

A new rapid resazurin-based microdilution assay for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*

Summary statistics

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Introduction

A detailed summary statistics that provides the basis for the numbers in the paper. This analysis ensures the reproducibility of the pipeline and creates the statistics from raw data.

Software and packages

R version and package dependencies that were used

```
print(session)
```

```
## R version 3.3.2 (2016-10-31)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows >= 8 x64 (build 9200)
##
## locale:
## [1] LC_COLLATE=English_United Kingdom.1252 LC_CTYPE=English_United Kingdom.1252 LC_MONETARY=Engl.
##
## attached base packages:
## [1] grid      stats      graphics  grDevices utils      datasets  methods   base
##
## other attached packages:
## [1] reshape_0.8.6          XLConnect_0.2-12        XLConnectJars_0.2-12    gridExtra_2.2.1        str
##
## loaded via a namespace (and not attached):
## [1] splines_3.3.2          gender_0.5.1            gtools_3.5.0           assertthat_0.1          xlsxjars_0.6.1
## [30] TH.data_1.0-7          nnet_7.3-12             lazyeval_0.2.0          pbkrtest_0.4-6          NLP_0.1-9
## [59] dplyr_0.5.0            openNLPdata_1.5.3-2     rprojroot_1.1          rJava_0.9-8            stringi_1.1.2
```

Data description

Number of unique strains: 124

Strains and antimicrobials

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
training	84	84	84	84	84	84	84	588
validation	40	40	40	40	40	40	40	280
Sum	124	124	124	124	124	124	124	868

EUCAST classification

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
I	38	0	0	0	37	0	25	100
R	60	35	10	87	82	7	96	377
S	26	89	114	37	5	117	3	391
Sum	124	124	124	124	124	124	124	868

Dose response modelling

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
above limit of detection	4	0	0	0	7	4	0	15
quality ok	120	124	124	124	117	120	124	853
Sum	124	124	124	124	124	124	124	868

	training	validation	Sum
above limit of detection	6	9	15
quality ok	582	271	853
Sum	588	280	868

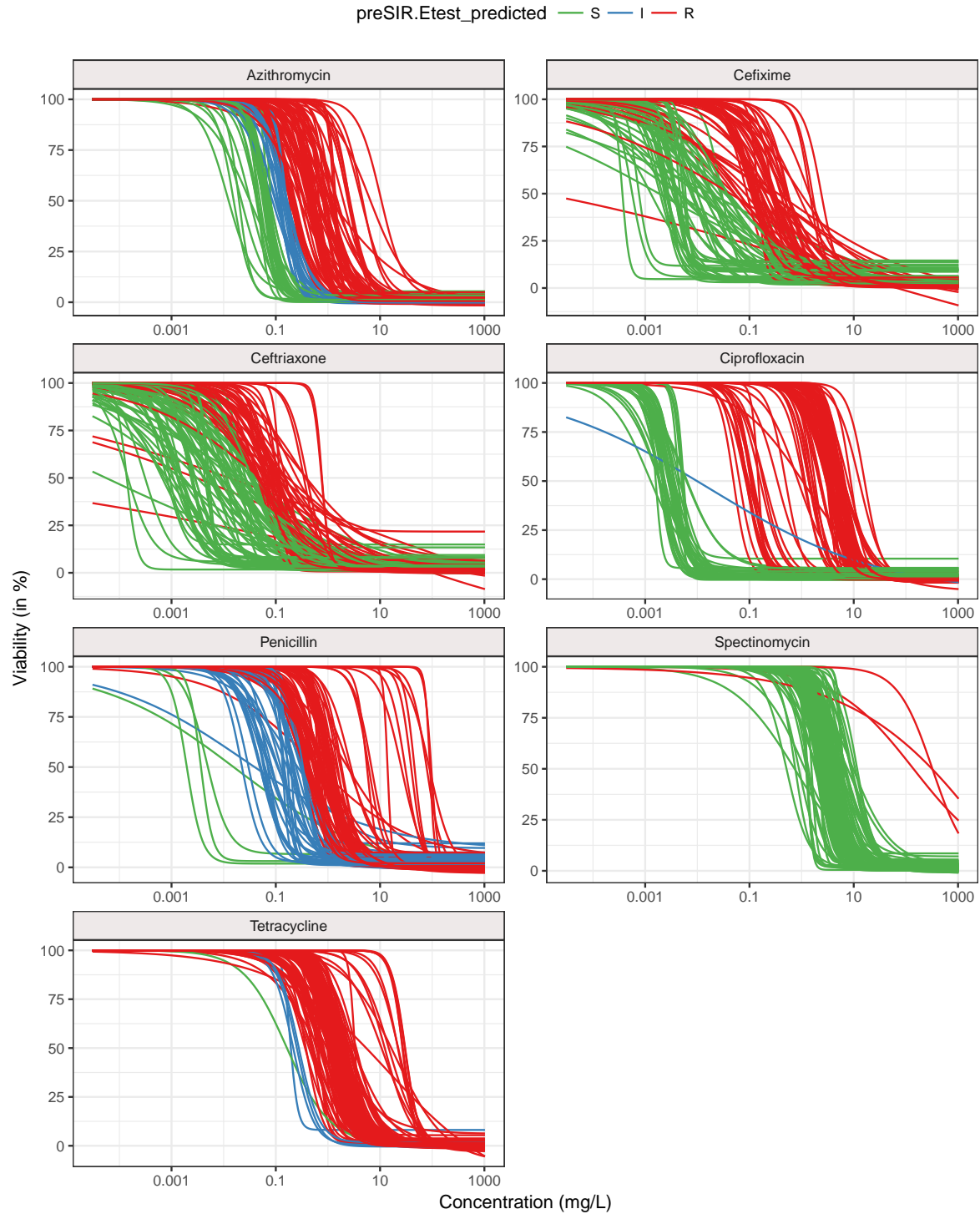


Figure 1. Potency shift of antimicrobials across different strains of *N. gonorrhoeae*. Dose response curves for all strains and antimicrobials are shown (except samples above limit of detection). Strains that were classified as susceptible according to EUCAST 2016 MIC breakpoints⁴⁶ were coloured in green, intermediate resistant strains in blue and resistant strains in red. The gradual shift of the potencies (EC₅₀) towards higher

concentrations can be observed for all antimicrobials.

Regression analysis

Note: esti is already in natural logs, therefore also the standard deviation

```
## [[1]]
## [[1]]$Estimates
##
## Call:
## lm(formula = log(etest) ~ (esti))
##
## Coefficients:
## (Intercept)      esti
##      1.101      1.001
##
##
## [[1]]$Matrix
## [[1]]$Matrix[[1]]
##      (Intercept)      esti
## (Intercept) 0.0023234883 0.0003695523
## esti      0.0003695523 0.0002613105
##
## [[1]]$Matrix$Summary
##
## Call:
## lm(formula = log(etest) ~ (esti))
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -4.1999 -0.6237  0.0616  0.7230  3.0847
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  1.10145    0.04820   22.85  <2e-16 ***
## esti        1.00057    0.01617   61.90  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 1.014 on 569 degrees of freedom
## Multiple R-squared:  0.8707, Adjusted R-squared:  0.8705
## F-statistic: 3831 on 1 and 569 DF, p-value: < 2.2e-16
```

Pearson's correlation coefficient

```
[1] 0.9331071
```

Outlier

The column “deviation” displays doubling dilutions deviation of predicted values from MIC. The column “compare” displays the EUCAST to the predicted classification.

ID	strain	antibiotic	MIC	Etest_predicted	deviation	compare
1_Ceftriaxone_40strains1.txt	Fluorometric1	Ceftriaxone	0.002	0.3302	7.367	S_to_R
11_Ceftriaxone_40strains2.txt	Fluorometric11	Ceftriaxone	0.002	0.4692	7.874	S_to_R
14_Cefixime_40strains2.txt	Fluorometric14	Cefixime	0.016	1.083	6.081	S_to_R
17_Ceftriaxone_40strains2.txt	Fluorometric17	Ceftriaxone	0.004	2.387	9.221	S_to_R
18_Cefixime_40strains2.txt	Fluorometric18	Cefixime	0.016	9.451	9.206	S_to_R
18_Ceftriaxone_40strains2.txt	Fluorometric18	Ceftriaxone	0.002	0.4898	7.936	S_to_R
18_Penicillin_80strains3.txt	Fluorometric18	Penicillin	32	1.923	-4.057	R_to_R
33_Ceftriaxone_80strains5.txt	Fluorometric33	Ceftriaxone	0.004	0.1736	5.44	S_to_R
34_Ceftriaxone_80strains5.txt	Fluorometric34	Ceftriaxone	0.004	0.06407	4.002	S_to_S
37_Ceftriaxone_80strains5.txt	Fluorometric37	Ceftriaxone	0.008	0.2569	5.005	S_to_R
4_Penicillin_40strains1.txt	Fluorometric4	Penicillin	3	118.8	5.307	R_to_R
4_Penicillin_80strains1.txt	Fluorometric4	Penicillin	4	78.77	4.3	R_to_R
5_Penicillin_40strains1.txt	Fluorometric5	Penicillin	3	91.36	4.929	R_to_R
5_Spectinomycin_40strains1.txt	Fluorometric5	Spectinomycin	1024	25974	4.665	R_to_R
57_Cefixime_80strains11.txt	Fluorometric57	Cefixime	0.016	0.3266	4.352	S_to_R
60_Cefixime_80strains8.txt	Fluorometric60	Cefixime	0.016	1.067	6.059	S_to_R
66_Ceftriaxone_80strains9.txt	Fluorometric66	Ceftriaxone	0.004	0.000183	-4.45	S_to_S
73_Cefixime_80strains9.txt	Fluorometric73	Cefixime	0.016	0.2623	4.035	S_to_R
73_Ceftriaxone_80strains9.txt	Fluorometric73	Ceftriaxone	0.008	0.1709	4.417	S_to_R

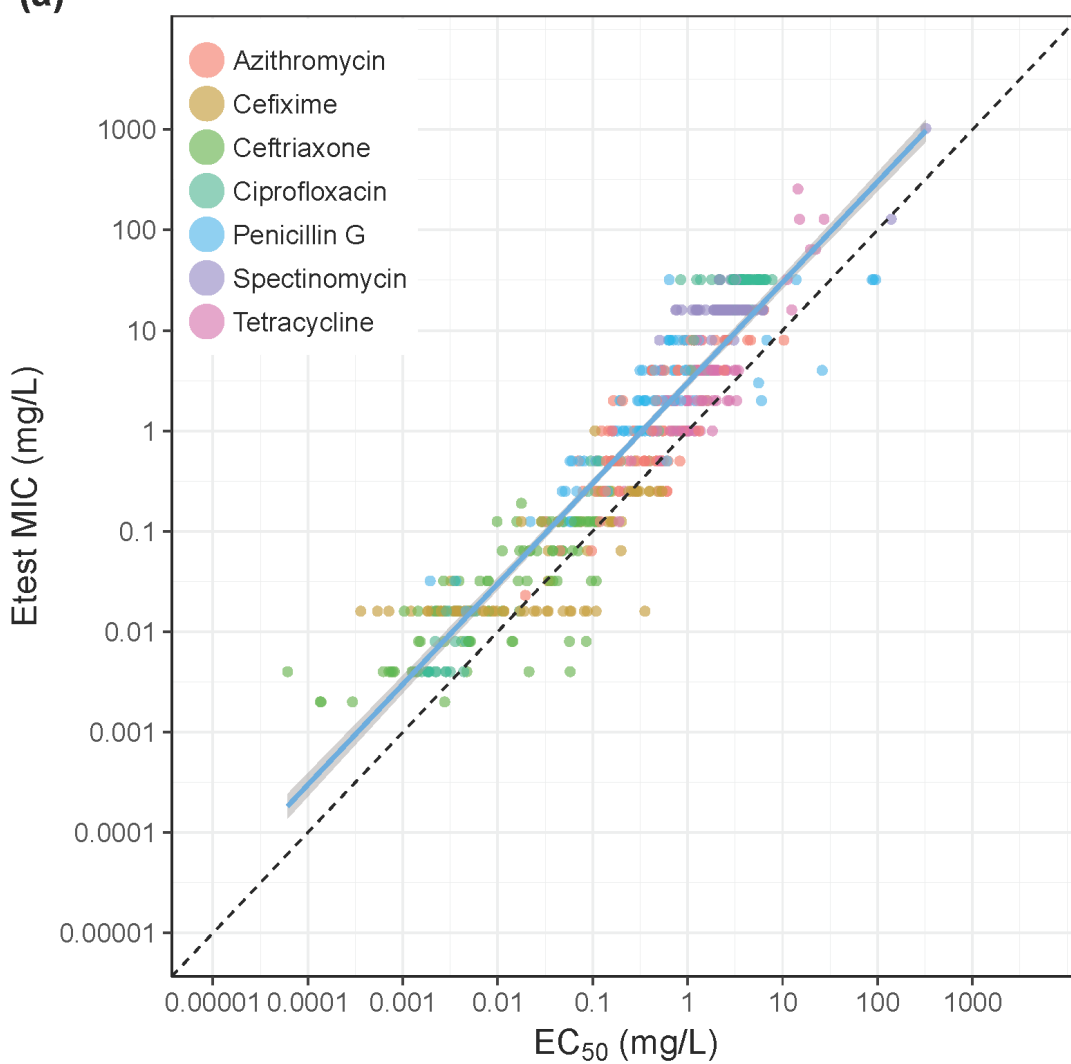
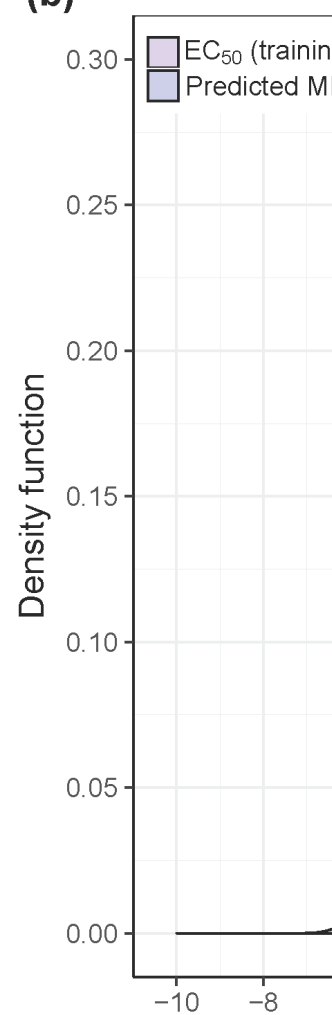
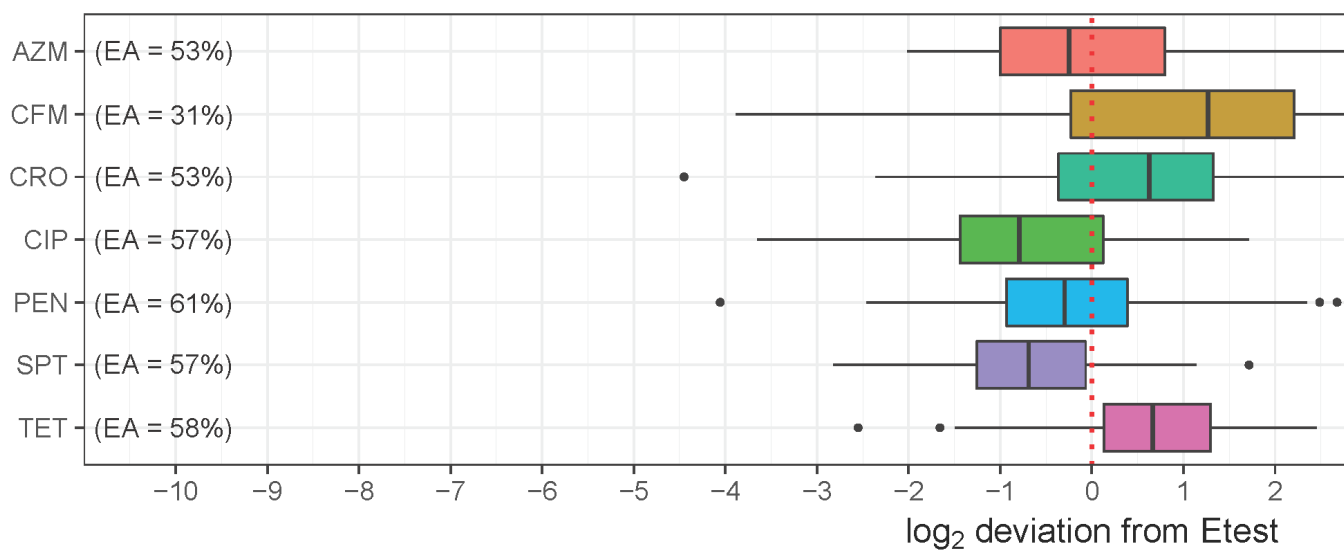
(a)**(b)****(c)**

Figure 2. Correlation and deviations between the Etest MICs and predicted MICs. (A) Linear regression between EC50 and Etest MIC for the training data (84 strains). The Pearson's correlation coefficient for the linear regression (blue line) was 0.93 and the confidence interval highlighted in grey. Slope and intercept for a perfect correlation was drawn as dashed black line for comparison. (B) The kernel density function of the EC50 values for the training data (n=269) is shown in red (median -1.63). The kernel density of the predicted MICs for training and validation data (n=837) is shown in purple (median -0.015). (C) Deviations of predicted MICs from Etest MIC per antimicrobial (n=837). The boxplots show the median and 25%-75% quartiles. The whiskers span the range from the bottom 5% to the highest 95% of the data. The essential agreement (EA) is written below the boxplots.

Categorical agreement with Etest

Categorical agreement, absolute and in percentage

EUCAST as rows, predicted categories as columns

	S	I	R	Sum
S	307	8	76	391
I	13	42	45	100
R	1	12	364	377
Sum	321	62	485	868

	S	I	R	Sum
S	0.354	0.009	0.088	0.45
I	0.015	0.048	0.052	0.115
R	0.001	0.014	0.419	0.434
Sum	0.37	0.071	0.559	1

Categorical agreement, by antibiotics, absolute and in percentage

EUCAST_to_predicted_categories

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
I_to_I	9	0	0	0	30	0	3	42
I_to_R	17	0	0	0	7	0	21	45
I_to_S	12	0	0	0	0	0	1	13
R_to_I	6	0	0	0	6	0	0	12
R_to_R	54	35	9	87	76	7	96	364
R_to_S	0	0	1	0	0	0	0	1
S_to_I	5	0	0	1	1	0	1	8
S_to_R	5	29	40	0	0	0	2	76
S_to_S	16	60	74	36	4	117	0	307
Sum	124	124	124	124	124	124	124	868

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
I_to_I	0.01	0	0	0	0.035	0	0.003	0.048
I_to_R	0.02	0	0	0	0.008	0	0.024	0.052

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
I_to_S	0.014	0	0	0	0	0	0.001	0.015
R_to_I	0.007	0	0	0	0.007	0	0	0.014
R_to_R	0.062	0.04	0.01	0.1	0.088	0.008	0.111	0.419
R_to_S	0	0	0.001	0	0	0	0	0.001
S_to_I	0.006	0	0	0.001	0.001	0	0.001	0.009
S_to_R	0.006	0.033	0.046	0	0	0	0.002	0.088
S_to_S	0.018	0.069	0.085	0.041	0.005	0.135	0	0.354
Sum	0.143	0.143	0.143	0.143	0.143	0.143	0.143	1

CI spanning over two categories (without limit of detection)

	Azithromycin	Cefixime	Ceftriaxone	Ciprofloxacin	Penicillin	Spectinomycin	Tetracycline	Sum
0	91	93	88	115	87	110	116	700
1	29	19	36	5	30	10	8	137
Sum	120	112	124	120	117	120	124	837

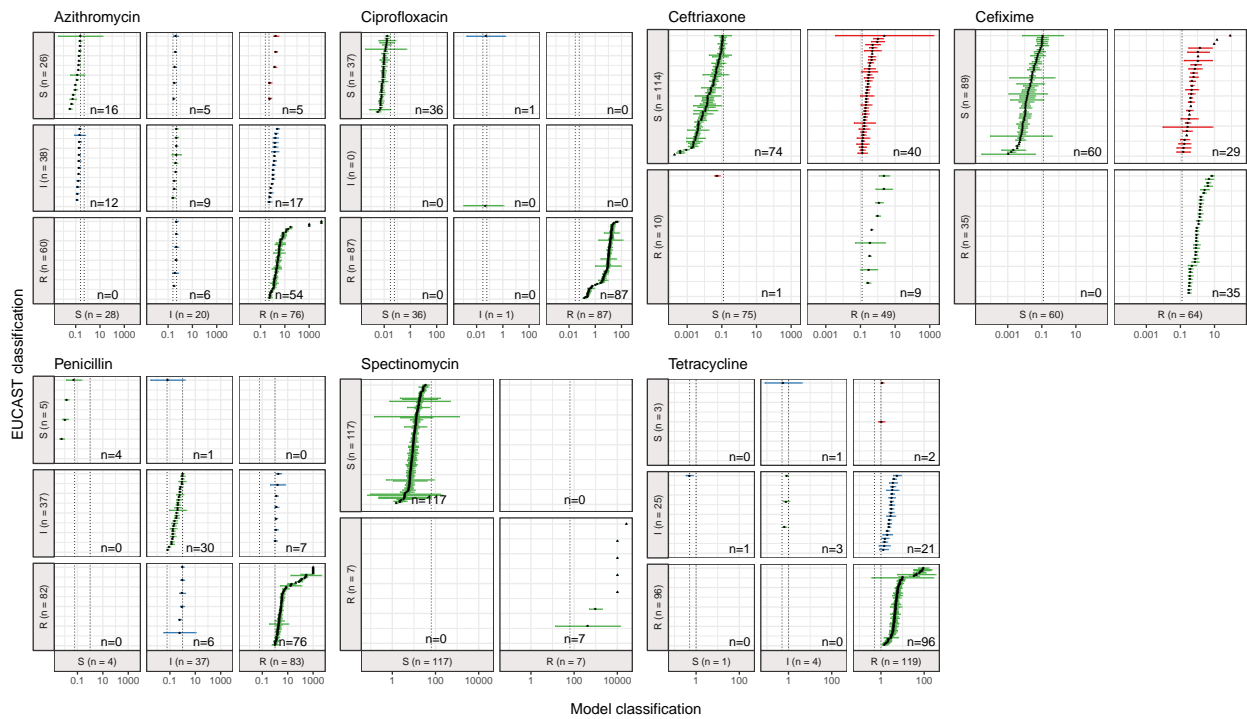


Figure 3. Contingency table with categorical errors of model predicted MICs. Etest MIC data were classified into the categories resistant (R), susceptible (S) and intermediate resistant (I) according to the EUCAST 2016 criteria. The cutoff values (mg/L) are shown as dashed black lines. Predicted MIC values (n=868) are shown as point estimates (black dots) with 95% confidence interval (colored dashes). For some estimates no confidence interval could be calculated (limit of detection), those were drawn as triangles. Correctly classified strains are drawn in green. Minor errors resulting from misclassifications of intermediate strains are drawn in blue. Major errors (S to R) were found for ceftriaxone (n=42), cefixime (n=30), azithromycin (n=5) and tetracycline (n=2). One very major error (R to S) was found for ceftriaxone (red). A high number of estimates (n=140) has confidence intervals spanning two categories.

Specificity and sensitivity

Note: I counted as R

Categorical agreement summary

EUCAST as rows, predicted categories as columns

	R	S
R	463	14
S	84	307

R_to_R	R_to_S	S_to_R	S_to_S
463	14	84	307

Sensitivity

$$\frac{R-to-R}{R-to-R+R-to-S} = 0.9706$$

95% CI

```
binom.test(463, 477, p = 0.5, conf.level = 0.95)
```

```
##
## Exact binomial test
##
## data: 463 and 477
## number of successes = 463, number of trials = 477, p-value < 2.2e-16
## alternative hypothesis: true probability of success is not equal to 0.5
## 95 percent confidence interval:
## 0.9512455 0.9838631
## sample estimates:
## probability of success
## 0.9706499
```

Specificity

$$\frac{S-to-S}{S-to-S+S-to-R} = 0.7852$$

95% CI

```
binom.test(307, 389, p = 0.5, conf.level = 0.95)
```

```
##
## Exact binomial test
##
## data: 307 and 389
## number of successes = 307, number of trials = 389, p-value < 2.2e-16
## alternative hypothesis: true probability of success is not equal to 0.5
## 95 percent confidence interval:
## 0.7452549 0.8286867
## sample estimates:
```

```
## probability of success
## 0.7892031
```

Essential agreement with Etest

Etest_deviation	AZM	CFM	CRO	CIP	PEN	SPT	TET
onedeviation	53	30	52	57	61	57	58
twodeviation	95	67	79	93	83	89	94
fourdeviation	100	96	93	100	97	99	100

Supplementary Material

Timecourse

8 WHO reference strains were followed over a time-course of 0-15 hours and measured every 3 hours (one repli-

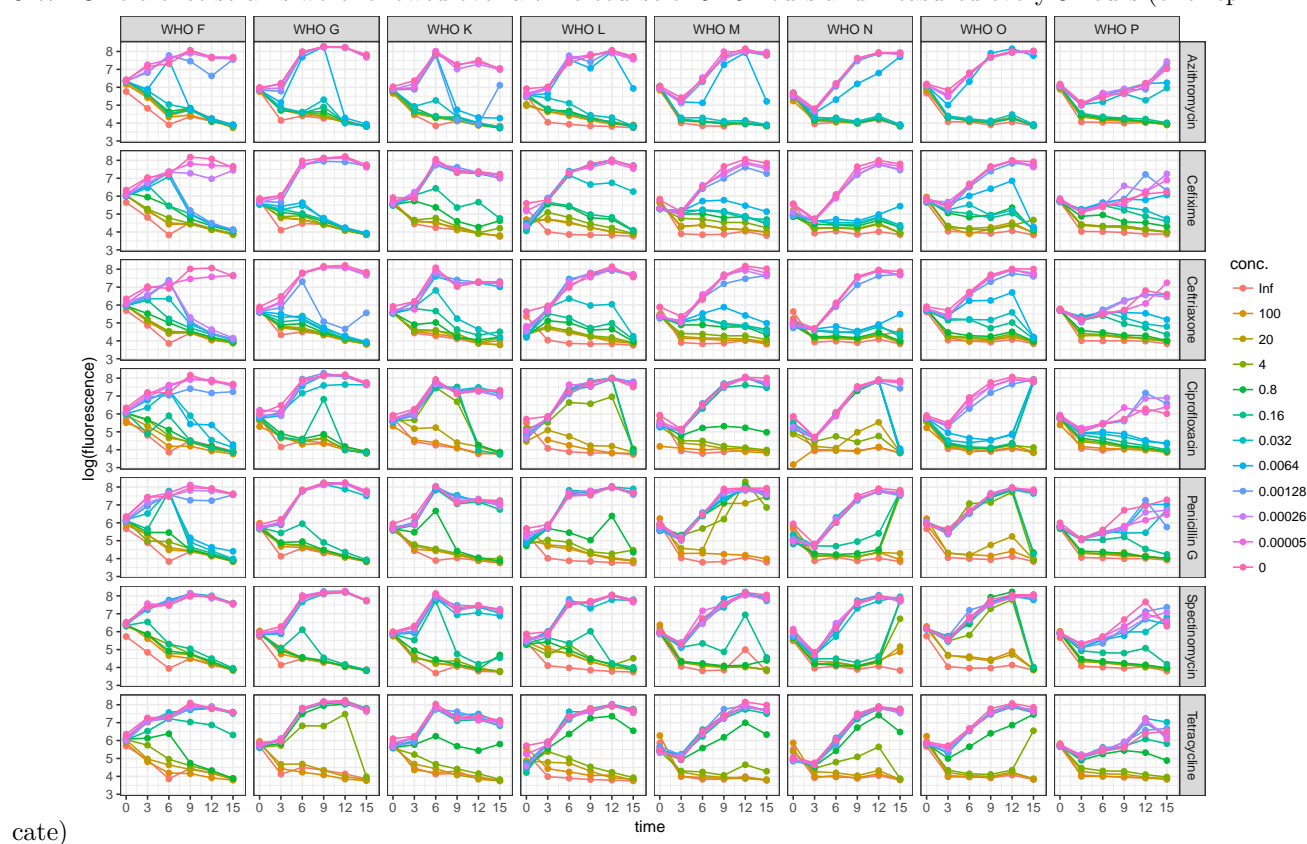


Figure S1. Fluorescence based time-kill curves. Logarithmized fluorescence values are plotted against the time (h). Ten different dilutions of each antimicrobial, positive control (Inf) and negative control (conc. 0) were tested on eight WHO reference panel strains. Start concentrations were calibrated to approximately 107 CFU/ml which corresponds to a log fluorescence of 6. From 0-3 hours negative controls without antimicrobial resulted in decreased bacterial numbers, at 6 hours all samples show increased fluorescence.

Coefficient of variation

8 Reference strains were used in this analysis. 3 replicates with seven antimicrobials (n=56) were used to calculate the coefficient of variation

strain	antibiotic	MIC	mean	sd	CV
WHO F	Azithromycin	0.125	0.01394	0.009803	0.7031
WHO F	Cefixime	<0.016	0.0005485	4.743e-05	0.08647
WHO F	Ceftriaxone	<0.002	0.0002095	0.0001515	0.7231
WHO F	Ciprofloxacin	0.004	0.002442	0.0003445	0.141
WHO F	Penicillin	0.032	0.005978	0.0005169	0.08648
WHO F	Spectinomycin	16	3.141	2.253	0.7174
WHO F	Tetracycline	0.25	0.04333	0.02345	0.5413
WHO G	Azithromycin	0.25	0.03174	0.0008395	0.02645
WHO G	Cefixime	<0.016	0.003609	6.18e-05	0.01712
WHO G	Ceftriaxone	0.008	0.00183	0.000874	0.4776
WHO G	Ciprofloxacin	0.125	0.04593	0.003962	0.08626
WHO G	Penicillin	0.5	0.1495	0.03925	0.2625
WHO G	Spectinomycin	16	1.556	1.256	0.807
WHO G	Tetracycline	32	9.979	2.121	0.2126
WHO K	Azithromycin	0.25	0.04507	0.005532	0.1227
WHO K	Cefixime	0.25	0.1223	0.01594	0.1303
WHO K	Ceftriaxone	0.064	0.02189	0.01038	0.474
WHO K	Ciprofloxacin	>32	9.845	1.578	0.1603
WHO K	Penicillin	2	0.7465	0.07489	0.1003
WHO K	Spectinomycin	16	2.669	1.447	0.542
WHO K	Tetracycline	2	1.368	0.1663	0.1215
WHO L	Azithromycin	0.5	0.04175	0.007214	0.1728
WHO L	Cefixime	0.125	0.06644	0.007194	0.1083
WHO L	Ceftriaxone	0.25	0.05039	0.02726	0.5409
WHO L	Ciprofloxacin	>32	4.47	0.626	0.1401
WHO L	Penicillin	2	0.9015	0.0676	0.07498
WHO L	Spectinomycin	16	1.919	1.076	0.5605
WHO L	Tetracycline	2	1.187	0.2155	0.1815
WHO M	Azithromycin	0.25	0.0404	0.003365	0.08328
WHO M	Cefixime	<0.016	0.00291	0.0003033	0.1042
WHO M	Ceftriaxone	0.012	0.001849	0.0008431	0.4558
WHO M	Ciprofloxacin	2	0.2936	0.03352	0.1142
WHO M	Penicillin	8	22.13	1.932	0.08727
WHO M	Spectinomycin	16	2.266	1.16	0.5119
WHO M	Tetracycline	2	0.9742	0.09473	0.09724
WHO N	Azithromycin	0.25	0.02093	0.00204	0.0975
WHO N	Cefixime	<0.016	0.004648	0.0003874	0.08336
WHO N	Ceftriaxone	0.004	0.001196	0.0007731	0.6463
WHO N	Ciprofloxacin	4	1.588	0.03178	0.02001
WHO N	Penicillin	8	2.304	1.996	0.8663
WHO N	Spectinomycin	16	1.162	0.8212	0.7065
WHO N	Tetracycline	16	6.36	0.4289	0.06742
WHO O	Azithromycin	0.25	0.04498	0.007163	0.1592
WHO O	Cefixime	0.016	0.009882	0.004161	0.4211
WHO O	Ceftriaxone	0.032	0.004744	0.003545	0.7473
WHO O	Ciprofloxacin	0.008	0.002171	0.0003707	0.1707
WHO O	Penicillin	>32	10.29	4.058	0.3944
WHO O	Spectinomycin	>1024	462.4	167.8	0.3629

strain	antibiotic	MIC	mean	sd	CV
WHO O	Tetracycline	2	0.9739	0.1611	0.1655
WHO P	Azithromycin	4	0.2521	0.006027	0.0239
WHO P	Cefixime	<0.016	0.003542	0.0006906	0.195
WHO P	Ceftriaxone	0.004	0.001126	0.0005669	0.5037
WHO P	Ciprofloxacin	0.004	0.001845	0.0001956	0.106
WHO P	Penicillin	0.25	0.09521	0.001573	0.01652
WHO P	Spectinomycin	8	2.168	1.272	0.5867
WHO P	Tetracycline	1	0.4477	0.1042	0.2327

```
print(summary(cv$CV))
```

```
##      Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
## 0.01652 0.09743 0.16810 0.29190 0.50570 0.86630
```

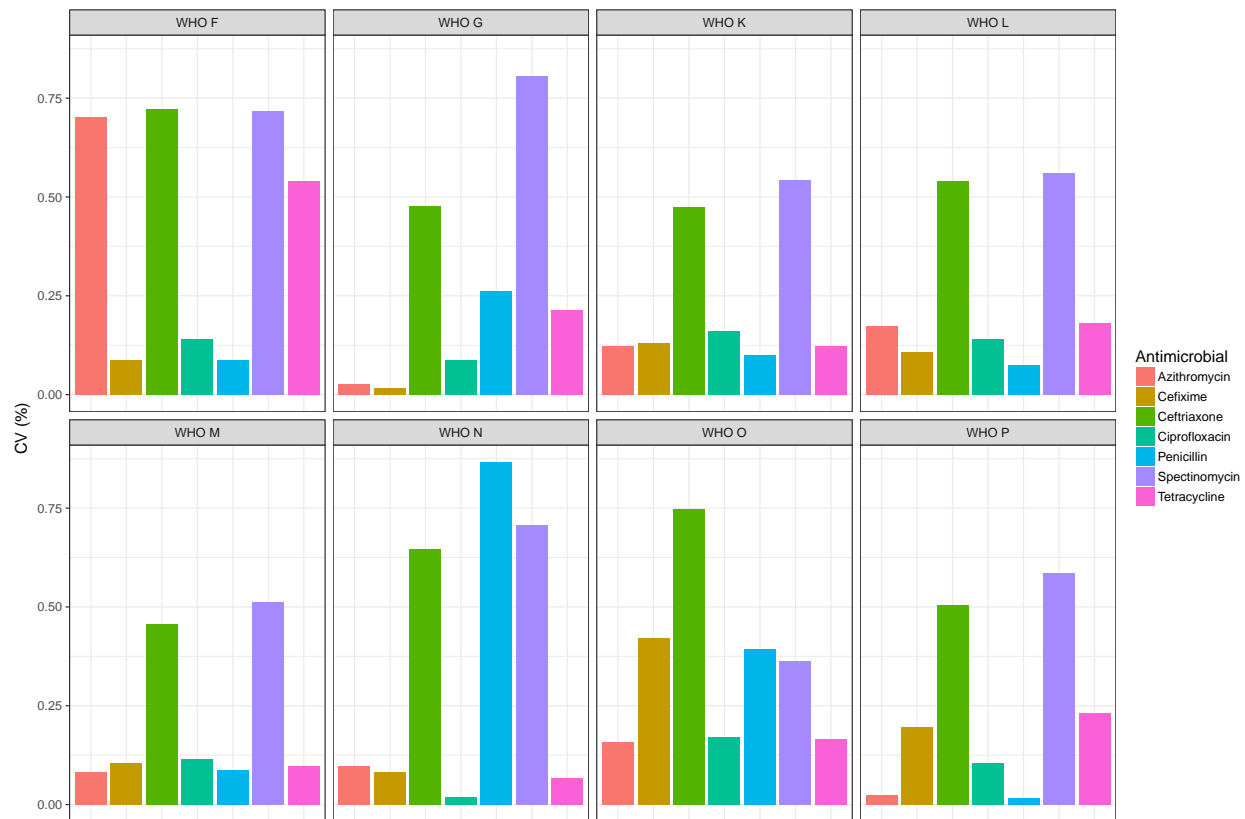


Figure S2. Intra assay coefficient of variation. To test the reproducibility of the resazurin MIC assay seven antimicrobials were tested on eight WHO reference strains (n=56). The mean and standard deviation of three independent experiments was calculated. The coefficient of variation (ratio of standard deviation over the mean) was calculated for sample. Barplots are shown for each sample. The mean of the coefficient of variation (intra assay CV) is 0.29.

Hill coefficient statistics

It was tested if the Hill coefficient was significantly different (t-test) between antimicrobials and strains.

	antibiotic	mean	sd	min	max
3	Ceftriaxone	1.621	1.277	0.1166	7.455
2	Cefixime	1.916	1.539	0.1029	7.151
7	Tetracycline	2.091	0.8737	0.4026	7.818
5	Penicillin	2.498	1.674	0.3353	10.47
1	Azithromycin	2.598	1.494	0.6032	13.44
4	Ciprofloxacin	2.704	1.186	0.2596	7.733
6	Spectinomycin	2.899	1.713	0.3138	9.158

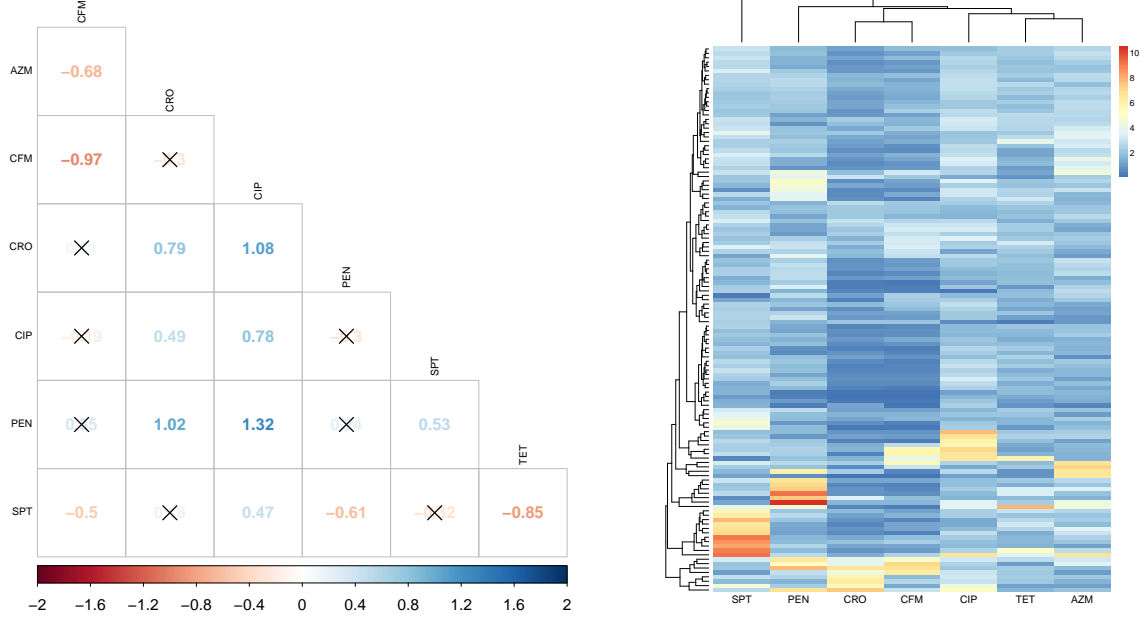


Figure S3. Difference of Hill coefficients. (a) The difference between the mean of 124 Hill coefficients (124 clinical strains examined) is shown for each antimicrobial combination. High values are shown in an increasingly intense blue colour gradient and low values in red. A pairwise t-test was performed and non-significant differences (p value > 0.05) marked with a black cross. (b) Hierarchical clustering of Hill coefficients. Rows represent Hill coefficients for different strains (N=124) and columns antimicrobials. The beta-lactams penicillin G, ceftriaxone and cefixime are more similar to each other than to the other antimicrobials.

Example for Biphasic curves

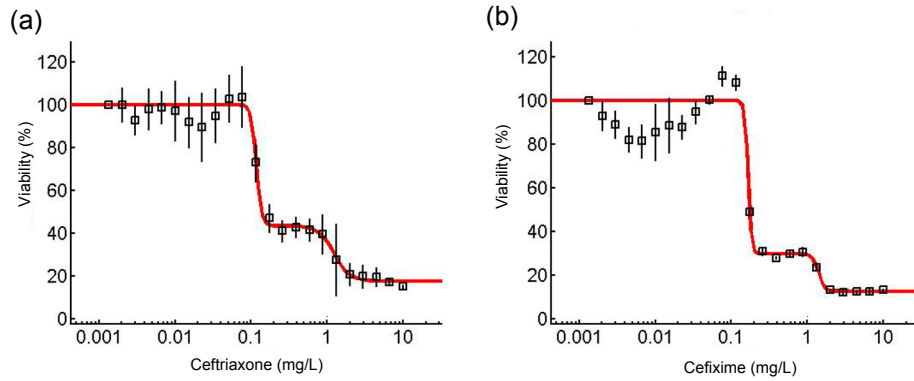


Figure S4. Biphasic dose response curves. The viability (%) was plotted against 24 different antimicrobial concentrations. Mean and standard error of three independent experiments are shown. (a) Ceftriaxone in Strain 11 (validation data). A biphasic model (red curve) fits the model better (bic=563) than a monophasic model (bic=794).¹ The first EC₅₀ is at 0.12 mg/L and the second at 1.21 mg/L (Etest MIC=0.125 mg/L). (b) Cefixime in Strain 11 (validation data). A biphasic model (red curve) fits the model better (bic=850) than a monophasic model (bic=8574). The first EC₅₀ is at 0.16 mg/L and the second at 1.39 mg/L (Etest MIC=0.25 mg/L).