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

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Objectives: Rapid, cost-effective and objective methods for antimicrobial susceptibility testing of *Neisseria gonorrhoeae* would greatly enhance surveillance of antimicrobial resistance. Etest, disc diffusion and agar dilution methods are subjective, mostly laborious for large-scale testing and take ∼24 h. We aimed to develop a rapid broth microdilution assay using resazurin (blue), which is converted into resorufin (pink fluorescence) in the presence of viable bacteria.

Methods: The resazurin-based broth microdilution assay was established using 132 *N. gonorrhoeae* strains and the antimicrobials ceftriaxone, cefixime, azithromycin, spectinomycin, ciprofloxacin, tetracycline and penicillin. A regression model was used to estimate the MICs. Assay results were obtained in ∼7.5 h.

Results: The EC_{50} of the dose–response curves correlated well with Etest MIC values (Pearson's $r = 0.93$). Minor errors resulting from misclassifications of intermediate strains were found for 9% of the samples. Major errors (susceptible strains misclassified as resistant) occurred for ceftriaxone (4.6%), cefixime (3.3%), azithromycin (0.6%) and tetracycline (0.2%). Only one very major error was found (a ceftriaxone-resistant strain misclassified as susceptible). Overall the sensitivity of the assay was 97.1% (95% CI 95.2–98.4) and the specificity 78.5% (95% CI 74.5–82.9).

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 is an effective low-cost method to evaluate novel antimicrobials and for high-throughput screening, and expands the currently available methodologies for surveillance of antimicrobial resistance in gonococci.

Introduction

Neisseria gonorrhoeae is a very fastidious bacterium that causes the sexually transmitted infection gonorrhoea. Gonorrhoea is a public health concern globally[1,2] and *N. gonorrhoeae* has developed resistance to all antimicrobials introduced for treatment.[3] Accordingly, enhanced surveillance of antimicrobial susceptibility in *N. gonorrhoeae* is imperative globally.[1] Ideally, this surveillance should be performed using methods determining the MICs of relevant antimicrobials. MIC-based methods are also valuable to directly inform treatment after laboratory results are available and evaluate *in vitro* efficacy of novel antimicrobials.

Owing to the lack of any appropriate broth medium for MIC determination, MIC-based susceptibility testing of *N. gonorrhoeae* has been limited to disc diffusion, Etest and the agar dilution method (gold standard). Essential agreement with the agar dilution method is defined as ±1 doubling dilution and should ideally be >90% for diagnostic purposes where the same resistance breakpoints are applied.[4] Etest has shown excellent agreement with the agar dilution method in many settings.[4–7] However, discordant results have been found, particularly when different growth media were used.[8] A multicentre international study revealed that the categorical agreement between Etest and agar dilution was ≥88%, but was very poor for disc diffusion.[9] Unfortunately, all these methods are relatively slow (∼24 h), subjective, require expertise and/or are expensive. Faster methods that allow results to be obtained on the same day have been developed in the past for other bacteria,[10,11] but are not available for *N. gonorrhoeae*.

For many bacterial species, broth microdilution is the reference method due to accuracy, low costs and high versatility.[12,13] Several attempts have been made to develop a broth microdilution method also for *N. gonorrhoeae*, but none of these has 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 (Hill models), as this allows the estimation of the effective concentration (EC) at a specified response level.[24] Furthermore, EC values on a continuous scale take the variability of the data into account by calculating CIs. In the field of toxicology the lower CI is defined as the non-toxic concentration. This so-called benchmark dose approach has largely replaced methods that rely on dense dose spacing because of its statistical superiority and reduction of animal use.[25–28] Furthermore, the shape of the dose–response curve can provide additional valuable information on the compounds being tested.[24] The Hill coefficient can provide information about the pharmacodynamic properties of an antimicrobial and has been used in modelling studies of single and dual antimicrobial effects.[21,22,29–31] However, the interpretation and significance of the Hill coefficient has been unclear in previous studies and laborious colony counting has limited these studies to a few strains.

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. Methods in which OD (at e.g. OD_{600} or OD_{450}), resazurin (Alamar blue), MTT, luciferase (ATP levels) and lactate dehydrogenase are measured are widespread, with readouts that correlate with the number of cells.[32] Resazurin is a blue dye that is converted into pink-fluorescent resorufin in the presence of metabolically active cells.[33,34] Unlike OD, a measure of growth inhibition, it reflects the viability of cells and is potentially suitable for time–kill assays. Resazurin has an excellent signal-to-noise ratio and has been used previously in screening for toxicity testing,[35] high-throughput applications,[36] biofilm screening[37] and MIC testing.[33,38–40]

The aim of this study was to develop a resazurin-based broth microdilution assay for antimicrobial susceptibility testing of *N. gonorrhoeae* that is rapid, objective, scalable, quantitative and inexpensive. Three datasets were generated in this study. The 2008 WHO *N. gonorrhoeae* reference strains ($n = 8$)[41,42] were studied to ensure the reproducibility of the assay and to compare multiple measurement endpoints between 0 and 15 h. Training data consisting of 84 *N. gonorrhoeae* strains were used to develop a regression model for estimating the MIC from dose–response curves. Finally, a panel of 40 strains with blinded MICs was used for validation.

Materials and methods

Bacterial strains, culture and broth microdilution assay

The variability and reproducibility of the assay were validated in eight WHO reference strains (three replicates).[41,42] Additionally, 84 gonococcal strains were used as training data to develop a regression model for estimating the MIC after 6 h of incubation time (one replicate). The assay was finally validated with 40 gonococcal strains with blinded MICs (one replicate). The blinded strains were selected to represent a wide variety of antibiograms. The strains were preserved in glycerol stocks at −80°C. All strains were subsequently cultured on Chocolate Agar PolyViteX (bioMérieux, Marcy-l'Étoile, France) at 37°C in a humid 5% CO_2-enriched atmosphere for 16–18 h and then sub-cultured once for 16 h. A McFarland standard of 0.5 was prepared for each strain and 1 mL of bacterial suspension was further diluted to $\sim 1 \times 10^7$ cfu/mL in 15 mL of heated (37°C) GW broth.[20] A volume of 90 μL of this suspension was added to 96-well round-bottom microtitre plates (360 μL wells), with each well containing 10 μL of a previously prepared dilution series. Dilution series of the antimicrobials were prepared in GW medium. Positive control (GW medium containing 1% Triton X-100) and negative control (10 μL of GW medium) were added to the first and last well, respectively. The plates were incubated for 6 h at 37°C in a humid 5% CO_2-enriched atmosphere. Detailed standard operating procedures, including Figure S1, are available as Supplementary data at *JAC* Online.

Resazurin readouts

Resazurin powder (Sigma–Aldrich, China) was diluted in PBS (pH 7.4) to a final concentration of 0.1 mg/mL. We ensured that the pH of the highest antimicrobial concentration was neutral in all samples to avoid artefacts. After incubation of the broth microdilution plates, 50 μL of the dye was added to each well and mixed using an electronic multichannel dispenser. The plates were incubated for 75 min at 37°C. Fluorescence was then measured at 560 and 590 nm excitation in a plate reader (Varioskan Flash, Thermo Scientific).

Etest MIC

The Etest MICs (bioMérieux) were determined in accordance with the manufacturer's instructions, on gonococcal resistance agar plates (GCRAPs) [3.6% Difco GC Medium Base agar (BD Diagnostics, Sparks, MD, USA) supplemented with 1% haemoglobin (BD Diagnostics) and 1% IsoVitalex (BD Diagnostics)].

Dose–response modelling

The antimicrobial effect on the different bacterial strains was quantified with dose–response curves. We first subtracted the background fluorescence resulting from dead bacteria in the positive control wells from the resazurin readout. We then fitted a sigmoidal dose–response curve to the fluorescence data of each antimicrobial–strain combination:[43,44]

$$f(x) = u + \frac{l - u}{1 + e^{H(x - \ln(EC_{50}))}} \quad (1)$$

where $f(x)$ is the fluorescence, x is the natural logarithm of the antibiotic concentration, and u and l describe the upper and lower asymptote, respectively. The EC_{50} is the antibiotic concentration at which the effect is half-maximal and H denotes the slope of the sigmoidal function, i.e. the Hill coefficient. Next, the data were divided by u to normalize all dose–response curves to 100% viability. Hill coefficient differences across antimicrobials were tested with pairwise t-tests. Hierarchical complete linkage clustering was used to compare antimicrobial similarity.[45]

Samples were considered to be above the limit of detection, and therefore categorized as resistant, if the antibiotic, at its highest concentration, reduced viability by <50%. This was the case for six samples in the training data ($n = 588$) and nine samples in the validation data ($n = 280$). Excluding samples that were above or below the limit of detection (including Etest MICs beyond the limit of detection) resulted in 571 evaluable samples in the training data and 266 samples in the validation data. Reference strain data were not included to avoid bias from replicate testing of these samples. The relationship between EC_{50} and Etest was analysed for the training data by log-transforming both values and fitting a linear regression:

$$\ln(\text{Etest}) = \alpha + \beta \ln(EC_{50}) + \varepsilon \quad (2)$$

where ε is a normally distributed error. The slope and intercept of this regression were then used to predict the MIC from the EC_{50} values for the blinded strains. 95% CIs for each predicted MIC were calculated using 10^5 bootstrap samples taking into account the uncertainty from the sigmoidal model and the linear regression model. The analysis pipeline, descriptive statistics and raw data are available from GitHub (https://github.com/sunnivas/ResazurinMIC).

Essential agreement with Etest

Essential agreement was defined as the percentage of strains with predicted MICs that did not deviate by more than ± 1 doubling dilution from Etest MICs. Deviations from the Etest MICs were calculated as $\log_2$ differences from the predicted MIC (837 evaluable samples for training and validation data). Reference strain data were not included to avoid bias from replicate testing of these samples.

Categorical agreement with Etest

The strains were categorized as susceptible (S), intermediate (I) or resistant (R) to each antimicrobial in accordance with the EUCAST 2016 guidelines.[46] As previously described,[47] minor errors were defined as misclassifications of intermediate strains as susceptible or resistant. Major errors were susceptible strains misclassified as resistant. Very major errors were resistant strains that were misclassified as susceptible. The EC_{50} values are read on a continuous scale and therefore nearly identical values around a resistance breakpoint (e.g. 0.125 and 0.126) can result in categorical errors. Sensitivity and specificity of the assay were calculated as previously described[48] for the resistant strains (true positive samples), intermediate strains (true positive samples) and susceptible strains (true negative samples).

Results

Dose–response modelling

The 2008 WHO reference strains ($n = 8$) were exposed to ceftriaxone, cefixime, azithromycin, spectinomycin, ciprofloxacin, tetracycline or penicillin for a time course from 0–15 h (Figure S2). After 6 h, the difference between dead and viable gonococcal cells was sufficiently pronounced to fit dose–response curves to the data. For this endpoint of 6 h, the coefficient of variation (CV) was calculated for the EC_{50} of three independent experiments. The CV ranged from 1.7% to 87% and the intra-assay CV was 29% ($n = 56$) (Figure S3). Dose–response curves were gradually shifted towards higher concentrations, indicating decreased potency of the antimicrobials against the intermediate and resistant strains compared with susceptible strains (Figure 1). There was a clear separation of susceptible and resistant strains for ciprofloxacin and spectinomycin. For the β-lactam antimicrobials ceftriaxone, cefixime and penicillin the Hill coefficients (slopes) were more heterogeneous than for the other samples (Figure 1). The mean (±SD) of this parameter gradually increased from ceftriaxone (1.6±1.3) to cefixime (1.9±1.5), tetracycline (2.1±0.9), penicillin (2.5±1.7), azithromycin (2.6±1.5), ciprofloxacin (2.7±1.2) and spectinomycin (2.9±1.7). A pairwise t-test showed that the differences between the antimicrobials were significant ($P < 0.005$) when the distance between the means was >0.5 (Figure S4a). Furthermore, hierarchical clustering showed a high similarity of the Hill coefficient for the β-lactam antimicrobials ceftriaxone, cefixime and penicillin compared with the other antimicrobials (Figure S4b).

For the training data (84 strains), Pearson's correlation between the Etest MICs and EC_{50} values of all antimicrobials was 0.93 (Figure 2a). Compared with the Etest values, the EC_{50} values were systematically lower, with a median deviation of −1.68 doubling dilutions (Figure 2b). The regression parameters α ($\hat{\alpha} = 1.10$; $SD_{\hat{\alpha}} = 0.048$) and β ($\hat{\beta} = 1.00$; $SD_{\hat{\beta}} = 0.016$) of the linear log–log regression were used to predict the 837 MICs of the training and validation data. The deviation of the predicted MIC from Etest followed a normal distribution with a median of −0.015 and 95% of the deviations ranged between −4.45 and 9.22. Outliers were mostly attributed to the β-lactam antimicrobials penicillin (e.g. overestimation in β-lactamase-producing strains) and cefixime and ceftriaxone (e.g. potentially biphasic or triphasic curves with large CIs). One example of a strain with biphasic curves for ceftriaxone and cefixime was studied in detail (Figure S5).[49] The 75% quartiles for the deviations were larger for azithromycin, cefixime and ceftriaxone compared with ciprofloxacin, penicillin, spectinomycin and tetracycline (Figure 2c). The essential agreement between the Etest MICs and the predicted MICs was 53% for all antimicrobials, being lowest for cefixime (31%) and highest for penicillin (61%).

Categorical agreement

The Etest and predicted MICs ($n = 868$) were classified as susceptible, intermediate resistant and resistant according to the EUCAST 2016 resistance breakpoints[46] (Figure 3). The sensitivity of the assay was 97.1% (95% CI 95.2–98.4). Minor errors resulting from misclassifications of intermediate resistant strains were found for 9% of the data. For penicillin, spectinomycin and ciprofloxacin no major errors were identified. False-positive misclassifications (S misclassified as R), i.e. major errors, occurred for tetracycline (0.2%), azithromycin (0.6%), cefixime (3.3%) and ceftriaxone (4.6%) for a total of 9% of the data. One very major error (R misclassified as S) occurred for ceftriaxone (Etest MIC 0.19 versus 0.053 mg/L). Many predicted MIC values (16.5%) had 95% CIs spanning two categories. The overall specificity of the assay was 78.5% (95% CI 74.5–82.9).

Discussion

The developed resazurin-based broth microdilution assay was able to discriminate between resistant and susceptible strains relatively reliably, was faster (~7.5 h for results) than currently available MIC methods for *N. gonorrhoeae* and had an excellent sensitivity of 97.1% (95% CI 95.2–98.4). The gold standard MIC-based method agar dilution and the Etest method are both based on subjective, visual readouts and are therefore limited to a relatively low

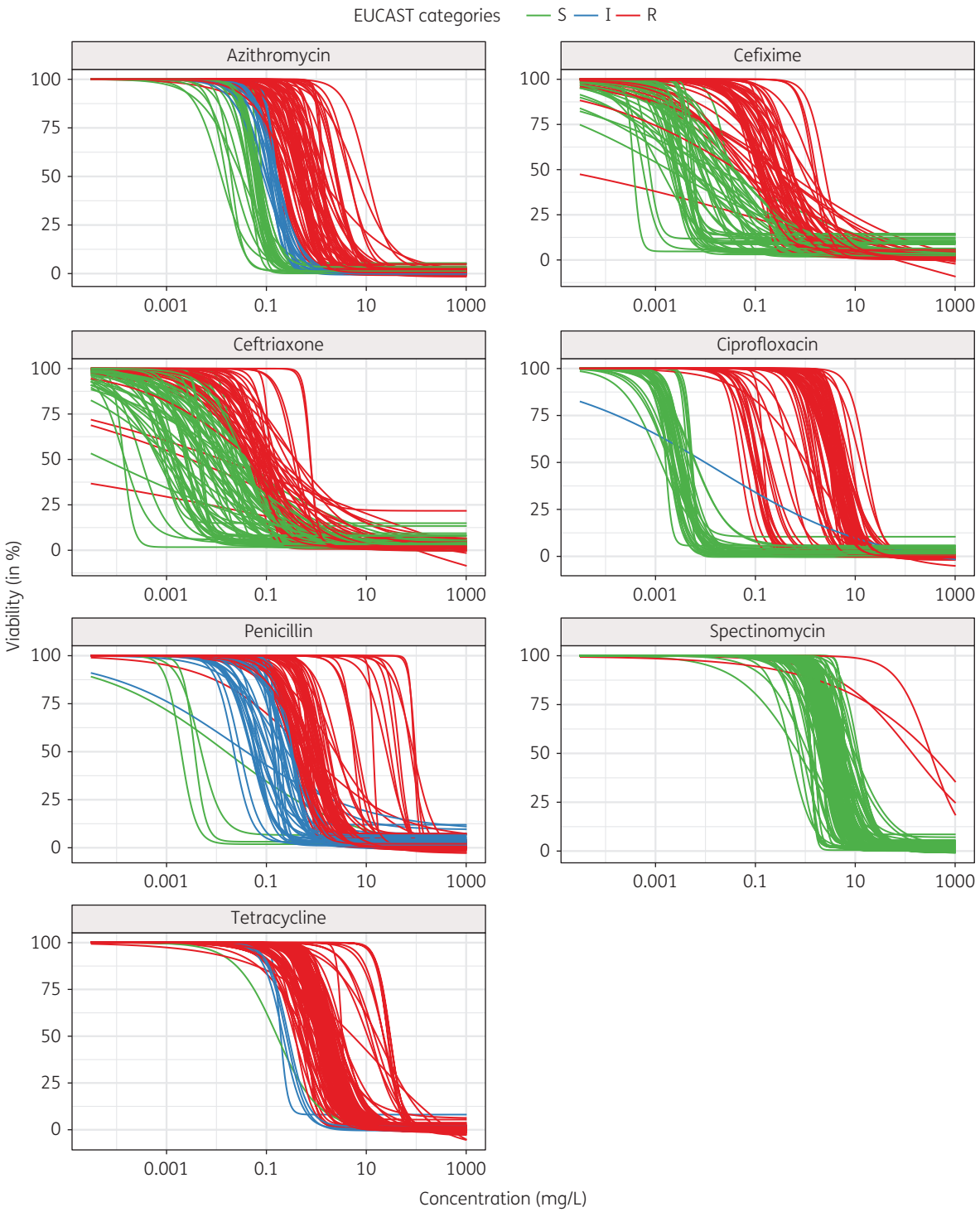

Figure 1. Potency shift of antimicrobials across different strains of *N. gonorrhoeae*. Dose–response curves for all strains and antimicrobials are shown (except samples above the limit of detection). Strains that were classified as susceptible according to EUCAST 2016 MIC breakpoints[46] are coloured green, intermediate strains blue and resistant strains red. The gradual shift of the potencies (EC_{50}) towards higher concentrations can be observed for all antimicrobials.

throughput. Dose–response modelling allows the precise estimation of the EC_{50} of antimicrobials from a continuous scale and provides CIs rather than having the precision limited by doubling dilutions. It is inherently difficult to apply resistance breakpoints that were designed for doubling dilution-based methods to dose–response curve-based MICs. This was reflected by many categorical errors resulting from estimates that had CIs overlapping two S/I/R categories. The performance of the assay was excellent for

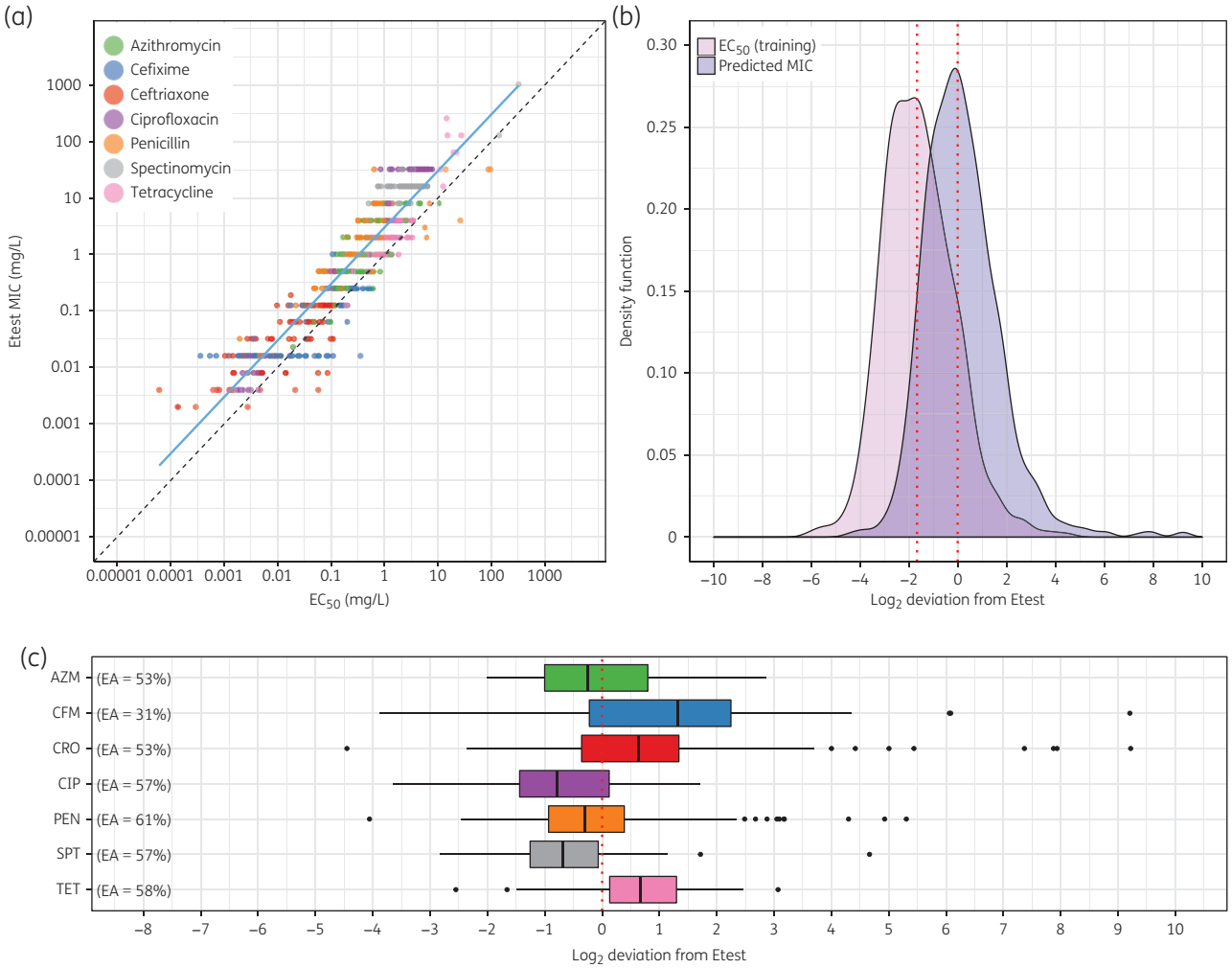

Figure 2. Correlation and deviations between Etest MICs and predicted MICs. (a) Linear regression between EC_{50} and Etest MIC for the training data ($n = 571$). Pearson's correlation coefficient for the linear regression (blue line) was 0.93. Slope and intercept for a perfect correlation are drawn as a dashed black line for comparison. (b) The kernel density function of the EC_{50} values for the training data ($n = 571$) is shown in pink (median −1.68). The kernel density of the predicted MICs for the training and validation data ($n = 837$) is shown in purple (median −0.015). (c) Deviations of predicted MICs from Etest MIC for each antimicrobial ($n = 837$). Boxplots show the median and IQR. Whiskers span the range from the bottom 5% to the highest 95% of the data. Essential agreement (EA) is indicated next to the boxplots. AZM, azithromycin; CFM, cefixime; CRO, ceftriaxone; CIP, ciprofloxacin; PEN, penicillin; SPT, spectinomycin; TET, tetracycline.

ciprofloxacin, penicillin and spectinomycin (no major errors) and acceptable for azithromycin (0.6% major errors) and tetracycline (0.2% major errors).

The deviations of resazurin-based MICs from Etest MICs followed a normal distribution. Outliers were mainly attributed to the β-lactam antimicrobials and contributed to the suboptimal essential agreement and assay specificity of only 78.5% (95% CI 74.5–82.9). For penicillin, substantially higher MICs were measured with the resazurin assay, e.g. for β-lactamase-producing strains. For cefixime and ceftriaxone there were many false-positive results and consequently an overestimation of resistance was measured. The complex mechanism of action and evolution of resistance to these antimicrobials is not fully understood and involves several resistance determinants in multifaceted interactions (*penA*, *penB*, *mtrR*, factor X).[3,50] The correlation of EC_{50} and MIC has been previously shown to be not strictly linear and was largely influenced by different PBPs in *Streptococcus pneumoniae*.[51] The binding kinetics of a mechanism involving several targets and/or resistance determinants can result in dose–response curves that are biphasic and potentially triphasic (Figure S5).[22,49] In these cases the correlation between EC_{50} and Etest MIC differs from those in dose–response curves with only one inflection point and can result in false-positive results.

Performing the regression analysis for the different antimicrobials separately might improve the assay specificity, particularly for the β-lactam antimicrobials. An endpoint of 6 h provided only a snapshot of the antimicrobial properties and examining many more timepoints, more starting inocula and a very large number of strains, covering a wide range of MICs and ideally including *in vitro*-selected resistant strains, might also provide valuable data for

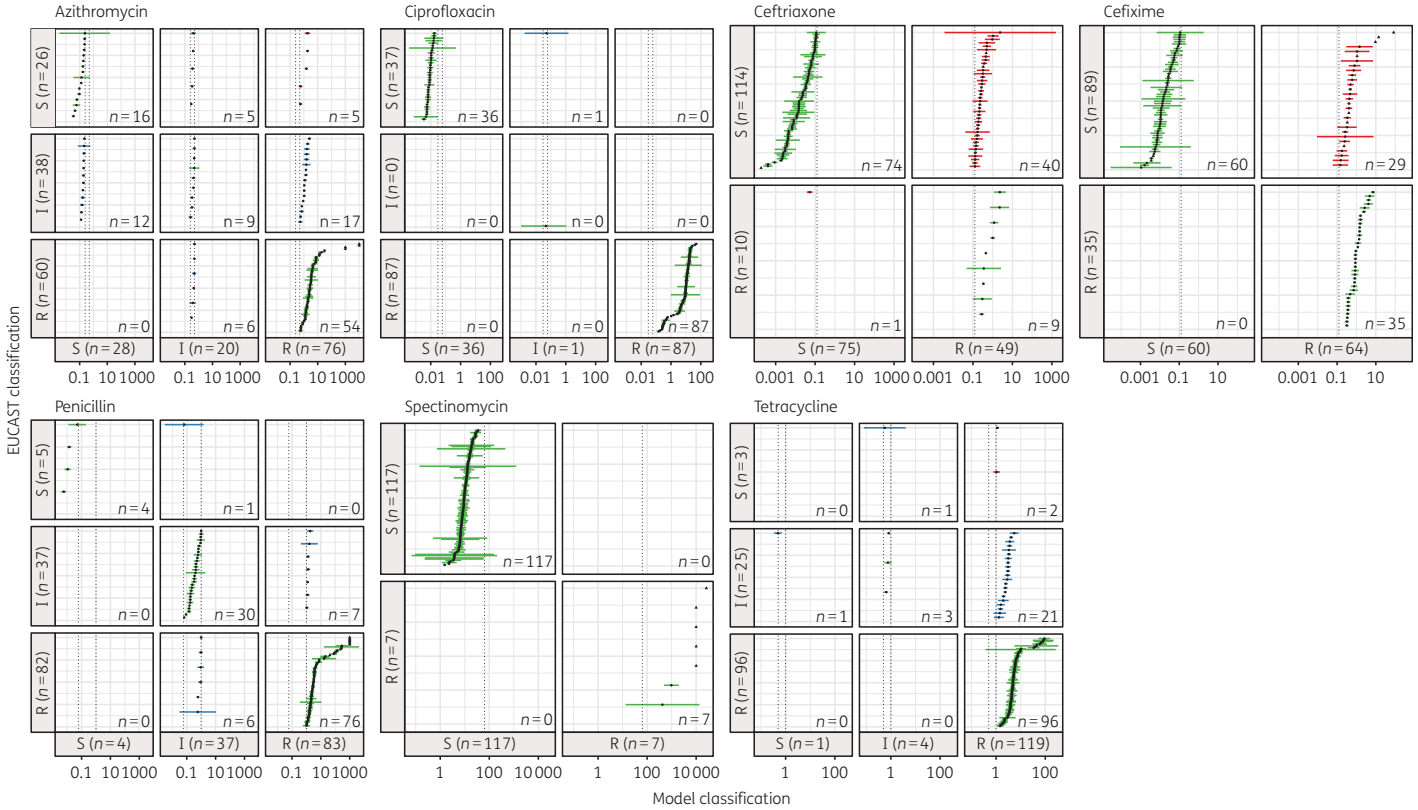

Figure 3. Contingency table with categorical errors of model-predicted MICs. Etest MIC data were classified into the categories R, I and S according to the EUCAST 2016 criteria.[46] The cut-off values (mg/L) are shown as dashed black lines. Predicted MIC values ($n = 868$) are shown as point estimates (black dots) with 95% CI (coloured dashes). For some estimates no CI could be calculated (limit of detection); these are drawn as triangles. Correctly classified strains are drawn in green. Minor errors resulting from misclassifications of intermediate strains are shown in blue. Major errors (S misclassified as R) were found for ceftriaxone ($n = 40$), cefixime ($n = 29$), azithromycin ($n = 5$) and tetracycline ($n = 2$) and are shown in red. One very major error (R misclassified as S) was found for ceftriaxone (red). A high number of estimates ($n = 138$) have CIs spanning two categories.

improvements. Scaling up the assay to a robotic platform might be necessary for appropriate examination of all these parameters and strains.

Despite these limitations, the developed rapid resazurin-based broth microdilution assay was highly objective (avoiding visual subjective readout) and employs a standardized algorithm reducing operator bias, which can be especially valuable in multicentre studies. These properties, and the low price of resazurin, are especially valuable when screening large libraries of new compounds, antimicrobials or antimicrobial combinations. Frequently, the question that needs to be answered is the potency of antimicrobials relative to each other rather than absolute numbers. The β-lactam antimicrobials cefixime, ceftriaxone and penicillin displayed significantly lower Hill coefficients than the other antimicrobials. Information about this parameter is useful for research questions beyond susceptibility testing, such as combination therapy and pharmacodynamic modelling.

In summary, the developed resazurin-based broth microdilution assay is a rapid, objective, high-throughput, quantitative and cost-effective new tool for studying *N. gonorrhoeae* in liquid culture. The Hill coefficient could be compared for a large number of strains, highlighting differences between antimicrobials. The new assay opens up avenues for high-throughput synergy testing, evaluation of novel antimicrobials and surveillance of resistance.

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Transparency declarations

None to declare.

Supplementary data

The standard operating procedures and Figures S1–S5 are available as Supplementary data at *JAC* Online.

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