



Challenges in Antimicrobial Susceptibility Testing of fastidious organisms

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Fastidious organisms

- Microorganisms that will grow only if special nutrients are present in the culture medium.
- Fastidious = Excessively particular, critical, or demanding; hard to please

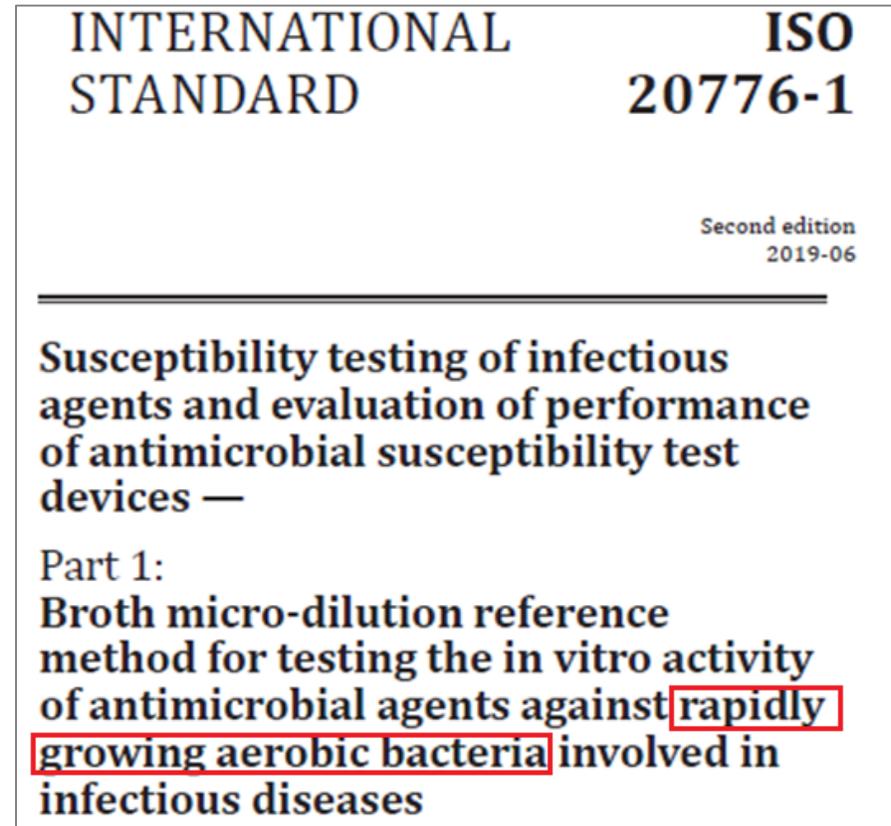
Fastidious organisms with EUCAST methods

- Streptococci
 - Streptococcus groups A, B, C and G
 - *Streptococcus pneumoniae*
 - Viridans group streptococci
- *Haemophilus influenzae*
- *Moraxella catarrhalis*
- *Listeria monocytogenes*
- *Pasteurella* spp.
- *Campylobacter jejuni* and *C. coli*
- *Corynebacterium* spp.
- *Aerococcus sanguinicola* and *A. urinae*
- *Kingella kingae*

EUCAST methods:

- Broth microdilution
- Disk diffusion

Reference broth microdilution (BMD) method



Annex D (informative) Special test situations

D.2.1 Fastidious aerobic and facultative anaerobic bacterial species (e.g. Haemophilus)

Two methods are currently used and have not been fully compared. Haemophilus Test Medium is documented by CLSI^[4], and Mueller-Hinton Fastidious (MH-F) Broth is documented by EUCAST^[16]. Readers are referred to the most recent versions of CLSI and EUCAST documents for information on medium preparation, testing and quality control results. When comparative testing of these methods has been completed and validated, this document will be updated to include reference antimicrobial susceptibility test method(s) for these micro-organisms.

Broth microdilution of fastidious organisms

Organism	Medium	Inoculum	Incubation
Fastidious organisms except <i>Campylobacter</i>	MH-F broth	5×10^5 CFU/mL	Air (sealed panels) 35°C 16-20 h*
<i>Campylobacter</i>	MH-F broth	5×10^5 CFU/mL	Microaerobic 41°C 24 ± 1 h*

* Prolonged incubation is only validated for *Corynebacterium*, *Aerococcus*, *Kingella kingae* (40-44 h) and *Campylobacter* (40-48 h).

MH-F broth = Cation-adjusted Mueller Hinton broth + 5% lysed horse blood and 20 mg/L β-NAD

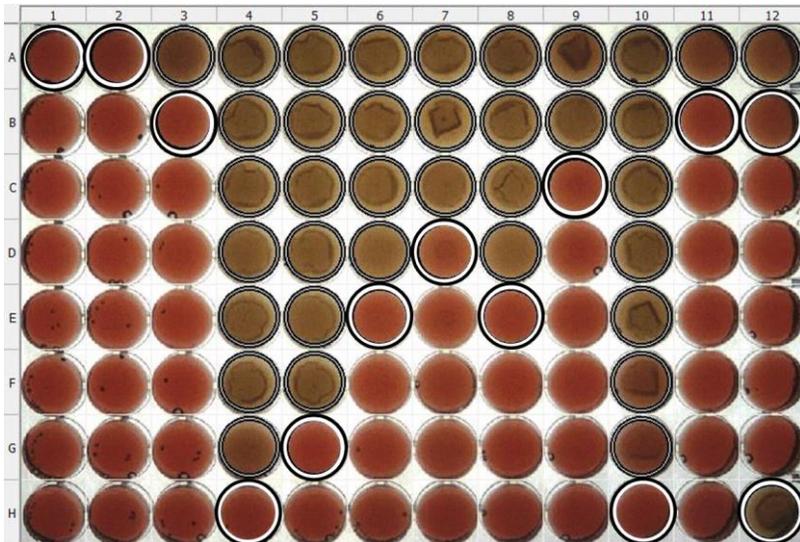
Preparation of lysed horse blood for MH-F broth

- The horse blood must be lysed by repeated freezing and thawing
 - Freeze the blood at -20°C overnight and thaw
 - Repeat the cycle until the cells are completely lysed (at least 3 cycles)
- A clear solution is essential for reading
 - Clarify the lysed horse blood by centrifugation
 - Repeating the centrifugation may improve the clarity of the solution

https://www.eucast.org/ast_of_bacteria/media_preparation

Reading BMD endpoints

- Read the MIC as the lowest concentration of the agent that completely inhibits growth of the organism as detected by the unaided eye.
- Read MICs only when there is sufficient growth, *i.e.* obvious button or definite turbidity, in the positive growth control.
- Results should be read manually. The use of a mirror may facilitate reading.



Haemolysis

- For fastidious organisms tested in MH-F broth, haemolysis of the blood can be seen. This is often accompanied by turbidity or a deposit of growth (pellet).
- Read inhibition of growth and not inhibition of haemolysis.
- Haemolysis with turbidity or pellet should be regarded as growth when determining endpoints.



Disk diffusion of fastidious organisms

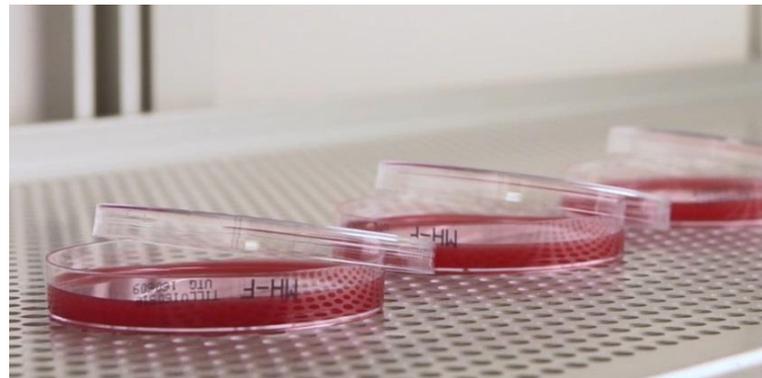
Organism	Medium	Inoculum	Incubation
Fastidious organisms except <i>Campylobacter</i>	MH-F agar	McF 0.5	5% CO ₂ 35°C 16-20 h*
<i>Campylobacter</i>	MH-F agar	McF 0.5	Microaerobic 41°C 24 ± 1 h*

* Prolonged incubation is only validated for *Corynebacterium*, *Aerococcus*, *Kingella kingae* (40-44 h) and *Campylobacter* (40-48 h).

MH-F agar = Mueller Hinton agar + 5% defibrinated horse blood and 20 mg/L β-NAD

MH-F plates and excess humidity

- The surface of the agar should be dry before use. Excess moisture may cause fuzzy zone edges and/or haze within zones.
 - No drops of water should be visible on the surface of the agar or inside the lid. This is often seen with plates stored in plastic bags or sealed containers.
 - To reduce problems related to too humid agar plates, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min.
- MH-F plates should always be dried to reduce swarming when testing *Campylobacter*.



Drying plates at 35°C
with the lid removed

MH-F and fuzzy zone edges

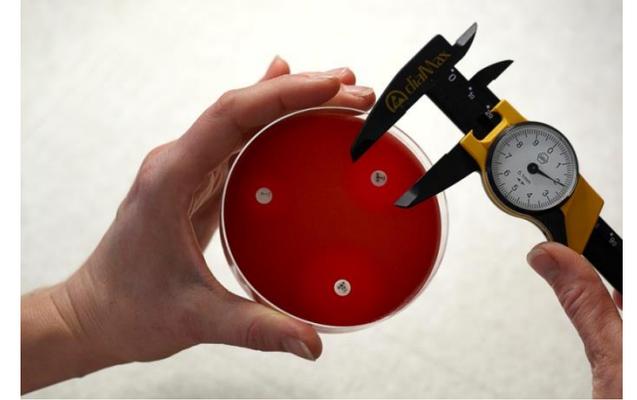


Streaking of plates and application of disks

- Streaking of plates
 - Gram-negative bacteria
 - Remove excess fluid by pressing and turning the swab against the inside of the tube to avoid over-inoculation.
 - Gram-positive bacteria
 - Do not remove excess fluid.
 - Take particular care when streaking plates to ensure that there are no gaps between streaks.
- Limit the number of disks on each plate to allow good growth and to avoid overlapping of zones.

Reading zones

- Read MH-F plates from the front with the lid removed illuminated with reflected light.
- Zone edges should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye.
- Holding the plate at a 45-degree angle to the work bench may facilitate reading when zone edges are difficult to define.
 - Particularly important for viridans group streptococci and *Campylobacter*



Fuzzy zone edges

Streptococcus pneumoniae

- Small colonies that are visible when the plate is held about 30 cm from the naked eye at a 45-degree angle to the work bench should be taken into account when reading zones.
- The presence of small colonies close to the zone edge may be related to excess humidity in the MH-F media, and may be reduced by drying the plates prior to use.



Reading of zones with fuzzy zone edges for *S. pneumoniae*.

Growth or haemolysis?

- It is sometimes difficult to differentiate between haemolysis and growth.
- Read inhibition of growth and not inhibition of haemolysis.
- See the EUCAST Reading Guide for examples.

β -haemolysis

- Tilt the plate back and forth to better differentiate between haemolysis and growth.
- β -haemolysis is usually free from growth.



S. pyogenes



Streptococcus group C

α -haemolysis

- Tilt the plate back and forth to better differentiate between haemolysis and growth.



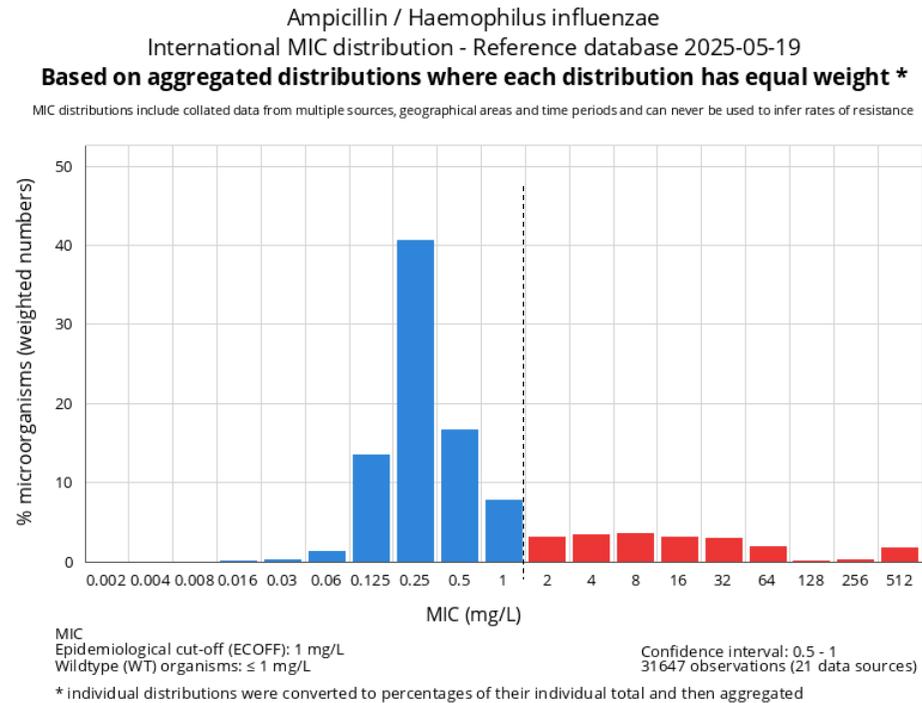
There is usually growth in the whole area of α -haemolysis.



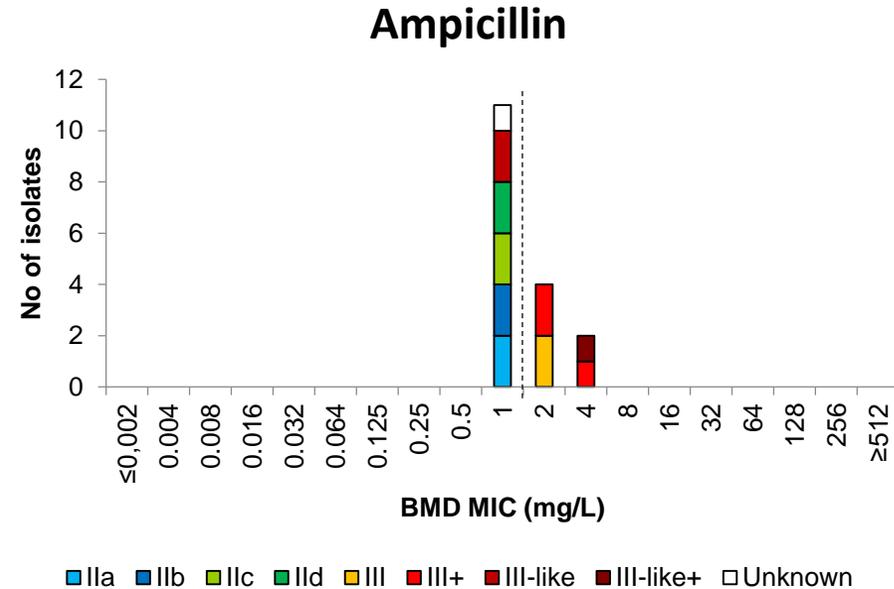
For some organisms, there is additional α -haemolysis without growth. Tilt the plate to differentiate between haemolysis and growth.

Challenges with *Haemophilus influenzae*

- Challenges in antimicrobial susceptibility testing are often related to beta-lactam agents and resistance due to PBP mutations

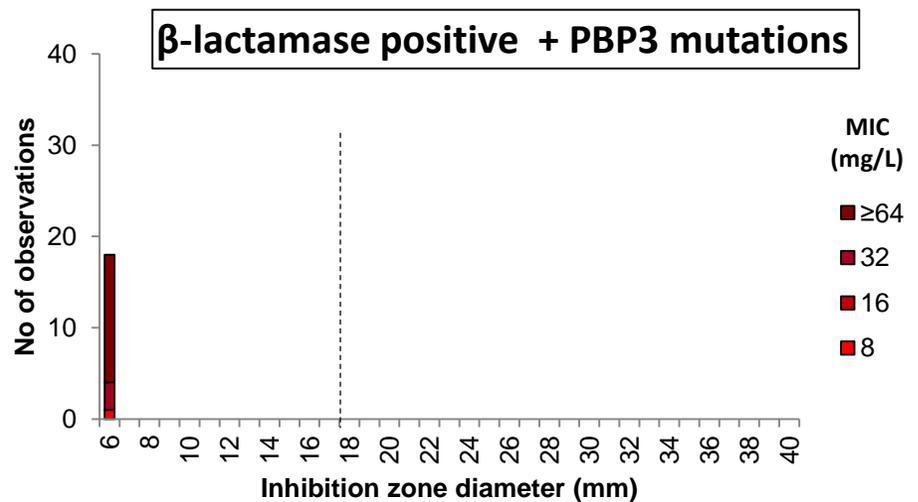
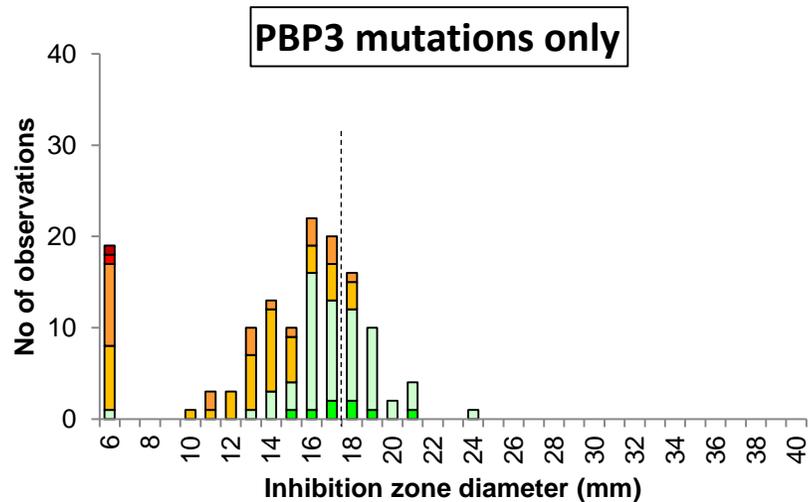
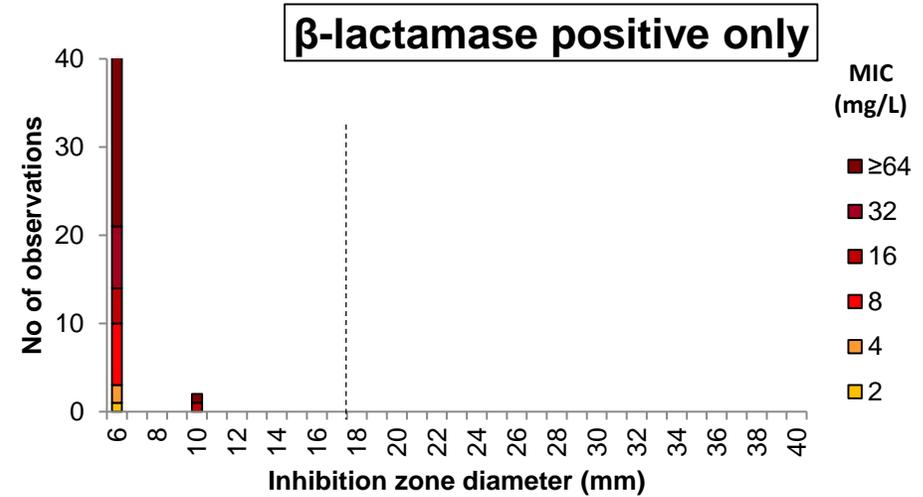
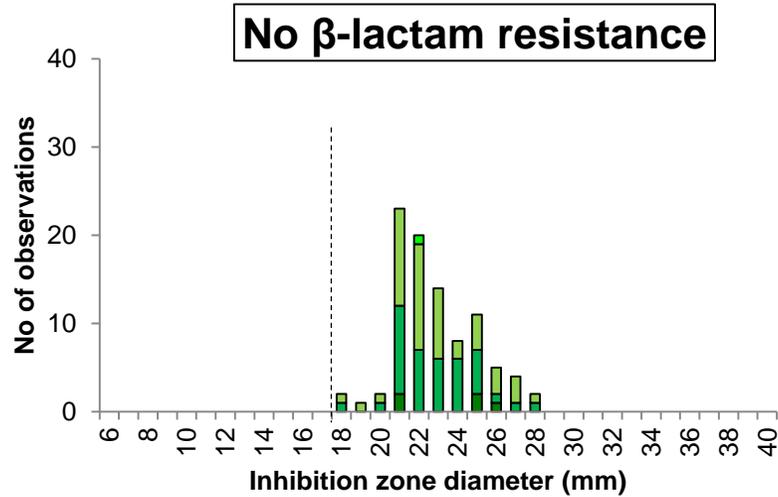


17 *H. influenzae* – all with PBP3 mutations



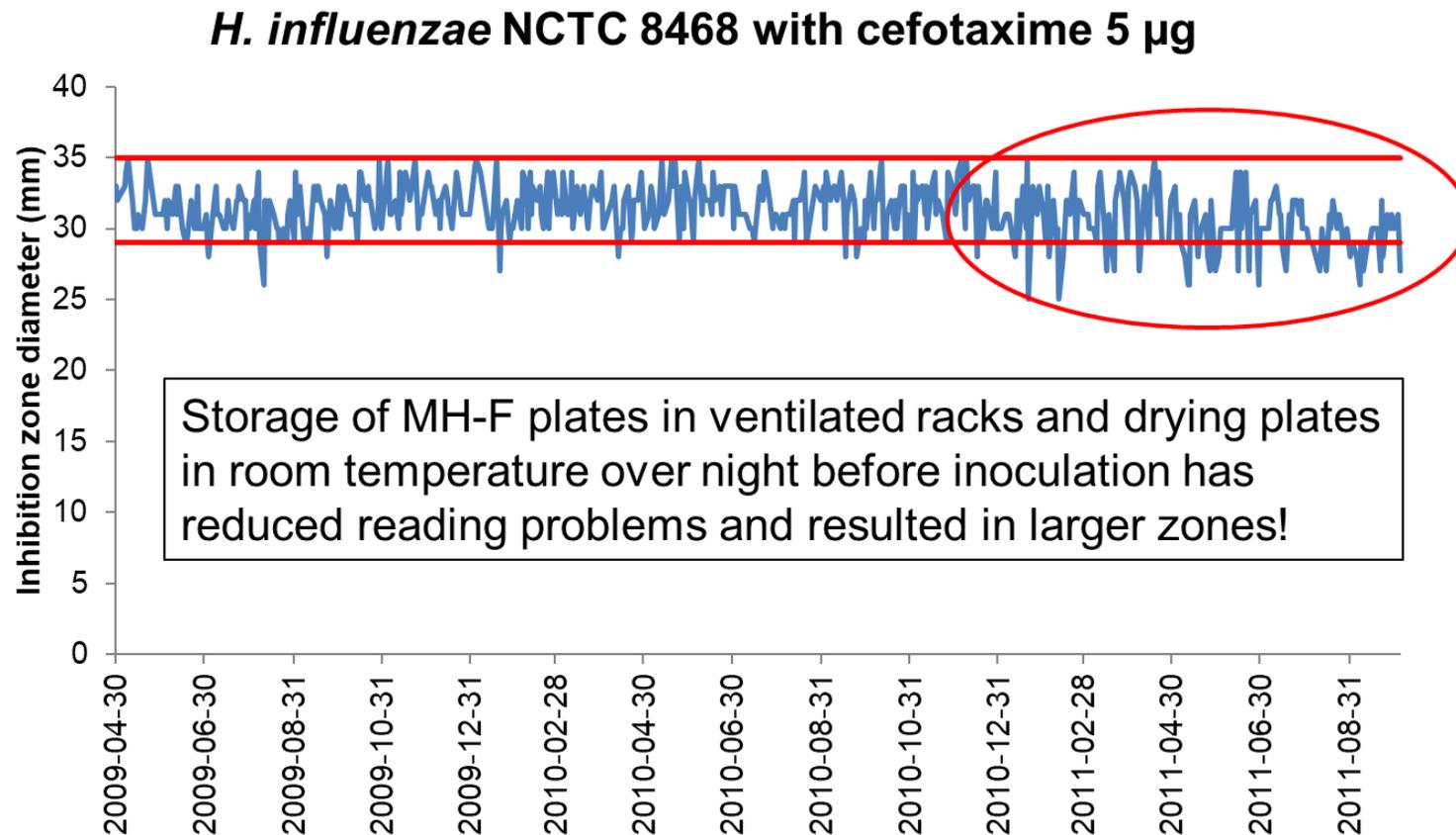
H. influenzae

Ampicillin 2 µg disk diffusion per beta-lactam resistance mechanism



H. influenzae and humid MH-F plates

- Problems with too humid MH-F plates causing haze within zones and fuzzy zone edges



Reading of zones for *H. influenzae*

- For beta-lactam agents: Read the outer edge of zones where an otherwise clear inhibition zone contains an area of growth around the disk



Tips for disk diffusion of fastidious organisms

- Dry the MH-F plates!
 - Always for *Campylobacter*
- *H. influenzae*
 - Remove excess fluid from the cotton swab before inoculating the plates
- Viridans group streptococci, *Corynebacterium*, *Aerococcus*, *Campylobacter*, *Listeria* (and *Kingella kingae*)
 - Do not remove excess fluid
 - Take particular care when streaking plates to ensure that there are no gaps between streaks
 - Limit the number of disks per plate
- Read zone diameters with the plate held at a 45-degree angle to the work bench
 - Particularly important for viridans group streptococci and *Campylobacter*
- Poor growth after 16-20 h incubation?
 - Repeat the test with carefully streaked plates and less disks per plate

