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

Sunniva Förster[1,2,3,4]*, Valentino Desilvestro[5], Lucy Hathaway[3], Nicola Low[1], Christian Althaus[1] and Magnus Unemo[2]

[1]Institute of Social and Preventive Medicine, University of Bern, Bern, Switzerland; [2]WHO Collaborating Centre for Gonorrhoea and other STIs, Örebro University, Örebro, Sweden; [3]Institute for Infectious Diseases, University of Bern, Bern, Switzerland; [4]Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland; [5]World Trade Institute (WTI), University of Bern, Bern, Switzerland

*Corresponding author. Institute of Social and Preventive Medicine, University of Bern, Finkelhubelweg 11, 3012, Bern, Switzerland. Tel.: xxxxxxx; Fax: xxxxxxx; E-mail: sunniva.foerster@ifik.unibe.ch

Running title: Microdilution susceptibility assay for gonococci

Word count: Synopsis: xxx words, Main text: xxxx words

Objectives: Rapid, cost-effective and objective methods for antimicrobial susceptibility testing of *Neisseria gonorrhoeae* would greatly enhance surveillance of antimicrobial resistance. Etest, disc diffusion or agar dilution methods are subjective, laborious for large-scale testing, and take 16-20 hours. We developed a rapid broth microdilution assay using resazurin (blue), which is converted to resorufin (pink fluorescence), to analyse dose-response curves for antimicrobials in gonococcal isolates.

Methods: The resazurin-based broth microdilution assay was established using the 2008 WHO gonococcal reference strains (n=8) and the antimicrobials ceftriaxone, cefixime, azithromycin, spectinomycin, ciprofloxacin, gentamicin, tetracycline, and penicillin G. An initial dataset including 84 blinded gonococcal strains was then used to develop a regression model for estimating the MIC after six hours incubation time. The assay was finally validated with 40 blinded gonococcal strains.

Results: Results were obtained in approximately 7.5 hours. The EC_{50} of the dose-response curves correlates linear with Etest MIC values ($R^2 = 0.87$). Only one very major error was found for a resistant ceftriaxone strain misclassified as susceptible. Minor errors resulting from misclassifications of intermediary resistant strains were found for 3.8% of the samples. Major errors occurred for ceftriaxone (34%), cefixime (24%), azithromycin (4%) and tetracycline (2%). Overall the sensitivity of the assay was 97.13% (CI: 95.22-98.42) and the specificity 79.27 % (CI: 74.84-83.23).

Conclusions: A rapid, objective, high-throughput, quantitative and cost-effective broth microdilution assay was established for gonococci. For use in routine diagnostics without confirmatory testing, the specificity might remain suboptimal for ceftriaxone and cefixime. However, the assay could be an effective low-cost method to evaluate novel antimicrobials, for high throughput screenings, and expands the currently available methodologies for surveillance of antimicrobial resistance in gonococci.

Introduction

53   *Neisseria gonorrhoeae* is a very fastidious bacterium that causes the sexually transmitted

54   infection gonorrhoea. Gonorrhoea is a public health concern globally,[1,2] and *N. gonorrhoeae*

55   has developed resistance to all antimicrobials introduced for treatment.[3] Accordingly, enhanced

56   surveillance of antimicrobial susceptibility in *N. gonorrhoeae* is imperative globally.[1] Ideally,

57   this surveillance should be performed using methods determining the MICs of relevant

58   antimicrobials. MIC-based methods are also valuable to directly inform treatment after

59   laboratory results are available- and evaluate *in vitro* efficacy of novel antimicrobials.

60   Due to the lack of any appropriate broth medium, MIC-based susceptibility testing of *N.*

61   *gonorrhoeae* has been limited to disk diffusion, Etest and agar dilution method (gold standard).

62   Essential agreement with the agar dilution method is defined as ±1 doubling dilution and should

63   ideally be above 90% for diagnostic purposes where the same resistance breakpoints are

64   applied.[4] Etest has shown excellent agreement with the agar dilution method in many settings.[4–

65   7] However, discordant results have been found particularly when different growth media were

66   used.[8] A multicentre study revealed that the overall agreement within a method was above 70%

67   categorical agreement between Etest and agar dilution was ≥ 88% but was very poor for disk

68   diffusion.[9] Unfortunately, these methods are relatively slow (~24 hours), subjective, require

69   expertise, and/or are expensive. Faster methods that allow results to be obtained on the same

70   day have been developed in the past for other bacteria[10,11], but are not available for *N.*

71   *gonorrhoeae*.

72   For many bacterial species, broth microdilution is the reference method due to accuracy,

73   low costs and high versatility.[12,13] Several attempts have been made to develop a broth

74   microdilution method also for *N. gonorrhoeae* but none of the developed methods have been

particularly accurate and suitable for routine use.[14–16] It is difficult to synchronize the growth of different *N. gonorrhoeae* strains and effects such as autolysis occur when the bacteria enter the stationary phase.[17–19] Chemically defined Graver-Wade (GW) broth[20] supports the growth of phylogenetically diverse auxotypes and clinical isolates and might be a suitable medium for susceptibility testing.[21,22]

Unfortunately, MIC values based on doubling dilution series are left, interval, or right censored discrete data which makes error statistics challenging.[23] The potency of drugs in pharmacology is frequently measured with dose-response curves, as this allows the estimation of the effective concentration (EC) at a specified response level. Furthermore, EC values on a continuous scale take the variability of the data into account by calculating model based confidence intervals (CIs). In the field of toxicology the upper border of the confidence interval is defined as non-toxic, this benchmark dose approach (BMD) has largely replaced methods that rely on dense dose spacing because of its statistical superiority and reduction of animal use.[24–27] Furthermore, the slope of the pharmacodynamic curve potentially provides additional valuable information on the compounds being tested.[28] The Hill coefficient can potentially provide information about the pharmacodynamic properties of an antimicrobial and has been used in modelling studies of single and dual antimicrobial effects.[21,22,42–44] However, the interpretation and significance of the Hill slope has been unclear in previous studies and laborious colony counting limited these studies to few strains. Theoretically a steep Hill slope indicates that small increases in antimicrobial concentrations result in more effective killing.

The biological response to a compound can be measured using different readouts. Traditionally the MIC is defined as the concentration of an antimicrobial that inhibits visual growth but methods to quantify the number of bacterial cells more objectively are available nowadays. Measuring the optical density (at e.g. OD_{600} or OD_{450}), resazurin (Alamar blue), 3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), luciferase (ATP levels)

4

100 and lactate dehydrogenase are widespread methods where readouts indirectly correlate with the

101 number of cells.[29] Resazurin is a blue dye that is converted to pink fluorescent resorufin in the

102 presence of metabolically active cells.[30,31] Unlike optical density that is a measure of growth

103 inhibition, it reflects the viability of cells and is potentially suitable for time-kill assays as well.

104 Resazurin has an excellent signal to noise ratio and has been used previously in screenings for

105 toxicity testing, high throughput applications and MIC testing.[30,32]

106   The aim of the present study was to develop a resazurin-based broth microdilution assay

107 for antimicrobial susceptibility testing of *N. gonorrhoeae* that is rapid, objective, scalable,

108 quantitative and inexpensive. Three datasets were generated in this study. A panel of reference

109 strains (n=8) was studied to ensure the reproducibility of the assay and compare multiple

110 measurement endpoints in a time-course between 0-15 hours. Training data consisting of 84

111 strains were used to develop a predictive model for estimating the MIC from dose-response

112 curves. Finally, a panel of forty strains with blinded MICs was used to validate the prediction.

113

114 **Material and methods**

115 *Bacterial strains, culture and broth microdilution assay*

116 The resazurin assay was established using the 2008 WHO gonococcal reference strains

117 (n=8)[33,34] and the antimicrobials ceftriaxone, cefixime, azithromycin, spectinomycin,

118 ciprofloxacin, gentamicin, tetracycline, and penicillin G. The shortest possible endpoint for

119 measuring the MIC was determined by measuring the fluorescence of the reference panel

120 strains during a time-course from 0-15 hours. The reference panel of eight WHO strains was

121 examined in three independent experiments with an endpoint of six hours to test the intra-assay

122 coefficient of variation of the assay as previously described[35]. A training dataset of 84 blinded

123 gonococcal strains was then used to develop a regression model for estimating the MIC after

124 six hours incubation time (one replicate). The assay was finally validated with 40 blinded

125   gonococcal strains (one replicate). The blinded strains were selected to represent a wide variety

126   of antibiograms. The strains were preserved in glycerol stocks at -80°C. All strains were

127   subsequently cultured on Chocolate agar PolyViteX (Biomerieux, Marcy l'Etoile, France) at

128   37°C in a humid 5% CO_2-enriched atmosphere for 16-18 hours and then sub-cultured once for

129   16 hours. A McFarland standard of 0.5 was prepared for each strain and 1 mL further diluted

130   to approximately $1x10^7$ CFU/mL in 15 mL heated (37°C) GW broth.[20] A volume of 90 µL of

131   this suspension was added to 96-well round bottom microtiter plates (360 µL wells) with each

132   well containing 10 µL of a previously prepared dilution series. Dilution series of the

133   antimicrobials were prepared in GW medium. Positive control (GW medium containing 1%

134   TritonX-100) and negative control (10 µL GW medium) were added to the first and last well,

135   respectively. The plates were incubated for 6 hours at 37°C, humid 5% CO_2-enriched

136   atmosphere.

137

138   ***Resazurin readouts***

139   Resazurin powder (Sigma Aldrich, China) was diluted in PBS (pH 7.4) to a final concentration

140   of 0.1 mg/mL. It was ensured that the pH of the highest antimicrobial concentration was neutral

141   in all samples to avoid artefacts. After incubation of the broth microdilution plates, 50 µL of

142   the dye was added to each well and mixed using an electronic multichannel dispenser. The

143   plates were incubated for 75 minutes at 37°C. Fluorescence was then measured at 560 and 590

144   nm excitation in a plate reader (Varioskan Flash, Thermo Scientific).

145

146   ***Etest MIC***

147   The Etest MICs (bioMérieux) were determined in accordance with the manufacturer´s

148   instructions, on GCRAP agar plates (3.6% Difco GC Medium Base agar [BD, Diagnostics,

149   Sparks, MD, USA] supplemented with 1% haemoglobin [BD, Diagnostics] and 1%

150   IsoVitalex [BD, Diagnostics]).

151

152   ***Dose-response modelling***

153   The antimicrobial effect on the different bacterial strains was quantified with dose-response

154   curves. Firstly background fluorescence resulting from dead bacteria in the positive control

155   wells was subtracted. A sigmoidal model was fit to the viability data of each antimicrobial-

156   strain combination[39]:

157    $$f(x) = u + \frac{l-u}{1+EC50^{H(x-EC50)}} \qquad \text{(Eq. 1)}.$$

158

159   Four parameters describe the curve, the lower model asymptote *l*, the upper model asymptote

160   *u*, the slope of the curve *H* and the EC_{50}. The background resulting from dead bacteria (positive

161   controls) was subtracted. Next, the data were divided by *u* to normalize all dose response curves

162   to 100% viability. Pairwise t-test of the mean of the hill slopes for each of the tested

163   antimicrobials were made to test if the differences found in this parameter are significant.

164   Hierarchical complete linkage clustering of the hill slopes was used to compare the

165   antimicrobials[21].

166   The EC_{50} was measured for each of the antimicrobial-strain combinations. Samples

167   were considered to be above the limit of detection, and therefore categorized as resistant, if the

168   antibiotic, at its highest concentration, reduced viability by less than 50%. This was the case

169   for 6 samples in the training data (n=672), 9 samples in the validation data (n=320) and 3

170   reference strain samples (n=192). Excluding Etest MICs that were above or below limit of

171   detection and samples where EUCAST resistance breakpoints are not available to date

172   (gentamicin) resulted in 571 evaluable samples in the training data, 269 samples in the

173   validation data and 137 in the reference strain data.

174   The relationship between EC_{50} and Etest was analysed for the training data by log

175   transforming both values and fitting a linear regression:

176        $\log(Etest) = \alpha + \beta \log(EC_{50}) + \varepsilon$ (Eq. 2).

177   Slope and intercept of this regression were used to predict the MIC from the EC_{50} values.

178   Confidence intervals (CI) for each predicted MIC were calculated using bootstrapping in order

179   to take in account estimation error in both sigmoidal and linear regression models. The EC_{50}

180   and its standard deviation from the sigmoidal model were used as parameters in the normal

181   distribution used to resample 10^5 EC_{50}. Similarly, 10^5 values for α and β in Eq.2 were obtained

182   by resampling from a two dimensional normal distribution $N_2([\alpha, \beta], \text{cov}(\alpha, \beta))$. The 0.025 and

183   0.975 percentiles of the resulting 10^5 predicted MICs distribution consist in the 95%

184   bootstrapped CIs. The analysis pipeline and data are available from github (xxxx).

185   Essential agreement was defined as the percentage of strains with predicted MICs that do

186   not deviate more than ±1 doubling dilution from Etest MICs. Deviations from the Etest MICs

187   were calculated as $\log_2$ differences from the predicted MIC (840 evaluable samples for training

188   and validation data). Reference strain data were not included to avoid bias from replicate testing

189   of these samples.

190

191   *Categorical agreement with Etest*

192   The strains were categorized as S (susceptible), I (intermediate), and R (resistant) to each

193   antimicrobial in accordance with the EUCAST 2016 guidelines.[37] As previously described,[38]

194   minor errors were defined as misclassifications of intermediate strains as susceptible or

195   resistant. Major errors were susceptible strains misclassified as resistant. Very major errors

196   were resistant strains that were misclassified as susceptible. The EC_{50} values are read on a

197   continuous scale, therefore nearly identical values around a resistance breakpoint (e.g. 0.125

198   and 0.126) can result in categorical errors. Sensitivity and specificity of the assay were

199   calculated as previously described[39], for the resistant (true positive samples), intermediary

200   strains (true positive samples) and susceptible strains (true negative samples).

201

202   **Results**

203   *Dose-response modelling*

204   The 2008 WHO reference strains (n=8) were exposed to ceftriaxone, cefixime, azithromycin,

205   spectinomycin, ciprofloxacin, gentamicin, tetracycline, and penicillin G for a time course from

206   0 to 15 hours (Figure S1). After six hours, the difference between dead and viable gonococcal

207   cells was sufficiently pronounced to fit dose-response curves to the data. For this endpoint of

208   six hours, the coefficient of variation was calculated for the EC_{50} of three independent

209   experiments. The coefficient of variation (CV) ranged from 1.7% to 100%, the intra-assay CV

210   was 28% (n=64) (Figure S2). Dose-response curves were gradually shifted towards higher

211   concentrations, indicating increased potency of the antimicrobials in the intermediary resistant

212   and resistant strains compared to susceptible strains (Figure 1). There was a clear separation of

213   susceptible and resistant strains for ciprofloxacin and spectinomycin. For the β-lactams

214   ceftriaxone, cefixime and penicillin G the hill coefficients (slopes) were more heterogeneous

215   than for the other samples. The mean of this parameter gradually increased from ceftriaxone

216   (1.8) to cefixime (2), tetracycline (2.1), penicillin G (2.3), azithromycin (2.5), spectinomycin

217   (2.9) and was highest for gentamicin (3.3) (Figure S3A). A pairwise *t*-test showed that the

218   differences between the antimicrobials were significant (p-value <0.005) when the distance

219   between the means was larger than 0.5 (Figure S3B). Furthermore, hierarchical clustering

220   shows a high similarity of the hill coefficient for the β-lactams ceftriaxone, cefixime and

221   penicillin G compared to the other antimicrobials. The correlation between the Etest MICs and

222   EC_{50} values for all antimicrobials was 0.87 (Figure 2A). Compared to the Etest values, the EC_{50}

223   values were systematically lower with a median deviation of -1.68 doubling dilutions (Figure

224 2B). Slope (1) and intercept (1.1) of the linear log-log regression were used to predict the 840

225 MICs of training and validation data. This prediction shifted the median deviation from Etest

226 MICs to 0.004 doubling dilutions. The 75% percent quartiles for the deviations were larger for

227 azithromycin, cefixime and ceftriaxone compared to ciprofloxacin, penicillin G, spectinomycin

228 and tetracycline (Figure 1C). The essential agreement between the Etest MICs and the

229 predicted MICs was 47% for all antimicrobials, being lowest for cefixime (29%) and highest

230 for penicillin G (61%).

231

232 *Categorical agreement*

233 The Etest and predicted MICs were classified as susceptible, intermediate and resistant

234 according to the EUCAST 2016 resistance breakpoints[30] (Figure 3). According Etest MIC 55%

235 of the samples were classified as resistant, 23% as intermediary resistant and xx% as resistant.

236 For spectinomycin, tetracycline and penicillin G, no major errors were identified. False positive

237 misclassifications (S to R), i.e. major errors, occurred for ceftriaxone (34%), cefixime (24%),

238 azithromycin (4%), and tetracycline (2%). False negative misclassifications (R to S), i.e. very

239 major errors, were very rare and only identified for one ceftriaxone sample (Etest MIC 0.19

240 mg/L vs. 0.053 mg/L). A high number of misclassifications (38 cases) was due to MIC values

241 close to the breakpoints and had 95% CIs spanning two categories. Overall the sensitivity of

242 the assay was 97.13% (CI: 95.22-98.42) and the specificity 79.27 % (CI: 74.84-83.23).

243

244 **Discussion**

245 The developed resazurin-based broth microdilution assay was able to discriminate between

246 resistant and susceptible strains reliably in an assay time, considerably shorter (approximately

247 7.5 hours) than those of the currently available MIC methods for *N. gonorrhoeae*. The gold

248 standard methods agar dilution and Etest are both based on subjective, visual readouts and are

249   therefore limited to a relatively low throughput. Dose-response modelling allows the estimation

250   of the EC_{50} of antimicrobials from a continuous scale and therefore allows calculation of a

251   precise estimates including confidence intervals rather than having the precision limited by

252   doubling dilutions. Continuous values from dose-response curves are inherently difficult to

253   compare to resistance breakpoints designed for doubling dilution-based methods. This was

254   reflected by many categorical errors resulting from estimates that have CIs overlapping two

255   SIR categories. The overall sensitivity including errors resulting from misclassified

256   intermediary strains was 97.13% (CI: 95.22-98.42). The performance of the assay was

257   excellent for ciprofloxacin, penicillin G and spectinomycin (no major errors) and acceptable

258   for azithromycin (4% major errors) and tetracycline (2% major errors). For cefixime and

259   ceftriaxone many false positive results and consequently an overestimation of resistance was

260   measured. The complex mechanism of action of these antimicrobials is not well understood

261   and involves several resistance determinants (*PenA*, *PenB*, *mtrR*, *ponA, factorX)*. The

262   correlation of EC_{50} and MIC has been previously shown to be largely influenced by different

263   penicillin binding proteins in *Streptococcus pneumoniae*.[40] This might explain the strain

264   dependent heterogeneity of hill coefficients (Figure S3) and dose response curves that are

265   biphasic (Figure S4).[22,41] To model such complex effects is not possible with the simple four

266   parameter hill function employed in this this study and therefore contributed to the poor

267   specificity of only 79.27 % (95% CI: 74.84-83.23). The deviation from Etest follows a normal

268   distribution, 95% of the values are within ± 4 doubling dilutions, outliers can be attributed to

269   the β-lactams penicillin G (large overestimation in beta lactamase producing strains), cefixime

270   and ceftriaxone (potentially biphasic or triphasic curves with large confidence intervals). The

271   essential agreement was suboptimal and might reflect why it has been difficult to establish a

272   broth microdilution assay for fastidious gonococci in the past. An endpoint of six hours

273   provided only a snapshot of the antimicrobial properties and examining much more time-

274 points, starting inocula, and very large number of strains might provide valuable data for

275 improvements. Furthermore, obtaining significantly more data, possibly by scaling the assay

276 to a robotic platform, would enable the regression analysis to be performed for the different

277 antimicrobials separately and also allow fitting a biphasic model.

278 Despite these drawbacks, the developed rapid resazurin-based broth microdilution assay

279 is highly objective (avoids visual subjective readout) and employs a standardized algorithm

280 reducing operator bias, which can be especially valuable in multicentre studies. These

281 properties, and the low price of resazurin, are especially valuable when screening large libraries

282 of new compounds, antimicrobials or antimicrobial combinations. Frequently, the question that

283 needs to be answered is the potency of antimicrobials relative to each other rather than absolute

284 numbers. While pH stripes are excellent for distinguishing acid from basic chemicals, complex

285 questions such as titration curves of a weak with a strong acid need more quantitative

286 approaches. The β-lactams cefixime, ceftriaxone and penicillin G had significantly lower hill

287 coefficients than the other antimicrobials for some strains. The macrolide azithromycin showed

288 similarity with tetracycline, both antimicrobials target protein translation. The hill coefficient

289 could be potentially useful for research questions beyond MIC such as combination therapy

290 and pharmacodynamic modelling.

291 In summary, the developed resazurin-based broth microdilution assay is a rapid, objective,

292 high-thoughput, quantitative and cost-effective new tool for studying *N. gonorrhoeae* in liquid

293 culture. The Hill coefficient could be compared for a large number of strains highlighting

294 differences between antimicrobials. The new assay opens up avenues for high-throughput

295 synergy testing, evaluation of novel antimicrobials and surveillance of resistance.

296

297 **Funding**

References

1. WHO. Global action plan to control the spread and impact of antimicrobial resistance in Neisseria gonorrhoeae. 2012. Available at: http://apps.who.int/iris/bitstream/10665/44863/1/9789241503501_eng.pdf. Accessed December 6, 2016.

2. Newman L, Rowley J, Hoorn SV, *et al.* Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PLOS ONE* 2015; **10**: e0143304.

3. Unemo M, Shafer WM. Antimicrobial Resistance in Neisseria gonorrhoeae in the 21st Century: Past, Evolution, and Future. *Clin Microbiol Rev* 2014; **27**: 587–613.

4. Biedenbach DJ, Jones RN. Comparative assessment of Etest for testing susceptibilities of Neisseria gonorrhoeae to penicillin, tetracycline, ceftriaxone, cefotaxime, and ciprofloxacin: investigation using 510(k) review criteria, recommended by the Food and Drug Administration. *J Clin Microbiol* 1996; **34**: 3214–7.

5. Liu H, Taylor TH, Pettus K et al. Assessment of Etest as an alternative to agar dilution for antimicrobial susceptibility testing of Neisseria gonorrhoeae. J Clin Microbiol 2014; 52: 1435–40.

6. Singh V, Bala M, Kakran M et al. Comparative assessment of CDS, CLSI disc diffusion and Etest techniques for antimicrobial susceptibility testing of Neisseria gonorrhoeae: a 6-year study. BMJ Open 2012; 2: e000969.

7. Gose S, Kong CJ, Lee Y et al. Comparison of Neisseria gonorrhoeae MICs obtained by Etest and agar dilution for ceftriaxone, cefpodoxime, cefixime and azithromycin. J Microbiol Methods 2013; 95: 379–80.

8. Liao C-H, Lai C-C, Hsu M-S et al. Antimicrobial susceptibility of Neisseria gonorrhoeae isolates determined by the agar dilution, disk diffusion and Etest methods: comparison of results using GC agar and chocolate agar. Int J Antimicrob Agents 2010; 35: 457–60.

9. Ison CA, Martin IMC, Lowndes CM et al. Comparability of laboratory diagnosis and antimicrobial susceptibility testing of Neisseria gonorrhoeae from reference laboratories in Western Europe. J Antimicrob Chemother 2006; 58: 580–6.

333   10. Kelly MT, Leicester C. Evaluation of the Autoscan Walkaway system for rapid
334   identification and susceptibility testing of gram-negative bacilli. *J Clin Microbiol* 1992; **30**:
335   1568–71.

336   11. Godsey JH, Bascomb S, Bonnette T, *et al.* Rapid antimicrobial susceptibility testing of
337   gram-negative bacilli using Baxter MicroScan rapid fluorogenic panels and autoSCAN-W/A.
338   *Pathol Biol (Paris)* 1991; **39**: 461–5.

339   10. Reller LB, Weinstein M, Jorgensen JH et al. Antimicrobial susceptibility testing: a review
340   of general principles and contemporary practices. Clin Infect Dis 2009; 49: 1749–55.

341   11. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the
342   minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008; 3:
343   163–75.

344   12. Takei M, Yamaguchi Y, Fukuda H et al. Cultivation of Neisseria gonorrhoeae in liquid
345   media and determination of its in vitro susceptibilities to quinolones. J Clin Microbiol 2005;
346   43: 4321–7.

347   13. Geers TA, Donabedian AM. Comparison of broth microdilution and agar dilution for
348   susceptibility testing of Neisseria gonorrhoeae. Antimicrob Agents Chemother 1989; 33:
349   233–4.

350   14. Shapiro MA, Heifetz CL, Sesnie JC. Comparison of microdilution and agar dilution
351   procedures for testing antibiotic susceptibility of Neisseria gonorrhoeae. J Clin Microbiol
352   1984; 20: 828–30.

353   15. Dillard JP, Seifert HS. A peptidoglycan hydrolase similar to bacteriophage endolysins
354   acts as an autolysin in Neisseria gonorrhoeae. Mol Microbiol 1997; 25: 893–901.

355   16. Elmros T, Burman LG, Bloom GD. Autolysis of Neisseria gonorrhoeae. J Bacteriol 1976;
356   126: 969–76.

357   17. Chan YA, Hackett KT, Dillard JP. The lytic transglycosylases of Neisseria gonorrhoeae.
358   Microb Drug Resist 2012; 18: 271–9.

359   29. Wade JJ, Graver MA. A fully defined, clear and protein-free liquid medium permitting
360   dense growth of Neisseria gonorrhoeae from very low inocula. FEMS Microbiol Lett 2007;
361   273: 35–7.

362   18. Foerster S, Golparian D, Jacobsson S et al. Genetic resistance determinants, in vitro time-
363   kill curve analysis and pharmacodynamic functions for the novel topoisomerase II inhibitor
364   ETX0914 (AZD0914) in Neisseria gonorrhoeae. Front Microbiol 2015; 6: 1377.

365   19. Foerster S, Unemo M, Hathaway LJ et al. Time-kill curve analysis and pharmacodynamic
366   functions for in vitro evaluation of antimicrobials against Neisseria gonorrhoeae. BMC
367   Microbiol 2016; 16: 216.

368   20. Kassteele J van de, Santen-Verheuvel MG van, Koedijk FDH et al. New statistical
369   technique for analyzing MIC-based susceptibility data. Antimicrob Agents Chemother 2012;
370   56: 1557–63.

371 21. Slob W. Benchmark dose and the three Rs. Part I. Getting more information from the
372 same number of animals. Crit Rev Toxicol 2014; 44: 557–67.

373 22. Slob W. Benchmark dose and the three Rs. Part II. Consequences for study design and
374 animal use. Crit Rev Toxicol 2014; 44: 568–80.

375 23. Davis JA, Gift JS, Zhao QJ. Introduction to benchmark dose methods and U.S. EPA's
376 benchmark dose software (BMDS) version 2.1.1. Toxicol Appl Pharmacol 2011; 254: 181–
377 91.

378 24. Filipsson AF, Sand S, Nilsson J et al. The benchmark dose method--review of available
379 models, and recommendations for application in health risk assessment. Crit Rev Toxicol
380 2003; 33: 505–42.

381 25. Sampah MES, Shen L, Jilek BL et al. Dose–response curve slope is a missing dimension
382 in the analysis of HIV-1 drug resistance. Proc Natl Acad Sci U S A 2011; 108: 7613–8.

383 26. Rampersad SN. Multiple applications of alamar blue as an indicator of metabolic function
384 and cellular health in cell viability bioassays. Sensors 2012; 12: 12347–60.

385 27. Khalifa RA, Nasser MS, Gomaa AA et al. Resazurin microtiter assay plate method for
386 detection of susceptibility of multidrug resistant Mycobacterium tuberculosis to second-line
387 anti-tuberculous drugs. Egypt J Chest Dis Tuberc 2013; 62: 241–7.

388 28. Palomino J-C, Martin A, Camacho M et al. Resazurin microtiter assay plate: simple and
389 inexpensive method for detection of drug resistance in Mycobacterium tuberculosis.
390 Antimicrob Agents Chemother 2002; 46: 2720–2.

391 26. Schmitt DM, Connolly KL, Jerse AE et al. Antibacterial activity of resazurin-based
392 compounds against Neisseria gonorrhoeae in vitro and in vivo. Int J Antimicrob Agents 2016;
393 48: 367–72.

394 33. Unemo M, Golparian D, Sánchez-Busó L, *et al.* The novel 2016 WHO Neisseria
395 gonorrhoeae reference strains for global quality assurance of laboratory investigations:
396 phenotypic, genetic and reference genome characterization. *J Antimicrob Chemother* 2016;
397 **71**: 3096–108.

398 34. Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J. Phenotypic and genetic
399 characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended for
400 global quality assurance and quality control of gonococcal antimicrobial resistance
401 surveillance for public health purposes. *J Antimicrob Chemother* 2009; **63**: 1142–51.

402 35. Schultheiss, O.C., Stanton, S.J. (2009). Assessment of salivary hormones. In: Harmon-
403 Jones, E., Beer, J.S., eds. Methods in Social Neuroscience. New York: Guilford Press.

404 36. Anon. Bioassay Analysis Using R | Ritz | Journal of Statistical Software. Available at:
405 https://www.jstatsoft.org/article/view/v012i05. Accessed March 16, 2016.

406 37. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint
407 tables for interpretation of MICs and zone diameters. 2016.

408 38. CLSI, Wayne, PA, USA. Clinical and Laboratory Standards Institute. Development of In
409 Vitro Susceptibility Testing Criteria and Quality Control Parameters, 2nd edn. Approved
410 Guideline M23-A2. 2001. Available at:
411 http://shop.clsi.org/site/Sample_pdf/M23A3_sample.pdf. Accessed December 7, 2016.

412 39. Parikh R, Mathai A, Parikh S, Chandra Sekhar G, Thomas R. Understanding and using
413 sensitivity, specificity and predictive values. *Indian J Ophthalmol* 2008; **56**: 45–50.

414 40. Kocaoglu O, Tsui H-CT, Winkler ME, Carlson EE. Profiling of β-Lactam Selectivity for
415 Penicillin-Binding Proteins in Streptococcus pneumoniae D39. *Antimicrob Agents Chemother*
416 2015; **59**: 3548–55.

417 41. Di Veroli GY, Fornari C, Goldlust I, *et al.* An automated fitting procedure and software
418 for dose-response curves with multiphasic features. *Sci Rep* 2015; **5**: 14701.

419 42. Regoes RR, Wiuff C, Zappala RM, Garner KN, Baquero F, Levin BR. Pharmacodynamic
420 functions: a multiparameter approach to the design of antibiotic treatment regimens.
421 *Antimicrob Agents Chemother* 2004; **48**: 3670–6.

422 43. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape.
423 *Pharmacol Res Perspect* 2015; **3**. Available at:
424 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4492765/. Accessed November 17, 2015.

425 44. Yu G, Baeder DY, Regoes RR, Rolff J. Combination Effects of Antimicrobial Peptides.
426 *Antimicrob Agents Chemother* 2016; **60**: 1717–24.

427 45. Schmitt DM, Connolly KL, Jerse AE, Detrick MS, Horzempa J. Antibacterial activity of
428 resazurin-based compounds against Neisseria gonorrhoeae in vitro and in vivo. *Int J*
429 *Antimicrob Agents* 2016; **48**: 367–72.
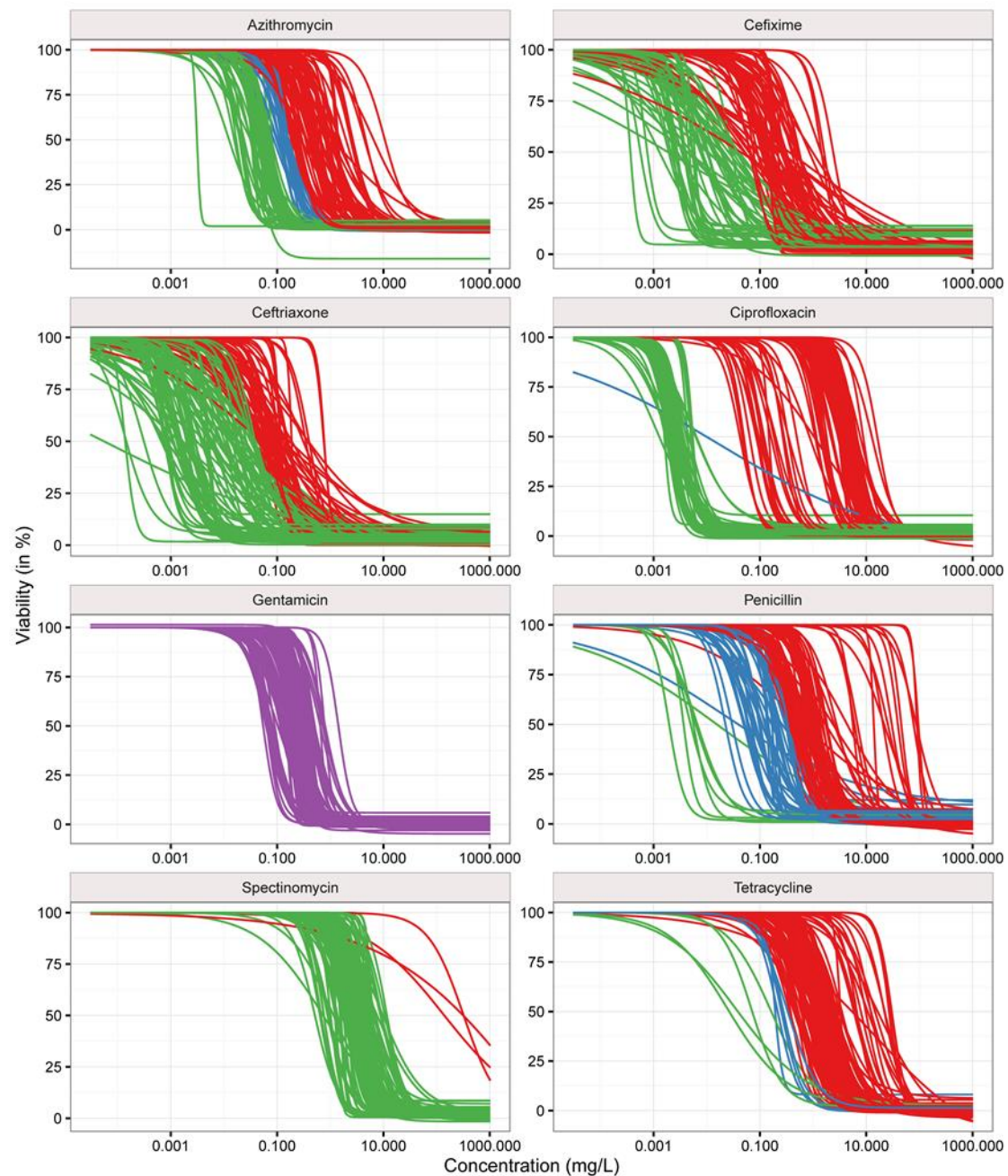
Figure 1. Potency shift of antimicrobials across different strains. Dose response curves for all strains and antimicrobials (without samples above limit of detection). Strains that were classified as susceptible according to EUCAST 2016 criteria were coloured in green, intermediary resistant strains in blue and resistant strains in blue. No EUCAST criteria are defined for gentamicin (purple). The gradual shift of the potencies (EC_{50}) towards higher concentrations can be observed for all antimicrobials.
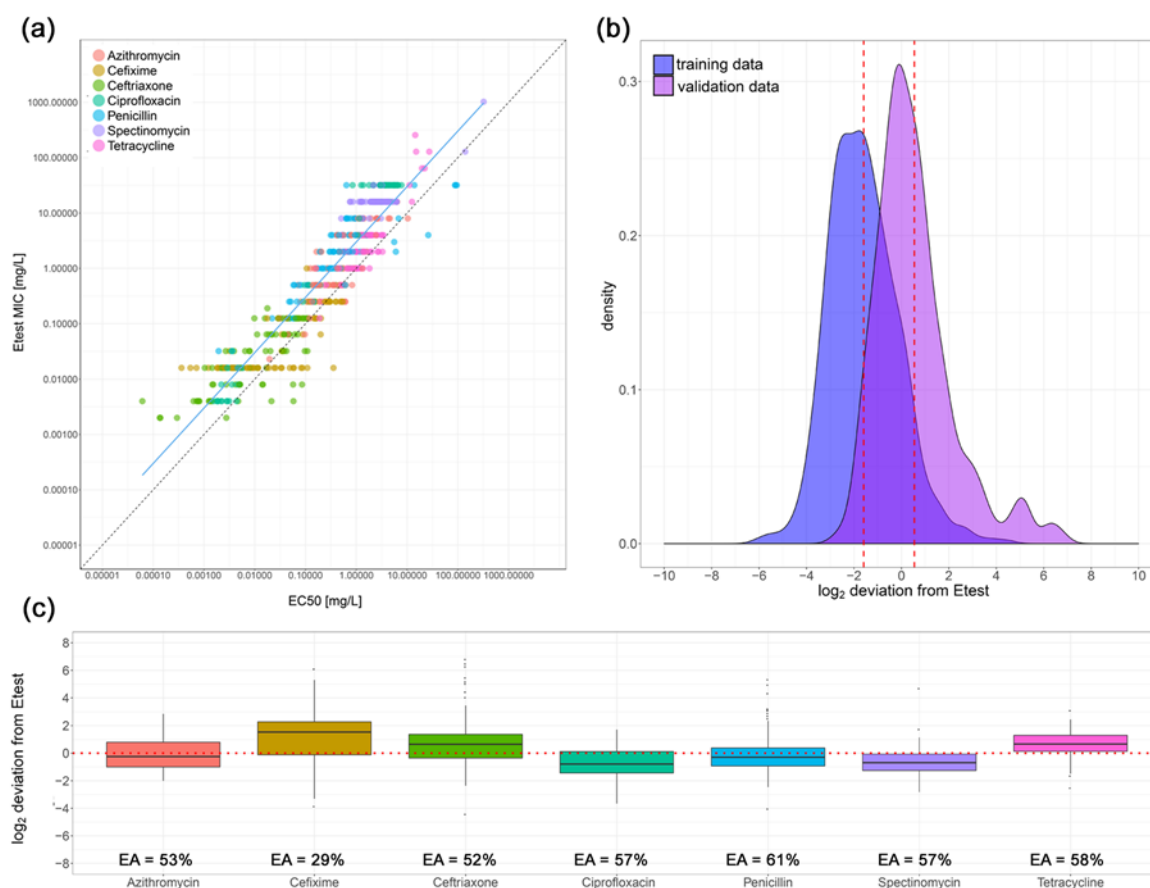
Figure 1. Correlation and deviations between the Etest and predicted MICs. (A) The correlation of Etest MIC and EC_{50} for the dataset used for developing the regression model (84 blinded strains examined) is shown for log-log transformed values. The Pearson's correlation coefficient for the linear regression was 0.83. Slope and intercept for a perfect correlation (1) was drawn as dashed black line for comparison. (B) The kernel distribution of the values for these 84 strains is drawn in blue (median -1.8). The kernel distribution of the MICs predicted with the slope and intercept of the 84 strains is highlighted in purple (median 0.11). (C) Deviations of predicted MICs (124 clinical strains examined) from Etest MIC are shown for seven antimicrobials. 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) defined as less than ±1 doubling dilution from Etest for each antimicrobial is written below the boxplots.

456
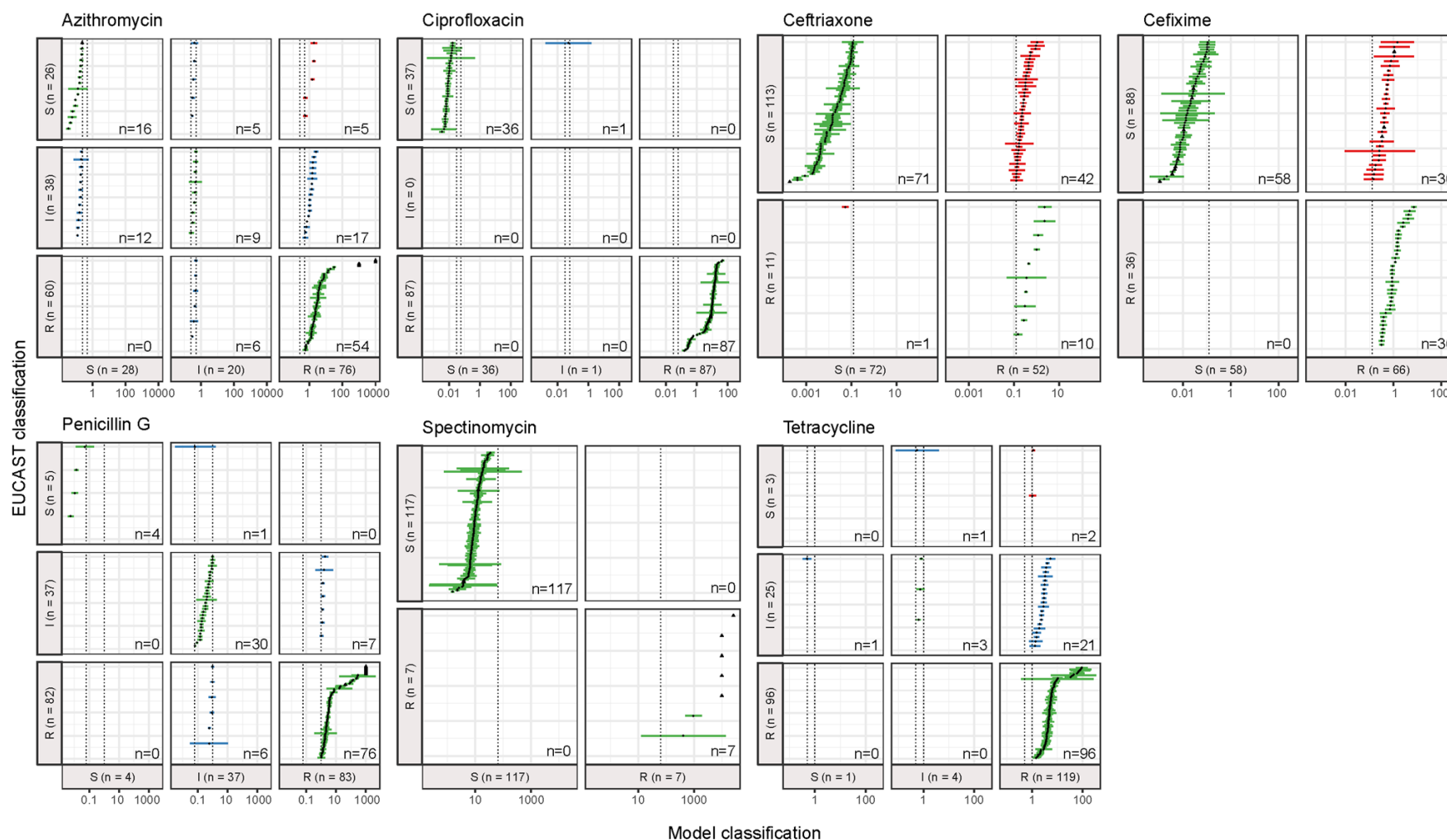
Figure 1. Contingency table with categorical errors of model predicted MICs. Etest MIC data were classified into the categories resistant (R),

susceptible (S) and intermediate (I) according to the EUCAST 2016 criteria.[30] The cutoff values (mg/L) are shown as dashed black lines. Predicted

459   MIC values are shown as point estimate with 95% confidence interval. Correctly classified strains are depicted as green. Major errors (S to R) and

460   very major errors (R to S) are shown in red. Minor errors resulting from misclassifications of intermediate strains are drawn in blue. Data below

461   or above limit of detection were not included. Gentamicin and Spectinomycin were excluded from this analysis.

462