK/PD analysis using an in vitro simulation model.

Unlike the United States regimen, that frequently used as the primary treatment for gonorrhea in Japan involves multiple and lower doses of quinolones, which results in the exposure of *N. gonorrhoeae* clinical strains to low concentrations of the agents. It has been suggested that the administration of large amounts of quinolones for clinical use will enhance the prevalence of quinolone resistance (11). However, the viable CFU counts of a genetically characterized quinolone-resistant strain (R-4/5/1) were reduced below the limit of detection by a simulated serum concentration after oral dosing of ciprofloxacin according to the Japanese clinical regimen (200 mg t.i.d.). These results contradict the present situation observed in the clinical setting in Japan.

Moran and Levine proposed "therapeutic time" as a pharmacodynamic parameter for the treatment of gonorrhea, whereby the therapeutic time was defined as the interval between the time the peak plasma concentration was reached and the time the plasma concentration dropped to less than four times the MIC_{90} (18). According to that report, an effective gonococcal cure was expected when the value of the therapeutic time was 10 h or more according to all antimicrobial regimens, including the use of quinolones. The therapeutic time of the Japanese clinical regimen of ciprofloxacin against R-4/5/1 (MIC, 0.125 μg/ml) was 14.4 h. No discrepancy was observed between the prediction of the pharmacodynamic parameter (therapeutic time) and the results of the in vitro simulation model in this study. Therefore, in order to predict a clinical outcome, further detailed PK/PD analysis using an in vitro simulation model is still needed to determine the impact of the PK/PD parameters on bactericidal activity and resistance selectivity.

The use of in vitro PK/PD dynamic models using FB is likely to give the optimum pharmacodynamic parameters for the application of quinolone in the treatment of gonorrhea. It is hoped that such an approach will render it possible to propose an effective regimen of quinolones without risking increased microbial resistance.

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