**A NEW RAPID RESAZURIN-BASED MICRODILUTION ASSAY FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *NEISSERIA GONORRHOEAE***

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**Objectives**

Rapid and objective methods for antimicrobial susceptibility testing of *Neisseria gonorrhoeae* would greatly enhance surveillance of antimicrobial resistance. Susceptibility testing using Etest, disc diffusion or agar dilution methods takes 16-20 hours. We developed a rapid microdilution assay using the blue dye resazurin, which is converted to pink fluorescent resorufin in the presence of metabolically active bacteria, to measure the minimum inhibitory concentration (MIC) of different antimicrobials in *N. gonorrhoeae* isolates.

**METHODS**

A broth microdilution assay was established for the antimicrobials azithromycin, gentamicin, ciprofloxacin, ceftriaxone, cefixime, tetracycline, penicillin G and spectinomycin. A training dataset of 84 *N. gonorrhoea* strains was used to develop a model for estimating the MIC after six hours incubation time using fluorescent resazurin as readout. The assay was validated with 40 blinded strains and 8 WHO reference strains.

**RESULTS**

The new method correctly distinguished the category of “resistant” and “susceptible” in all cases for ciprofloxacin, spectinomycin, penicillin G and tetracycline. Minor errors occurred for azithromycin that were wrongly misclassified as resistant. Major errors were found for cefixime and ceftriaxone. Gentamicin could not be compared since resistance no resistance breakpoints are available for this antimicrobial.

**CONCLUSIONS**

A rapid and cost efficient broth microdilution assay was established for *N. gonorrhoeae*.Categorical agreement was excellent in general but major classification errors were found for ceftriaxone and cefixime. The assay could be a low-cost method to evaluate novel antimicrobial compounds and for high throughput screening to enhance surveillance of gonococcal resistance.

**Keywords:** **Gonorrhea, resazurin, broth microdilution, minimal inhibitory concentration, dose-response curve**

**Running title: Broth microdilution assay for antimicrobial susceptibility testing of *Neisseria gonorrhoeae***

**INTRODUCTION**

*Neisseria gonorrhoeae* is a gram-negative, fastidious bacterium that causes the sexually transmitted disease gonorrhoea. Methods to determine the MIC of new isolates are crucial to monitor the epidemiology of resistant clones, diagnose treatment failures, discover resistance determinants and describe the effects of novel compounds.

The MIC is defined as the concentration of a compound that inhibits visible growth. Due to the lack of suitable broth media susceptibility testing in *N. gonorrhoeae* has been mainly limited to either Etest, disc diffusion or agar dilution. Agar dilution is the gold standard for susceptibility testing of in this fastidious organism. Essential agreement with this method is defined as +/- one doubling dilution and should be above 90% for diagnostic purposes where the same resistance breakpoints are applied1. Etest has been shown to be in excellent agreement with the reference method in most settings1–4. The most discordant results were found when different growth media where used, MICs are lower when GC agar is used compared to the richer chocolate agar5. A multicentre study revealed that overall agreements between the different methods is >70%and Etest was in better agreement with agar dilution than disk diffusion6. Unfortunately these methods are expensive, require expertise and are relatively slow (~24 hours).

We aimed to develop a broth microdilution assay for *N. gonorrhoea* that is rapid, quantitative and inexpensive. In many bacterial species broth microdilution is the reference method due to low costs and high versatility7,8. Several attempts were made to develop a broth microdilution method also for *N. gonorrhoea* in the past but none of these is used nowadays9–11. Due to the fastidious nature of these bacteria, using liquid culture growth is more challenging than solid agar media. Growth is more difficult to synchronize between different strains and effects such as autolysis occur when the bacteria enter stationary phase12–14. We used the chemically defined Graver-Wade broth in a time-kill assay. It supports reliably the growth of all tested phylogenetically diverse auxotypes and clinical isolates and might be a suitable medium for MIC testing15,16.

The potency of drugs in pharmacology is most frequently measured with dose-response curves, as this allows the estimation of the effective concentration (EC) at a specified (arbitrary) response level. MIC values based on doubling dilution series are left, interval, or right censored discrete data which makes error analysis challenging. 17. In contrast EC values on a continuous scale take the variability of the data into account by calculating model based confidence intervals and do not rely on doubling dilutions. Furthermore, the shape of the pharmacodynamics curve provides additional valuable information on the test compound18. The superiority of this approach has been statistically well established and the benchmark dose is now strongly recommended for toxicity testing19–22.

The response to the tested compounds can be measured with different readouts. Measuring the optical density at (e.g. OD600 or OD450), resazurin (Alamar blue), MTT, Luciferase (ATP levels) and Lactat dehydrogenase are widespread methods that indirectly correlate with cell numbers23.

Resazurin is a blue dye that is converted to pink fluorescent resazurin in the presence of metabolically active cells24,25. Unlike the optical density that measures growth inhibition it reflects the viability of cells and can decrease in response to bactericidal compounds. It has been previously used in screenings for toxicity testing, high throughput applications and MIC testing in bacteria24,26.

We hypothesize that this allows the measurement of the MIC at earlier time-points and follow the viability of bacteria over time. A panel of eight reference strains was exposed to the antimicrobials azithromycin, gentamicin, ciprofloxacin, ceftriaxone, cefixime, tetracycline, penicillin and spectinomycin for a time course from 0 to15 hours. Growth was monitored using OD600 and Resazurin as read-out. A panel of 124 strains was exposed to the same antimicrobials for six hours and a regression analysis made to study how the methods correlates with conventional MICs measured with Etest.

**MATERIAL AND METHODS**

**Bacteria and Cultures**

124 clinical isolates and eight WHO quality control strains (F, G, K, L, M, N, O, P) were studied. Strains were taken from -80°C Glycerol stocks and streaked out on Choc-Agar Plates (Biomerieux). They were cultured at 37°C and 5% CO2 in a humid atmosphere for 16-18 hours and then sub-cultured once for 16 hours. A McFarland standard of 0.5 was prepared for each strain and 1 ml diluted to approximately 1x107 CFU/ml in 15 ml warm (37°C) GW broth27. A volume of 90 ul of bacterial suspensions were added to 96-well plates (Sarstedt) with each well containing 10 ul of a previously prepared dilution series. For the antimicrobials azithromycin, gentamycin, ciprofloxacin, ceftriaxone, cefixime, penicillin and tetracycline a stock solution of 1000 mg/L was prepared and for spectinomycin 10000 mg/L. Dilution series of the antimicrobials were prepared in medium.. Positive control (1% TritonX in EtOH abs.) and negative control (10 ul..) were added to the first and last well, respectively. The plates were incubated for 6 hours at 37°C, 5 % CO2 in a humid atmosphere. Resazurin powder (Sigma Aldrich, China) was diluted in PBS (pH 7.4) to a final concentration of 0.1 mg/ml, 50 µl of the dye was added to each well and mixed using an electronic multichannel dispenser. The plates were incubated for 75 minutes at 37°C. Fluorescence was then measured at 560 nm excitation and 590 nm in a plate reader (Varioskan Flash, Thermo Scientific). Three independent experiments were performed for the WHO reference strains and one experiment for the 124 clinical isolates.

**Etest MIC**

Etest method (bioMérieux, Marcy l'Etoile, France) was done in accordance with the manufacturer´s instructions, on GCRAP agar plates (3.6% Difco GC Medium Base agar [BD, Diagnostics] supplemented with 1% hemoglobin [BD, Diagnostics] and 1% IsoVitalex [BD, Diagnostics]).

**Dose response modelling**

A sigmoidal dose response curve was fit to the viability data of each strain antibiotic combination using a self-starter four parameter model28.

Background fluorescence was subtracted from each sample and the untreated controls were normalized (100 % viability).. Samples were considered to be above the limit of detection and therefore categorized as resistant if the antibiotic, at its highest level, reduced viability by less than 20%. Similarly, the sample was considered below limit of detection and categorized susceptible, if the lowest concentration reduced viability by more than 80%. All antimicrobials were analysed separately. The effective concentration (EC) for each point in the curve from 1% reduction to 99% reduction in viability (1% increments) was calculated. The pearson’s correlation coefficients resulting from linearly regressing log transformed Etest MICs and log transformed ECx were extracted for each of the 99 possibilities (Figure S1). The following analysis were done, for each antimicrobial, using the ECmax that resulted in the maximal correlation. Hierarchical complete linkage clustering was used to compare the antimicrobials15,29. Pairwise t-test were used to test for differences in the Hill slope parameters across antibiotics.

The relationship between the EC50 and Etest was analysed using log-log regression. For each EC50 a corresponding Etest was predicted using the regression parameters, which are defined for unique antimicrobials. The analysis of the precision of this prediction needs to take in account the estimation error of the EC50 parameter in the dose response method. Bootstrapping was therefore used to compute appropriate confidence intervals. Bootstrapping consists in estimating the properties of an estimator by resampling, with replacement, from a set of observations. The key assumption is that the observed distribution of a given sample approximates the underling population distribution from which the sample is drawn. Therefore, correct CI are derived by resampling both the EC50 and the parameters or the log-log model.

The resampling of the EC50 is performed in two steps as for some observations the standard deviation in the dose response is relatively high, resulting in negative EC50. This leads to NAs when taking logs. Therefore, a first sample of size 1e5\*2 is drawn from a normal distribution with the EC50 and its standard deviation as parameters. Subsequently, after dropping the negative values, 1e5 observations are finally drawn with replacement. It should be noted that dropping the negative EC50 introduces a bias.

Firstly, the variance-covariance of the regression parameters was estimated.

Subsequently a sample of 1e50 combinations of linear regression parameters were drawn from a 2 dimensional normal distribution, using as parameters the variance-covariance matrix of the log-log regression. Combining the two yields a sample of 1e50 predicted Etest values, from which the 0.025 and 0.975 percentiles are computed to estimate 95% CI.

**Regression analysis of Etest MIC and ECmax**

The lfinear regression parameters

**Predictive modelling**

**Validation of the predicted MIC estimates**

Match the S, I, R classes to see sensitivity and specificity. Calculate the coefficient of variation between three independent experiments and the fold change deviation from Etest.

**Benchmarking the predicted MIC estimates**

**RESULTS**

**Time kill curve analysis**

The WHO panel contains well characterized strains of resistant and susceptible nature. Each strain was exposed for six hours to azithromycin, gentamicin, ciprofloxacin, ceftriaxone, cefixime, tetracycline, penicillin and spectinomycin. No visual turbidity was identified in the untreated controls, after adding resazurin a bright pink fluorescent colour developed. Wells containing the positive control or lethal concentrations of antimicrobials remained dark blue.

**Regression analysis**

Dose response curves were analysed for 124 *N. gonorrhoea*e strains in eight antimicrobials. The slopes of the pharmacodynamic functions (*k*) were compared across all samples. The median of this parameter gradually increased from ceftriaxone (1.1) to cefixime (1.3), tetracycline (1.95), penicillin G (2.1), azithromycin (2.1), spectinomycin (2.4) and was highest for gentamicin (3.1). A pairwise t-test showed that the differences between those groups were significant except for those with very similar means such as penicillin G and azithromycin. Furthermore, hierarchical clustering revealed that data can be grouped in three main clusters. The beta lactams ceftriaxone, cefixime and penicillin G can be distinguished from the other antimicrobials. Ciprofloxacin and gentamicin were found in the second cluster. Tetracycline and spectinomycin were similar and in the same group as azithromycin. No significant differences between resistant strains and susceptible strains were found.

A linear regression was fit for each effect level from EC1 to EC99 for azithromycin, ciprofloxacin, ceftriaxone, cefixime, tetracycline and penicillin G. The pearson’s correlation coefficient was calculate for each of these values (Figure S1). Meaningful linear regressions could not be fit for spectinomycin and gentamicin because the panel contained 94.4% and 100% susceptible strains respectively. Pearson correlation coefficients were highest for ciprofloxacin (0.98) at ECxx, tetracycline (0.88) at ECxx and for penicillin at ECxx (0.85). Lower pearson’s correlation coefficients were measured for ceftriaxone (0.77) at ECxx, azithromycin (0.73) at ECxx and cefixime (0.70) at ECxx. The slopes for these regressions were shallower for ceftriaxone, cefixime and penicillin compared to the other antimicrobials. The deviation from Etest expressed in doubling dilutions was –xxx and was lower for each of the antimicrobials. When slope and intercept of the regressions were used to predict the MIC equivalent after 24 hours none of the regressions

**Predicted Etest**

The hill function was used to estimate the potency of the antimicrobials in eight WHO reference strains and 80 clinical isolates. In resistant strains the curves were shifted towards higher concentrations, indicating increased potency (EC50). Some highly resistant strains did not follow a sigmoidal dose-response curve and therefore were considered above limit of detection. It was further assessed which effective concentration correlates optimal with the gold standard methods Etest and agar dilution. At EC25 the correlation with gold standard methods was optimal, indicated by the highest point in the polynomial model fit to the pearson’s correlation coefficient from EC1-EC99 (Figure2A). The intercept of the linear regression fitted to the log-log transformed data was 1.6 and the slope 0.58. The parameters were used to predict the MIC, which shifts the dataset left towards higher values more similar to the gold standard. The regression line through the predicted MIC values approximates an intercept of zero. The fold change deviation from conventional MICs is smaller than 1 (within a doubling dilution) for 77% of the cases (Figure 3). Only two outliers with a fold change deviation higher than five were identified, in both cases WHO N treated with gentamicin. The data were sorted according to the three classes, susceptible, intermediary and resistant (Table 1). The categories susceptible (107 strains) and resistant (53 strains) were classified correctly in all cases corresponding to a sensitivity CI of 93.15%-100.00% and a specificity of 96.61% to 100.00%. Eleven strains (of 22) were correctly classified as intermediary resistant in the conventional method, seven were misclassified as resistant and three as susceptible. Eight resistant and four susceptible strains were recognized as intermediary resistant with the new method. The coefficient of variation was calculated to estimate the variability between three independent (Figure 4). All coefficients of variation were below 1 (mean 0.3).

**DISCUSSION**

The EC and the slope (*k*) were the only parameters that varied freely, the data were normalized to the untreated controls and background corrected therefore the lower and the lower asymptote was approximately zero.

The resazurin assay was able to discriminate between resistant and susceptible strains reliably in an assay time considerably shorter than those of currently available MIC methods for *N. gonorrhoeae*. The gold standard methods Etest and agar dilution are both based on visual readouts and therefore are limited to a relatively low throughput. Both assays are based on doubling dilutions which for Etest often results in values below or above limit of detection. For agar dilution the range is more dynamic but has to be determined for each compound prior to the experiment. Employing a hill function allows to estimate the effective concentrations of antimicrobials from a continuous scale and therefore allows to calculate a precise estimate and confidence interval rather than having the precision limited by doubling dilutions. The variability between three independent experiments was 30% on average and the maximal deviation found 100%, magnitude seems to be comparable to those the gold standard methods.

Choosing 1:5 dilutions instead of doubling dilutions allows to cover a wider range of concentrations. However the viability of highly resistant strains is not fully reduced after exposure to antimicrobials which makes it impossible to fit a sigmoidal hill function in these cases. Avoiding visual readout and employ a standardized algorithm additionally reduces operator bias, which can be especially valuable in a setting that involves more than one laboratory. These properties, and the low price of resazurin powder, are especially valuable when screening large libraries of new compounds or antimicrobial combinations. Frequently the questions that needs to be answered is the potency of antimicrobials relative to each other rather than absolute numbers. While EC25 values are much lower than MIC values determined with the gold standard methods they correlate very well. This allows to transform the EC25 values to predict MIC concentrations and classify the results according to three categories, susceptible, intermediary resistant and resistant. Most notably all resistant strains have been recognized correctly by the predictor. The predictor we established should be validated using a large number of strains. Some of the intermediary resistant strains were classified wrong. This is not unexpected because the cutoff criteria were designed for Etest, based on doubling dilutions that were rounded up to the next higher dilution.

A systematic comparisons between different reference laboratories revealed that the intra laboratory reproducibility of all three methods is excellent but remains challenging between laboratories.

In summary the new assay shows an excellent performance in the selected quality control strains and antimicrobials. GW medium is a colourless and defined medium that can be used for classical MIC testing in liquid broth, in low resource settings without plate reader. Our resazurin based method reduces the assay time significantly and opens up avenues for larger scale experiments in basic research.

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