

Methods for nitrofurantoin susceptibility testing

Diverse Genetic Determinants of Nitrofurantoin Resistance in UK *Escherichia coli*

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Table of contents

1. Nitrofurantoin Etests	2
Materials	2
Procedure	2
2. Isolating subpopulations of heterogeneous <i>E. coli</i> samples.....	4
Materials	4
Procedure	4
References.....	6

1. Nitrofurantoin Etests

This experiment aimed to validate predictions of nitrofurantoin susceptibility and explore effects of novel mutations in *ribE*.

Materials

- 30 *E. coli* isolates or strains (Table 4 in the manuscript):
 - 27 test isolates from the HPRU collection plus three control isolates or strains.
 - Each isolate or strain was kept as single-colony stock and saved in a -80°C freezer.
- 30 MIC test strips: [Liofilchem](#)[®] (product code: 92022) NITROFURANTOIN F 0.032-512.
- Shiranee Lab:
 - Mueller-Hinton (MH) broth 2, cation adjusted (Sigma-Aldrich[®], [90922-500G](#))
 - 29 MH agar plates (Thermo Fisher Scientific[™], [PO0152A](#))
- Charing Cross Hospital: 61 MH agar plates (Thermo Fisher Scientific[™], [PO0152A](#))
 - 30 plates for MIC testing.
 - 30 plates for counting inoculum CFUs (quality check).
 - One plate for growing control strain ATCC25922 from stock saved under -80°C.

Procedure

Day 1
<p>Growing EC1069B in MH broth 2 (Hammersmith Campus, afternoon)</p> <ol style="list-style-type: none">1. Prepared fresh 10 mL MH broth 2 of pH 7.3 ± 0.1<ol style="list-style-type: none">a. Rinsed the glass bottle with distilled water to remove any detergent before adding the brothb. Sent the broth to autoclave2. Took stock of isolate EC1069B out of the -80°C freezer3. Transferred one or more beads from the cryovial to a 50-mL Falcon tube containing 7.5 mL broth.4. Incubated the tube aerobically at 37°C with gentle shaking overnight (>16 h). <p>Note: Since EC1069B showed lower viability than other isolates, it was inoculated to broth rather than agar in an attempt to revive it from our frozen stock.</p>
Day 2
<p>Growing isolate IN09 and 28 HPRU <i>E. coli</i> isolates on MH agar (Hammersmith Campus, morning):</p> <ol style="list-style-type: none">1. Checked growth of isolate EC1069B in the Falcon tube.2. Assuming EC1069B grows, then streaked out the broth on an MH plate.3. Saved rest of stock of EC1069B on beads and kept them frozen under -80°C.4. Prepared 27 MH agar plates5. Took stock of the other 27 isolates out of the -80°C freezer6. Took one bead from each cryovial and streaked out its bacteria on a plate.7. Inverted these plates and incubated them aerobically overnight at 37°C.

Growing control strain ATCC25922 on MH agar (Charing Cross Hospital, afternoon):

1. Took one MH agar plate
2. Took stock of strain ATCC25922 out of the -80°C freezer
3. Streaked out the strain from one bead on an MH agar plate
4. Inverted the plate and incubated them aerobically at 37°C overnight.

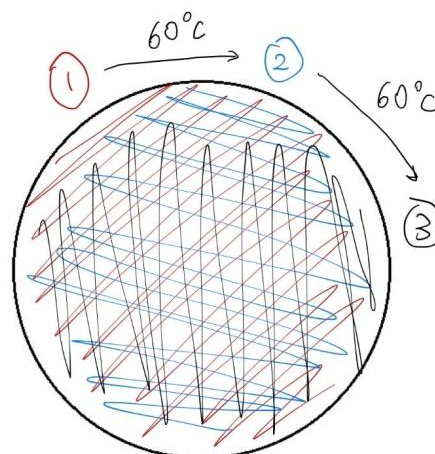
Day 3

Sending the 29 agar plates to Charing Cross Hospital (Hammersmith Campus, morning)

1. Checked bacterial growth on the plates
2. Wrapped and sealed each plate with two pieces of paraffin papers
3. Sent plates to Charing Cross Hospital

NIT strip tests (Charing Cross Hospital, afternoon)

1. Received the 29 plates from Hammersmith.
2. Checked conditions of all the 30 plates (including the plate of control strain ATCC25922).
3. Prepared 60 MH agar plates.
4. Made an inoculum from a sweep of 3–4 well separated colonies of each strain.
 - a. Suspended the sweep in 0.85% sterile saline.
 - b. Adjusted turbidity to 0.5 McFarland.
5. Inoculated each adjusted inoculum onto an MH agar plate for MIC testing.
 - a. Soaked a sterile swab in bacterial suspension.
 - b. Spread the suspension on the agar plate evenly via streaking *three times* per plate, with two rotations of the plate by approximately 60 degrees each time, as illustrated in the picture.
 - c. Applied the nitrofurantoin MIC strip to the agar surface, with the scale facing upwards. Pressed the strip with a sterile disposable forceps on the agar surface to ensure the whole strip was in complete contact with the agar surface.
 - d. Inverted plates after they are properly dried and incubate them under 35°C for 18 hours.
6. Plated out 10 µL of each inoculum for counting its CFU as a measure of quality control.
 - a. Inverted plates after they were properly dried and incubated them under 35°C overnight.



Day 4

Reading MICs and inoculum densities (Charing Cross Hospital)

1. Read inoculum density from each plate for CFU counting. (Desirable: $1-2 \times 10^8$ CFU/mL)
2. Read the nitrofurantoin MIC from each plate
 - a. The MIC was read at where the relevant inhibition ellipse intersects the strip, as per product instructions and reading guide for bactericidal antimicrobials (1–3).

- b. Since nitrofurantoin is a bactericidal antimicrobial, the MIC should be read at the point of complete inhibition of all growth. Haze and microcolonies or microcolonies within 3 mm from the strip should be read as growth.
- c. MICs were rounded to the conventional two-fold series of concentration increments (2, 4, 8, ... mg/L).
- d. Took a photo of each plate for review.

2. Isolating subpopulations of heterogeneous *E. coli* samples

This experiment aimed to confirm and obtain subpopulations of *E. coli* isolates EC394_9, EC0026B, and EC0880B that resulted in heterogeneous susceptibility to nitrofurantoin in the Etests. Materials were prepared and steps were carried out as per *EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing Version 8.0*.

Materials

- Isolates EC394_9, EC0026B, and EC0880B from the HPRU collection, isolate IN09 (positive control), and strain ATCC25922 (negative control).
 - Each isolate or strain was kept as single-colony glycerol stock and saved in a -80°C freezer.
- Thermo Fisher Scientific™ Oxoid™ nitrofurantoin discs (catalogue number: [CT0034B](#), 100 µg nitrofurantoin per disc).
- Sigma-Aldrich® Mueller-Hinton (MH) broth 2, cation adjusted (product code: [90922-500G](#)).
- Sterile MH 2 agar plates (90-mm diameter) prepared in the lab.
- McFarland turbidity standard
- Sterile 0.85% saline

Procedure

Day 1
Growing bacteria in MH broth 2 <ol style="list-style-type: none"> 5. Retrieved stock of bacteria from the -80 °C freezer and placed cryovials in ice. 6. Transferred more than 250 µL stock from each cryovial to a 50-mL Falcon tube containing 7.5 mL MH broth 2. 7. Incubated inoculated broth aerobically at 35 °C with 225-RPM (revolutions per minute) shaking for 18 hours.
Day 2
Disc-diffusion tests for confirming heterogeneous nitrofurantoin susceptibility <ol style="list-style-type: none"> 1. For each isolate or strain, transferred 150 µL broth culture into a 13-mL Falcon tube. 2. Prepared an inoculum of each isolate or strain by gradually diluting its stock with saline till the turbidity reaches 0.5 McFarland standard.

3. Inoculated two plates for each of EC394_9, EC0026B, and EC0880B and one plate for each control*.
 - a. Evenly spread the inoculum in three directions (rotated the plate by 60° each time) on the plate with a sterile cotton swab
 - b. Dried the plate for 1 minute and orthogonally applied four nitrofurantoin discs per plate for EC394_9, EC0026B, and EC0880B or one disc per plate of controls (IN09 and ATCC25922).
4. Incubated plates at 35 °C for 18–20 hours.
5. For each of EC394_9, EC0026B, and EC0880B, mixed 1 mL broth culture with 1 mL 100% glycerol and saved the mixture in the -80 °C freezer.

* More discs were applied to test isolates EC394_9, EC0026B, and EC0880B than those for controls in order to identify plausible resistant subpopulations of test isolates if these subpopulations were rare.

Day 3

Reading diameters of inhibition zones and growing plausible resistant subpopulations in MH broth 2

7. Used digital callipers to measure the diameter of the most regular inhibition zone on each agar plate three times and took the mean of measurements as the diameter of the chosen zone.
8. Determined nitrofurantoin susceptibility using EUCAST breakpoints v10.0.
9. Grew resistant subpopulations in MH broth 2
 - c. For each plate of EC0880B and EC0026B (neither plate of EC394_9 showed colonies growing within inhibition zones), used inoculating loops to pick up a well separated small colony and large colony within one or two inhibition zones (Notably, the chosen colonies were not necessarily located within the same inhibition zone). In total, two colonies were picked up per plate.
 - d. An isolate name was assigned to each chosen colony.
 - e. Suspended each chosen colony in 7.5 mL MH broth contained in a 50-mL Falcon tube.
10. Retrieved negative control ATCC25922 again from the freezer and inoculated it in a 50-mL Falcon tube containing 7.5 mL MH broth 2.
11. Incubated bacteria at 35 °C with 225-RPM (revolutions per minute) shaking for 20 hours*.

* 20-hour incubation time was chosen to ensure sufficient growth of small-colony isolates.

Day 4

Disc-diffusion tests for plausible resistant isolates

3. Transferred 200 µL broth culture* of each isolate of strain to a 13-mL Falcon tube.
4. Made an inoculum of each isolate or strain by diluting and adjusting the stock to a turbidity of 0.5 McFarland standard with sterile 0.85% saline and the original stock.
5. Evenly spread the inoculum on the plate with a sterile cotton swab as described for Day 2.
6. Dried plates for two minutes and applied two nitrofurantoin discs on opposite sides†.
7. Incubated plates at 35 °C for 18 hours.

8. Saved every non-control isolate in glycerol and the -80 °C freezer as described for Day 2.

* This volume was chosen based on turbidity of the broth culture, so it seems inconsistent with the 150-μL volume of broth culture taken on Day 2.

† Since the purpose of this step is to determine nitrofurantoin susceptibility of each isolate rather than identifying population heterogeneity, only two discs were applied per plate for cross validation.

Day 5

Reading diameters of inhibition zones

1. Used digital callipers to measure the diameter of the more regular inhibition zone on each agar plate three times and took the mean of measurements as the diameter of the chosen zone.
2. Determined nitrofurantoin susceptibility using EUCAST breakpoints v10.0.

References

1. [Product instructions](#) (MTS™ PI - Rev.1, Jan 2020, F00045) for Liofilchem's Etest strips.
2. [Liofilchem® MIC Test Strip Photographic Guide](#) (2015).
3. [MIC Test Strip Technical Sheet Cidal-Static](#) (MTS30 Rev.3 / 08.06.2015).