EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Broth microdilution methodology with focus on reading of MICs - Linezolid as an example

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Standardisation of antimicrobial susceptibility testing

- Results change with changed parameters.
  - It is crucial to adhere to the methodology to get reproducible and reliable results!

- Standardisation of:
  - Antimicrobial agents
    - Potency, solvents, diluents, two-fold dilutions
  - Media
    - Type of media, supplements, pH etc.
  - Inoculum
  - Incubation
  - Reading of results

MIC – the gold standard for AST

Minimum Inhibitory Concentration

- “The lowest concentration that, under defined *in vitro* conditions, prevents visible growth of bacteria within a defined period of time” (ISO 20776-1).

INTERNATIONAL STANDARD

ISO 20776-1

Second edition
2019-06

Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices —

Part 1:

≤200 µL

Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases

Broth microdilution (BMD)
ISO 20776-2, 2019

- Rapidly growing aerobic bacteria
- Fresh or frozen panels (<-60°C)
- Microdilution: ≤200 µL per well
- Standardised procedures for
  - Antimicrobial agents
    - Solvents, diluents, 2-fold dilutions
  - Inoculum
  - Incubation time and temperature
  - Reading of MIC endpoints (standard reading instructions)
- Referral to EUCAST and CLSI documents for
  - Testing of fastidious organisms
  - Specific reading instructions

EUCAST recommendations for BMD

- Perform BMD according to the ISO standard
  - Final inoculum of 5×10^5 CFU/mL
  - Sealed panels
  - Incubation in air, at 35°C for 16-20 h

  Non-fastidious and fastidious organisms

- Commercial panels with freeze-dried antimicrobials are accepted by EUCAST if they produce expected results
  - BUT many commercial products include few dilutions, resulting in:
    - Truncated MICs in both the lower and upper end
    - Products difficult to verify and control

EUCAST media for BMD

- Broth media
  - Non-fastidious organisms
    - Un-supplemented Mueller-Hinton broth (ISO 20776-1)
  - Fastidious organisms
    - MH-F broth: Mueller-Hinton broth with 5% lysed horse blood and 20 mg/L β-NAD (EUCAST media preparation, https://www.eucast.org/ast_of_bacteria/media_preparation)

Preparation of inoculum

- Final inoculum of 5×10^5 CFU/mL
- Start with McF 0.5 (~ 1-2 x 10^8 CFU/mL for *E. coli* ATCC 25922)
  - Dilutions depend on:
    - Volume of broth in wells (fresh/frozen or freeze-dried plates) and volume to be dispensed in each well
- Microtiter plates for BMD must contain one well without antimicrobials to serve as a growth control.
  - A check of the purity and the size of the inoculum can be performed from the growth control well before incubating the plates

Incubation

- Seal panels with a tight lid or adhesive seal to avoid evaporation
- To avoid uneven heating, microdilution trays should not be stacked more than four high
  - By placing an empty tray at the top of each stack, condensation on the inside of the adhesive cover is reduced, which facilitates reading.
- Incubate at 35°C in air for 16-20 h
  - Glycopeptides should be read after 24 h incubation
  - Prolonged incubation (40-48 h) is only allowed when specified in the EUCAST Breakpoint Table
    - *Corynebacterium, Aerococcus, Kingella kingae* and *Campylobacter*

Reading BMD endpoints

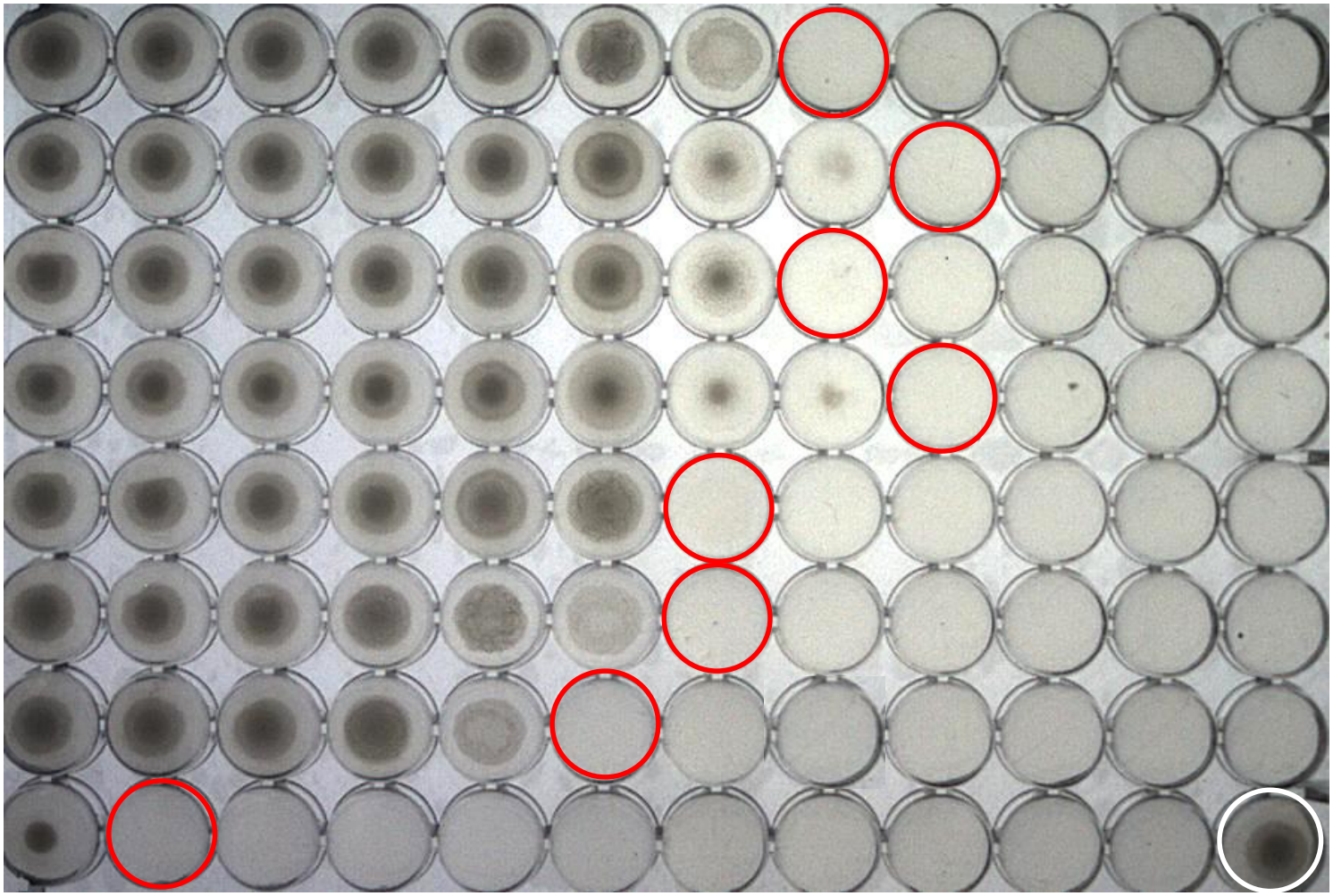
- Results should be read manually. The use of a mirror may facilitate reading.
  - If an automated reader or camera system is used, it must be calibrated to manual reading.

Reading BMD endpoints

- Growth appears as turbidity or as a deposit of cells at the bottom of the well (*i.e.* a pellet). The appearance of growth differs depending on the organism and the antimicrobial agent tested.
- Read MICs only when there is sufficient growth, *i.e.* obvious button or definite turbidity, in the positive growth control.
- **Unless otherwise stated, read the MIC as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye.**

Reading of BMD panels

Unless otherwise stated, endpoints should be read at complete inhibition of growth.

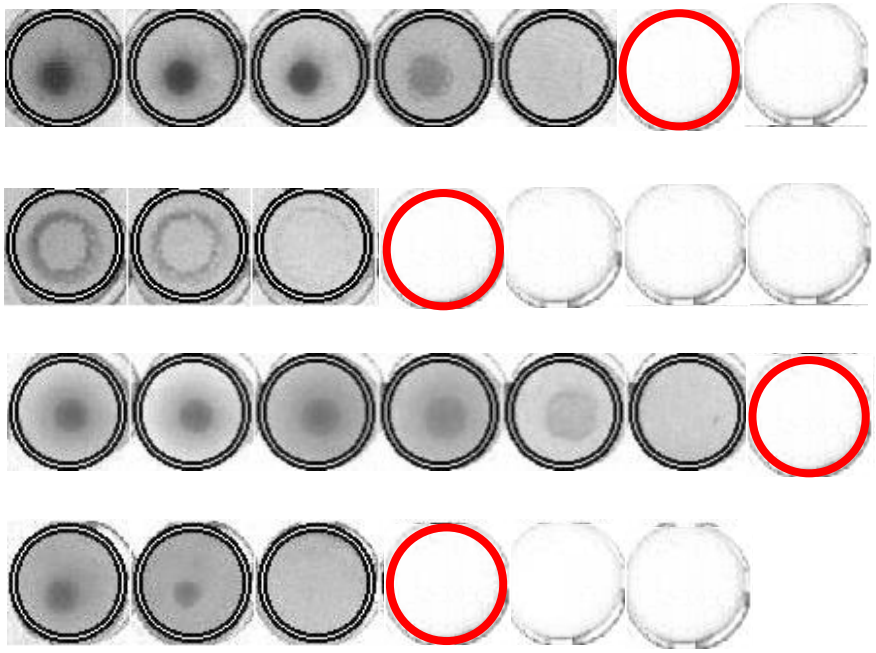


Positive control

Antimicrobial concentration →

Turbidity without pellet

- Haze or turbidity without a pellet is often seen for *Pseudomonas* spp. and *Acinetobacter* spp. This should be regarded as growth and the endpoint read at the first well with complete inhibition (clear broth).



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).

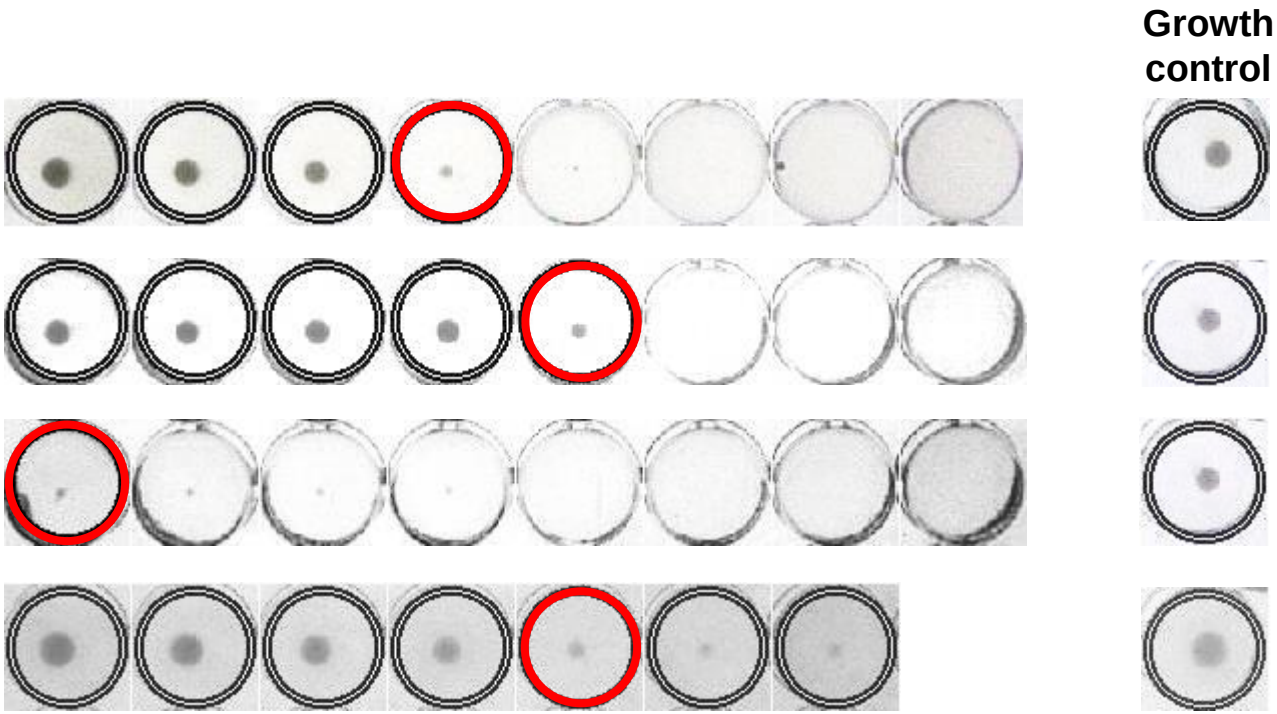
- Haemolysis with turbidity or pellet should be regarded as growth when determining endpoints.

Specific reading instructions related to trailing endpoints

- The following antimicrobial agents require specific reading instructions:
  - Trimethoprim and trimethoprim-sulfamethoxazole (all organisms)
  - Bacteriostatic antimicrobial agents, both with Gram-positive and Gram-negative organisms
  - Cefiderocol
    - Specific testing conditions (iron-depleted MH broth) and specific reading instructions, see EUCAST Guidance document https://www.eucast.org/eucastguidancedocuments)

Trimethoprim and trimethoprim-sulfamethoxazole

Read the MIC at the lowest concentration that inhibits ≥80% of growth as compared to the growth control.



Gram-positive cocci with bacteriostatic antimicrobial agents

- Disregard pinpoint growth (tiny buttons) when trailing growth occurs.

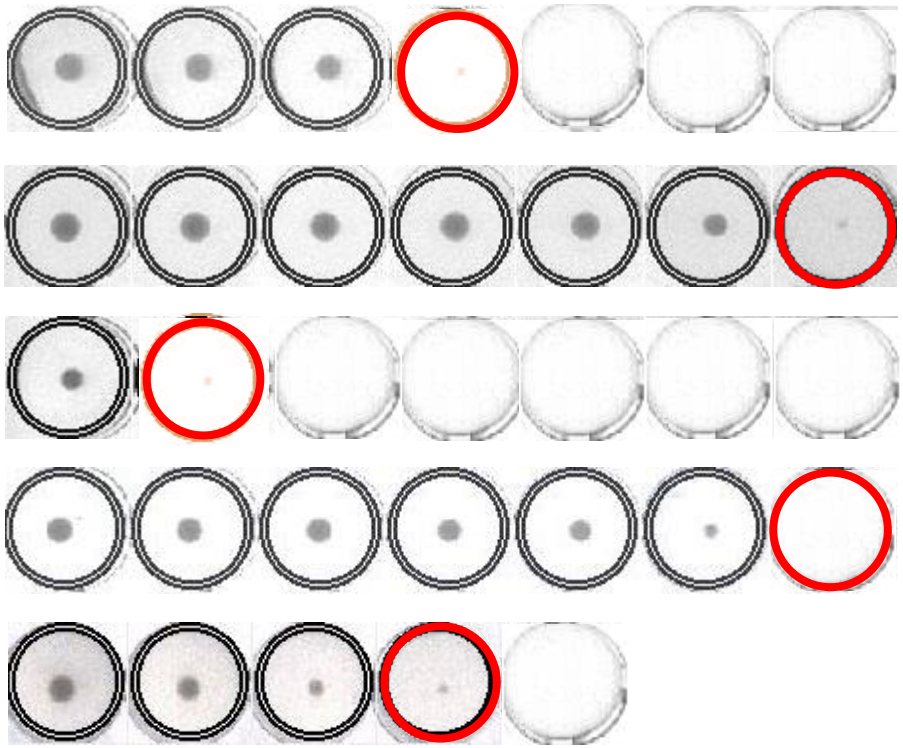
Doxycyline

Fusidic acid

Tetracycline

Doxycyline

Linezolid



CLSI M07, 10th edition, 2015

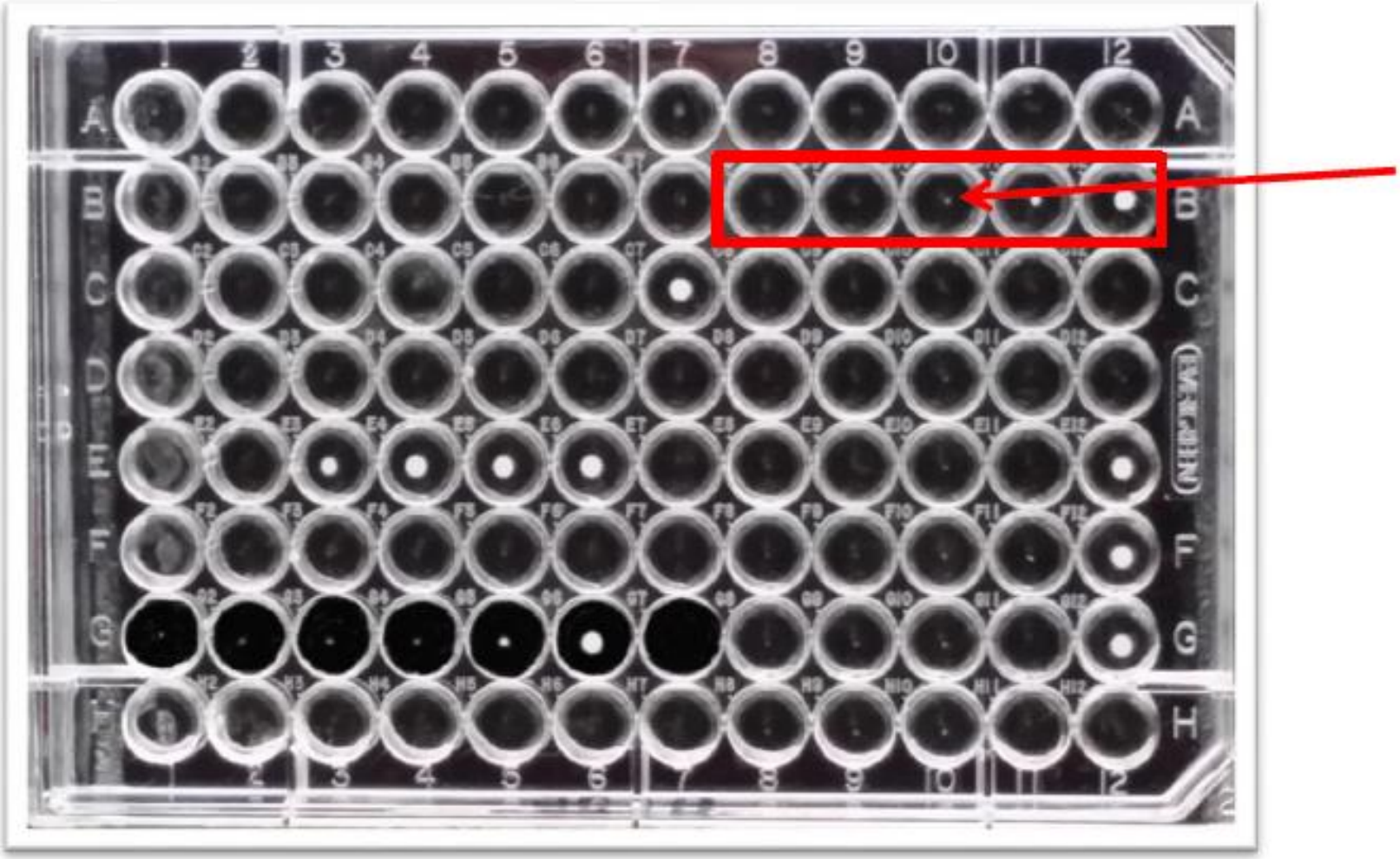
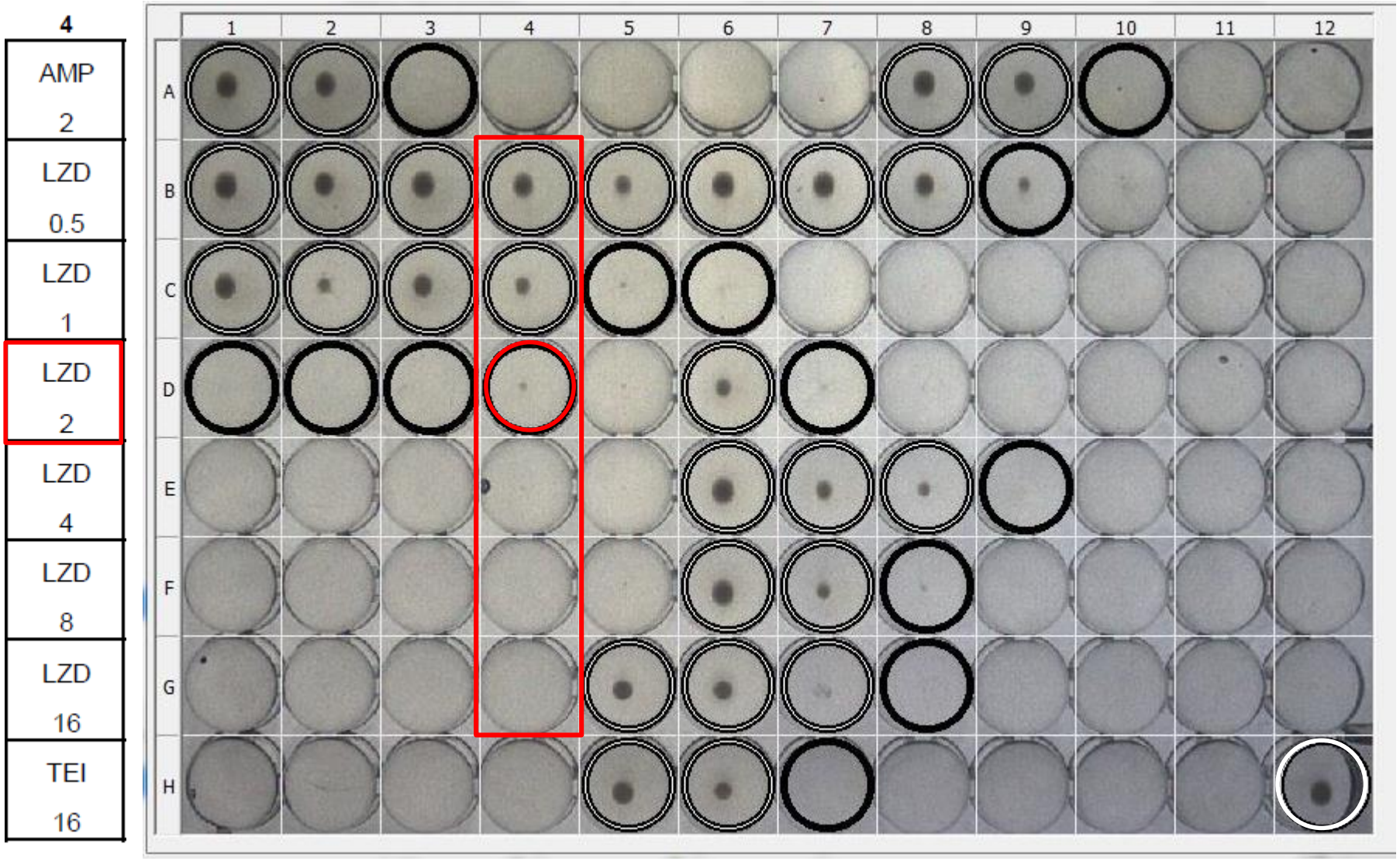


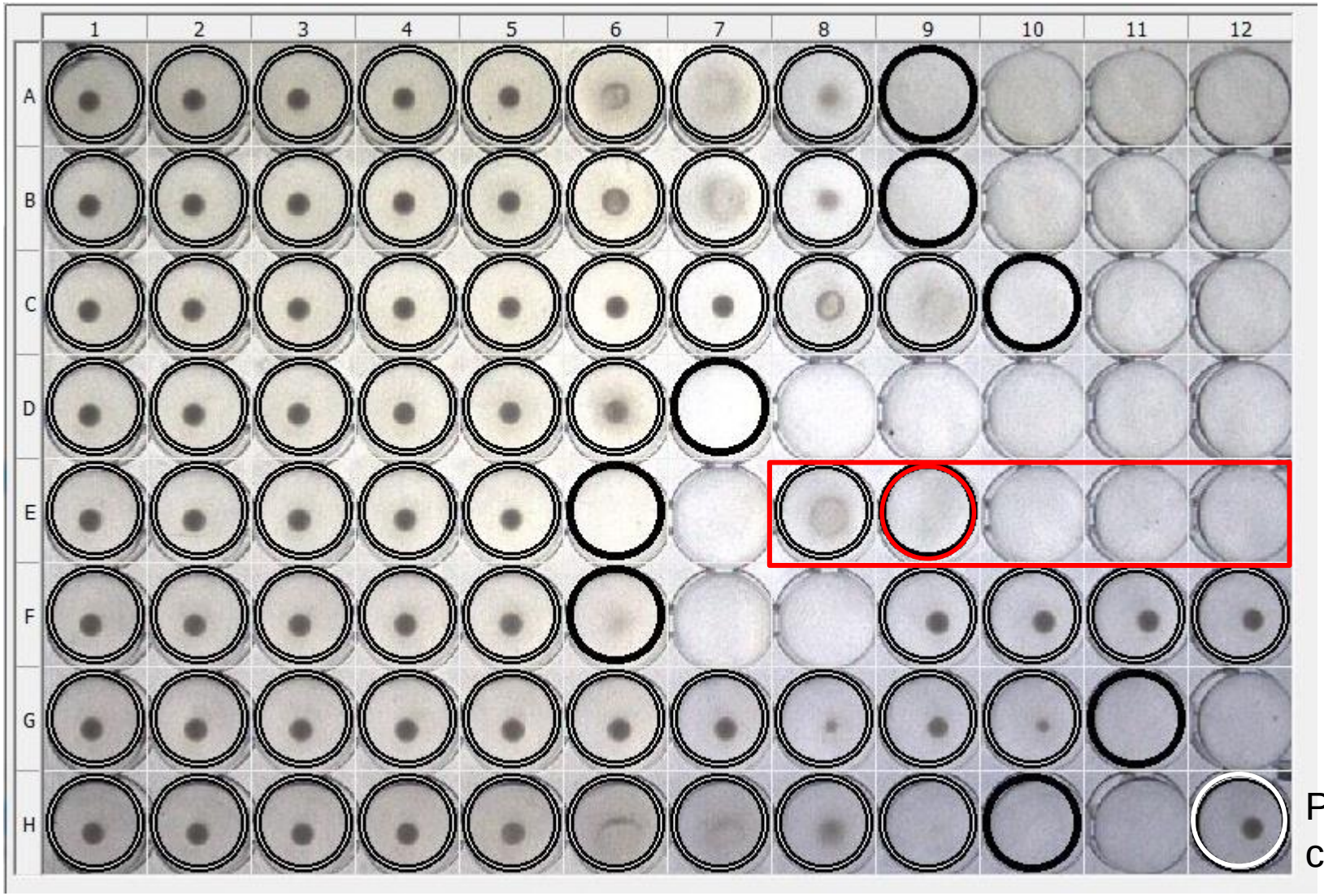
Figure 4. Linezolid: Trailing End Points (B8–B12, 16–1 µg/mL), MIC = B10

S. aureus ATCC 29213 and linezolid
Range 1-4 mg/L, Target 2 mg/L



Positive control

E. faecalis ATCC 29212 and linezolid
Range 1-4 mg/L, Target 2 mg/L

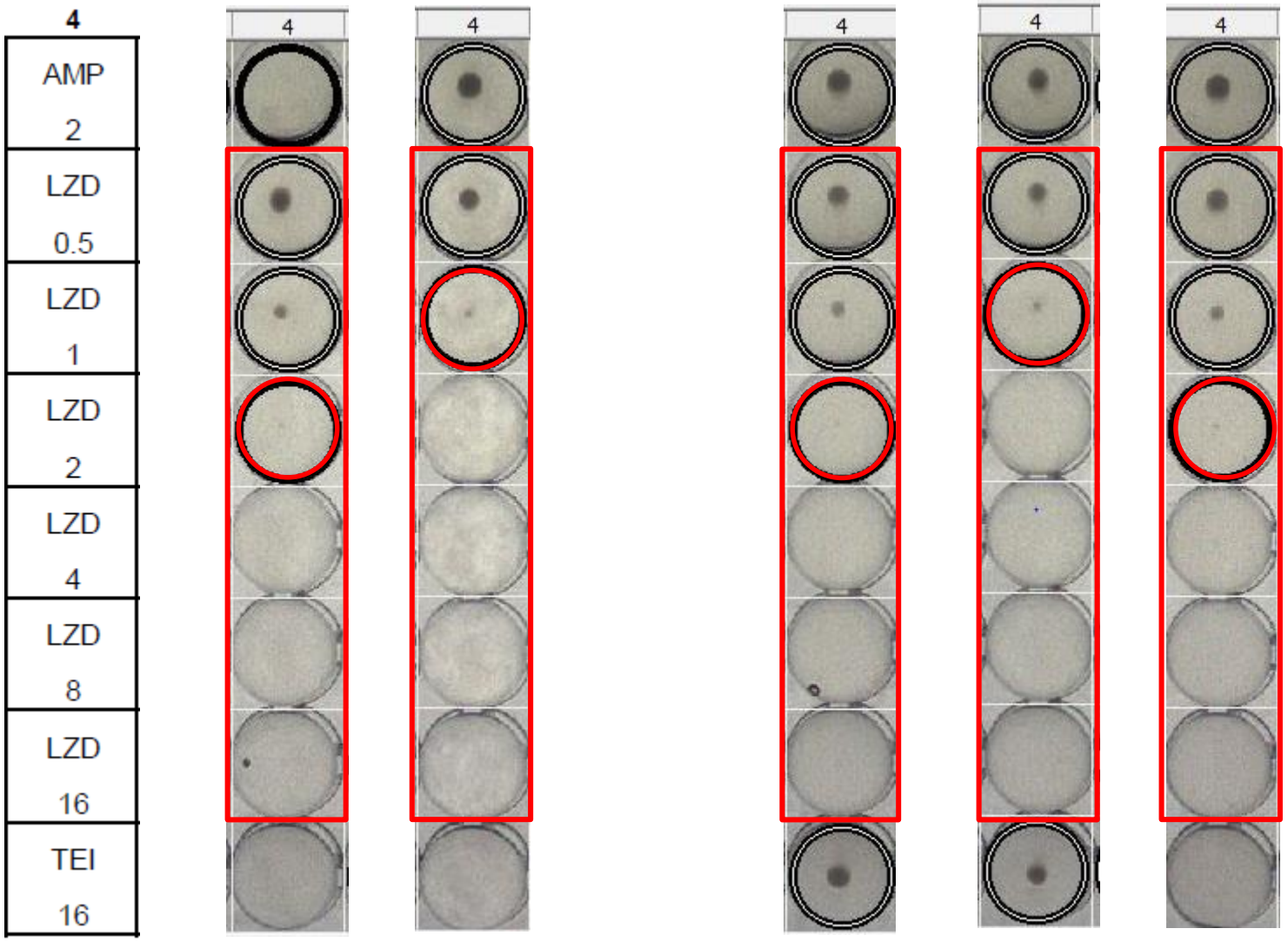


Positive control

E	CLA 0.03	CLA 0.06	CLA 0.12	CLA 0.25	CLA 0.5	CLA 1	CLA 2	LZD 0.5	LZD 1	LZD 2	LZD 4	LZD 8

Clinical isolates and linezolid

S. aureus *E. faecium*



Summary of BMD methodology
ISO 20776-1

Inoculum	5 x 10^5 CFU/mL
Media	MH broth (non-fastidious organisms) MH-F broth* (fastidious organisms)
Incubation	35°C in air (sealed panels) 16-20 h (24 h for glycopeptides)
Reading	Read the MIC as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye. • For specific reading instructions, see EUCAST and CLSI documents.

* Mueller-Hinton broth with 5% lysed horse blood and 20 mg/L beta-NAD (EUCAST recommended media for fastidious organisms)

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search term

AST of bacteria

Organization
Consultations
EUCAST News
New definitions of S, I and R
Clinical breakpoints and dosing
Rapid AST in blood cultures
Expert rules and expected phenotypes
Resistance mechanisms
Guidance documents
SOP
MIC and zone distributions and ECOFFs
AST of bacteria
Media preparation
MIC determination
Disk diffusion methodology
Disk diffusion implementation
Breakpoint tables
Quality Control
Strains with defined susceptibility
Calibration and validation
Warnings!

... MIC determination

MIC determination of non-fastidious and fastidious organisms

The EUCAST recommendations for MIC determination for non-fastidious organisms are in complete agreement with the recommendations from the International Standards Organisation (→ ISO).

For fastidious organisms (streptococci including *S. pneumoniae*, *H. influenzae*, *Moraxella catharrhalis*, *Listeria monocytogenes*, *Pasteurella* spp, *Kingella kingae*, *Aerococcus* spp, *Campylobacter* spp, and others), EUCAST recommends the same methodology but with the use of MH-F broth (MH broth with lysed horse blood and beta-NAD), see EUCAST media preparation file.

Media preparation (agar and broth for non-fastidious and fastidious organisms) (v 6.0, 1 January, 2020).

Broth microdilution - EUCAST reading guide v 4.0 (1 January 2022)

Guidance on cefiderocol MIC testing - see guidance document.

There are a number of commercially available surrogate MIC determination methods, such as commercial broth microdilution methods, gradient tests, semi-automated devices, etc. It

Information in EUCAST Breakpoint Tables

Staphylococcus spp.

MIC determination (broth microdilution according to ISO standard 20776-1 except for fosfomycin where agar dilution is used)
Medium: Mueller-Hinton broth
Inoculum: $5x10^5$ CFU/mL
Incubation: Sealed panels, air, 35±1ºC, 18±2h
Reading: Unless otherwise stated, read MICs at the lowest concentration of the agent that completely inhibits visible growth. See ”EUCAST Reading Guide for broth microdilution” for further information.
Quality control: *Staphylococcus aureus* ATCC 29213. For agents not covered by this strain, see EUCAST QC Tables.

Streptococcus pneumoniae

MIC determination (broth microdilution according to ISO standard 20776-1)
Medium: Mueller-Hinton broth + 5% lysed horse blood and 20 mg/L β-NAD (MH-F broth)
Inoculum: $5x10^5$ CFU/mL
Incubation: Sealed panels, air, 35±1ºC, 18±2h
Reading: Unless otherwise stated, read MICs at the lowest concentration of the agent that completely inhibits visible growth. See ”EUCAST Reading Guide for broth microdilution” for further information.
Quality control: *Streptococcus pneumoniae* ATCC 49619. For agents not covered by this strain, see EUCAST QC Tables.

Quality control

- Reference methods don’t automatically give the correct results just because they are reference methods.
- For reproducible and reliable results, methodology must be strictly adhered to.
- Quality control must be performed for all materials used (BMD panels, broth etc).
  - Commercial BMD panels must often be tested with several strains to cover all agents

QC and reproducibility of linezolid BMD

	S. aureus ATCC 29213	*E. faecalis* ATCC 29212	*S. pneumoniae* ATCC 49619
	2	2	2
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	2	1
	2	1	2
	2	1	1
	2	1	1
	2	1	2
	2	2	1
	2	2	1
	2	2	1
	2	2	1
Target	**2**	**2**	**0.5-1**
Range	**1-4**	**1-4**	**0.25-2**

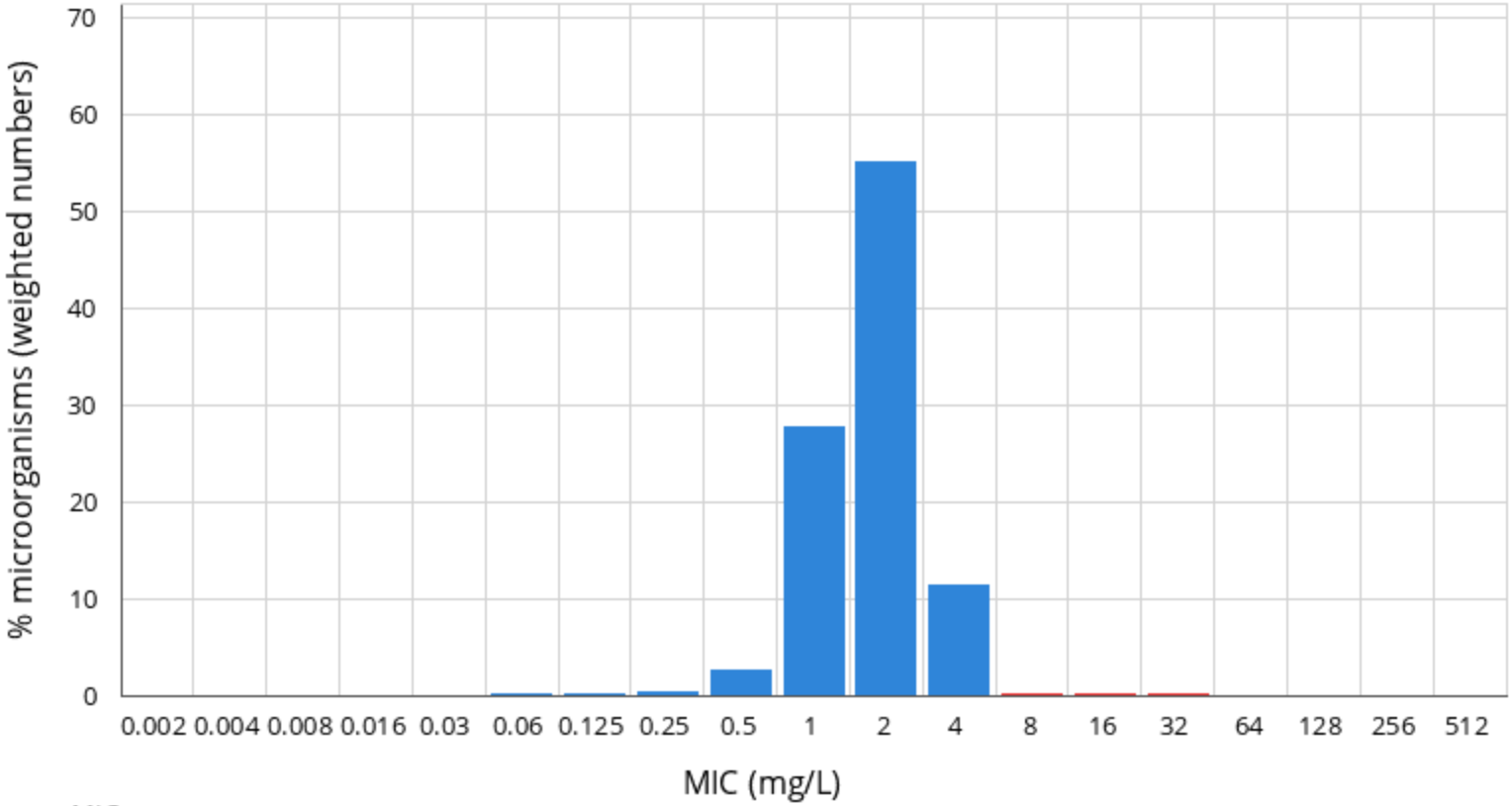
On target
Upper limit
Lower limit
Out of range

Linezolid / Staphylococcus aureus

International MIC distribution - Reference database 2022-06-01

Based on aggregated distributions where each distribution has equal weight *

MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance



MIC
Epidemiological cut-off (ECOFF): 4 mg/L
Wildtype (WT) organisms: ≤ 4 mg/L

Confidence interval: 2 - 4
67615 observations (29 data sources)

* individual distributions were converted to percentages of their individual total and then aggregated

Questions and/or problem?

Contact us!

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