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METHOD ARTICLE

REVISED Construction and optimization of a 'NG Morbidostat' - An automated continuous-culture device for studying the pathways towards antibiotic resistance in *Neisseria gonorrhoeae* [version 2; peer review: 2 approved]

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Abstract

To obtain a detailed picture of the dynamics of antibiotic resistance development in *Neisseria gonorrhoeae*, we built a morbidostat according to the protocol of Toprak *et al.*, adjusted to the specific characteristics required for the growth of *N. gonorrhoeae*. In this article we describe the adaptations, specifications and the difficulties we encountered during the construction and optimization of the NG morbidostat. As a proof of concept, we conducted a morbidostat experiment by increasing concentrations of azithromycin in response to bacterial growth. We started the experiment with two *N. gonorrhoeae* reference strains WHO-F and WHO-X. These strains were grown in 12 mL GC Broth supplemented with IsoVitaleX™ (1%) and vancomycin, colistin, nystatin, trimethoprim (VCNT) selective supplement for 30 days in a 6% CO_2 environment at 36°C. Samples of the cultures were taken 2-3 times a week and minimal inhibitory concentrations (MICs) of azithromycin were determined using E-test. The initial MICs of WHO-F and WHO-X were 0.125 μg/mL and 0.25 μg/mL, respectively. In less than 30 days, we were able to induce high level azithromycin resistance in *N. gonorrhoeae*, with a 750 and 1000 fold increase in MIC for WHO-F and WHO-X, respectively.

Keywords

morbidostat, Neisseria gonorrhoeae, antibiotics, macrolide resistance, antimicrobial resistance, sexual transmitted infection

Open Peer Review

Reviewer Status ✓ ✓

	Invited Reviewers	
	1	**2**
version 2 (revision) 08 Jan 2020	✓ report	✓ report
version 1 26 Apr 2019	? report	? report

1. **William M. Shafer**, Emory University, Atlanta, USA

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Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the Antimicrobial Resistance collection.

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Author roles: Verhoeven E: Data Curation, Investigation, Software, Validation, Visualization, Writing – Original Draft Preparation; **Abdellati S**: Investigation, Methodology, Writing – Review & Editing; **Nys P**: Methodology, Resources; **Laumen J**: Writing – Review & Editing; **De Baetselier I**: Supervision, Writing – Review & Editing; **Crucitti T**: Conceptualization, Methodology, Project Administration, Supervision, Writing – Review & Editing; **Kenyon C**: Conceptualization, Funding Acquisition, Methodology, Project Administration, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Introduction

Gonorrhoea is a sexual transmitted infection (STI) caused by the obligate human pathogen *Neisseria gonorrhoeae* (gonococcus), a Gram-negative diplococcus[1,2]. Transmission of the gonococcus occurs primarily by direct contact between the mucosal membranes in the urogenital tract, anal canal and oropharynx, usually during sexual activity[2].

Gonorrhoea is one of the most commonly reported STIs[3]. The World Health Organization (WHO) estimated an incidence of 87 million new cases for gonorrhoea (individuals aged 15–49 years) in 2016, worldwide[4]. An increase in cases of gonorrhoea has been reported in several European countries: 11% rise from 2014 to 2015 in the United Kingdom[5] and a doubling of cases in 2013 and 2015 among men who have sex with men (MSM) in France[6].

There is a growing global concern about antimicrobial resistance in *N. gonorrhoeae*, with resistance reported to almost all antimicrobials previously and currently available for treatment[7]. In response to this concern, the Centers for Disease Control and Prevention (CDC) and the European guidelines for treatment of gonorrhoea introduced dual antimicrobial therapy with azithromycin (oral) and ceftriaxone (injectable) for uncomplicated gonorrhoea in 2012[2,8]. Dual therapy was also recommended by WHO in 2016[9]. However, the prevalence of ceftriaxone and azithromycin-resistant strains is increasing in certain areas[10,11].

Therefore, more research is required to investigate how *N. gonorrhoeae* develops antibiotic resistance so rapidly. A better understanding of the pathways to resistance may enable novel strategies to prevent the emergence of resistance in the future[12]. In order to evaluate the dynamics of resistance development, Toprak *et al.*[13] developed a microbial culture device called a 'morbidostat'. A morbidostat is a bioreactor that continuously monitors bacterial growth and adjusts antibiotic concentration to induce bacterial resistance against the drug[13,14]. Experiments using a morbidostat can give us insights into the evolution of resistance and the nature, order and speed at which mutations arise. Whole-genome sequencing can be used to characterize the sequential mutation steps in the resistance genesis in great detail[14,15].

The aim of this study was to build a morbidostat according to the protocol of Toprak *et al.*[13]. We report here on the construction, optimization and a proof of concept of the NG morbidostat, a morbidostat adjusted to the specific characteristics required for the growth of *N. gonorrhoeae*.

Methods

Principle of the morbidostat

The morbidostat measures growth rates based on the intensity of back-scattered light through a bacterial suspension using an optical detection system. The bacteria are suspended in a fixed volume of liquid medium with continuous stirring. At fixed time intervals, the culture is diluted with a fixed volume. Depending on turbidity measurements and growth rate, an algorithm defines dilution with fresh medium or fresh medium containing antibiotic. Fresh medium with antibiotic is injected if the turbidity exceeds a threshold and when the net growth rate of the bacteria is positive. This threshold is set in the mid-log phase of the growth curve. At this point, there is a high metabolic activity present in the bacteria and the population is not nutrient limited. To allow *N. gonorrhoeae* to adapt to the environment, a threshold for fresh medium is set as well. Over time, the concentration of antibiotic added to these vials increases and drug resistance in bacteria evolves. During the entire experiment, the volume in the morbidostat culture vials is kept constant using a 16-channel suction pump (Ismatec, ISM938D). Morbidostat experiments are continued until a diminishing rate of increase in drug resistance is observed[13,14].

Construction of the morbidostat

The NG morbidostat consisted of two parts: one part containing 15 independent culture vials was built within a CO_2 incubator (Figure 1), the other part, outside the incubator, contained the suction pumps and controlling equipment (Figure 2).

We modified Toprak's protocol[13] and included one drug pump for each culture vial instead of two. This resulted in 30 peristaltic pumps: 15 medium pumps and 15 drug pumps for a capacity of

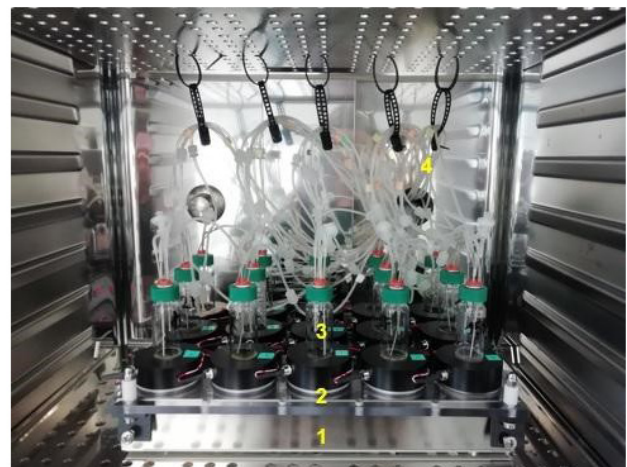**Figure 1. Neisseria gonorrhoeae morbidostat set-up inside the CO_2 incubator.** (1) the magnetic stirrer plate with 15 positions. Upon this, (2) the vial holder array with the optical detection system placed upon the magnetic stirrer plate. (3) morbidostat culture vials that are placed and connected with silicone tubes going through a hole (4) at the back of the incubator to the peristaltic pumps outside the incubator.
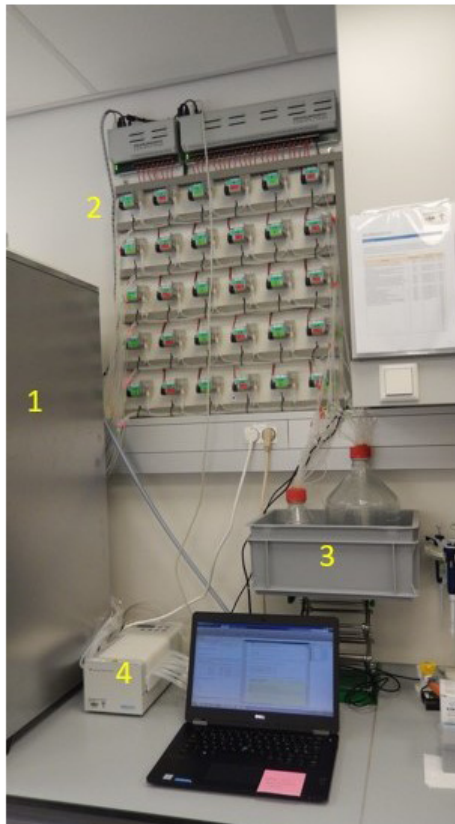
Figure 2. (1) CO$_2$ incubator with the culture vials and the vial holder array as shown in Figure 1. Silicone tubes connected to the peristaltic pumps, which are all placed against a plate fixed to the wall (2), (3) reservoirs bottles for plain media and media with antibiotic. (4) The 16-channel suction pump for waste.

15 morbidostat culture vials. Consequently, we manually increased the drug concentrations by changing the drug reservoirs after a period of time, usually every three days. This modification made the morbidostat substantially cheaper.

The construction of the morbidostat proceeded in three steps. Firstly, the construction of the morbidostat culture vials. Secondly, the construction of the vial holder array and its optical detection system. These two parts were placed on a magnetic stirrer in an incubator, providing the growing conditions of *N. gonorrhoeae*: an atmosphere of 6% CO$_2$ and a temperature of 36°C. Finally, each part was connected with silicone tubes and computer controlled peristaltic pumps for liquid transfer[13].

(i) Morbidostat culture vials. The morbidostat culture vial was a flat-bottom glass vial with a volume of 40 mL (Chemglass, CG-4902-08). It was closed at the top by an open-top GPI cap (Chemglass, CV-3750-0024) containing a Teflon insert with four holes (Euro-scientific). These holes were used for liquid injections (medium and antibiotics), waste extraction and for filtered air intake. Therefore, four pieces of PolyEtherEtherKetone (PEEK) tubing (BGB, 211609-25) were placed through the holes of the Teflon insert[16]. The PEEK tubing ends were connected to high temperature-resistant silicone tubing (Carl

Roth, 9556.1) on the outside of the culture vial. Different parts of silicone tubing were connected using Luer connectors (male and female) (Nordson Medical, MTLL004-6005 and FTLL004-6005). Each culture vial contained a magnetic stirrer bar (Carl Roth, PK75.1), to maintain a constant stirring of the culture. All materials used for assembling, were temperature-resistant and autoclavable[13].

(ii) Vial holder array and its optical detection system. When the morbidostat was in operation mode, the morbidostat culture vials were placed in the holder array, a figure of the vial holder array with all its dimensions is shown in Figure 3. In the tube holder, infrared (IR) light emitting diodes (LEDs) (Velleman, L-7113E3BT) and photodetectors (Velleman, L-7113P3C) were machined in a black Delrin material by two openings, positioned at an angle of 135° to maximize scattered light detection. The LEDs and photodetectors were the optical detection system, and measured the optical density or turbidity in the morbidostat culture vials at set time intervals. The LEDs were connected to a relay-interface device, that could switch ON/OFF to a voltage of approximately 6V. The photo-detectors were connected with a data acquisition device (DAQ) card (Measurement Computing, USB-1616FS). This DAQ card recorded the analog voltage readings across the photo-detectors and sent a digital signal to the computer. The vial holder array sat on top of the 15-position magnetic stirrer (Carl Roth, EHY9.1) that ensured continuously stirring at a speed of 200 rotations per minute (rpm) in each culture vial[13].

(iii) Assembling of the controlled peristaltic pump array. The last part of the construction was the connection of silicone tubes and computer controlled peristaltic pumps for liquid transfer to/from the morbidostat culture vials. All different parts with silicone tubes were connected to each other using Luer connectors. These silicone tubes exited the culture vials via the incubator (Memmert, ICO150med) through a hole at the backside. The 30 peristaltic pumps and the 16-channel suction pump were controlled with a relay interface device (Measurement Computing, USB-ERB08 and USB-ERB24), that is connected to a computer. The pumps were computer controlled using an algorithm coded in MATLAB software (MATLAB R2015b). The control software for the NG morbidostat is available from GitHub (see Software availability). The whole code was based on the code Toprak *et al.* used[13]. The code '*ExperimentController.m*' was the main class file, which ran the whole experiment by controlling '*PumpController.m*', '*DataReceiver.m*' and '*DataMonitor.m*' in parallel. '*PumpController.m*' controlled the 30 peristaltic pumps and the 16-channel suction pump. The file '*DataReceiver.m*' contained the code that read the voltage measurements by the photo-detectors. It converted the voltage readings automatically to turbidity measurements, depending on the calibration parameters. The file '*DataMonitor.m*' monitored the real-time data to the user while the experiment was running. Algorithm parameters like dilution time, growth time and mixing time could be set for the experiment. The 16-channel peristaltic pump was used to remove the waste/excess volume in the morbidostat culture vials.

To make sure the whole system was sterile, all tubing was autoclaved at 121°C for 21 minutes followed by a washing cycle
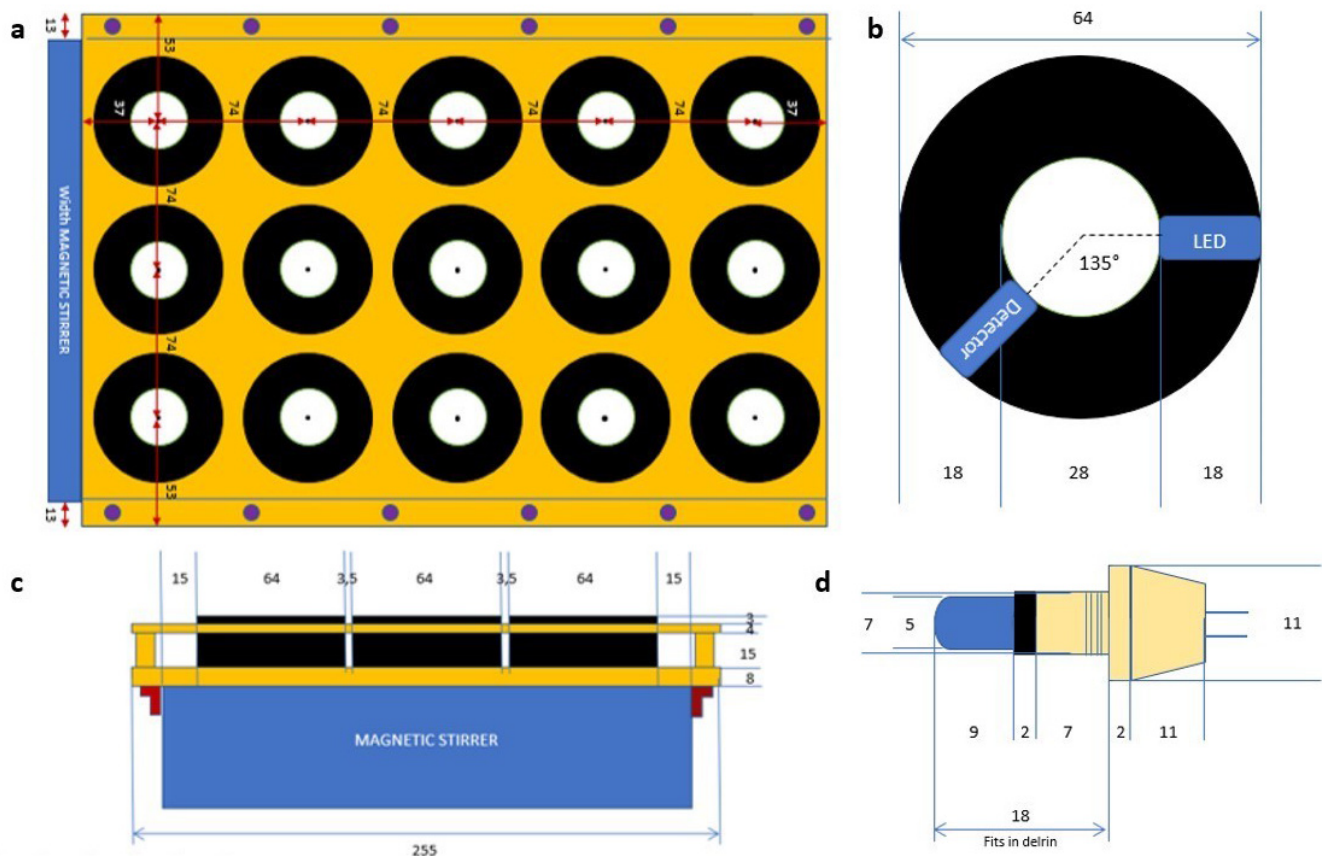
Figure 3. Dimensions of the vial holder array. (**a**) Top view of the vial holder array sitting on the magnetic stirrer plate, (**b**) Top view of the Delrin ring with the light emitting diode (LED) and detector, (**c**) Side view of the vial holder array which sits on the magnetic stirrer plate and (**d**) the dimensions of the LED/detector used.

with bleach (7%), sterile water, ethanol (70%), sterile water and growth medium, respectively, before use[13].

Proof-of-concept

We used an inoculation volume of 10 μL from a *N. gonorrhoeae* bacterial suspension (WHO-F and WHO-X) of 4.0 McF in 12 mL GC Broth supplemented with 1% IsoVitaleX (BD BBL™) enrichment for each morbidostat culture vial. All culture vials were autoclaved at 121°C for 20 minutes before use. *N. gonorrhoeae* grew in the morbidostat in cycles of 21 minutes and after each cycle, depending on turbidity measurements and growth rate, an algorithm in the software diluted the suspension with 1 mL fresh medium or with 1 mL fresh medium containing antibiotics. We set 1.3 McF as a threshold for addition of fresh medium, to allow *N. gonorrhoeae* to adapt to the environment without being diluted. Fresh medium with antibiotic was injected when a threshold of 2.0 McF was exceeded and the net growth was positive, otherwise fresh medium was injected. The algorithm used, is shown in Figure 4. After 800 seconds, a 16-channel suction pump removed the excess liquid. We took samples every 2–3 days by disconnecting the culture vials and swiping an inoculating loop through the suspension. Samples were then grown on blood agar plates and after approximately 24 hours, we stored these cultures from the blood agar plates into skim

milk at -80°C[14]. Azithromycin susceptibility testing was performed using E-tests (Biomerieux). E-tests were performed on GC-agar plates and ATCC strain 49226 and strain WHO-X were used for quality control. In Figure 5 the influence of bacterial growth when adding antibiotic is shown.

Results & discussion
Optimization

N. gonorrhoeae is very sensitive to environmental changes and requires a nutrient rich growth medium, we encountered a few issues during the optimization of our NG morbidostat.

i. Temperature optimization. After the incubator was set and validated on a temperature of 36.5°C, we were not able to maintain proper growth at each position of the morbidostat. A particularly problematic position was the middle part of the vial holder. Temperature measurements using temperature probes inside the morbidostat culture vials, revealed temperatures ranging from 38°C to 41°C, which is too hot for optimal growth of *N. gonorrhoeae*. To solve this problem, we had to remove the upper plexi plate of the vial holder to maintain a homogenous and adequate temperature distribution between the culture vials. In addition, we changed the setpoint temperature in the incubator itself to 35°C, as the motor of the magnetic stirrer plate
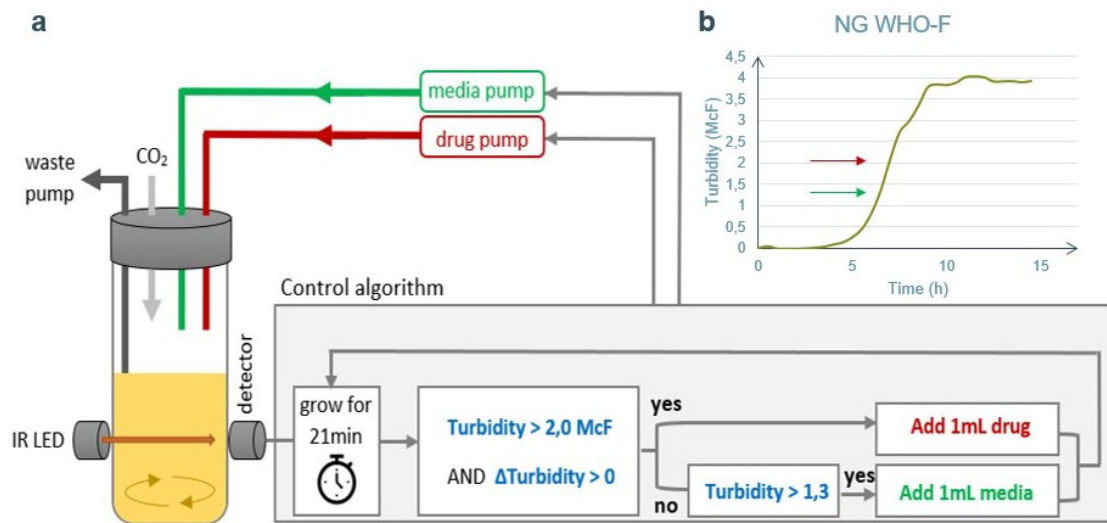
Figure 4. (**a**) Control algorithm used in the NG morbidostat. Thresholds used in this algorithm were based on values obtained from the (**b**) growth curve of *N. gonorrhoeae,* green and red arrows at the mid-log phase correspond with the thresholds for medium and antibiotic injections, respectively.
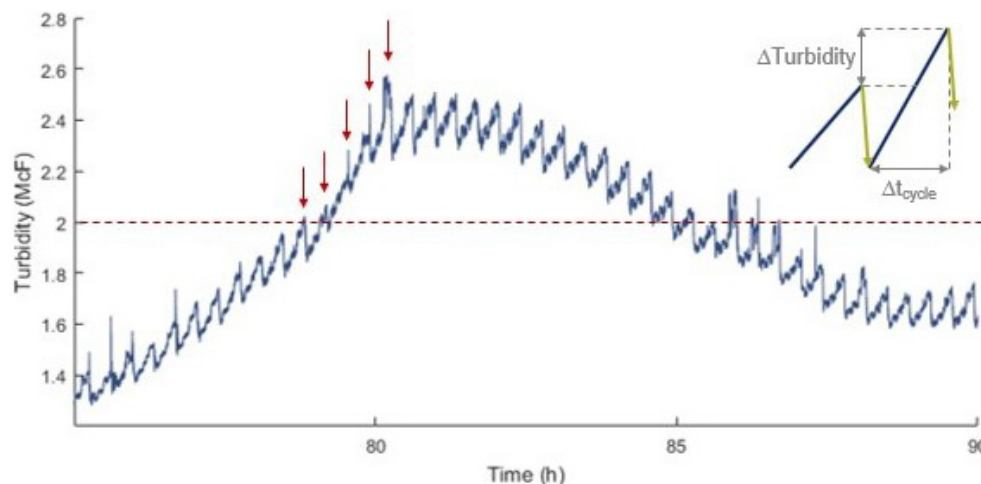

Figure 5. Representative bacterial growth in the NG morbidostat. Red arrows indicate where antibiotics were added to the culture. Every small peak in this curve corresponds to a cycle of 21 minutes.

generates heat in its direct environment and *N. gonorrhoeae* is very sensitive to these environmental changes. With these adjustments, temperatures measured in the morbidostat culture vials were in the right temperature range (approximately 36°C) for all 15 vials.

ii. Contamination. Compared to previous morbidostat experiments described in literature[11,13,15,17], where a minimal growth medium is used, *N. gonorrhoeae* requires a nutrient rich growth medium (GC Broth) which in its turn increases the risk of contamination.

The most common contaminant of our culture media were *Bacillus* species. This rapidly growing contaminant resulted in two major problems. First, the nutrients available in the medium

were used by these contaminants thus insufficient for the gonococcus growing in the culture vials. Second, when we used 0.2 μm filters between (a) the media reservoir and the peristaltic pump and (b) the peristaltic pump and the morbidostat culture vial, filters were clogged very rapidly. As a consequence (a) the peristaltic pumps could no longer draw the medium into the system or (b) pressure overload occurred in the tubing between the peristaltic pump and the morbidostat culture vial, causing a disconnection between the peristaltic pumps and the tubing. Therefore, we do not recommend to work with these filters when there is high risk of contamination in the culture medium. Our next step was to limit contamination by placing the medium reservoir in a UV-light-box, but in less than 24 hours contamination reoccurred. Ultimately the only way we found to prevent this contamination was to add a vancomycin,

colistin, nystatin and trimethoprim selective supplement (VCNT) to the growth medium. VCNT inhibits the growth of most other micro-organisms excluding *N. gonorrhoeae*[18]. This step solved the contamination problem.

Proof of concept – *in vitro* evolution of resistance to azithromycin

As proof of concept, we used the NG morbidostat to generate resistance to azithromycin. In this pilot experiment we used single vials with two strains of *N. gonorrhoeae* (WHO-F and WHO-X) exposed to increasing concentrations of azithromycin. We used the same two strains of *N. gonorrhoeae* contemporaneously exposed to growth medium only, as controls.

We observed the highest bactericidal rate soon after the first azithromycin exposure. After this initial exposure, the population needed a longer time to return to their log phase than later on in the experiment. Figure 6 shows a growth curve of the population during the experiment.

The initial minimal inhibitory concentrations (MICs) of WHO-F and WHO-X were 0.125 µg/mL and 0.25 µg/mL respectively. In the first week, the MICs of WHO-F and WHO-X increased approximately 24-fold and 48-fold, respectively. By the end of the experiment (30 days), the MICs of WHO-F and WHO-X had increased more than 750-fold and 1000-fold, respectively (Table 1; Figure 7).
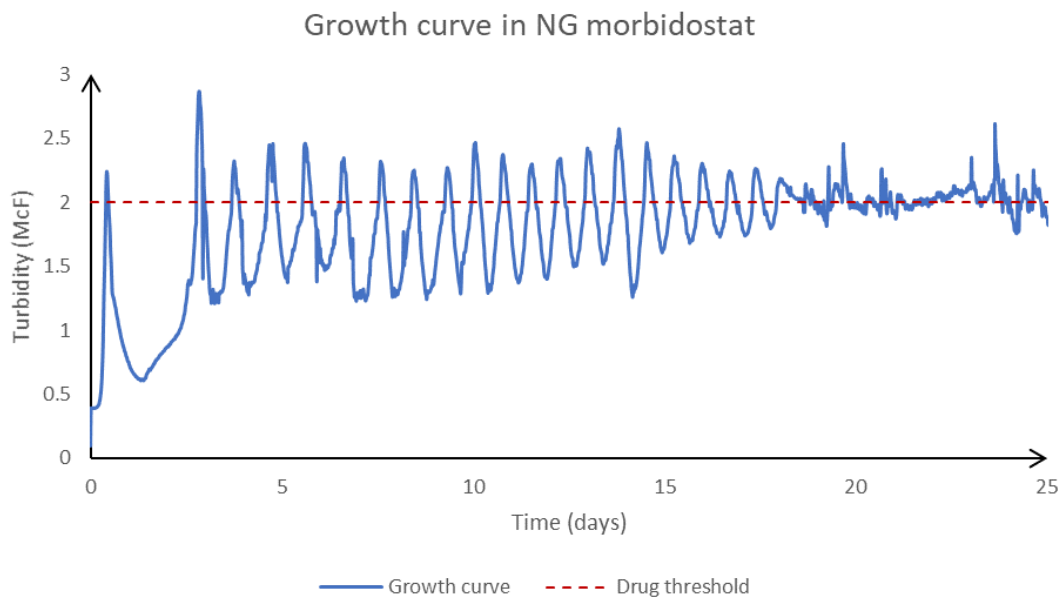

Figure 6. Growth curve of the population during a morbidostat experiment.

Table 1. Minimum inhibitory concentrations (MICs) measurements in morbidostat over time, using E-test.

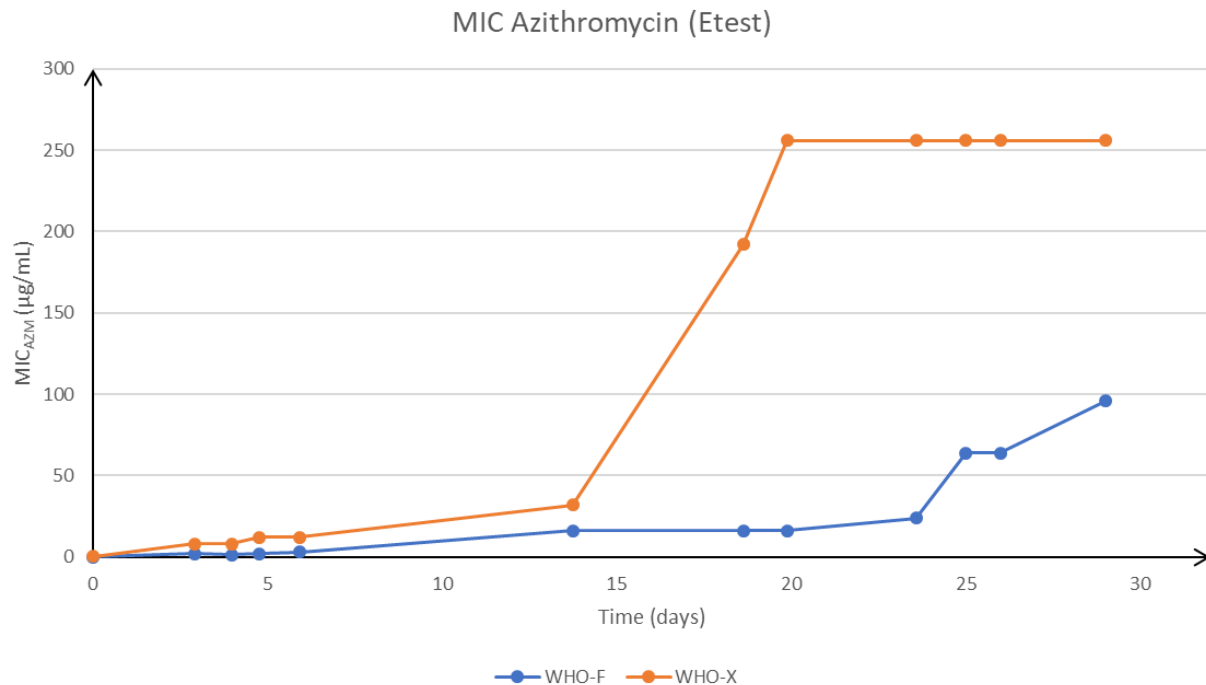| time (days)	MIC$_{AZM}$ (µg/mL)	
	WHO-F (v1)	WHO-X (v6)
0	0.125	0.25
3	2	8
4	1.5	8
5	2	12
6	3	12
14	16	32
19	16	192
20	16	>256
24	24	>256
25	64	>256
26	64	>256
29	96	>256

MIC Azithromycin (Etest)

Figure 7. Evolution in azithromycin-resistance in morbidostat of Neisseria gonorrhoeae strain WHO-F and WHO-X over time using E-test.

Conclusion

In conclusion, we described how to adapt the morbidostat device developed by Toprak *et al.*[13] to a version that is able to maintain *N. gonorrhoeae* growth under constant antibiotic pressure. The key adaptations involved building the morbidostat within a CO_2 incubator, fine-tuning the positioning of the vial holder array and the use of VCNT. This enabled us to induce high level azithromycin resistance in *N. gonorrhoeae* within 30 days. We plan to repeat these experiments for azithromycin and ceftriaxone in triplicate and perform whole-genome sequencing on those samples to characterize the sequential mutation steps in the resistance genesis. In future experiments we also plan to use the NG Morbidostat to evaluate how different antimicrobial combinations (including antiseptic products[19] and bacteriophages) may be used to prevent the emergence of antimicrobial resistance in *N. gonorrhoeae*.

Data availability

Underlying data

Figshare: data_proof_of_concept. https://dx.doi.org/10.6084/m9.figshare.7987169[20]

This project contains the following underlying data:

- data_proof_of_concept_experiment.xlsx (Proof of concept – *in vitro* evolution of resistance to azithromycin underlying data)

- raw_data_figure5.xlsx (Data underlying Figure 5)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Software availability

Source code: https://github.com/everhoeven/NG_Morbidostat

Archived source code: http://doi.org/10.5281/zenodo.2643437[21]

Licence: MIT

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Open Peer Review

Current Peer Review Status: ✓ ✓

Version 2

Reviewer Report 09 April 2020

https://doi.org/10.5256/f1000research.23861.r58407

✓ **Olusegun Soge**

Neisseria Reference Laboratory, Departments of Global Health and Medicine, University of Washington, Seattle, WA, USA

The authors have satisfactorily addressed my review comments. I recommend acceptance of the paper.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antimicrobial resistance in Neisseria gonorrhoeae

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 January 2020

https://doi.org/10.5256/f1000research.23861.r58408

✓ **William M. Shafer**

Department of Microbiology and Immunology, Emory University, Atlanta, GA, USA

I am satisfied with the responses and revisions and recommend acceptance.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of research deals with antibiotic resistance and Neisseria

gonorrhoeae.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 01 August 2019

?

Olusegun Soge
Neisseria Reference Laboratory, Departments of Global Health and Medicine, University of Washington, Seattle, WA, USA

This interesting manuscript describes the construction and optimization of the 'NG Morbidostat' for *in vitro* selection of antimicrobial resistance in *Neisseria gonorrhoeae*. The NG Morbidostat would be valuable for real-time investigation of the evolution of antimicrobial resistance in *N. gonorrhoeae*. I have a few comments for the authors to consider.

1. What is the rationale for selecting the two WHO strains used for the proof of concept experiment?

2. Did the authors perform species confirmatory tests on the presumptive *N. gonorrhoeae* colonies cultured over the period of the experiment (30 days)?

3. Please mention the media and QC strains used for Etest.

4. I would recommend performing agar dilution antimicrobial susceptibility testing with a standard panel of antimicrobials to confirm that there was no cross-resistance to other antimicrobials.

5. It has been described previously that high-level azithromycin resistance can develop rapidly in the laboratory in *N. gonorrhoeae* isolates with a single mutated 23S rRNA allele.[1] What are the genetic and phenotypic characteristics of the two WHO strains? Could the presence of multiple resistance-conferring mutations, including a single-base-pair deletion within a 13-bp inverted-repeat sequence in the *mtrR* promoter of WHO X[2] have contributed to the higher azithromycin MICs obtained when compared to WHO F?

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Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antimicrobial resistance in Neisseria gonorrhoeae

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Sep 2019
Jolein Laumen, Institute of Tropical Medicine, Antwerp, Belgium

We would like to thank the reviewers for their valuable comments. Please find our responses below:

1.What is the rationale for selecting the two WHO strains used for the proof of concept experiment?
For the proof of concept experiment we chose WHO-F because this strain is susceptible to the major relevant anti-gonococcal antibiotics (penicillin G, tetracycline, ciprofloxacin, azithromycin, cefixime, ceftriaxone and spectinomycin)[1]. It thus enables us to study the evolution of AMR from a wildtype strain. The WHO-X strain was chosen because it is already resistant to multiple antibiotics, including penicillin G, tetracycline, ciprofloxacin, ceftriaxone

(MIC 2) and cefixime (MIC 4)[1]. WHO-X also has the 13 bp inverted repeat in its mtrR promoter and has a slightly elevated azithromycin MIC (0.25). These features may enhance the development of resistance to azithromycin faster or via a different pathway.

2.Did the authors perform species confirmatory tests on the presumptive *N. gonorrhoeae* colonies cultured over the period of the experiment (30 days)?
The presumptive *N. gonorrhoeae*colonies of the proof of concept experiment were not confirmed by additional testing. However, in a soon to be published detailed follow-up experiment we performed whole genome sequencing of the obtained samples that confirmed the identity of the isolates as *N. gonorrhoeae.*

3.Please mention the media and QC strains used for Etest.
E-tests were performed on GC-agar plates and ATCC strain 49226 and strain WHO-X were used for quality control.

4.I would recommend performing agar dilution antimicrobial susceptibility testing with a standard panel of antimicrobials to confirm that there was no cross-resistance to other antimicrobials.
Thank you for this useful comment. We have just finished a more detailed follow-up experiment wherein we will report the antimicrobial susceptibility of different antimicrobials (tested via agar dilution).

5.It has been described previously that high-level azithromycin resistance can develop rapidly in the laboratory in *N. gonorrhoeae* isolates with a single mutated 23S rRNA allele.[1]What are the genetic and phenotypic characteristics of the two WHO strains? Could the presence of multiple resistance-conferring mutations, including a single-base-pair deletion within a 13-bp inverted-repeat sequence in the *mtrR* promoter of WHO X[2] have contributed to the higher azithromycin MICs obtained when compared to WHO F?
The WHO-F strain has a wild type genetic profile whereas the WHO-X strain contains various mutants, including the 13-bp inverted repeat sequence in the *mtrR*promoter. The WHO-X strain does not have any mutations in the 23S rRNA gene. In our more detailed follow-up experiment we saw that the WHO-F strain also reached a MIC of >256 (determined by E-test). However, as in the current paper, the WHO-X strain developed high level resistance faster than WHO-F. This faster acquisition could be due to the genetic profile of WHO-X. Whole genome sequence analysis of this follow-up experiment will be described in a subsequent paper.

References
1. Unemo M, Golparian D, Sánchez-Busó L, Grad Y, Jacobsson S, Ohnishi M, Lahra MM, Limnios A, Sikora AE, Wi T, Harris SR. The novel 2016 WHO Neisseria gonorrhoeae reference strains for global quality assurance of laboratory investigations: phenotypic, genetic and reference genome characterization. Journal of Antimicrobial Chemotherapy. 2016 Jul 17;71(11):3096-108.

Reviewer Report 25 June 2019

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? **William M. Shafer**

Department of Microbiology and Immunology, Emory University, Atlanta, GA, USA

This manuscript is an important contribution to the field of antibiotic resistance development in *Neisseria gonorrhoeae*. The "NG Morbidostat" detailed by the authors has many advantages for studies on evolution of antibiotic resistance in culture. The construction and use of the system is well described. I have a few technical questions that require the attention of the authors:

1. Please indicate the colony type of the two strains employed (state of piliation and opacity).

2. For azithromycin, Etest strips can give variable results so it is important to verify the deduced MIC by the more accepted agar dilution method. Also, was the Etest assay performed on outgrowth of single colonies or the batch culture? Did the authors check single colonies?

3. It is not clear as to why blood agar plates were employed as opposed to the more conventional GC agar?

4. It would seem that the progression in azithromycin MICs represented initial mutations in the mtr locus followed by 23S rRNA genes, This could be checked quickly by PCR/sequencing.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of research deals with antibiotic resistance and Neisseria gonorrhoeae.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Sep 2019

Jolein Laumen, Institute of Tropical Medicine, Antwerp, Belgium

We would like to thank the reviewers for their valuable comments. Please find our responses below:

1.Please indicate the colony type of the two strains employed (state of piliation and opacity).
This article describes the construction and optimization of the NG morbidostat. Its primary goal is to share how we constructed/optimized the morbidostat, and in particular how we overcame stumbling-blocks along the way. We thought this would be a useful contribution to the field as it may help other groups who may wish to construct in-vitro models to test various theories about gonococcal AMR selection. The article also includes a proof of concept experiment. We have recently completed larger scale experiments where we have conducted the various steps suggested by the reviewers such as describing the colony type, confirming MIC results with agar dilution and performed whole genome sequencing of the isolates every 3 to 5 days. These results will be described in a forthcoming paper.

2.For azithromycin, Etest strips can give variable results so it is important to verify the deduced MIC by the more accepted agar dilution method. Also, was the Etest assay performed on outgrowth of single colonies or the batch culture? Did the authors check single colonies?
Because this was a proof of concept experiment, we performed E-tests on the batch culture. We have previously compared the results of azithromycin E-tests with agar dilution in our laboratory and found them to be comparable, as was also found by others (Liu H, Taylor TH, Pettus K, Trees D. Assessment of Etest as an alternative to agar dilution for antimicrobial susceptibility testing of Neisseria gonorrhoeae. Journal of clinical microbiology. 2014 May 1;52(5):1435-40).

3.It is not clear as to why blood agar plates were employed as opposed to the more conventional GC agar?

We used blood agar plates instead of GC agar plates because this allowed us to check for a broader spectrum of contaminants.

4.**It would seem that the progression in azithromycin MICs represented initial mutations in the mtr locus followed by 23S rRNA genes. This could be checked quickly by PCR/sequencing.**
The progression in azithromycin MIC indeed suggests initial mutations in the mtr locus followed by 23S rRNA genes. We are currently analyzing the whole genome sequencing data from our larger follow-up experiment and will then be able to provide this information in a subsequent paper.

Competing Interests: No competing interests were disclosed.

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