Susceptibility testing in antibacterial drug R&D

Guest speakers: Dee Shortridge & Rafael Cantón

Moderator: Christian Giske

Host: Astrid Pentz-Murr (GARDP)

2 March 2023

GARDP
Global Antibiotic Research & Development Partnership

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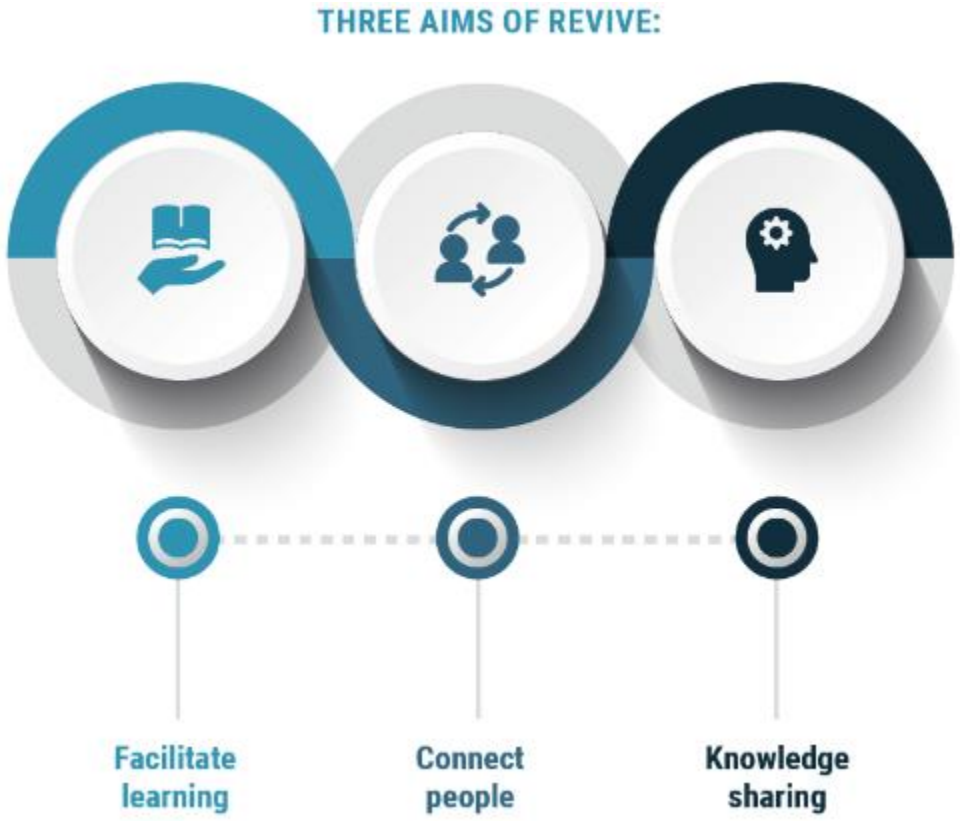

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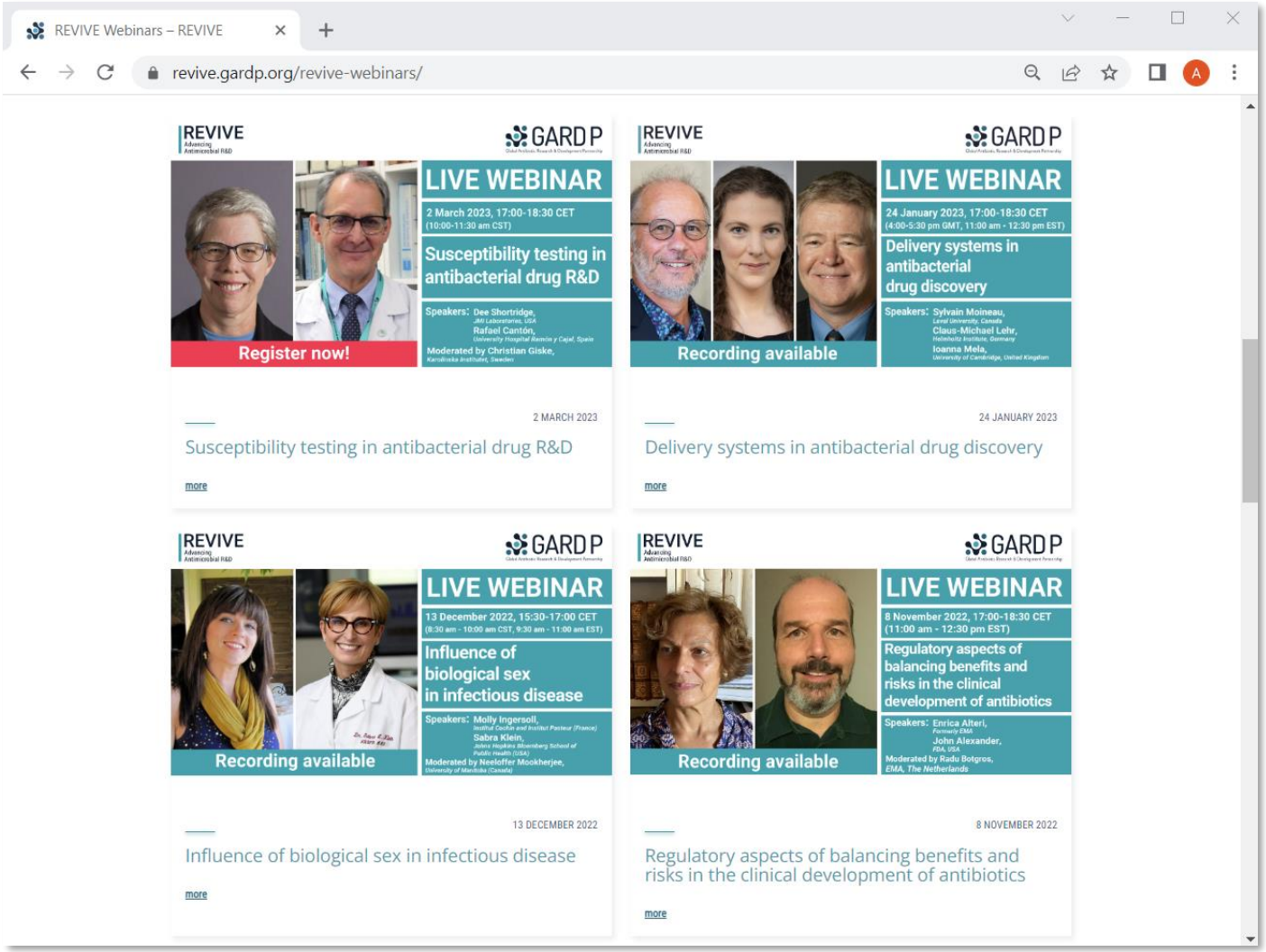

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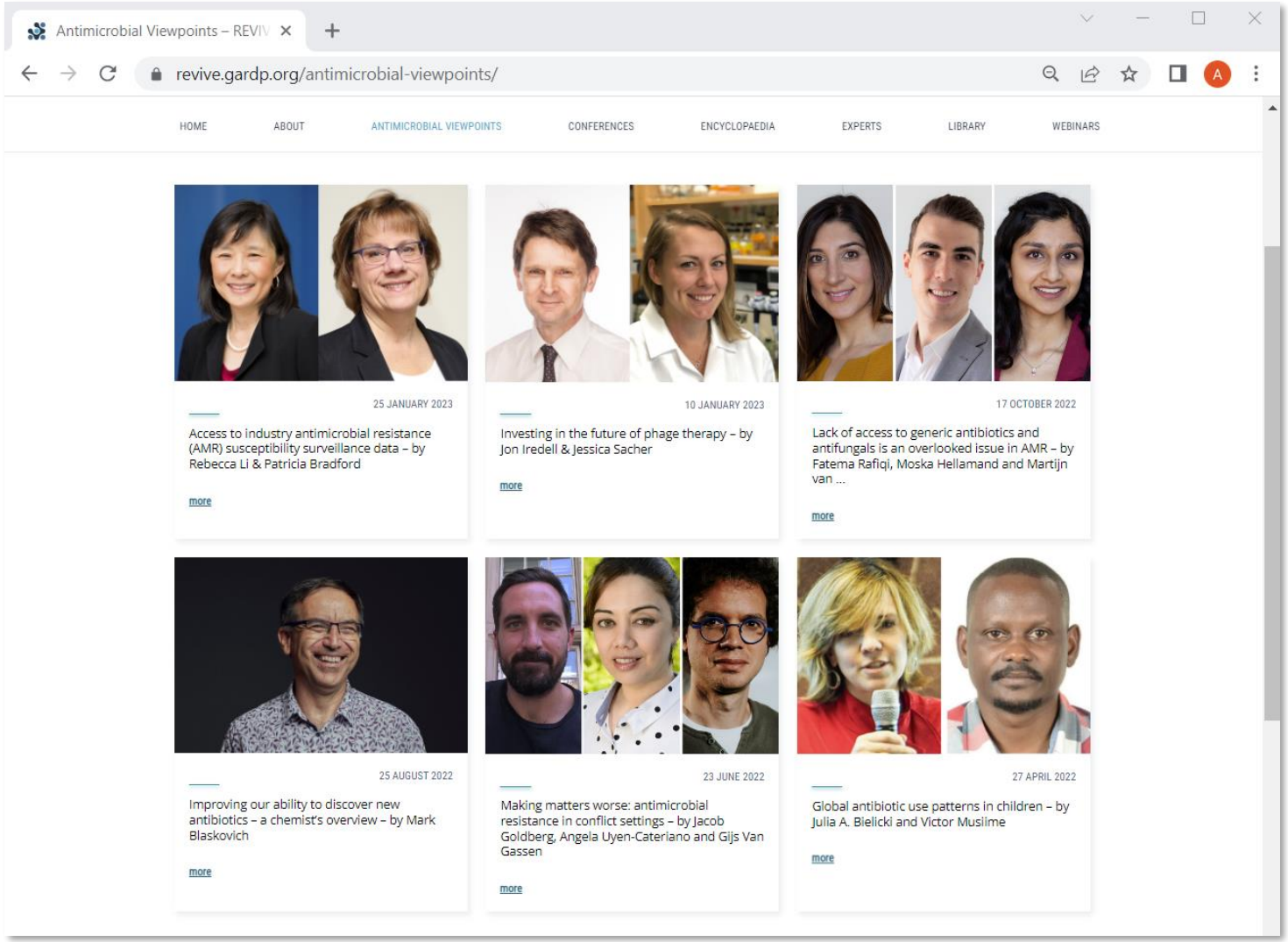
Antimicrobial Viewpoints



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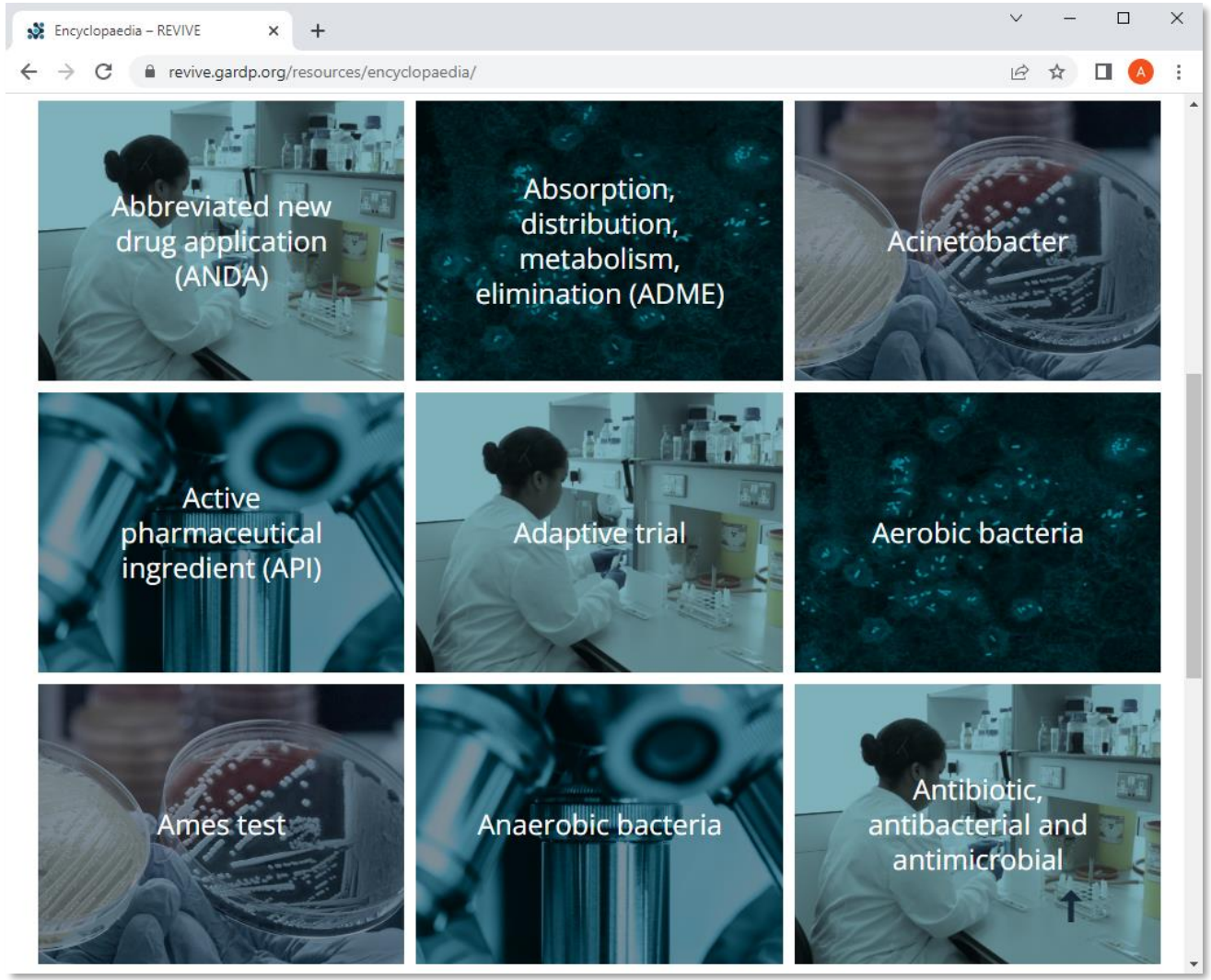
Antimicrobial Encyclopaedia



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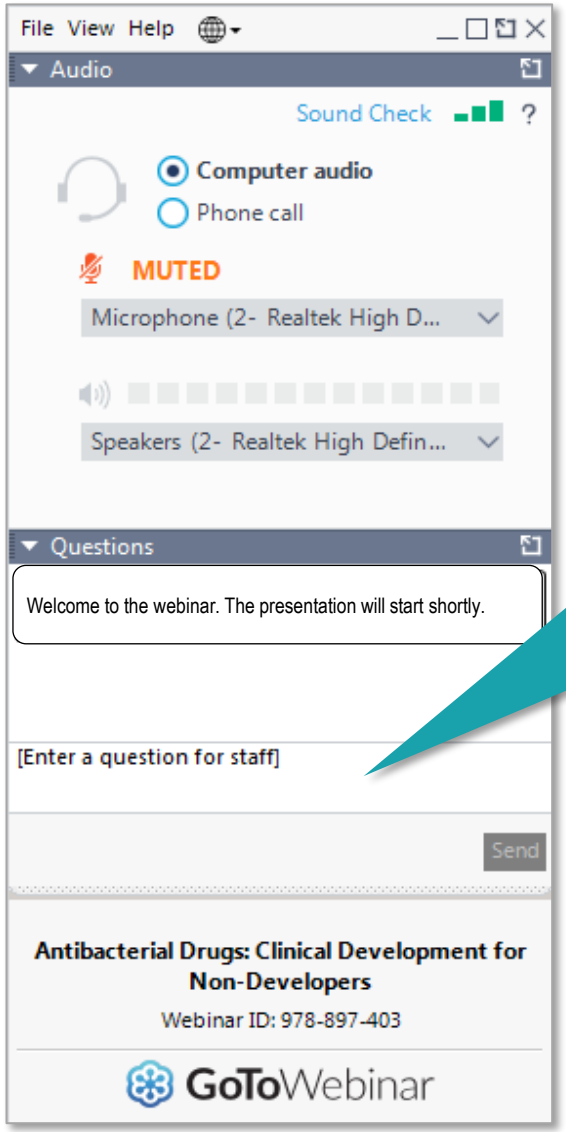
How to submit your questions

If your question is addressed to a specific speaker, please include their name when submitting the question.



The presentation will be followed by an interactive Q&A session.

Please submit your questions via the ‘questions’ window. We will review all questions and respond to as many as possible after the presentation.

Today’s speakers

Susceptibility testing in antibacterial drug R&D

Dee Shortridge
Senior Director
JMI Laboratories (USA)

Rafael Cantón
Head of Clinical Microbiology
Hospital Universitario Ramón y Cajal
Associate Profesor
Complutense University Madrid (Spain)

Moderator:
Christian Giske
Professor/Chief consultant physician
Department of Laboratory Medicine
Karolinska Institutet (Sweden)

Dee Shortridge

Dee Shortridge is a Senior Director for microbiology and diagnostics at JMI Laboratories, where she is responsible for overseeing new antibiotic development, surveillance projects, and diagnostic device development. Before her position at JMI Labs, Dee was director of R&D microbiology at bioMerieux, Inc. where she oversaw the development of ID and AST products for VITEK2 Systems. Before moving to bioMerieux, Dee held a senior group leader position for clinical microbiology at Abbott Laboratories, working on the clinical development of clarithromycin, cefdinir, cethromycin and delafloxacin. She is an ad hoc reviewer for the Journal of Clinical Microbiology, the Journal of Antimicrobial Agents and Chemotherapy and other journals. Dee completed her PhD in Microbiology and Immunology at the University of Colorado Health Sciences Center in Denver and a clinical postdoctoral education program fellowship at the University of Washington in Seattle.

Pre-clinical Antimicrobial Susceptibility Testing: Considerations and Challenges

Dee Shortridge, PhD

Len Duncan, PhD

JMI Laboratories, North Liberty, Iowa, USA

Disclosure

JMI Laboratories was contracted to perform services in 2022 for

AbbVie, Inc., AimMax Therapeutics, Amicrobe, Inc., Appili Therapeutics, Armata Pharmaceuticals, Astellas Pharma, Inc., Basilea Pharmaceutica AG, Becton, Dickinson and Company, bioMérieux, Biosergen AB, Bugworks, Cerba Research NV, Cidara Therapeutics, Cipla USA Inc., ContraFect Corporation, CorMedix Inc., Crestone, Inc., Curza Global, LLC, Diamond V, Discuva Ltd., Entasis Therapeutics, Enveda Biosciences, Evopoint Biosciences, Fedora Pharmaceuticals, Fox Chase Chemical Diversity Center, Genentech, Gilead Sciences, Inc., GSK plc, Institute for Clinical Pharmacodynamics, Iterum Therapeutics plc, Janssen Biopharma, Johnson & Johnson, Kaleido Biosciences, LifeMine Therapeutics, Medpace, Inc, Lysovant Sciences, Inc, Meiji Seika Pharma, Melinta Therapeutics, Menarini Group, Merck & Co., MicuRx Pharmaceutical Inc., Mundipharma International Ltd., Mutabilis, Nabriva Therapeutics, National Cancer Institute, National Institutes of Health, Ohio State University, Omnix Medical Ltd., Paratek Pharmaceuticals, Pfizer, PolyPid Ltd., PPD, Prokaryotics, Inc., Pulmocide Ltd, Qpex Biopharma, Revagenix, Roche Holding AG, Roivant Sciences, Scynexis, Inc., SeLux Diagnostics, Shionogi & Co., Ltd., Sinovent Pharmaceuticals, Inc., Spero Therapeutics, Sumitovant Biopharma, Inc., TenNor Therapeutics, ThermoFisher Scientific, U.S. Food and Drug Administration, VenatoRx Pharmaceuticals, Washington University, Watershed Medical, LLC, Wockhardt, and Zoetis, Inc.

Overview

- *In vitro* testing of pre-clinical compounds is used to
  - Determine the activity of a compound against species of interest
  - Compare activities between compounds and drug classes
  - Correlate *in vitro* activity with *in vivo* models and possible PK/PD targets
- This talk will cover
  - Determining MIC values for pre-clinical lead compounds (small molecules)
  - Developing a susceptibility testing method as the compound advances
- This talk will not discuss
  - Animal or PK/PD modeling
  - Breakpoint setting

Reference Method

- MIC testing for a pre-clinical candidate should be completed in standard cation-adjusted Mueller Hinton broth (CAMHB)
  - CAMHB is the most widely used and accessible testing medium
  - Indicated by CLSI, EUCAST, and ISO
  - Ca^{2+} and Mg^{2+} levels are maintained in a defined range
  - Broth microdilution preferred
- CAMHB supports the growth of most non-fastidious species of interest
  - Fastidious isolates need supplementation with blood
  - Anaerobes and other special organisms use different media
- If your compound has lower-than-expected activity with this method
  - That's a challenge
  - Need to understand your compound's characteristics

Solubility

- Is the compound soluble in a solvent at a sufficient level for MIC testing?
  - The standard working stock is 1280 mg/L (CLSI, M07)
  - The typical testing range is 32–0.015 mg/L, depending on activity
  - Testing below 0.001 mg/L may generate reproducibility issues
- Is your compound a neutral or salt form?
- Assess solubility by
  - Centrifugation/ analytical chemistry
  - Spectroscopy
- Consider the compound may precipitate
  - May exist but not be visible by eye
  - May occur at higher concentrations in an MIC test with reappearing growth

Solvent

- DMSO is the standard research solvent
  - Final concentration in MIC tests cannot exceed 1.0% (CLSI, M100)
- Determine early if another solvent would work
  - Water
  - Phosphate buffer
- Determine if additional tricks are needed
  - Heat
  - pH adjustment
  - Sonication
- Develop directions on how to solubilize

Testing Medium Stability of Compound

- Perform MIC determinations in CAMHB with the desired dilution range
- Test stability of working stock in (1280 mg/L)
  - Freshly prepared
  - Stored overnight at 4°C
- Determine MICs of standard ATCC strains
  - Freshly made medium
  - Medium made >1 week prior and stored at 4°C
  - Freshly made panels
  - Panels frozen overnight at -80°C, which contain diluted compound and CAMHB

Panel Stability

- Determine MIC panel stability
  - Test panels used above after storage at -80°C weekly for 4 weeks
  - Test panels monthly for 6 to 9 months
  - Graph MICs vs. time and look for trends
- If the compound will be combined with another agent
  - Test the combination working stock in buffer/water, fresh, and frozen
  - Test the combination with medium, both fresh and frozen, in relevant concentrations
  - Don’t assume that the combination will be compatible/stable just because each compound alone is stable

Spectrum of Activity

- Activity of compound in target and non-target organism groups
  - Gram-negative species: Enterobacterales, non-glucose fermenting species
  - Gram-positive species: staphylococci, enterococci, streptococci
- Activity of compound against multiple isolates per species
  - If initial testing was on stock isolates, use clinical isolates next
  - 10 isolates is okay, but 100 isolates is better to determine consistent activity
    - MIC range by genera and species
    - US FDA guidance
      - ≥100 isolates/key species collected in the last 3 years
      - 75% of which should be from USA
      - Usually much larger isolate sets are tested

Spectrum of Activity, continued

- Isolates should represent both wild-type and common resistant phenotypes
  - If available, test molecularly characterized isolates with common resistance mechanisms
  - See if the MIC range varies with isolates resistant to other drug classes
- Include CLSI/EUCAST QC isolates with each MIC run
- Include a relevant comparator with QC ranges
  - Comparator should be of the same or related class
  - Is a commonly used antimicrobial active against the target organisms

Variable Effect on *In vitro* MIC Testing

- Purpose
  - Study common variables and their effect on MIC values
  - Understand how robust your MIC test is
- Standard method
  - CAMHB microdilution
  - Triplicate testing, use median value
- Method variations
  - Inoculum: low, standard (10^5 CFU/mL), high
  - Incubation atmosphere: ambient, CO_2, microaerophilic, and anaerobic
  - Incubation time: 16, 18, or 24 hours
- Compare broth microdilution and agar dilution

Variable Effect on *In vitro* MIC Testing, continued

- pH
  - Low (pH 6), standard (pH 7.2), or high (pH 8)
  - Macrolides have less activity at lower pH
- Calcium and magnesium (lower and higher) vs standard CAMHB
  - Effect of other cations on compound activity might be useful
  - Depends on chemical class and mode of action
- Polysorbate-80
  - Addition of polysorbate-80 up to 0.002%
- CAMHB
  - MIC values can vary between MHB manufacturers
  - Compare 3 different MHB sources, if possible
  - MHB must be meet ISO criteria for use in susceptibility testing

Activity in Biological Fluids

Compound activity should be examined in

- Pooled human serum (25–50% v/v)
  - Heat-inactivated and non-heated (complement effect)
  - Novobiocin will show a large MIC shift against *S. aureus*
  - Activity will reflect the compound's protein binding
  - Some marketed drugs are highly protein bound but still efficacious
- Bovine lung surfactant (10% v/v)
  - Surrogate for human lung epithelial lining fluid
  - Daptomycin against *S. aureus* as a control, as it lacks activity in bovine surfactant
  - MICs can be difficult to read (use resazurin, if necessary)

Activity in Biological Fluids, continued

- Porcine mucin (2–10% v/v)
  - Of interest for large molecules
  - Colistin as a control, has decreased activity in 2% porcine mucin
- Lysed horse blood (2-5% v/v)
  - Standard medium additive for fastidious organisms
- Pooled human urine (80% v/v)
  - If the goal is to treat urinary tract infections

Activity in Biological Fluids, continued

To review,

The goal is to show that your compound's activity is not impacted by these fluids:

- Pooled human serum
- Lung surfactant
- Porcine mucin
- Lysed horse blood
- Pooled human urine

If MICs are >2 dilutions higher or lower, additional studies can be performed to determine the reason

Non-standard Medium

- If your compound has more activity (a lower MIC) in non-standard medium:
  - Do you develop your susceptibility test in that medium?
  - Is the MIC difference > than the +/- 1 doubling dilution variation of the method?
  - What is the difference in MIC values to your drug using a panel of isolates with a range of MICs, as well as on a larger set of wild-type isolates?
  - What percentage of isolates show the effect in non-standard medium?
- Does the lower MIC correlate much better with the *in vivo* PK/PD targets?

Non-standard Medium, continued

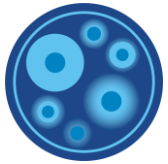
- Binding to plastic panels may be seen with larger molecules
  - Does polysorbate-80 lower MICs?
  - Compare glass tubes, polypropylene panels, polystyrene panels
- Using a non-standard method for your susceptibility test will add complexity to development (and cost)
  - Avoid whenever possible
  - Avoid just to increase *in vitro* potency
  - An alternate method may delay getting your compound on AST device menus
  - May delay adoption of testing by clinical laboratories

Discuss with CLSI/EUCAST early on if using a non-standard medium

Drugs Not Tested in Standard CAMHB

- See CLSI (M100)
  - Cefiderocol: iron-depleted CAMHB
  - Daptomycin: CAMHB + additional Ca^{2+}
  - Fosfomycin and mecillinam: agar dilution
  - Lipoglycopeptides: CAMHB + P-80 (0.002%)
    - Telavancin: CAMHB + P-80 (revision, with lowered breakpoints)
  - Oxacillin: CAMHB + 2% NaCl
  - Polymyxin: originally CAMHB + P-80 (now reversed to omit P-80)
  - Tigecycline: fresh medium

What About Disk Diffusion?



- Overall
  - Useful, qualitative test method for antimicrobials in a clinical setting
  - Does not work for polymyxins or vancomycin for staphylococci
- Determine if your compound is compatible with disk diffusion
  - Will it diffuse through agar?
  - Is it stable to drying on a paper disk?
- Early disk pilot with isolates that have known MIC values (low and high)
  - Make disks with a range of compound concentrations based on other agents in related classes
  - Correlate the diameter of inhibition around the disk with the MIC
  - Notice if there is a concentration that differentiates isolates with low and high MICs
- Formal disk development will be done through the CLSI/EUCAST Working Group

When to Go to Standards Organizations

When your compound is ready to move from pre-clinical to clinical,

plan when to go to CLSI and EUCAST with your compound and proposed method

- CLSI M23 describes what information is required
  - Need the completed studies discussed here (and others)
  - Begin discussions earlier if you have a non-standard reference method
  - Begin planning QC studies for broth microdilution and disk diffusion, if applicable
- Reference method for broth and disk
  - Changes to the standard method must be accepted by CLSI and EUCAST
  - The US FDA will also need to be aware of proposed changes
- Preliminary QC ranges will be required prior to clinical trials

When to think about Commercial AST

Consider for antimicrobials that will be used to treat infections caused by resistant bacterial pathogens, particularly in a hospital setting

- Devices
  - Disk diffusion first, if using
  - Then other *in vitro* devices, such as automated systems or gradient strips
- Remember
  - Development time varies and can be 3 to 4 years or more
  - Requirements for solvent, solubility, and stability vary by manufacturer
- Requires reference AST method with QC ranges
- If you have a non-standard method, more time will be required to determine if compatible

Conclusions

To develop a reproducible susceptibility test

Understand your compound's characteristics

- Mode of action
- Solubility
- Spectrum of activity
- Solvent
- Stability

Start the following studies (the earlier the better!)

- External MIC testing
- MHB comparisons
- Disk development
- M23 QC studies

Final Points

The goal is to make MIC testing with your compound as easy and reproducible as possible

- When determining MIC values, start with CAMHB first
  - If *in vitro* activity with reference broth microdilution doesn't correlate with *in vivo*
    - MIC method variation studies
    - An alternate MIC method is an option, but should be the last option
- The test should clearly and cleanly identify S vs R isolates
- If MIC endpoints are less than clear-cut to read
  - Develop reading guidelines with photos
  - Discuss how to read endpoint with external experts
  - Discuss with CLSI/EUCAST

References

- CLSI. M07Ed11. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 2018.
- CLSI. M23Ed5. Development of *in vitro* susceptibility testing criteria and quality control parameters. 2018.
- CLSI. M23SEd1. Procedure for optimizing disk contents (potencies) for disk diffusion testing of antimicrobial agents using harmonized CLSI and EUCAST criteria. 2020.
- CLSI M100Ed32. Performance standards for antimicrobial susceptibility testing. 2022.
- EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. V13. 2023.
- EUCAST. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. V13. 2023.
- ISO 16782. 2016. Clinical laboratory testing– Criteria for acceptable lots of dehydrated Mueller-Hinton agar and broth for antimicrobial susceptibility testing.
- ISO 20776. Part 1, 2019. Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacterial involved in infectious diseases.
- US FDA. Guidance for industry, microbiology data for systemic antibacterial drugs– development, analysis, and presentation. 2018.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Rafael Cantón

Rafael Cantón is the Head of the Microbiology Department at the Ramón y Cajal University Hospital and Associate Professor of Clinical Microbiology at the Complutense University (Madrid, Spain). His research work at the Instituto Ramón y Cajal de Investigación Sanitaria focuses on antimicrobial resistance mechanisms, new methods to study antimicrobial susceptibility and chronic respiratory infections. He is currently the clinical data coordinator of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and belongs to the advisory committee of the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR). He is president of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC) and EUCAST.

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LIVE WEBINAR

2 March 2023, 17:00-18:30 CET
(10:00-11:30 am CST)

Susceptibility testing in antibacterial drug R&D

GARD P
Global Antibiotic Research & Development Partnership

Pitfalls and opportunities of susceptibility testing in R&D and clinical trials of new antibiotics: Why is MIC needed?

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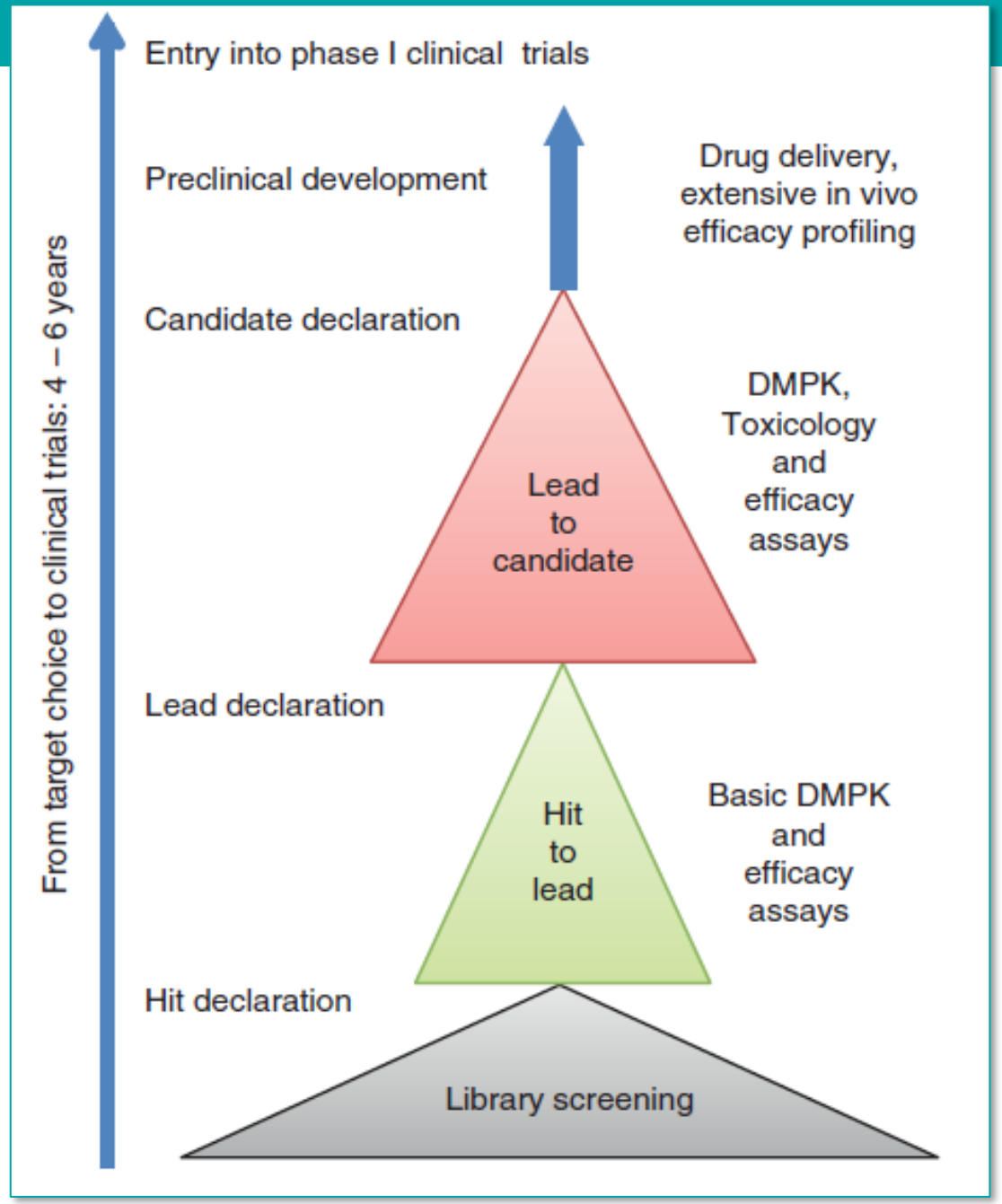
Pitfalls and opportunities of susceptibility testing in R&D and clinical trials of new antibiotics
Why are MIC needed?

Acknowledgements and disclosures

Antimicrobial drug discovery process



DMPK = drug metabolism and pharmacokinetics

Preclinical development

Lead optimization

In vitro activity

↓

MIC

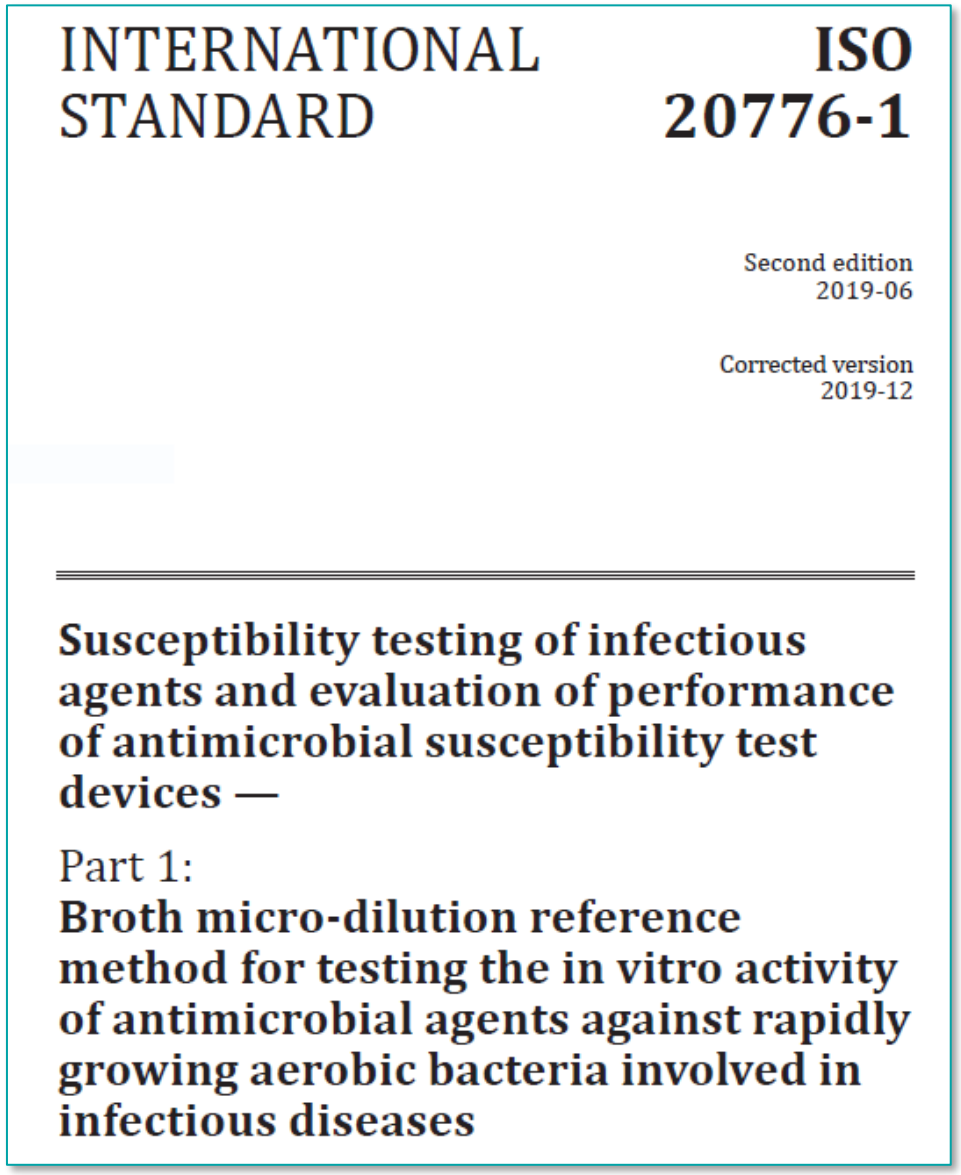
High-throughput screening

Assay development

Target validation

Hughes D, Karlén A. Discovery and preclinical development of new antibiotics. Ups J Med Sci. 2014; 119:162-9.

Pitfalls and opportunities of susceptibility testing in clinical trials of new antibiotics



INTERNATIONAL STANDARD

ISO 20776-1

Second edition
2019-06

Corrected version
2019-12

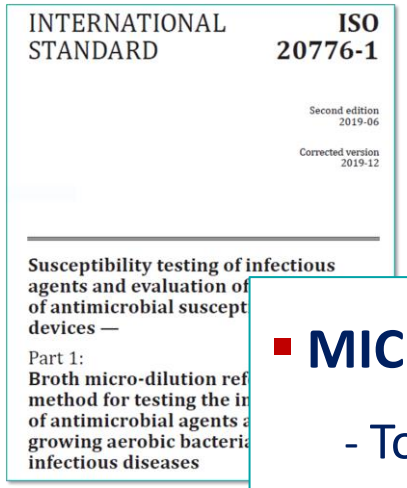
Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices —

Part 1:
Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases

Minimum inhibitory concentration (MIC)

Broth microdilution used as
reference method
by
breakpoint committees (EUCAST and CLSI)

Pitfalls and opportunities of susceptibility testing in clinical trials of new antibiotics


INTERNATIONAL STANDARD

ISO 20776-1

Second edition 2019-06

Corrected version 2019-12

Susceptibility testing of infectious agents and evaluation of of antimicrobial suscept devices —

Part 1:
Broth micro-dilution ref method for testing the in of antimicrobial agents a growing aerobic bacteri infectious diseases

- **MIC** reflects the activity of the drug under specific standardized test conditions and is used:
  - To guide **clinical use of antimicrobials** taking also into account other factors (PK/PD, resistance mechanisms, patient's characteristics, …)
  - To define **epidemiological cut-off values (ECOFFs / ECV)** to discriminate wild-type from non wild-type bacterial populations (without and with acquired resistance mechanisms, respectively)
  - **To compare activity of new antimicrobials** and **correlate with clinical outcomes in clinical trials**
  - In **susceptibility/resistance surveillance studies** in preclinical development and post-marketing authorization
  - To **calibrate alternative methods** (disc-diffusion, gradient tests, automatic susceptibility testing systems and new rapid system)

Reference value of in vitro activity in the drug discovery and in clinical trials

Antimicrobial drug discovery: *in vitro* activity

- **Antimicrobial hit**
  - A molecule that binds to a target that is important to a pathogen of interest and produce an antibacterial effect
- ***In vitro* antibacterial effect**
  - Use the **minimum inhibitory concentration (MIC)** as a reference value, reflecting the minimum concentration that inhibits the visible bacterial growth
  - MIC measure **inhibition of growth** (bacteriostatic) rather than **killing effect** (bactericidal)
  - Initially tested against a **short panel of isolates** (6-8) that might act as **biosensors** to initially define the antibacterial spectrum and potency
    - normally includes strains with no resistance mechanisms
    - suggested to include "hyper-susceptible" strains (i.g. defective efflux pumps: *E. coli* ΔrfaC/ΔtolC)
    - inclusion of a secondary panel with well characterized resistant strains
  - **Optimal MIC** for an antimicrobial hit is **≤1 mg/L**
  - MIC distribution of contemporary isolate to calculate MIC_{90} values (and ECOFF / ECV)

Hughes D, Karlén A. Ups J Med Sci. 2014; 119:162-9.; Muñoz KA, Hergenrother PJ. Acc Chem Res. 2021 16; 54:1322-33

Antimicrobial drug discovery: MICs of new antimicrobials

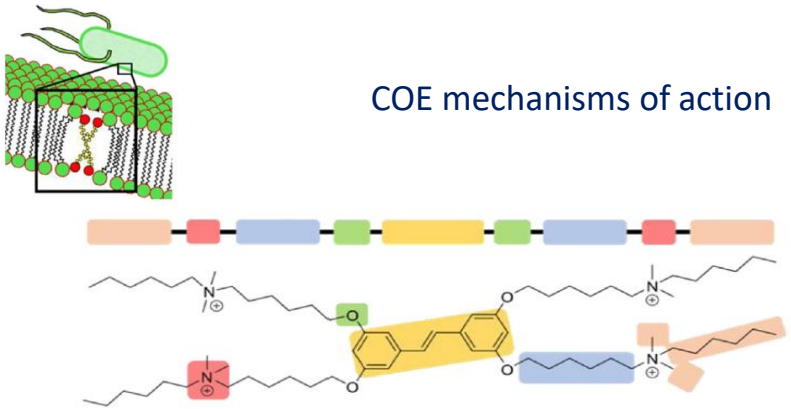
COE antibacterial activity (broth microdilution) against clinical isolates

Pathogen	Antibacterial activity MIC (µg/mL)						
	AZM		CIP		COE2-2hexyl	DSBN	COE2-3C
Gram-negative							
A. baumannii ATCC 19606	64	R	1	S	4	128	128
E. coli ATCC 25922	4	S	0.008	S	2	128	8
K. pneumoniae ATCC 13883	8	S	0.031	S	2	128	32
K. pneumoniae (CRE)[a]	256	R	128	R	4	256	64
N. gonorrhea ATCC 700825	0.031	S	0.002	S	0.5	4	0.5
N. gonorrhea ATCC 49226	0.063	S	0.002	S	1	16	1
P. aeruginosa ATCC 10145	128	R	0.125	S	8	>256	256
S. flexneri ATCC 29903	2	S	0.016	S	2	64	8
S. Typhimurium 14028	4	S	0.016	S	2	64	8
Y. pseudotuberculosis YPIII	8	S	0.008	S	1	128	16
Gram-positive							
MSSA Newman	1	S	0.25	S	1	1	1
MSSA blood isolate (MT3305)	>512	R	0.125	S	1	2	0.5
MRSA CA-USA300	128	R	0.5	S	1	1	0.5
MRSA blood isolate[a] (MT3302)	128	R	0.5	S	1	1	0.5
MRSA wound isolate (MT3315)	1	S	16	R	1	2	1
S. pneumoniae D39	0.031	S	0.5	I	4	32	8
S. pneumoniae Daw 1	8	R	0.5	I	8	32	4

MICs and susceptibility designations were determined by broth microdilution[26–28] ($n \geq 9$). S, susceptible; I, intermediate; R, resistant; AZM, azithromycin; CIP, ciprofloxacin; DSBN, distyrylbenzene oligoelectrolyte. [a]Bacterial isolate derived from a patient refractory to antibiotic therapy.

Conjugated oligoelectrolytes (COE)

- Small **amphiphilic molecules** sharing a modular structure that spontaneously interact with lipid bilayers
- Initially designed to insert into bacterial membranes functioning as electron transporters
- Some derivatives have antibacterial activity due to specific effects on membrane-associated functions (e.g. septation, motility, ATP synthesis, …)



COE mechanisms of action

Heithoff DM et al. EBioMedicine 2023 Feb 14:104461

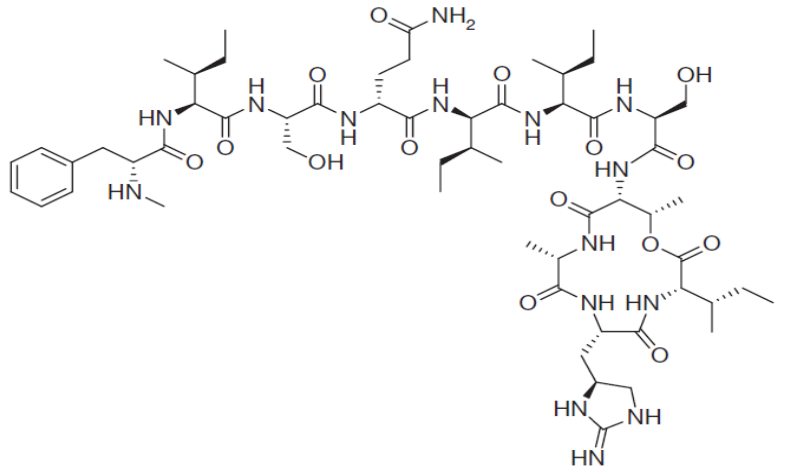
Antimicrobial drug discovery: MICs of new antimicrobials

Teixobactin antibacterial activity (broth microdilution*) against clinical isolates

Organism and genotype	Teixobactin MIC (μg ml^{-1})
S. aureus (MSSA)	0.25
S. aureus + 10% serum	0.25
S. aureus (MRSA)	0.25
Enterococcus faecalis (VRE)	0.5
Enterococcus faecium (VRE)	0.5
Streptococcus pneumoniae (penicillin^{R})	≤ 0.03
Streptococcus pyogenes	0.06
Streptococcus agalactiae	0.12
Viridans group streptococci	0.12
B. anthracis	≤ 0.06
Clostridium difficile	0.005
Propionibacterium acnes	0.08
M. tuberculosis H37Rv	0.125
Haemophilus influenzae	4
Moraxella catarrhalis	2
Escherichia coli	25
Escherichia coli (*asmB1*)	2.5
Pseudomonas aeruginosa	>32
Klebsiella pneumoniae	>32

*Cation adjusted MH broth supplemented with 0.002% polysorbate-80

- Depsipeptide that inhibit cell wall synthesis:
  - binds to lipid II (precursor of peptidoglycan)
  - form large supramolecular fibrils upon lipid II binding
- Bactericidal effect
- Tested with 0.002% polysorbate-80 to prevent drug binding to plastic surfaces



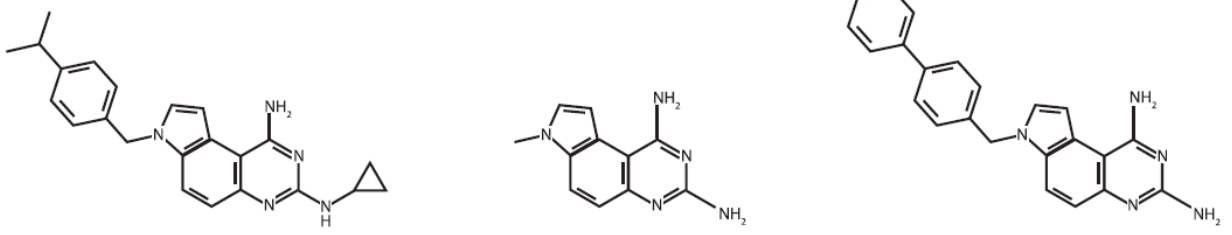
Teixobactin

Ling et al. Nature 2015; 517(7535):455-9

Antimicrobial drug discovery: MICs of new antimicrobials

Antibacterial activity (broth microdilution) of SCH-79797 and derivatives

Isolate	SCH-79797	IRS-10	IRS-16
E. coli lptD4213	3.13	0.78	0.02
B. subtilis 168	3.13	6.25	0.02
S. aureus MRSA	6.25	> 25	1.56
E. faecalis	2.00	N.D.	0.02
N. gonorrhoeae	4.00	N.D.	0.03
V. cholerae	6.25	N.D.	0.40



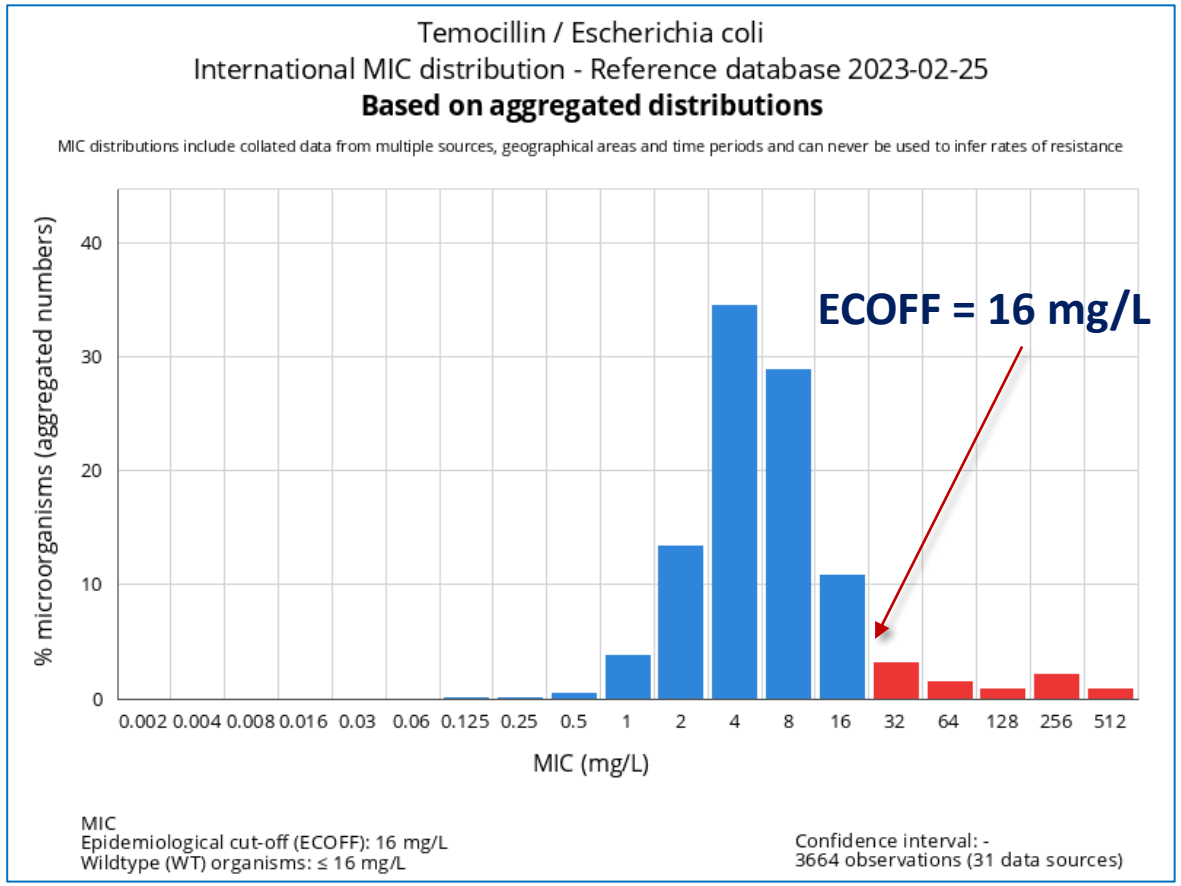
SCH-79797 IRS-10 IRS-16

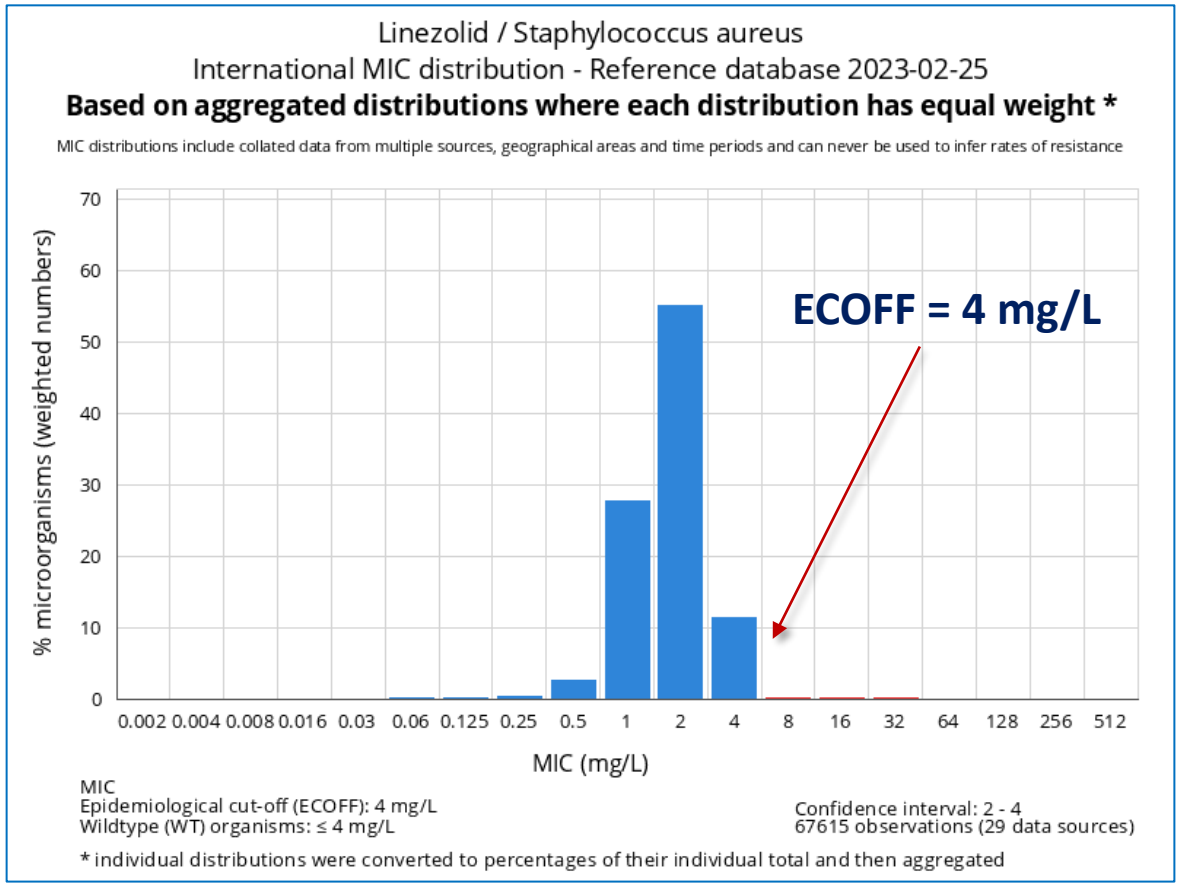
Irresistin-16

- SCH-79797 derivative with a dual mechanisms of action:
  - Interaction with folate metabolism (inhibition of dihydrofolate reductase)
  - Disruption of both membrane potential and integrity
- Bactericidal activity against both Gram-negative and Gram-positive bacteria

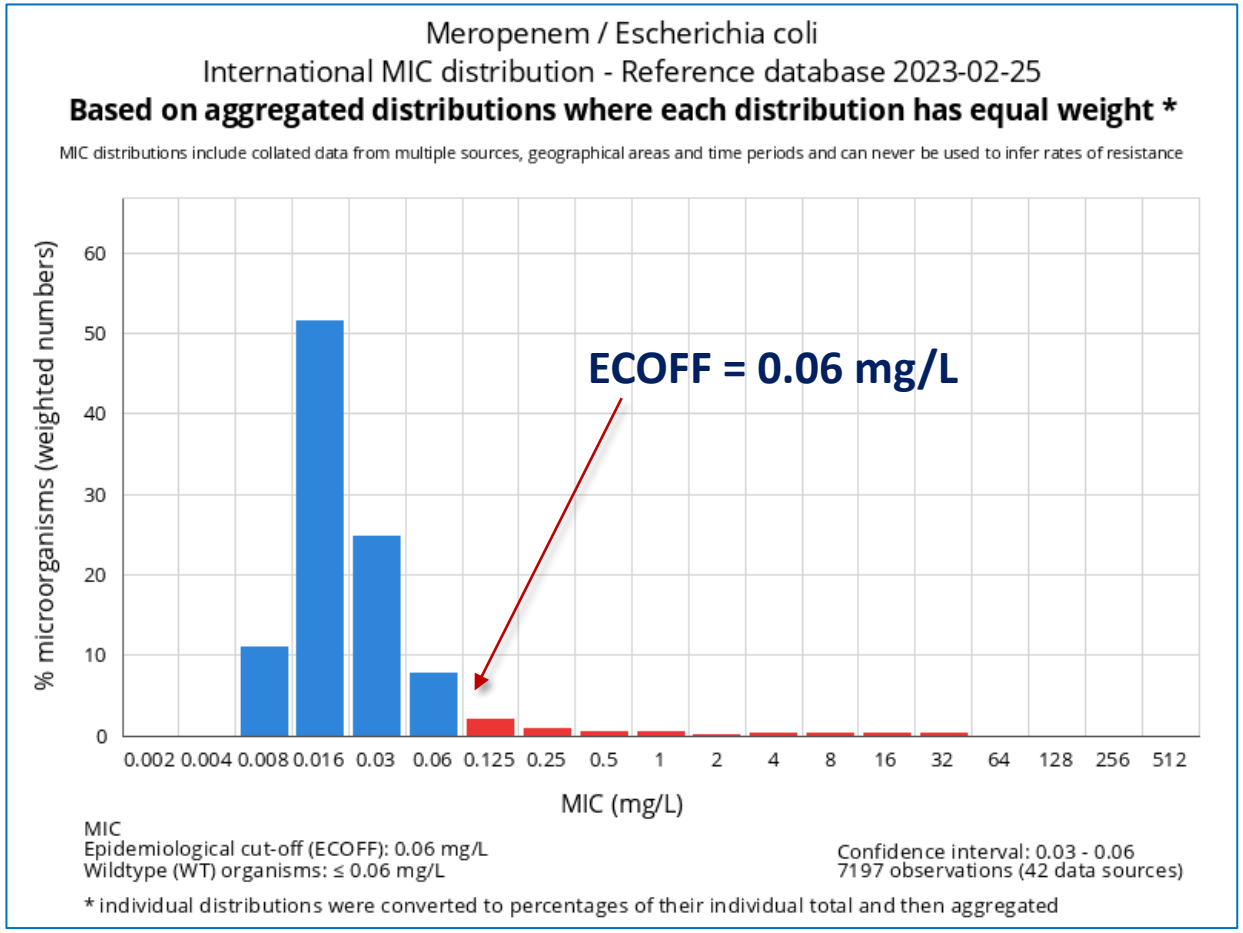
Martin JK et al. Cell 2020; 181:1518-32.e14.

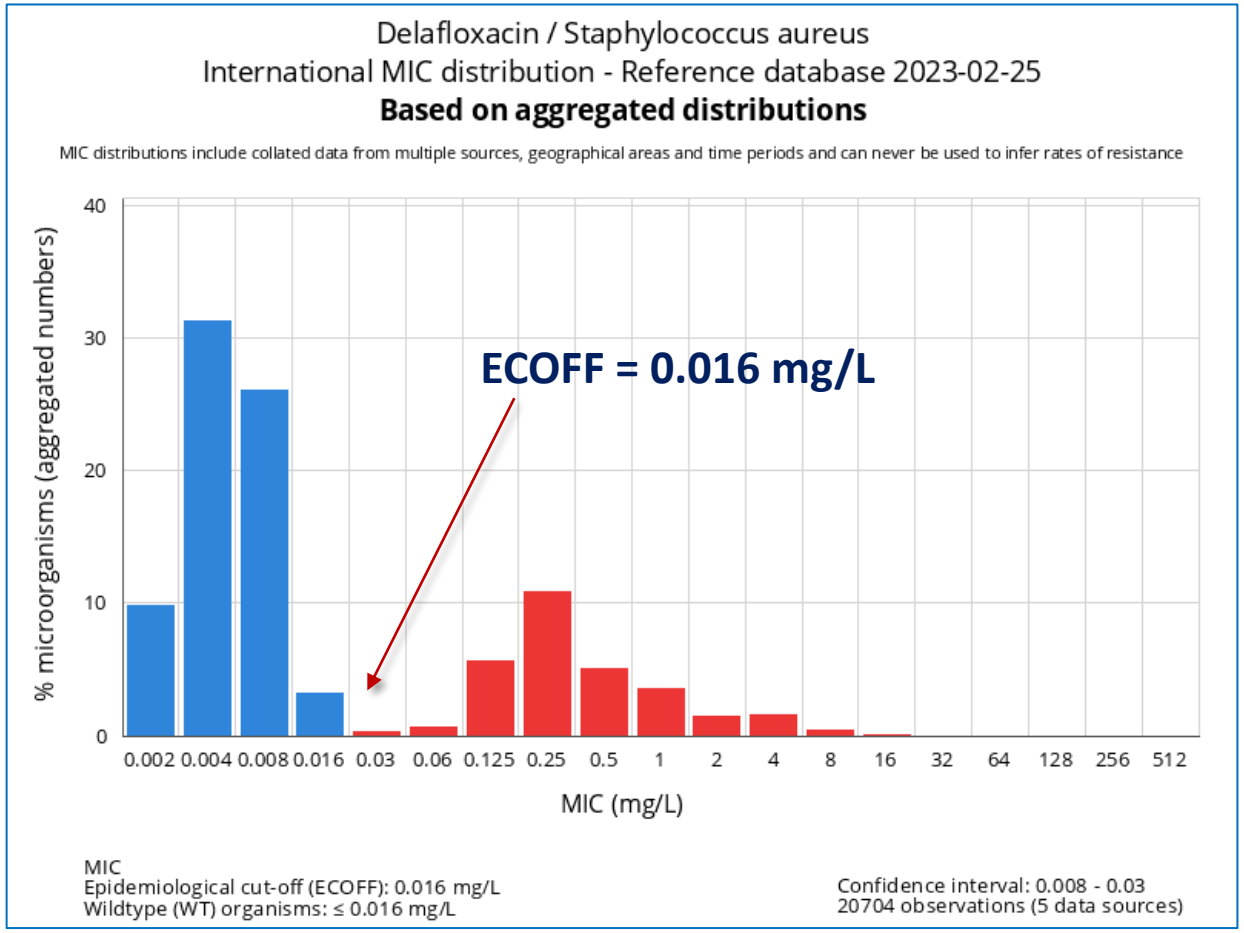
MIC and epidemiological cut-off values (ECOFF / ECV)





MIC and epidemiological cut-off values (ECOFF / ECV)

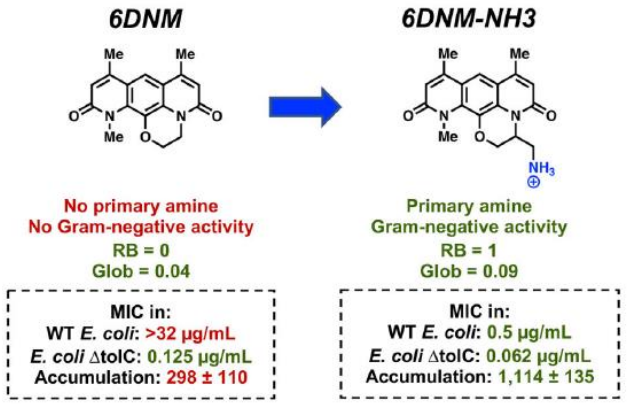




https://mic.eucast.org/search/

Antimicrobial susceptibility testing: enhancing permeability

- **MIC testing might fail when antimicrobials does not penetrate bacterial cell/outer membrane** (mainly in Gram-negatives) despite outstanding activity in biochemical assays yielding very high MICs
- Chemistry modification strategies facilitates enhancement of this activity allowing penetration and avoiding natural efflux pumps activity
- Measurement of compound accumulation rather than antibacterial activity should be initially tested to facilitate structure-activity relationship



eNTRy characteristics: presence of a ionizable **N**itrogen
enhanced **T**hree-dimensionality (measured as globularity)
Rigid structure (low number of rotatable bonds)

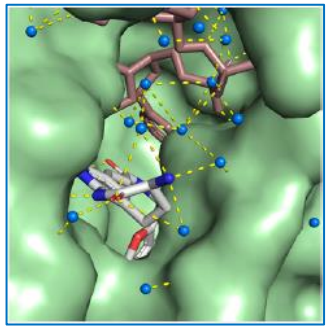
Comparative antimicrobial activity of the DNA gyrase inhibitor 6DNM-NH3

	Gram-positive		Gram-negative																				
	S. aureus		*E. coli*								*A. baumannii*						*K. pneumoniae*			*E. cloacae*			*P. aeruginosa*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
6DNM	1	0.06	>32	>32	>32	>32	>32	>32	>32	>32	16	>32	>32	16	16	>32	>32	>32	>32	32	32	>32	>32
6DNM-NH3	0.5	0.03	0.5	0.5	0.5	2	4	8	16	4	4	2	2	2	8	16	1	4	8	0.5	2	4	16
Cipro	0.125	32	0.008	0.004	0.008	64	16	>64	64	>64	0.5	32	16	16	64	64	<0.03	1	>64	<0.03	1	16	0.25

Modified from Muñoz KA, Hergenrother PJ. Acc Chem Res. 2021; 54:1322-33.

Antimicrobial susceptibility testing: enhancing permeability

- **Fabimycin:**
  - FabI inhibitor (enzyme catalyzing the rate-determining step in bacterial fatty acid biosynthesis)
  - Derived from Debio-1452 lead compound
  - Enhanced activity against ESKAPE Gram-negative pathogens and *Staphylococcus aureus*



Comparative antimicrobial activity of Fabimycin

	Reference strains			*E. coli*		*K. pneumoniae*				*A. baumannii*			
	S. aureus 29213	*E. coli* ΔtolC	*E. coli* MG1655	AR-0085	AR-0048	AR-0066	AR-0113	AR-0560	BAA-2472	AR-0033	AR-0273	AR-0299	AR-0313
Debio-1452	0.008	0.062	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Debio-1452-NH3	0.031	0.062	4	16	32	32	32	32	16	32	32	32	32
(S)-7, fabimycin	0.004	0.016	2	1	2	4	4	4	4	4†	4	2†	4†

Modified from Parker EN et al. ACS Cent Sci. 2022 Sep 28;8(9):1362

Antimicrobial susceptibility testing: MIC variability

- ISO-20776 recognized **MIC variability**
  - *Careful control and standardization are required for intra- and inter-laboratory reproducibility of broth MIC test*
  - *The MICs generally span 2-3 doubling dilutions with a dominant central values. For quality control (QC) strains can have a 4-dilution range*
  - Acceptable criteria for reproducibility:
    - one dilution from the mode for 95% of cases or range of at least **two 2-fold dilutions**

- **MIC:** endpoint representing the bacterial growth and bacteriostatic/bactericidal effects over time
- **Factor affecting MIC values**
  - **assay variation within and between laboratories (random and systematic errors)**
    - MH broth, pH conditions and supplements
    - Inoculum preparation and inoculum used
    - Atmosphere and incubation temperature
    - Time of incubation
    - MIC reading and observer (technician)
  - **biological variation** (variation between strains)

	$\log_2$ standard deviation around the $\log_2$
Intralaboratory	≈0.3 - 0.5
Interlaboratory	≈0.5 - 1

Turnidge J, Paterson DL. Clin Microbiol Rev 2007; 30:391-408; Mouton JW et al. J Antimicrob Chemother. 2018; 73:564-8; Mouton JW et al. J Antimicrob Chemother 2018; 73:2374-9

Antimicrobial susceptibility testing: MIC variability

Diagnostic Microbiology and Infectious Disease 78 (2014) 263–267

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Characterization of variables that may influence ozenoxacin in susceptibility testing, including MIC and MBC values☆,☆☆

Marta Tato [a], Yuly López [b], Maria Isabel Morosini [a], Ana Moreno-Bofarull [a], Fernando Garcia-Alonso [c], Domingo Gargallo-Viola [c], Jordi Vila [b,d], Rafael Cantón [a,*]

Comparison of ozenoxacin, ciprofloxacin and levofloxacin MIC values using **different pH conditions** and that of the standard (pH 7.4)

Antibiotic and test conditions	Number of isolates[a]	No. of strains with $\log_2$ difference							% agreement[b]
		≥−3	−2	−1	0	1	2	≥3	± 1 $\log_2$ dilutions
Ozenoxacin									
pH 5.4	55		5	18	6	24	2		87.3
pH 6.4	55	2	16	20	17				67.3
pH 7.4	55				55				100
pH 8.4	55				1	5[c]	20	30	10.9
Ciprofloxacin									
pH 5.4	48					4	14	30	8.3
pH 6.4	48			2	5	27	8	6	70.8
pH 7.4	48				48				100
pH 8.4	48	1	3	13	14	9	3	5	75
Levofloxacin									
pH 5.4	52					4	30[d]	18	7.7
pH 6.4	52				12	27	11	2	75
pH 7.4	52				52				100
pH 8.4	52			2	13	30	7[e]		86.5

Antimicrobial susceptibility testing: MIC variability

Diagnostic Microbiology and Infectious Disease 78 (2014) 263–267

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Comparison of ozenoxacin, ciprofloxacin and levofloxacin MIC values using **different inoculum and its preparation** and that of the standard (10^5 ufc/ml)

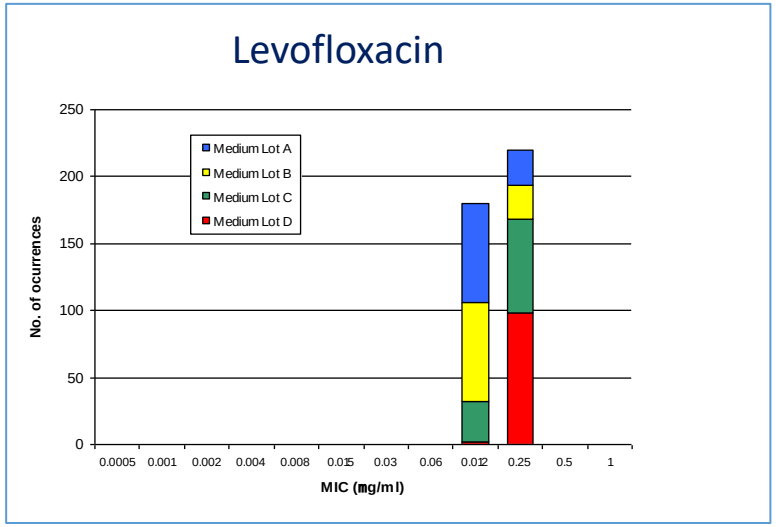
Antibiotic and test conditions	Number of isolates*	Nº of strains with log2 difference						
		≥-3	-2	-1	0	1	2	≥3
Ozenoxacin								
10^3 CFU/ml inoc.	15			7	8			
10^5 CFU/ml inoc.	15				15			
10^7 CFU/ml inoc.	15							15
Direct colony suspension	15				15			
Early growth-phase broth	15			7	7	1		
Overnight broth	15			4	9	2		
24 hours incubation	15				15			
48 hours incubation	15				9	6		
Ciprofloxacin								
10^3 CFU/ml inoc.	14			7	6	1		
10^5 CFU/ml inoc.	14				14			
10^7 CFU/ml inoc.	14							14
Direct colony suspension	14				14			
Early growth-phase broth	14			5	8	1		
Overnight broth	14			4	7	3		
24 hours incubation	14				14			
48 hours incubation	14				12	2		
Levofloxacin								
10^3 CFU/ml inoc.	15			5	10			
10^5 CFU/ml inoc.	15				15			
10^7 CFU/ml inoc.	15					1**		14
Direct colony suspension	15				15			
Early growth-phase broth	15			2	11	2		
Overnight broth	15			3	10	2		
24 hours incubation	15				15			
48 hours incubation	15				12	3		

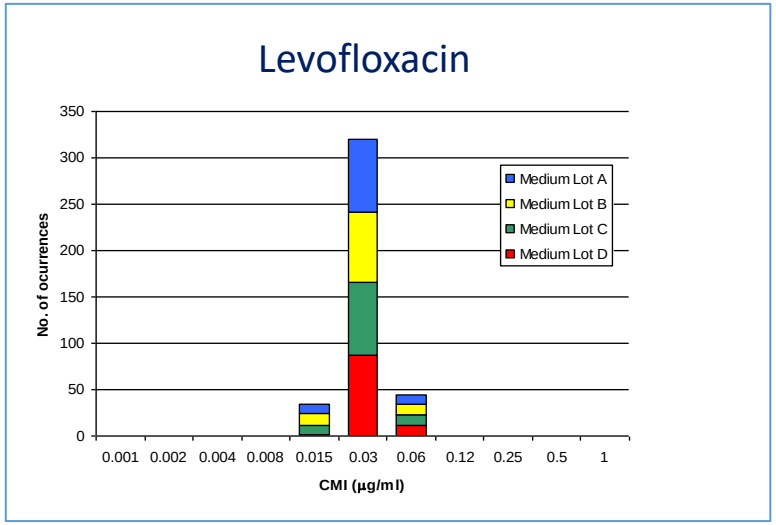
* Isolates with MICs ≥ 16 were excluded from the analysis; ** ≥1-$\log_2$ of difference

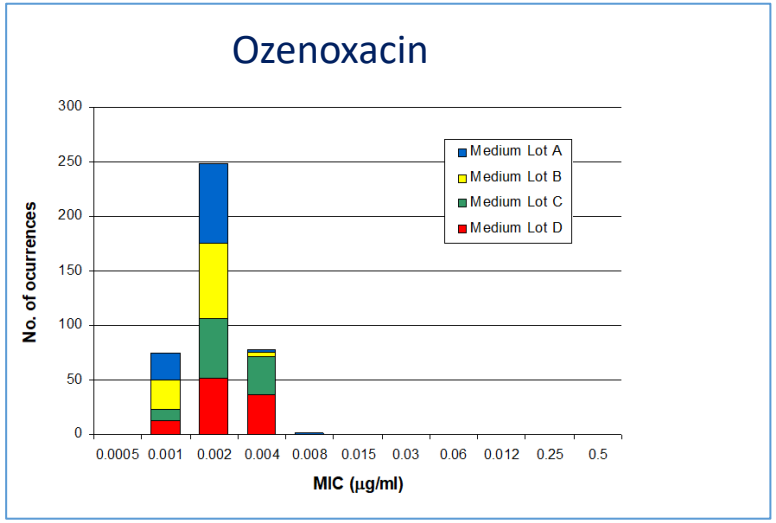
Antimicrobial susceptibility testing: MIC variability

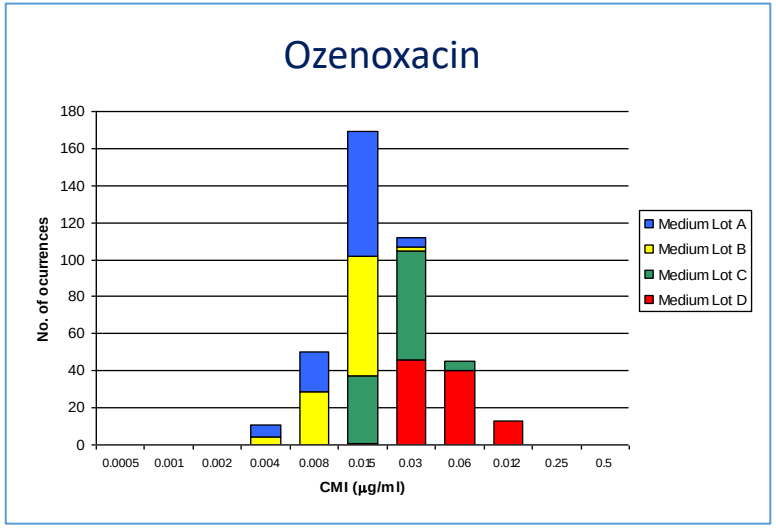
S. aureus ATCC 29213

E. coli ATCC 25922









Ozenoxacin M23 QC study

Multicenter study
10 laboratories

MIC values/strain
10 labs x 10 replicates x 4 media lots
= 400 MICs

Antimicrobial susceptibility testing: supplements to the testing media

Current antimicrobial agents with supplements in testing media

Antibiotic	Supplements	Microorganisms	Comments
Oxacillin	MH + ClNa 2% (microdilution and agar dilution)	*Staphylococcus* spp.	Improves detection of methicillin resistance in heteroresistant isolates
Daptomicin	MH Ca^{2+} 50 mg/L (microdilution)	Gram-positives	Improves interaction with membrane phospholipids
Oritavancin Dalbavancin	MH + polysorbate-80 (microdilution)	Gram-positives	Prevents drug binding to plastic surfaces
Fosfomycin	MH + 25 mg/L glucose-6-phosphate (G-6P) (agar dilution)	Enterobacterales, *Staphylococcus* spp.	Regulate competition of fosfomycin with G-6P during bacterial entry
Cefiderocol	Fe $^{2+}$ depleted MH broth and Ca^{2+}, Mg^{2+} and Zn^{2+} supplemented	Gram-negatives	Improves penetration binding residual Fe^{2+} (≈0.01 mg/L) and using iron transporters

CLSI. 2022. Performance standards for antimicrobial susceptibility testing, 30th ed. CLSI document M100. Clinical and Laboratory Standards Institute, Wayne, PA
EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. Version 13.0, 2023. http://www.eucast.org; Guidance document on broth microdilution testing of cefiderocol. Dec 2020. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Guidance_documents/Cefiderocol_MIC_testing_EUCAST_guidance_document_201217.pdf

Process of setting breakpoints: *The EUCAST approach*

- **Relevant factors in setting breakpoints** for antimicrobial agents

EUCAST SOP 1.4, 2 December 2021

1. Available formulation
2. Dosage (standard and maximum dosing)
3. Clinical indications and target organisms
4. Full MIC distributions for individual species
5. Pharmacokinetic (PK) data (humans): Cmax, AUC, T½, protein binding, Vd,…
6. Pharmacodynamic (PD) data
7. Modeling processes (Monte Carlo)
8. Clinical data relating outcomes to MIC values
9. Resistance mechanisms and their clinical significance, MIC for organisms expressing resistance mechanisms

→ MIC is a relevant value

AMR surveillance programs for the industry based on MIC values

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

19 May 2022
CPMP/EWP/558/95 Rev 3
Committee for Medicinal Products for Human Use (CHMP)

Guideline on the evaluation of medicinal products indicated for treatment of bacterial infections

4. Microbiological investigations

4.1. Non-clinical assessment of anti-bacterial activity

4.1.1. Spectrum of antibacterial activity

Every effort should be made to elucidate the mechanism of action of new antibacterial agents.

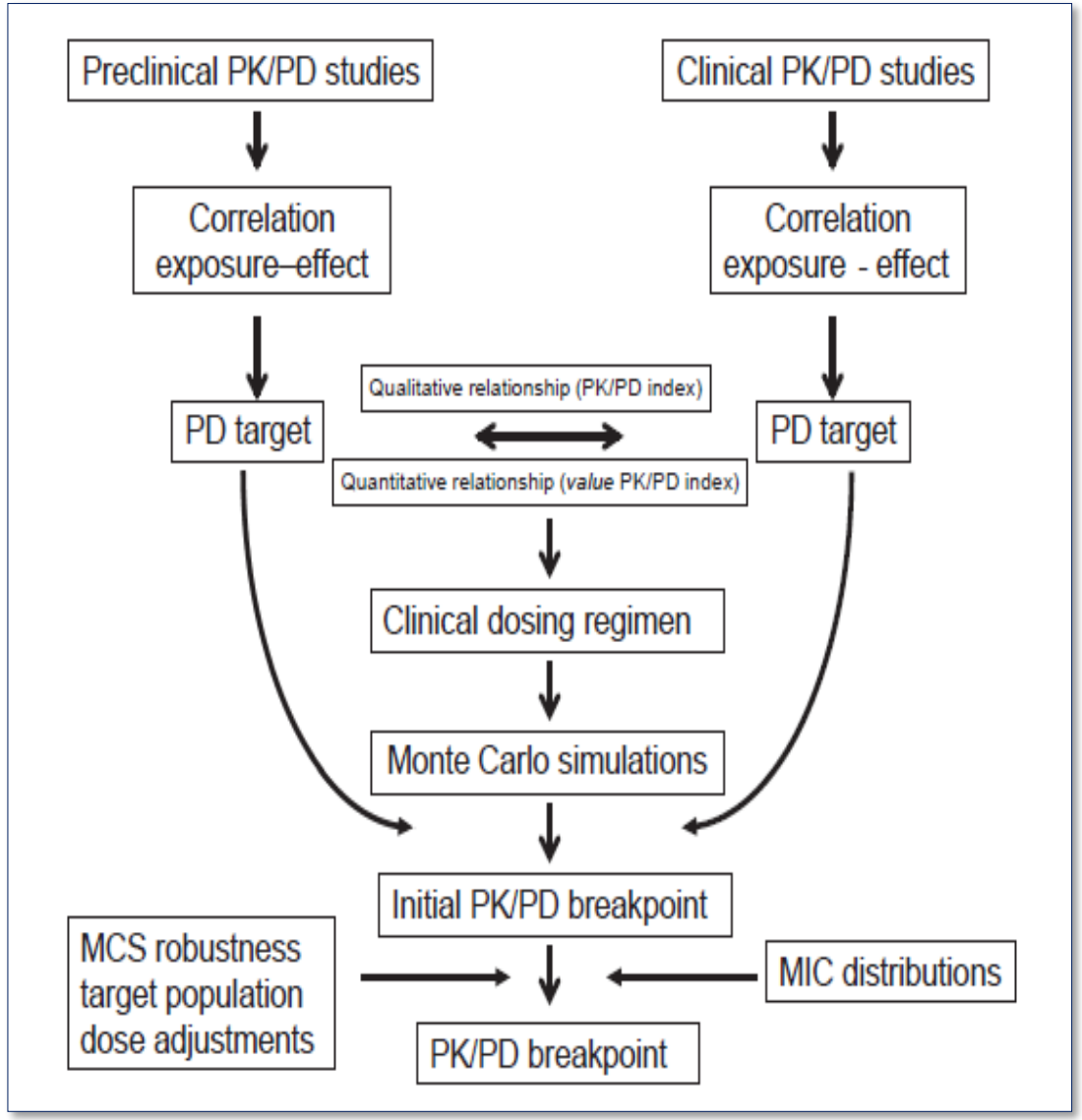
The methods used for determination of minimum inhibitory concentrations (MICs) should be described in detail and justified. Appropriate active controls should be included. The MIC_{50}, MIC_{90} and MIC range should be presented by species and, when appropriate, by sub-group (e.g. with and without specific resistance mechanisms) in tabular form. The MIC distributions should be presented in tables and in histograms.

The in-vitro activity of previously unlicensed antibacterial agents and of combinations of beta-lactams and beta-lactamase inhibitors (BL/BLIs; see further in section 4.1.3) should be determined against clinical isolates obtained within 5 years prior to filing an application dossier. These isolates should belong to pathogenic species relevant to the indication(s) sought and should be sourced from various countries and regions, including a representative sample from within the EU. For commonly encountered pathogens it should be possible to test several hundred isolates of each species, including representative numbers that demonstrate resistance to individual and multiple classes of antibacterial agents. For rare pathogens and strains with rarely encountered mechanisms of resistance or patterns of multi-drug resistance it is recommended that at least 10 organisms of each species or with each resistance mechanism/pattern are tested whenever possible.

Guideline on the evaluation of medicinal products indicated for treatment of bacterial infections
https://www.ema.europa.eu/en/evaluation-medicinal-products-indicated-treatment-bacterial-infections-scientific-guideline

Process of setting breakpoints: *The EUCAST approach*

PK/PD breakpoints



- Definition of the relationship between a ***PK/PD index*** and response to treatment (***pharmacodynamic target***)

  - *PK/PD indexes:*

    *f*AUC/MIC, Cmax/MIC, *f*T>MIC

  - *Pharmacodynamic target:*

    Minimum value aimed when treating patients based on preclinical and clinical drug/ microorganism exposure-response relationships

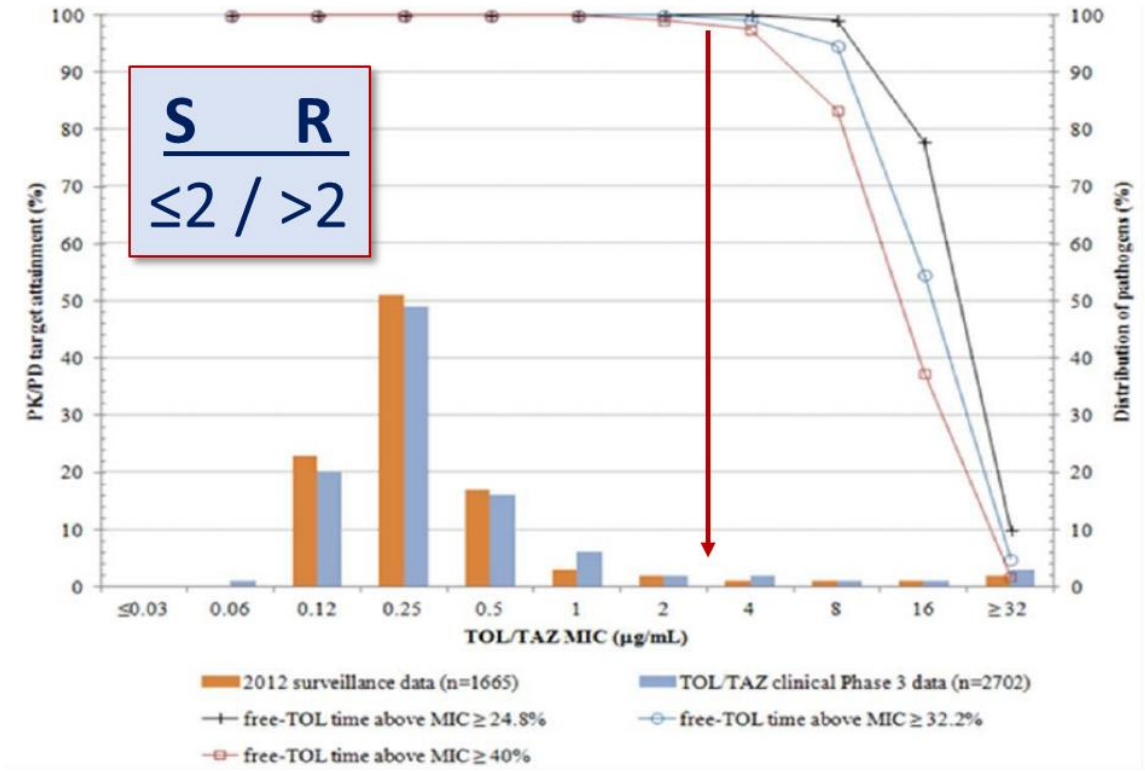
  - Probability of target attainment (PTA) using Monte Carlo Simulations (MCS) to avoid patients' variability

Mouton JW, et al. Clin Microbiol Infect 2012;18:E37-45

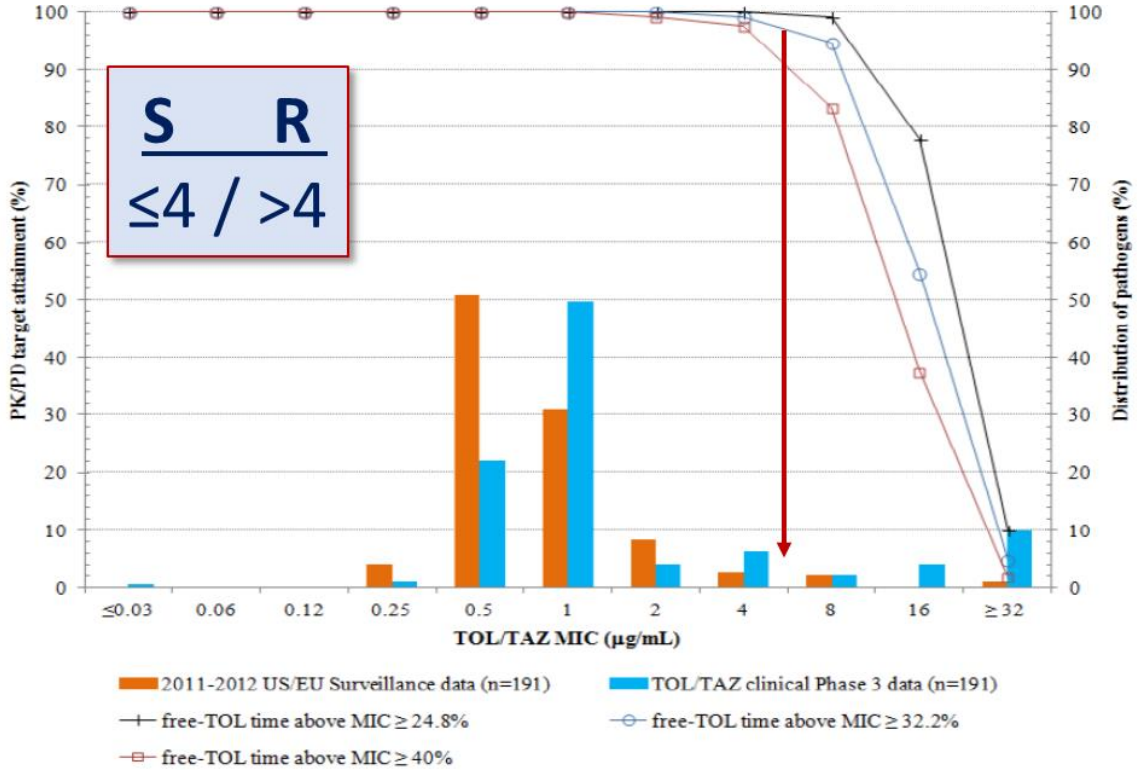
Ceftolozane-tazobactam breakpoints

- **PK models**: 1/0.5 g / 8 h (1-h infusion) for **cIAI and cUTI**, and 2/1 g every 8 h (1-h infusion) for **nosocomial pneumonia**
- ***f*T>MIC** 24.8% for bacteriostasis; 32.2% for a 1-log_{10} reduction and 40% for a 2-log_{10} reduction of CFU and a threshold concentration (C_T) of tazobactam of 1 mg/L determined in the mouse thigh model

Percentage Achieving Free-Drug %T>MIC for Enterobacterales



Percentage Achieving Free-Drug %T>MIC for *Pseudomonas aeruginosa*



EUCAST. Ceftolozane-tazobactam: rationale for the clinical breakpoints, version 1.0, 2020. https://www.eucast.org/documents/rd/

Ceftolozane-tazobactam breakpoints

Probability of target attainment at steady state based on renal function

Renal function category (CrCl, mL/min)	TOL/TAZ, mg (1-h infusion)	C_{max}, µg/mL median (range)	$AUC_{0-\infty}$, µg h/mL median (range)	PTA %*f*T > MIC MIC 2 mg/L			PTA %*f*T > MIC MIC 4 mg/L			PTA %*f*T > MIC MIC 8 mg/L		
				24.8%	32.2%	40.0%	24.8%	32.2%	40.0%	24.8%	32.2%	40.0%
ARC (>150 to ≤200)	1000/500	NA	NA	99	96	92	97	91	82	92	78	64
Normal (>90 to ≤150)	1000/500	72.8 (42–139)	231 (161–311)	100	98	96	99	96	91	96	89	79
Mild impairment (>50 to ≤90)	1000/500	93.4 (75.8–141)	315 (255–342)	100	100	99	100	99	97	99	97	92
Moderate impairment (≥29 to ≤50)	500/250	84.5 (64–136)	589 (306–900)	100	100	100	100	100	99	99	97	94
Severe impairment (≥15 to <29)	250/125	44.2 (30.2–60.6)	509 (429–762)	100	100	100	100	99	98	96	93	88
ESRD with hemodialysis	500/250; 100/50[a]	41.1 (17.5–56.4)	574 (287–1024)	100	100	100	100	100	100	100	100	100

No PK data were available from patients with ARC in the clinical trials, thus no observed values for C_{max} or AUC are available for those patients

ARC augmented renal clearance, $AUC_{0-\infty}$ area under the concentration–time curve extrapolated to infinity, C_{max} maximum concentration, *CrCl* creatinine clearance, *ESRD* end-stage renal disease, *fT > MIC* free-drug time above MIC, *MIC* minimum inhibitory concentration, *NA* not applicable, *PTA* probability of target attainment, *TOL/TAZ* ceftolozane/tazobactam

[a] 500/250 mg loading dose followed by 100/50 mg maintenance doses

Xiao AJ et al. Infect Dis Ther. 2017; 6:137-48

Ceftolozane-tazobactam: clinical data relating outcomes to MIC values

Pathogen Eradication Rates at Test of Cure by CTL-TAZ MIC Value from the Phase 3 cUTI Study in the microbiology evaluation at Test of Cure Population

Ceftolozane/ Tazobactam MIC (µg/mL)	Enterobacteriaceae n/N1 (%)	*E. coli* n/N1 (%)	*E. coli* (CTX-M-14/15) n/N1 (%)	*K. pneumoniae* n/N1 (%)	*K. pneumoniae* (CTX-M-14/15) n/N1 (%)	*P. mirabilis* n/N1 (%)	*P. aeruginosa* n/N1 (%)
0.06	2/2 (100)	2/2 (100)	-	-	-	-	-
0.125	65/73 (89.0)	64/72 (88.9)	-	-	-	-	-
0.25	141/151 (93.4)	128/138 (92.8)	4/5 (80.0)	6/6 (100)	1/1 (100)	3/3 (100)	-
0.5	37/44 (84.1)	22/27 (81.5)	11/14 (78.6)	5/6 (83.3)	0/1 (0)	7/7 (100)	1/2 (50.0)
1	10/13 (76.9)	6/8 (75.0)	4/5 (80.0)	2/3 (66.7)	0/1 (0)	-	1/1 (100)
2	5/8 (62.5)	1/2 (50.0)	0/1 (0)	3/4 (75.0)	2/3 (66.7)	-	-
4	1/3 (33.3)	0/1 (0)	-	-	-	-	-
8	2/3 (66.7)	0/1 (0)	0/1 (0)	2/2 (100)	1/1 (100)	-	-
16	2/3 (66.7)	-	-	1/1 (100)	1/1 (100)	-	1/1 (100)
32	-	-	-	-	-	-	-
64	1/1 (100)	-	-	1/1 (100)	-	-	-
>64	2/5 (40.0)	1/2 (50.0)	0/1 (0)	-	-	-	2/2 (100)

EUCAST. Ceftolozane-tazobactam: rationale for the clinical breakpoints, version 1.0, 2020
https://www.eucast.org/documents/rd/

Pitfalls and opportunities of susceptibility testing in R&D and clinical trials of new antibiotics
Why are MIC needed?

Conclusions

- **MIC** is the **reference value** to define in vitro antimicrobial activity in R&D of new antimicrobials
- There is a (consensus) **ISO document (ISO 20776)** defining its determination with broth microdilution as the reference method
- MICs might fail to define the activity of antimicrobials with problems to penetrate the outer membrane
- Different factors (**assay variation**) might affect MICs such as pH, inoculum and inoculum preparation, incubation time, …)
- Specific supplements are used with some antimicrobials to demonstrate the antibacterial activity
- During the process of setting breakpoints, MICs are used in
  - surveillance studies (pre-clinical development and post marketing authorization)
  - in PK/PD studies
  - in clinical outcome correlations
- MICS are used to monitor development of resistance

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Advancing
Antimicrobial R&D

LIVE WEBINAR

2 March 2023, 17:00-18:30 CET
(10:00-11:30 am CST)

Susceptibility testing in antibacterial drug R&D

GARD P
Global Antibiotic Research & Development Partnership

Pitfalls and opportunities of susceptibility testing in R&D and clinical trials of new antibiotics: Why is MIC needed?

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HOSPITAL RAMÓN Y CAJAL

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