Time-kill curve analysis and pharmacodynamic functions for *in vitro* evaluation of antimicrobials against *Neisseria gonorrhoeae*

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Gonorrhea is a sexually transmitted infection caused by the Gram-negative bacterium *Neisseria gonorrhoeae*. Resistance to first-line empirical monotherapy has emerged, so robust methods are needed to appropriately evaluate the activity of existing and novel antimicrobials against the bacterium. Pharmacodynamic functions, which describe the relationship between the concentration of antimicrobials and the bacterial net growth rate, provide more detailed information than the MIC only. In this study, a novel standardized *in vitro* time-kill curve assay was developed. The assay was validated using five World Health Organization *N. gonorrhoeae* reference strains and various concentrations of ciprofloxacin, and then the activity of nine antimicrobials with different target mechanisms were examined against a highly susceptible clinical wild type isolate (cultured in 1964). From the time-kill curves, the bacterial net growth rates at each antimicrobial concentration were estimated. Finally, a pharmacodynamic function was fitted to the data, resulting in four parameters that describe the pharmacodynamic properties of each antimicrobial. Ciprofloxacin resistance determinants shifted the pharmacodynamic MIC (zMIC) and attenuated the bactericidal effect at antimicrobial concentrations above the zMIC. Ciprofloxacin, spectinomycin and gentamicin had the strongest bactericidal effect during the first six hours of the assay. Only tetracycline and chloramphenicol showed a purely bacteriostatic effect. The pharmacodynamic functions differed between antimicrobials, showing that the effect of the drugs at concentrations below and above the MIC vary widely. In conclusion, *N. gonorrhoeae* time-kill curve experiments analyzed with pharmacodynamic functions have potential for *in vitro* evaluation of new and existing antimicrobials and dosing strategies to treat gonorrhea.

Keywords: *Neisseria gonorrhoeae*; gonorrhea; treatment; antimicrobial resistance; time-kill curve analysis; pharmacodynamic function

Antimicrobial resistance in *Neisseria gonorrhoeae* is a major public health problem. Strains of *N. gonorrhoeae* have developed resistance to all antimicrobials introduced for treatment and rare strains have been classified as superbugs. Clinical resistance to the last option for empirical antimicrobial monotherapy, ceftriaxone, was firstly described in 2009 (1, 2). Currently, treatment recommendations for gonorrhea and prediction of the efficacy of antimicrobials mainly rely on a single measurement: the MIC of the antimicrobial. However, antimicrobials that have different modes of action and lead to different treatment outcomes can have identical MICs (2). A better understanding of the *in vitro* pharmacodynamic properties of antimicrobials could be used to optimize dosing strategies and help prevent treatment failures (3).

Regoes et al. (4) introduced the concept of pharmacodynamic functions to study the relationship between bacterial net growth rates and the concentrations of antimicrobials, based on analyses of time-kill curves for a single laboratory strain of *Escherichia coli*. Mathematically, the pharmacodynamic function is based on a Hill function and characterized by four parameters: the maximal bacterial growth rate in the absence of antimicrobial (ψ_{max}), the minimal bacterial growth rate at high concentrations of antimicrobial (ψ_{min}), the Hill coefficient (κ), and the pharmacodynamic MIC (zMIC) (Figure 1). Information about the effects of antimicrobials at concentrations below and above the MIC is particularly valuable for pathogens like *N. gonorrhoeae*, for which there are limited data about the pharmacokinetic and pharmacodynamic effects of many antimicrobials. Furthermore, the pharmacodynamic properties of novel antimicrobials with known and unknown targets could be evaluated and directly compared to a set of mechanistically well-understood compounds.

There is no standardized and quality assured procedure for time-kill curve analysis of the fastidious obligate human pathogen *N. gonorrhoeae*. Published time-kill protocols for *N. gonorrhoeae* (5–7) are not generalizable, owing to the highly divergent growth requirements of different strains and interpretation of results generally relies on qualitative expert judgement. To study a wide range of *N. gonorrhoeae* strains, growth in the absence of antimicrobials must

71 be consistent and comparable and bacterial growth phases at the time of exposure to
72 antimicrobial need to be synchronized in early to mid-log phase.

73 In this study, a standardized *in vitro* time-kill curve assay for *N. gonorrhoeae* was developed
74 using Graver-Wade (GW) medium. GW medium is a chemically defined, nutritious, liquid
75 medium that supports growth of a wide range of *N. gonorrhoeae* auxotypes and clinical isolates
76 starting from very low inocula (8). The novel time-kill curve assay was validated on five World
77 Health Organization *N. gonorrhoeae* reference strains with fluoroquinolone resistance
78 determinants. A highly susceptible *N. gonorrhoeae* isolate (DOGK18, 1964, Denmark) was
79 subsequently studied in detail and time-kill curve analysis performed for nine antimicrobials
80 that have been or currently are used to treat gonorrhea. In a second step, we obtained the
81 pharmacodynamic functions for each antimicrobial from the *in vitro* time-kill data and studied
82 their pharmacodynamic properties against *N. gonorrhoeae*.

83

84 **MATERIALS AND METHODS**

85 ***Neisseria gonorrhoeae* isolates and media.** The five international *N. gonorrhoeae* reference
86 strains WHO G, WHO K, WHO L, WHO M, and WHO N with different ciprofloxacin
87 conferring mutations in *gyrA*, *parC* and *parE* (9) and a clinical antimicrobial susceptible 'wild-
88 type' isolate cultured in 1964 in Denmark (DOGK18), were studied. Isolates were cultured,
89 from frozen stocks (-70°C), on GCAGP agar plates (3.6% Difco GC Medium Base agar [BD,
90 Diagnostics, Sparks, MD, USA] supplemented with 1% haemoglobin [BD, Diagnostics], 1%
91 IsoVitalex [BD, Diagnostics] and 10% horse serum) for 18-20 hours at 37°C in a humid 5%
92 CO_2-enriched atmosphere. Gonococcal colonies were subcultured once more on GCAGP agar
93 for 18-20 hours at 37°C in a humid 5% CO_2-enriched atmosphere, before being transferred to
94 the liquid sterile GW medium, prepared as earlier described (4), for growth curve and time-kill
95 experiments.

Viable cell counts. Bacterial viability was measured using a modified Miles and Misra method as previously described (10). Growing bacteria were removed from 96-well plates at specified time points using a multichannel pipette and diluted in sterile phosphate buffered saline (PBS) in six subsequent 1:10 dilutions (20 µl culture in 180 µl diluent). Ten µl droplets of each dilution were spotted on GCRAP (3.6% Difco GC Medium Base agar [BD, Diagnostics] supplemented with 1% hemoglobin [BD, Diagnostics] and 1% IsoVitalex [BD, Diagnostics]). GCRAP plates were dried with open lid in a sterile environment for 30-60 minutes prior to their usage. After drying the droplets (approximately 5-10 minutes), plates were incubated for 24 hours at 37°C in a humid 5% CO_2-enriched atmosphere. For every concentration and time point, colonies were counted for the first dilution that resulted in a countable range of 3-30 colonies and the CFU/ml calculated.

Growth curves. Prior to growth curve experiments strains were subcultured on Chocolate agar PolyViteX (Biomerieux). A 0.5 McFarland inoculum was prepared and diluted to 100 CFU/ml ($1{:}10^6$) in GW Medium (35°C). A volume of 100 µl diluted bacteria per well was transferred to Sarstedt round bottom 96 well plates. The plates were tightly sealed and bacteria were grown shaking at 100 rpm at 35°C in a humid 5% CO_2-enriched atmosphere. Bacterial growth was monitored over a time-course of sixty hours (0, 2, 4, 6, 8, 10, 12, 20, 22, 24, 26, 28, 30, 32, 34, 40, 44, 48, 60 hours). For every sampled time point, the content of one well was removed and viable counts determined (10). Growth curves were analyzed by plotting the log CFU/ml against the time and fitting a Gompertz growth model to the data as implemented in the package *cellGrowth* (11, 12). Only lag, log and stationary phases were included in the analysis and the decline phase excluded.

Time-kill assay. Time-kill curve analyses were performed by culturing *N. gonorrhoeae* in GW medium (4), in the presence of eleven antimicrobial-concentrations in doubling dilutions ranging from 0.016×MIC to 16×MIC. For DOGK18, the MICs were determined prior to the experiment using Etest (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's

instructions. For all other strains, previously published MIC values were used (9). The antimicrobials examined were ciprofloxacin (Sigma Aldrich, China), gentamicin (Sigma Aldrich, Israel), spectinomycin (Sigma Aldrich, Israel), azithromycin (Sigma Aldrich, USA), benzylpenicillin (Sigma Aldrich, USA), ceftriaxone (Sigma Aldrich, Israel), cefixime (European pharmacopeia reference standard, France), chloramphenicol (Sigma Aldrich, China) and tetracycline (Sigma Aldrich, China). Growth curves were initially performed to confirm that all strains would reach a stable early- to mid-log phase after four hours of pre-incubation in antimicrobial-free GW medium. Subsequently, a 0.5 McFarland inoculum of *N. gonorrhoeae* was prepared in sterile PBS from cultures grown on GCAGP agar plates for 18-20 hours at 37°C in a humid 5% CO_2-enriched atmosphere. For each strain, 30 µl of the inoculum was diluted in 15 ml pre-warmed (37°C) antimicrobial-free GW medium and 90 µl per well was dispersed in round bottom 96-well Sarstedt microtiter plates. The plates were pre-incubated for 4 h shaking at 150 rpm, 35°C in a humid 5% CO_2-enriched atmosphere. To each well containing 90 µl of pre-incubated bacteria, 10 µl of one of the antimicrobial concentrations (or PBS) was added, resulting in eight identical rows (one row for each time-point) containing bacteria exposed to eleven different antimicrobial concentrations and one untreated control.

Estimating bacterial growth rates. The bacterial net growth rates (ψ) were determined from changes in the density of viable bacteria (CFU/ml) during the first six hours of the time-kill experiments. The bacterial populations were assumed to grow or die at a constant rate, resulting in an exponential increase or decrease in bacterial density:

$$N(t) = N_0 \times e^{\psi t}.$$

The net growth rate was estimated as the coefficient of a linear regression from the logarithm of the colony counts. Maximum likelihood estimation was used to account for the censored data (values below the limit of detection of 100 CFU/ml). The geometric mean of all measurements

147 at zero hours for a given antimicrobial as the first data point, was used. From the growth rate,
148 the bacterial doubling time can be calculated as follows:

149 $$T_{1/2} = \frac{\ln(2)}{\psi}.$$

150

151 **Pharmacodynamic function.** A pharmacodynamic function (Fig. 1) describes the
152 relationship between bacterial net growth rates (ψ) and the concentration of an antimicrobial
153 (a) (4):

154 $$\psi(a) = \psi_{\max} - \frac{(\psi_{\max} - \psi_{\min})\left(\frac{a}{\text{zMIC}}\right)^{\kappa}}{\left(\frac{a}{\text{zMIC}}\right)^{\kappa} - \frac{\psi_{\min}}{\psi_{\max}}},$$

155 where $\psi_{\max}$ is to the maximal bacterial growth rate in the absence of antimicrobial and $\psi_{\min}$ is
156 the minimal bacterial net growth rate at high concentrations of antimicrobial. zMIC is the
157 pharmacodynamic MIC where the bacterial growth rate is zero (ψ(zMIC) = 0). κ denotes the
158 Hill coefficient, which describes the steepness of the sigmoid relationship between bacterial
159 growth and antimicrobial concentration. For each antimicrobial, four parameters of the
160 pharmacodynamic function were estimated using a self-starter function, implemented in the
161 package *drc* (13) for the R software environment for statistical computing (14). All figures can
162 be reproduced with R code and data from the following GitHub repository:
163 https://github.com/sunnivas/PDfunction.

164

165 **RESULTS**

166 **Growth of *N. gonorrhoeae*.** Growth curves for the five different WHO *N. gonorrhoeae*
167 reference strains (Fig. S1 supplemental material) confirmed that growth was well supported in
168 GW medium. All strains could be grown from a starting inoculum of less than 10^3 CFU/ml and
169 had a lag phase of less than five hours. The stationary phase lasted until 36 hours for all strains,

followed by a steep declination phase. Growth was similar for all strains with WHO L the only strain that had a slightly slower growth.

Time-kill curves. Time-kill curves for ciprofloxacin using the WHO reference strains WHO G (MIC = 0.125 µg/ml), WHO K (MIC > 32 µg/ml), WHO L (MIC > 32 µg/ml), WHO M (MIC = 2 µg/ml), WHO N (MIC = 4 µg/ml) and DOGK18 (MIC = 0.008 µg/ml) are shown in Fig. 2. Ciprofloxacin induced a bactericidal effect in all six strains, but the onset of the bactericidal activity depended on the concentration of the antimicrobial and differed between strains. All strains with the exception of WHO M and WHO N were killed to below the limit of detection (100 CFU/mL) at the highest antimicrobial concentration (16×MIC). The susceptible DOGK18 strain experienced the most rapid killing during the first hour at high antimicrobial concentrations. For WHO G and WHO M, the bactericidal activity decreased during the six hours of the assay.

Time-kill curves for eight antimicrobials were made (spectinomycin, gentamicin, azithromycin, benzylpenicillin, ciprofloxacin, ceftriaxone, cefixime, chloramphenicol and tetracycline) using the highly antimicrobial susceptible DOGK18 strain (Fig. 3). Similar to the effect of ciprofloxacin (Fig. 2F), gentamicin and spectinomycin exhibited rapid killing during the first two hours of the assay for concentrations above MIC. Cefixime and ceftriaxone showed little effect from 0 hours to 3 hours, however, after that growth rate decreased rapidly. For benzylpenicillin and azithromycin, at concentrations above MIC, the killing started after one hour and decreased rapidly at later time points. The time-kill curves for tetracycline and chloramphenicol looked similar with almost no killing of bacteria within the assay time of four hours. Chloramphenicol showed a weak bactericidal effect at the highest antimicrobial concentration (Fig. 3).

Pharmacodynamic functions. The bacterial net growth rates were estimated from the time-kill curves by fitting a linear regression to the logarithm of the colony counts (Fig. 4A). The pharmacodynamic function was then fitted to the estimated growth rates at different

196 antimicrobial concentrations (Fig. 4B, solid line). Generally, the estimated net growth rates at
197 high antimicrobial concentrations reached a lower asymptote (ψ_{min}). In some cases, an
198 additional drop was observed in the estimated growth rates at very high antimicrobial
199 concentrations (Fig. 4B, dashed line). This phenomenon occurred at antimicrobial
200 concentrations that are likely to be toxic, so those data points were removed before estimating
201 the parameters of the pharmacodynamic function.

202 The pharmacodynamic functions for the six strains provided information on ciprofloxacin
203 resistance mechanisms (Fig. 4C and Table S3 in the supplemental material). The DOGK18
204 strain had a low pharmacodynamic MIC (zMIC) and a low net bacterial growth rate at high
205 antimicrobial concentrations (ψ_{min}), indicating the strong bactericidal effect of ciprofloxacin.
206 The five WHO reference strains showed that the ciprofloxacin resistance determinants not only
207 shifted the zMIC but also resulted in less killing at antimicrobial concentrations above the zMIC
208 (higher ψ_{min} compared to DOGK18 strain).

209 The pharmacodynamic functions for the nine antimicrobials in the DOGK18 strain
210 illustrated the different effects that antimicrobials induce on gonococcal growth of *N.*
211 *gonorrhoeae* (Fig. 4D and Table 1). The average of the maximal growth rate in the absence of
212 antimicrobials over all experiments was ψ_{max} = 0.77 h^{-1} (95% confidence interval [CI]: 0.71-
213 0.84 h^{-1}). This corresponds to a bacterial doubling time of $T_{1/2}$ = 54 min (95% CI: 49-59 min).
214 Ciprofloxacin, spectinomycin and gentamicin induced the strongest bactericidal effect with ψ_{min}
215 < -5 h^{-1}. Chloramphenicol and tetracycline exhibited almost no killing within the six hours of
216 the assay (ψ_{min} > -0.2 h^{-1}). The Hill coefficient κ ranged between 1.0 and 2.5. All four parameters
217 of the pharmacodynamic function were very similar for ceftriaxone, cefixime and the
218 bacteriostatic compounds chloramphenicol and tetracycline. Generally, the estimated zMIC
219 was in good agreement with the MIC measured by Etest but there were substantial deviations
220 for benzylpenicillin and cefixime.

221

DISCUSSION

A robust and reliable method to appropriately evaluate antimicrobial treatment options *in vitro* is one of the tools that is urgently needed to support tackling the problem of antimicrobial resistant *N. gonorrhoeae*. In this study, a standardized *in vitro* time-kill curve assay was developed and the resulting data were used to study pharmacodynamics parameters describing the relationship between the concentration of antimicrobials and the bacterial net growth rate. Our study applied the concept of pharmacodynamic functions (4) to *N. gonorrhoeae* for the first time. To our knowledge, this is also the first study to obtain and compare the pharmacodynamic functions of an antimicrobial in susceptible and resistant strains of the same pathogen species, opening up avenues into phenotypically understanding the effects of different resistance determinants.

The developed time-kill assay worked well for different *N. gonorrhoeae* strains, including highly resistant isolates. Growing the bacteria in microwell plates provided a high throughput and made it possible to study a wider range of antimicrobial concentrations in the same experiment. Growth curves confirmed reproducible growth in absence of antimicrobials, WHO L had a prolonged lag phase and grew slightly slower than the other strains. Therefore in the time-kill assays bacteria were grown for four hours before adding the antimicrobial. The assay time was limited to six hours and growth in absence of antimicrobials highly consistent and exponential for all strains. The five WHO reference strains tested have different combinations of ciprofloxacin resistance-conferring mutations in *gyrA*, *parC* and *parE*. These mutations resulted in a shift of the pharmacodynamic MIC (zMIC) and reduced the antimicrobial killing at the same time, showing that even high doses of ciprofloxacin had a limited effect on the growth of these strains. The analysis of time-kill data with a pharmacodynamic function confirmed the strong bactericidal effects of ciprofloxacin, gentamicin and spectinomycin. Ciprofloxacin is a prime example of a bactericidal antimicrobial, representing the class of topoisomerase II inhibiting fluoroquinolones (15). Spectinomycin inhibits protein translation

248 through blocking of tRNA translocation, whereas gentamicin induces translational misreading
249 (16–18). While spectinomycin is a well-recognised treatment option for gonorrhea and its
250 bactericidal action has been described previously (19, 20), gentamicin is only recommended
251 first-line treatment for gonorrhea in Malawi, where it is used together with doxycycline in the
252 syndromic management algorithm for urethritis (21). However, gentamicin has been suggested
253 for wider use in the treatment of gonorrhea (22–24). The cell wall inhibiting β-lactam
254 antimicrobials are known to have a time-dependent mode of action (25, 26). Therefore it was
255 not surprising that benzylpenicillin, ceftriaxone and cefixime were characterized by slower
256 killing ($-1.6\ h^{-1} < \psi_{min} < 0.6\ h^{-1}$). Although currently not used for treatment of *N. gonorrhoeae*,
257 chloramphenicol and tetracycline often act as model compounds for bacteriostatic effects (27,
258 28). These effects were confirmed by the estimates of the net bacterial growth rate at high
259 antimicrobial concentrations that were close to zero.

260 Regoes et al. (4) hypothesized that the Hill coefficient of the pharmacodynamic function (κ)
261 reflects the time and concentration dependency of antimicrobials. For example, ciprofloxacin
262 is thought to act in a concentration-dependent manner and a high value of κ would require the
263 antimicrobial concentrations to be at or above the zMIC. Tetracycline was considered a time-
264 dependent antimicrobial which could be characterized by lower values of κ. Our estimate for
265 tetracycline was not significantly different from that of the bactericidal ciprofloxacin and was
266 not as low as reported for *E. coli* (4). These discrepancies could reflect differences in time and
267 concentration dependency of antimicrobials in *N. gonorrhoeae* and *E. coli*, different
268 concentration ranges studied (up to 100x MIC) or could be simply due to the properties of the
269 specific strain Regoes et al. (4) studied.

270 There are some limitations of the methods used in the present study. First, the rapid
271 bactericidal effects of some antimicrobials occurred minutes after the compound was added
272 resulting in bacterial counts below limit of detection at the first time point. These effects can
273 make it challenging to estimate the minimal bacterial net growth rate at high concentrations of

antimicrobials (ψ_{min}), as observed for gentamicin for example. Second, exceedingly high antimicrobial concentrations can kill *N. gonorrhoeae* almost instantaneously. This might explain the observed outliers that deviated from the pharmacodynamic function (Fig. 4B). Third, the time-kill curves appeared to level off over time for bactericidal compounds in susceptible strains. Interestingly, this phenomenon might represent a physiological adaptation to those antimicrobials, often described as persister cell formation (29–34). This non-exponential killing makes it difficult to estimate the net growth rate with linear regression. The clinical relevance of persister cells has been demonstrated for chronic infections such as tuberculosis, cystic fibrosis and infections caused by S*taphylococcus aureus* (35–36). For *N. gonorrhoeae*, however, this phenomenon has not been previously described. Fourth, the proposed method allows appropriate evaluation of antimicrobials against *N. gonorrhoeae in vitro* only.

The *in vitro* pharmacodynamic parameters can provide relative comparisons across different strains and antimicrobials which can be extremely valuable in preclinical studies. However, deriving rational resistance breakpoints for different dosing schedules will have to come from clinical pharmacokinetic and pharmacodynamic (PK/PD) studies that include parameters such as serum concentrations and half-life time of the antimicrobial. For benzylpenicillin, ceftriaxone and cefixime the time of free antimicrobial above the MIC value should be maximized (37–40), suggesting that multiple dose treatment would be a rational strategy. However the longer serum half-life of ceftriaxone (41) compared to other β-lactam antimicrobials suggests that increasing the dose is still efficacious for most *N. gonorrhoeae* strains. Fluoroquinolones and aminoglycosides, which act in a concentration dependent and bactericidal manner should be given as a single high dose (42). This is typically achieved maximizing the AUC/MIC and peak serum concentration/MIC ratio (43-45). Our results suggest that this could be the case for ciprofloxacin, gentamicin and spectinomycin, which were found to be strongly bactericidal. Azithromycin has been described to be bacteriostatic in

Staphylococcus aureus, Streptococcus pneumoniae or *Haemophilus influenza* (46) but appears to act bactericidal on *Pseudomonas aeruginosa* (47). Our in-vitro pharmacodynamics parameters suggest that a clear classification of azithromycin as bacteriostatic or bactericidal is not possible for *N. gonorrhoeae*.

In summary, the present study shows that evaluation of the parameters of a pharmacodynamic function based on time-kill data can add valuable information beyond that of MIC values for different antimicrobials. The quantitative assessment of pharmacodynamic properties provides a more detailed picture about antimicrobial-induced effects on *N. gonorrhoeae*. The developed time-kill curve assay, in combination with the concept of the pharmacodynamic function, could be used for *in vitro* evaluation of new and existing antimicrobials and the effects of combining antimicrobials against *N. gonorrhoeae*.

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TABLE 1 Parameter estimates of the pharmacodynamic function for nine antimicrobials in the antimicrobial susceptible *Neisseria gonorrhoeae* strain DOGK18.

Antimicrobial	**Antimicrobial class**	κ [a]	ψ_{min} (h^{-1}) [a]	ψ_{max} (h^{-1}) [a]	**zMIC (µg/ml)** [a]	**MIC (µg/ml)** [b]
Ciprofloxacin	Fluoroquinolone	1.1±0.1	-8.9±2.2	0.7±0.4	0.002±0.0001	<0.004
Gentamicin	Aminoglycoside	2.0±0.3	-107.4±140.6	0.9±0.07	0.2±0.04	1
Spectinomycin	Aminocyclitol	1.0±0.6	-9.6±1	0.7±0.03	5±0.7	4
Azithromycin	Macrolide	2.5±0.2	-2.2±0.1	0.6±0.05	0.3±0.03	0.19
Benzylpenicillin	β-lactam	1.1±0.1	-1.6±0.6	0.9±0.2	0.004±0.002	0.032
Ceftriaxone	β-lactam	1.6±0.1	-0.6±0.2	0.8±0.07	0.0003±0.0001	<0.002
Cefixime	β-lactam	1.7±0.5	-0.8±0.2	0.8±0.1	0.0002±0.0002	0.016
Chloramphenicol	Chloramphenicol	1.8±0.4	-0.1±0.01	0.7±0.2	0.5± 0.1	0.19
Tetracycline	Tetracycline	1.0±0.2	-0.2±0.08	0.8±0.07	0.5±0.3	0.125

[a] *Estimates are given as arithmetic means and standard deviations from two independent experiments. Parameter estimates for each individual experiment are given in Table S2 of the supplemental material.*

[b] *MIC values measured with Etest in accordance with the manufacturer´s instructions.*

Figure legends

FIG 1 The pharmacodynamics function and four parameters is shown as described in (4). The bacterial growth rates (ψ) in response to each antimicrobial concentration are estimated from time-kill data with linear regression. The maximal bacterial growth rate ψ_{max}, the minimal bacterial net growth rate at high concentrations of antimicrobial ψ_{min}, the pharmacodynamic MIC (zMIC) and the Hill coefficient κ are shown and define the shape of the curve.

FIG 2 Time-kill curves for ciprofloxacin and six different *Neisseria gonorrhoeae* strains: WHO G (A), WHO K (B), WHO L (C), WHO M (D), WHO N (E) and DOGK18 (F). Controls without antimicrobials are shown in red. Twelve doubling dilutions are plotted, the

highest concentration (black line) corresponds to 16× MIC as measured with Etest. The limit of detection in the assay was 100 CFU/ml. Antimicrobial was added at time 0 h.

FIG 3 Time-kill curves for the *Neisseria gonorrhoeae* DOGK18 strain using eight different antimicrobials: gentamicin (A), spectinomycin (B), azithromycin (C), benzylpenicillin (D), ceftriaxone (E), cefixime (F), chloramphenicol (G) and tetracycline (H). Twelve doubling dilutions are plotted, the highest concentration (black line) corresponds to 16× MIC as measured with Etest. The limit of detection in the assay was 100 CFU/ml. Antimicrobial was added at time 0 h.

FIG 4 Pharmacodynamic functions for different antimicrobials and *Neisseria gonorrhoeae* strains. (A) Estimating growth rates (cefixime in DOGK18). Dashed lines represent linear regressions of the logarithm of the colony counts at different antimicrobial concentrations. The coefficient of the linear regression corresponds to the net bacterial growth rate (cefixime in DOGK18). (B) Fitting the pharmacodynamic function to estimated growth rates. Points correspond to the estimated net bacterial growth rates at different antimicrobial concentrations. The solid line shows the model fit after removing outliers at high antimicrobial concentrations. The dashed line indicates the model fit including all data points. The growth rate in absence of antimicrobial is shown in red at a concentration that is 10-fold lower than the lowest concentration. (C) Pharmacodynamics functions for ciprofloxacin in six *N. gonorrhoeae* strains (Low level resistance (LLR)=WHO G; High level resistance (HLR)=WHO K, WHO L; Resistance (R)=WHO M, WHO N; and Susceptible (S)= DOGK18) (D) Pharmacodynamic functions for nine different antimicrobials in DOGK18 strain. Each curve is based on the arithmetic mean of the estimated parameters from two independent time-kill experiments (as in Table 1).

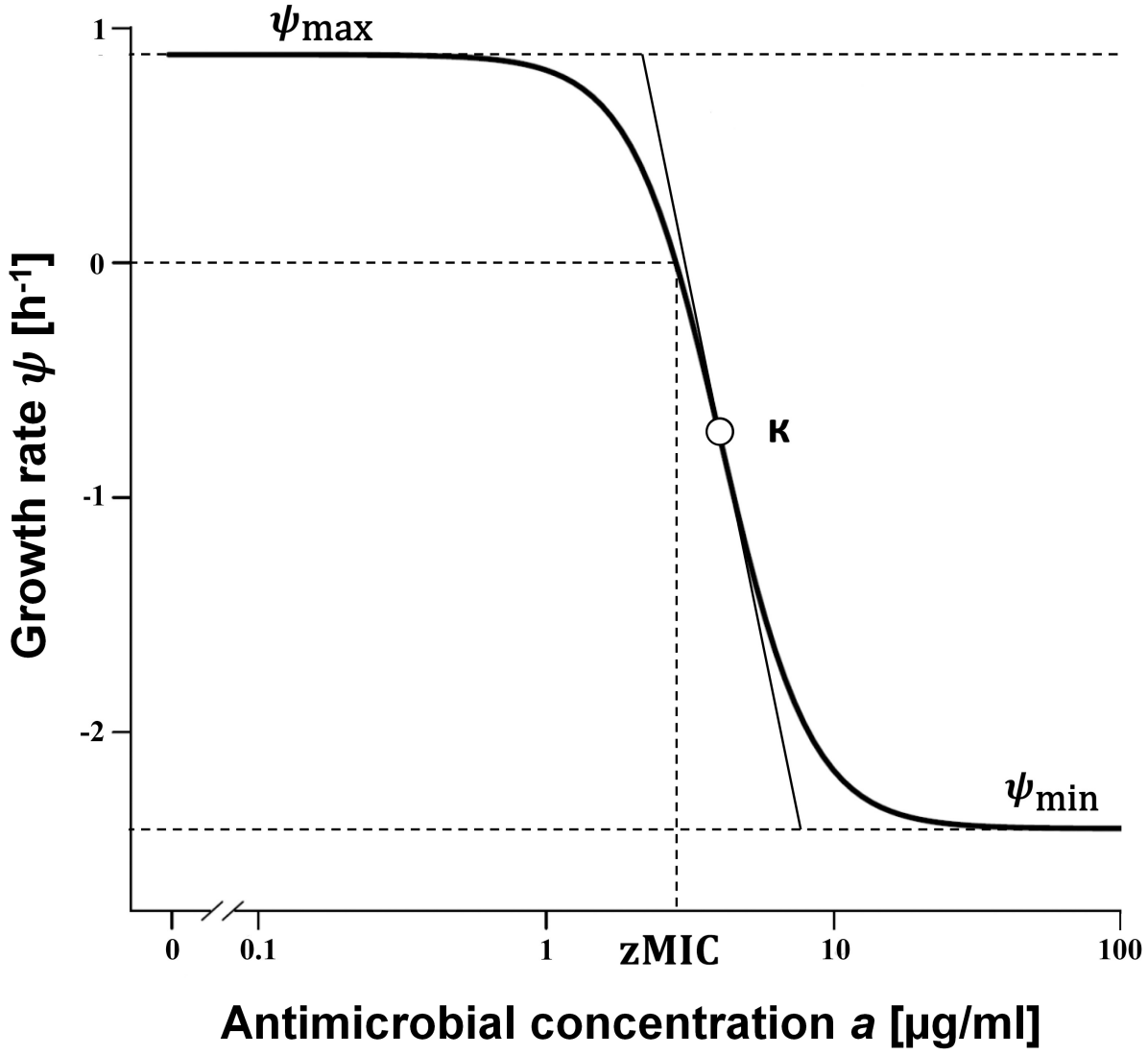


FIG 1 The pharmacodynamics function and four parameters is shown as described in (4). The bacterial growth rates (ψ) in response to each antimicrobial concentration are estimated from time-kill data with linear regression. The maximal bacterial growth rate $\psi_{\max}$, the minimal bacterial net growth rate at high concentrations of antimicrobial $\psi_{\min}$, the pharmacodynamic MIC (zMIC) and the Hill coefficient κ are shown and define the shape of the curve.

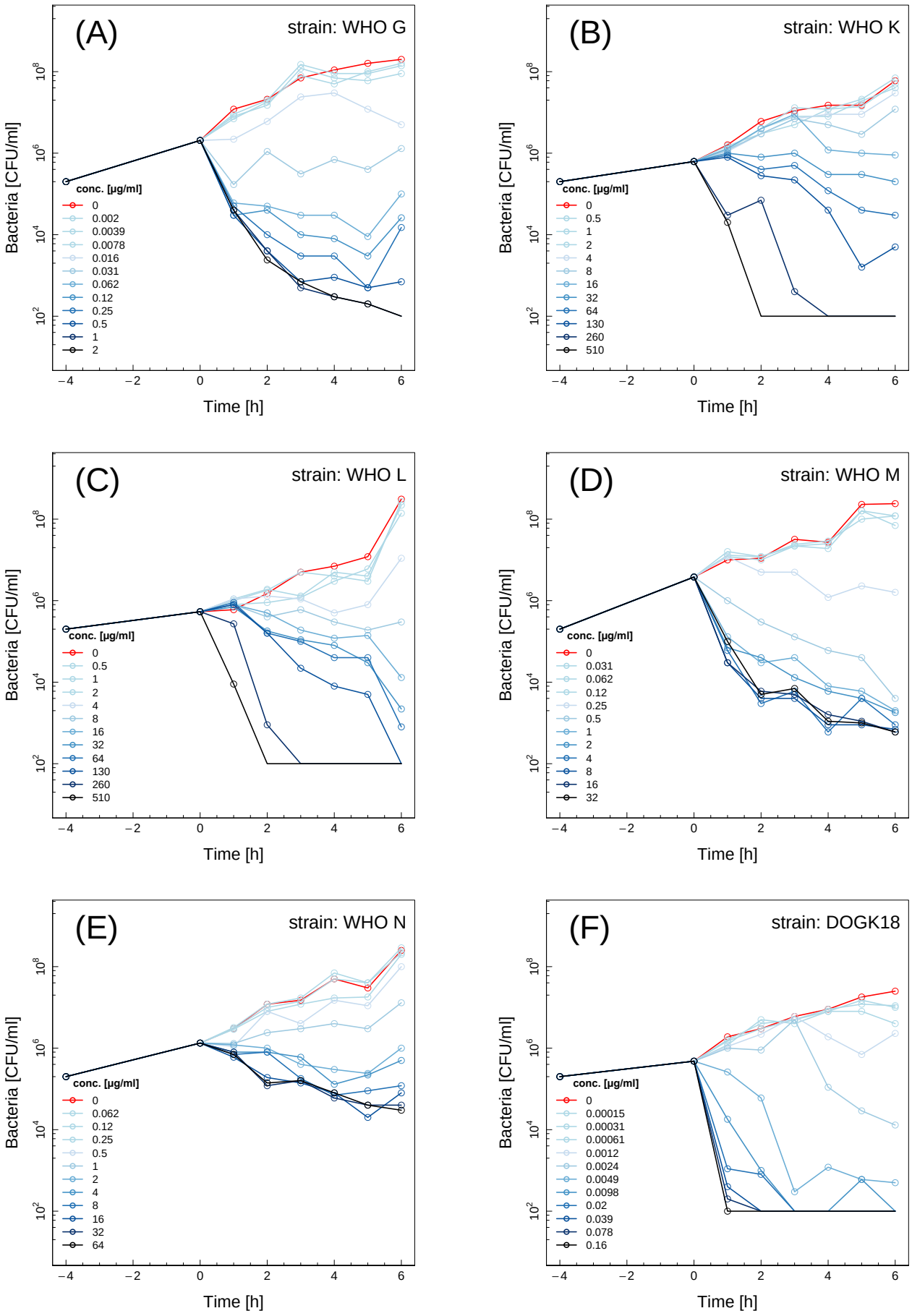


FIG 2 Time-kill curves for ciprofloxacin and six different *Neisseria gonorrhoeae* strains: WHO G (A), WHO K (B), WHO L (C), WHO M (D), WHO N (E) and DOGK18 (F). Controls without antimicrobials are shown in red. Twelve doubling dilutions are plotted, the highest concentration (black line) corresponds to 16 MIC as measured with Etest. The limit of detection in the assay was 100 CFU/ml. Antimicrobial was added at time 0 h.

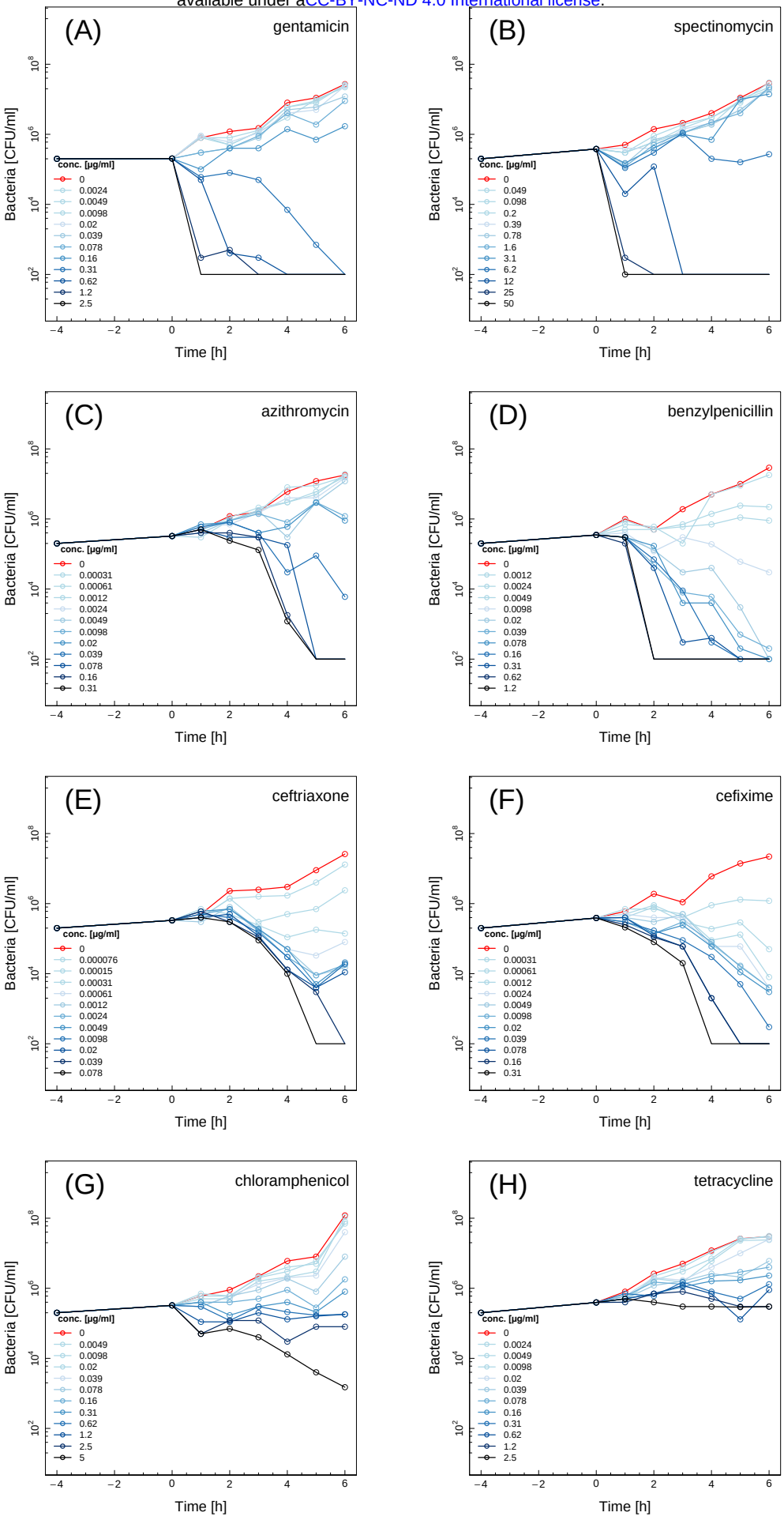


FIG 3 Time-kill curves for the *Neisseria gonorrhoeae* DOGK18 strain using eight different antimicrobials: gentamicin (A), spectinomycin (B), azithromycin (C), benzylpenicillin (D), ceftriaxone (E), cefixime (F), chloramphenicol (G) and tetracycline (H). Twelve doubling dilutions are plotted, the highest concentration (black line) corresponds to 16 MIC as measured with Etest. The limit of detection in the assay was 100 CFU/ml. Antimicrobial was added at time 0 h.

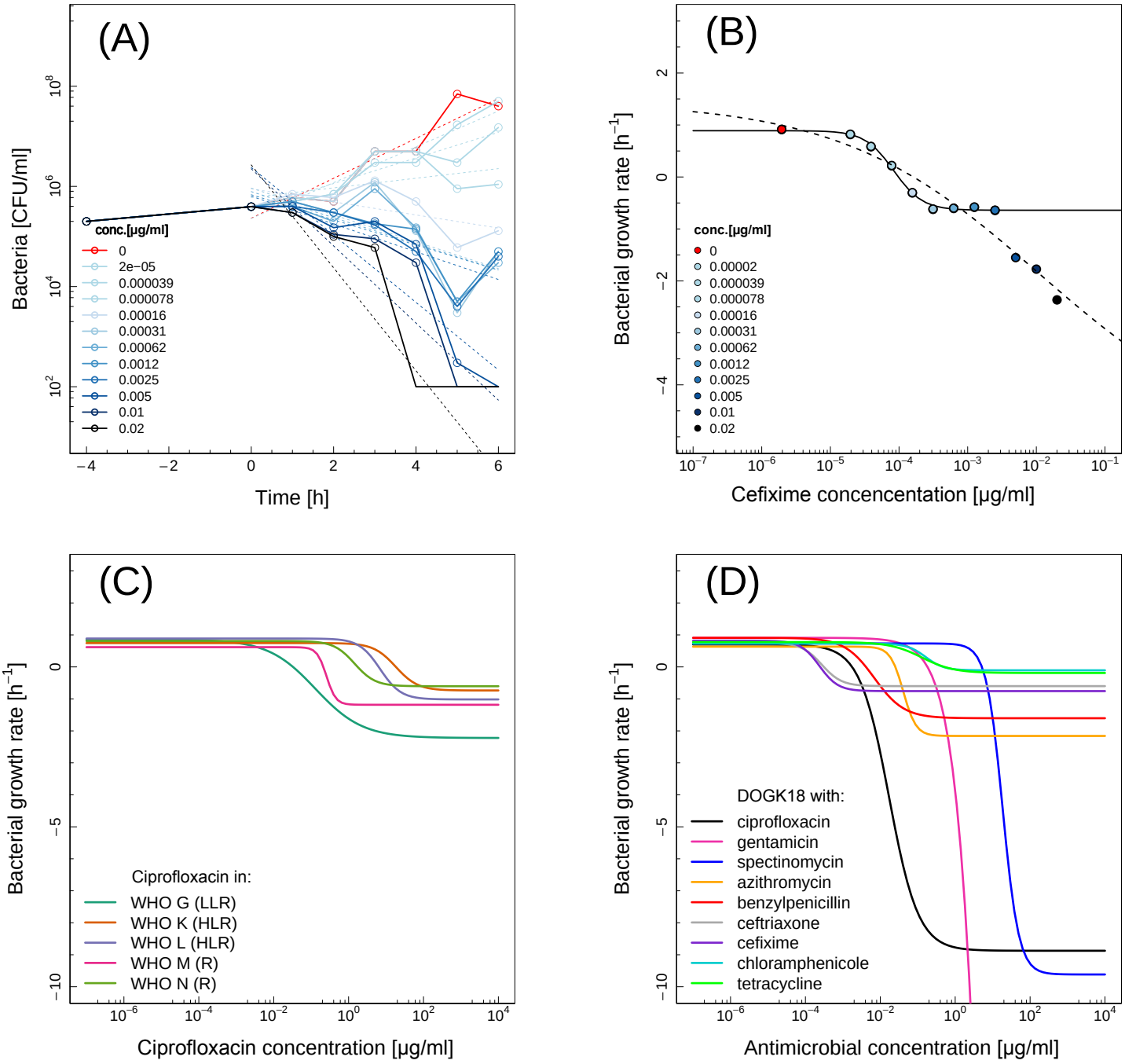
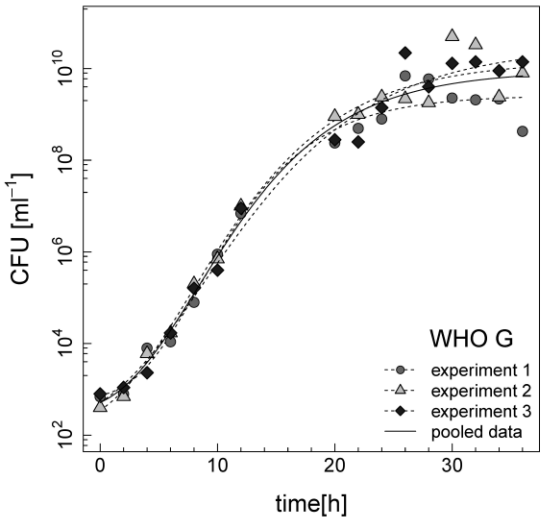
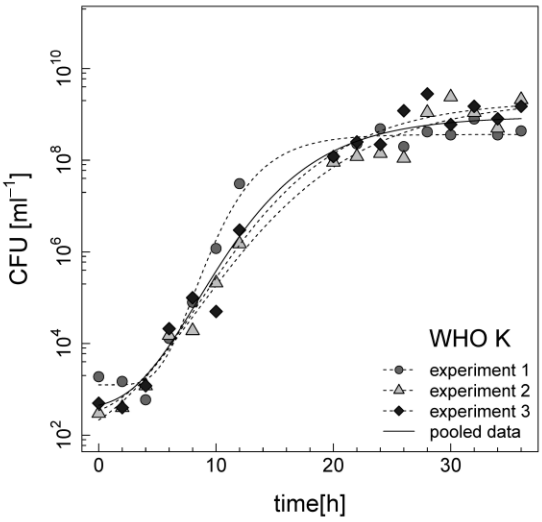
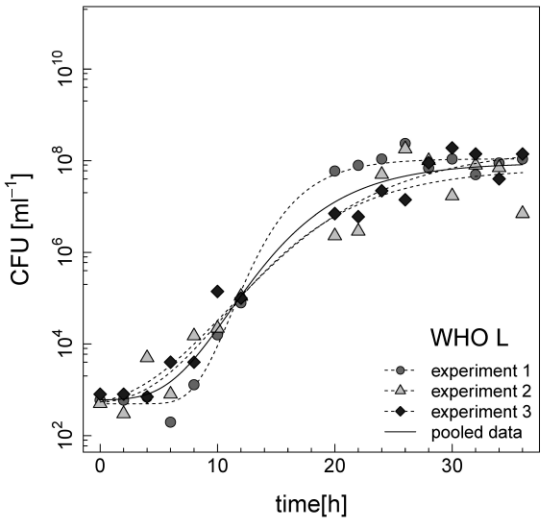


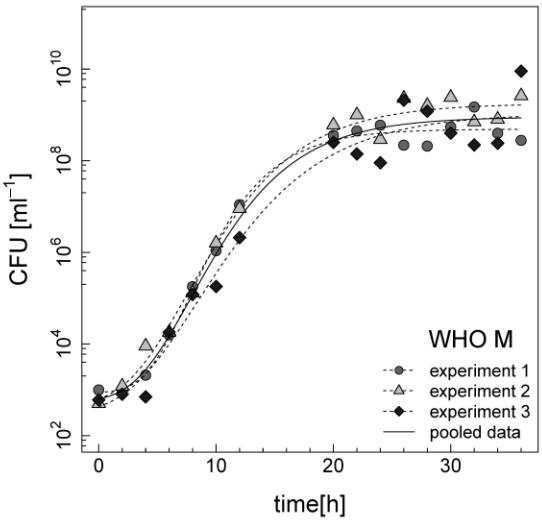
FIG 4 Pharmacodynamic functions for different antimicrobials and *Neisseria gonorrhoeae* strains. (A) Estimating growth rates (cefixime in DOGK18). Dashed lines represent linear regressions of the logarithm of the colony counts at different antimicrobial concentrations. The coefficient of the linear regression corresponds to the net bacterial growth rate (cefixime in DOGK18). (B) Fitting the pharmacodynamic function to estimated growth rates. Points correspond to the estimated net bacterial growth rates at different antimicrobial concentrations. The solid line shows the model fit after removing outliers at high antimicrobial concentrations. The dashed line indicates the model fit including all data points. The growth rate in absence of antimicrobial is shown in red at a concentration that is 10-fold lower than the lowest concentration. (C) Pharmacodynamics functions for ciprofloxacin in six *N. gonorrhoeae* strains (Low level resistance (LLR)=WHO G; High level resistance (HLR)=WHO K, WHO L; Resistance (R)=WHO M, WHO N; and Susceptible (S)= DOGK18) (D) Pharmacodynamic functions for nine different antimicrobials in DOGK18 strain. Each curve is based on the arithmetic mean of the estimated parameters from two independent time-kill experiments (as in Table 1).

Supplemental Material









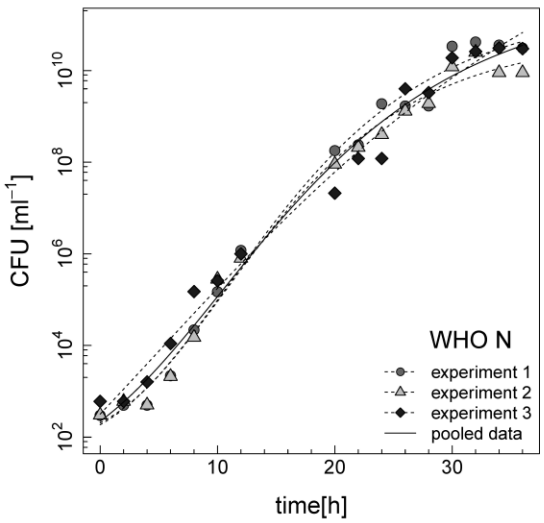


Figure S1. Growth curves for the 2008 WHO *Neisseria gonorrhoeae* reference strains WHO G (A), WHO K (B), WHO L (C), WHO M (D), WHO N (E). Data from three independent experiments are shown. CFU/ml for each time-point are shown in circles (experiment 1), triangles (experiment 2) and diamonds (experiment 3). A Gompertz growth model (1, 2) was fit to the data from three independent experiments (solid lane, pooled data). Individual fits from each of the experiments are shown as well in dashed lines. Growth rates were estimated in log phase between 2-20 hours (WHO G=0.75 [h^{-1}], WHO K=0.72 [h^{-1}], WHO L=0.57 [h^{-1}], WHO M=0.75 [h^{-1}], WHO N=0.70 [h^{-1}]). The maximal bacterial density was estimated as upper asymptote of the Gompertz model (WHO G=$9.74*10^9$ [CFU/ml], WHO K=$1.32*10^9$ [CFU/ml], WHO L=$6.57*10^7$ [CFU/ml], WHO M=$1.32*10^9$ [CFU/ml], WHO N=$5.32*10^{11}$ [CFU/ml]).

Table S1: Parameter estimates for nine different antimicrobials in *Neisseria gonorrhoeae* strain DOGK18 and model based standard errors.

Antimicrobial	κ	κ SE	ψ_{max} [h^{-1}]	ψ_{max} SE [h^{-1}]	ψ_{min} [h^{-1}]	ψ_{min} SE [h^{-1}]	zMIC [µg/ml]	zMIC SE [µg/ml]
Azithromycin	2.43	0.81	0.67	0.08	-2.25	0.17	0.0267	0.0050
Azithromycin	2.64	0.60	0.60	0.06	-2.07	0.11	0.0238	0.0033
Cefixime	2.07	0.23	0.89	0.04	-0.64	0.03	0.0001	0.0000
Cefixime	1.42	0.66	0.75	0.17	-0.87	0.10	0.0004	0.0001
Ceftriaxone	1.69	0.21	0.70	0.05	-0.74	0.03	0.0002	0.0000
Ceftriaxone	1.58	0.69	0.80	0.08	-0.46	0.09	0.0004	0.0001
Chloramphenicol	1.54	0.28	0.85	0.03	-0.12	0.04	0.3767	0.1099
Chloramphenicol	2.04	0.43	0.61	0.02	-0.10	0.03	0.5762	0.1461
Gentamicin	0.82	0.10	0.86	0.20	-206.80	397.96	0.1522	0.3604
Gentamicin	1.17	0.28	0.96	0.19	-7.96	1.60	0.2117	0.0474
Benzylpenicillin	1.19	0.29	0.77	0.15	-2.06	0.18	0.0053	0.0013
Benzylpenicillin	1.01	0.19	1.05	0.11	-1.15	0.09	0.0029	0.0005
Spectinomycin	2.41	0.11	0.76	0.04	-10.30	0.22	5.6452	0.2181
Spectinomycin	1.61	0.33	0.71	0.22	-8.94	0.96	4.6836	1.2304
Tetracycline	1.14	0.19	0.72	0.03	-0.25	0.05	0.3259	0.0860
Tetracycline	0.90	0.17	0.83	0.04	-0.13	0.07	0.7067	0.3721
Ciprofloxacin	1.04	0.21	0.98	0.25	-7.34	0.70	0.0017	0.0005
Ciprofloxacin	1.19	0.29	0.43	0.29	-10.40	1.27	0.0018	0.0009

Table S2: Parameter estimates for ciprofloxacin in five WHO *Neisseria gonorrhoeae* reference strains and DOGK18, and model based standard errors.

Strain	κ	κ SE	ψ_{max}	ψ_{max} SE	ψ_{min}	ψ_{min} SE	zMIC [µg/ml]	zMIC SE [µg/ml]
WHO G	0.69	0.14	0.86	0.12	-2.22	0.35	0.0327	0.0094
WHO K	1.47	0.29	0.75	0.03	-0.74	0.18	18.3111	3.9561
WHO L	1.56	0.27	0.89	0.06	-1.02	0.10	6.3510	0.7475
WHO M	3.49	0.77	0.62	0.05	-1.18	0.04	0.2122	0.0142
WHO N	1.58	0.24	0.81	0.04	-0.60	0.04	1.7405	0.1912
DOGK18	1.19	0.29	0.43	0.29	-10.40	1.27	0.0018	0.0009

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