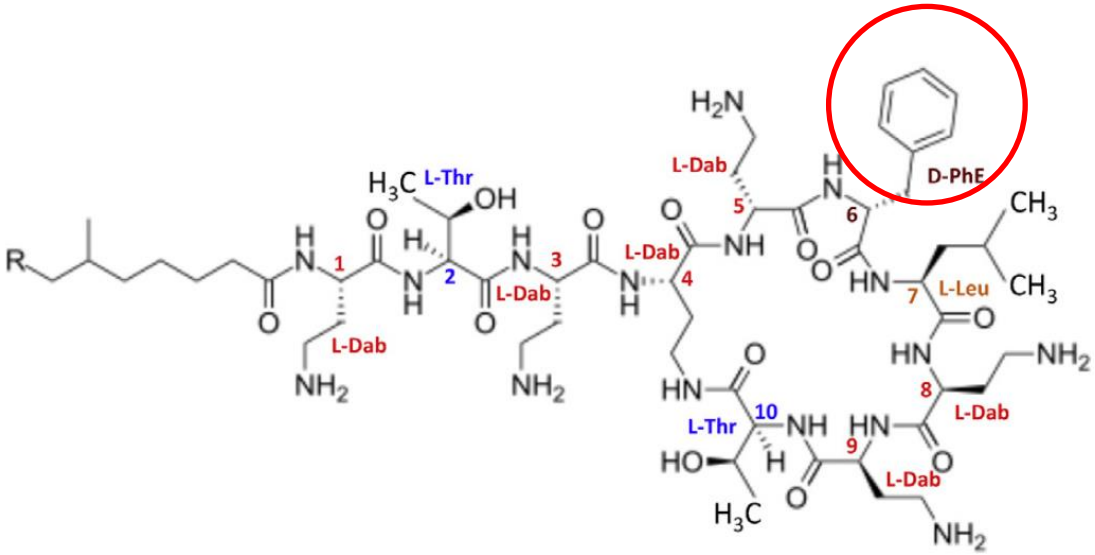
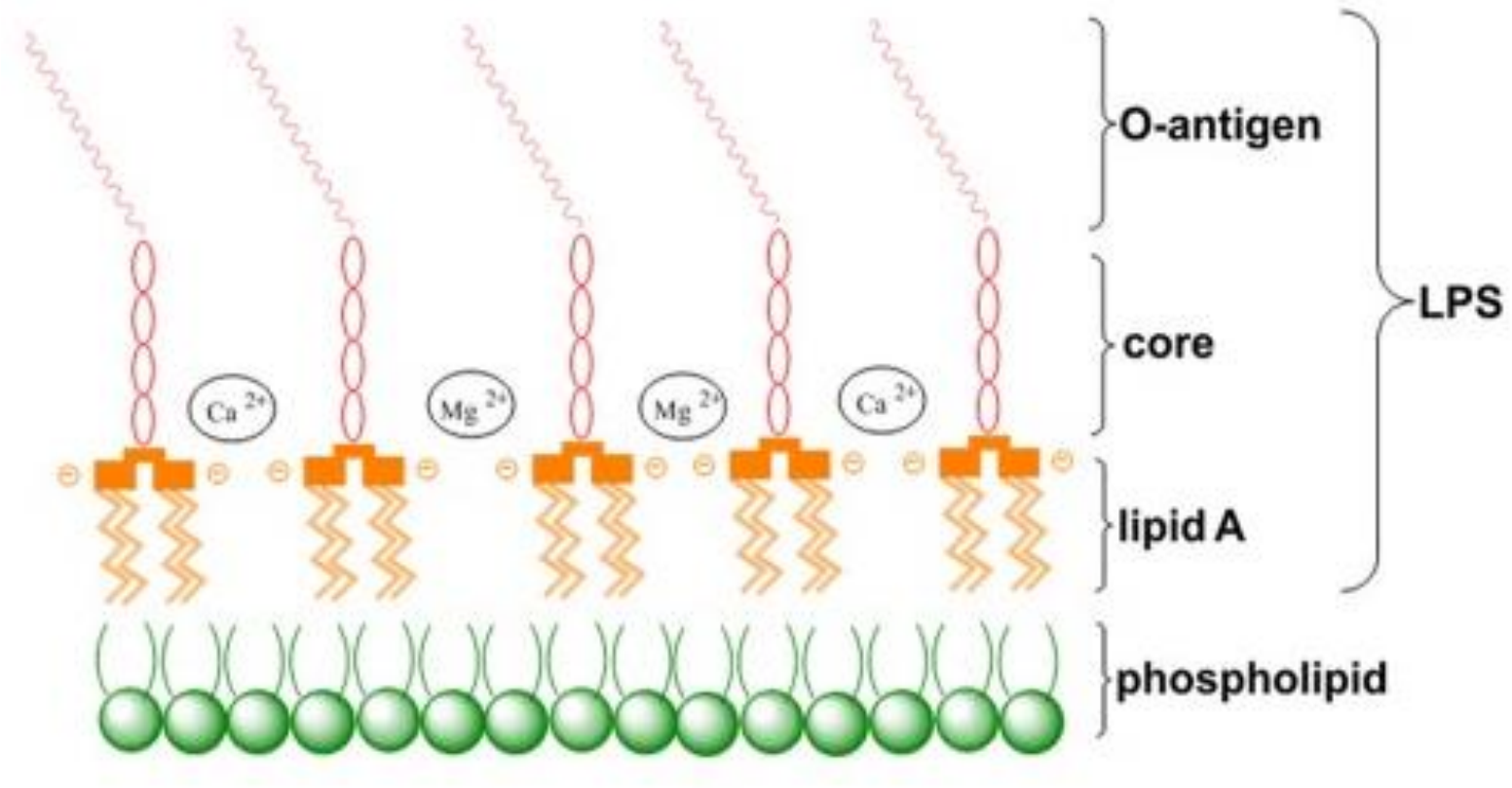
Recomendações atuais para testar a a sensibilidade às polimixinas. Como fazer microdiluição em caldo na rotina de um laboratório de microbiologia?

Jorge Sampaio
Membro do Comitê Gestor do BrCAST – 2016-2018
Sociedade Brasileira de Microbiologia

Polimixina B X Colistina (Polimixina E)

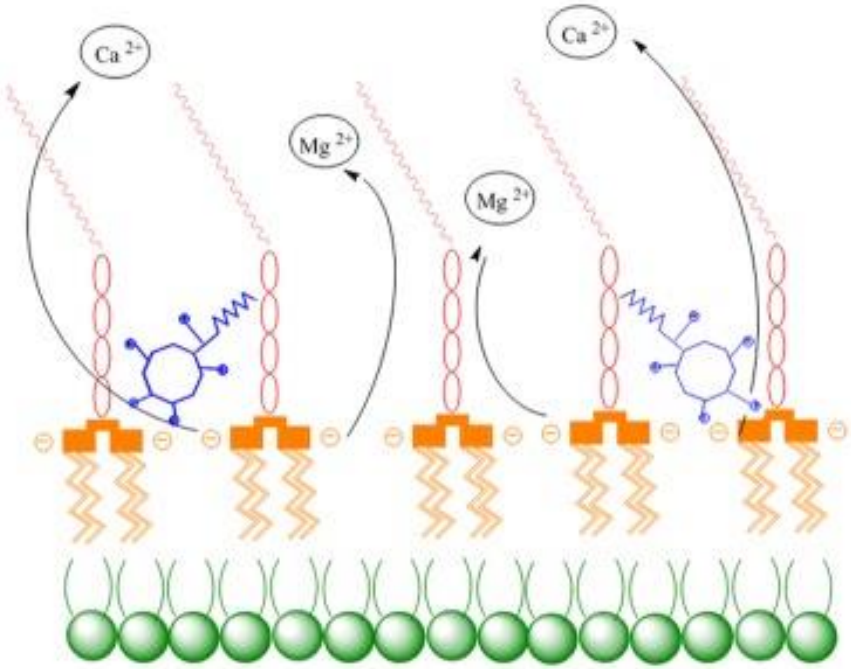


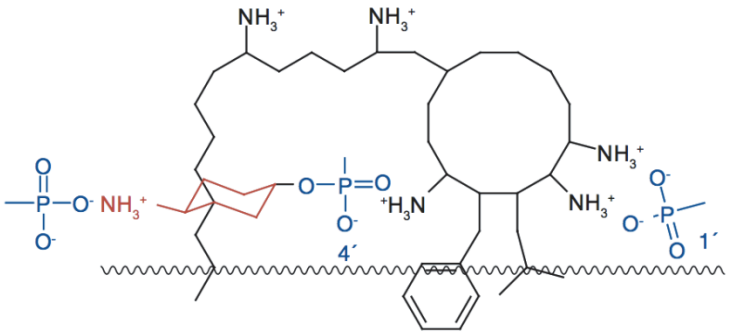
Mecanismo de ação das polimixinas



DERIS, Z. Z. et al. Bioconjugate Chem (2014)

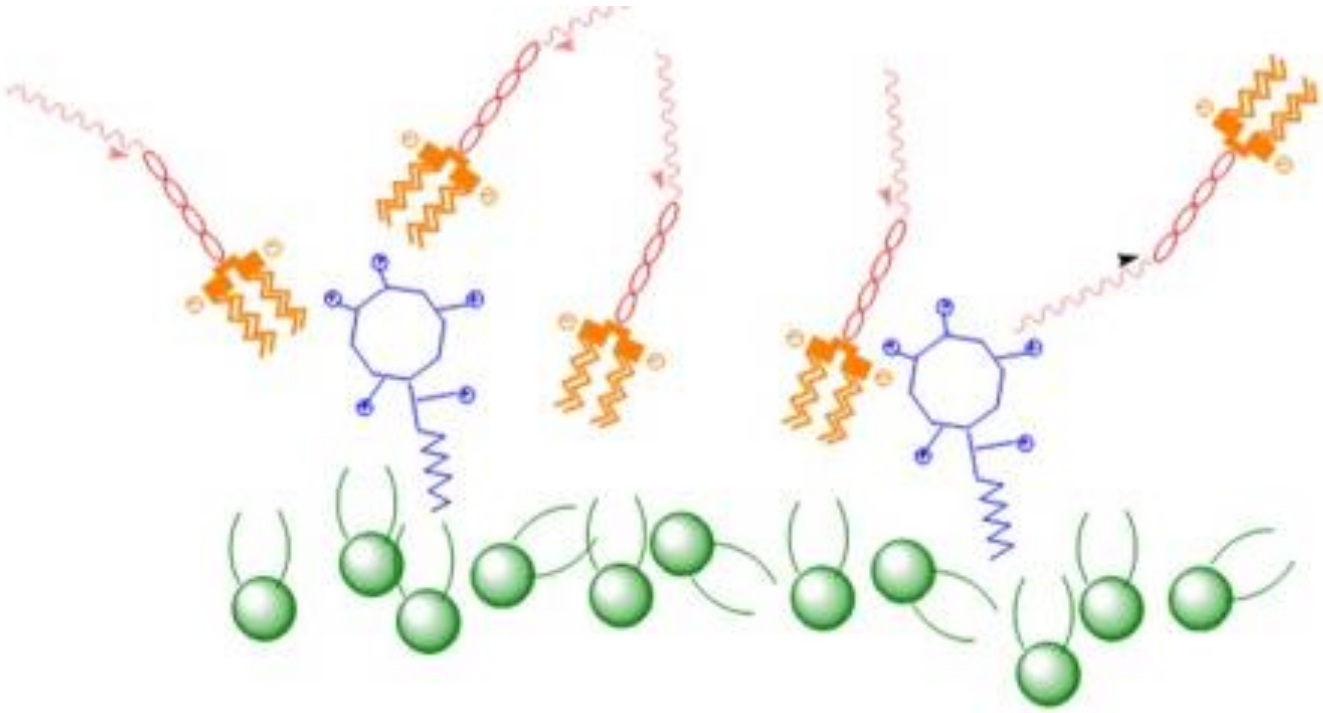
Mecanismo de ação das polimixinas





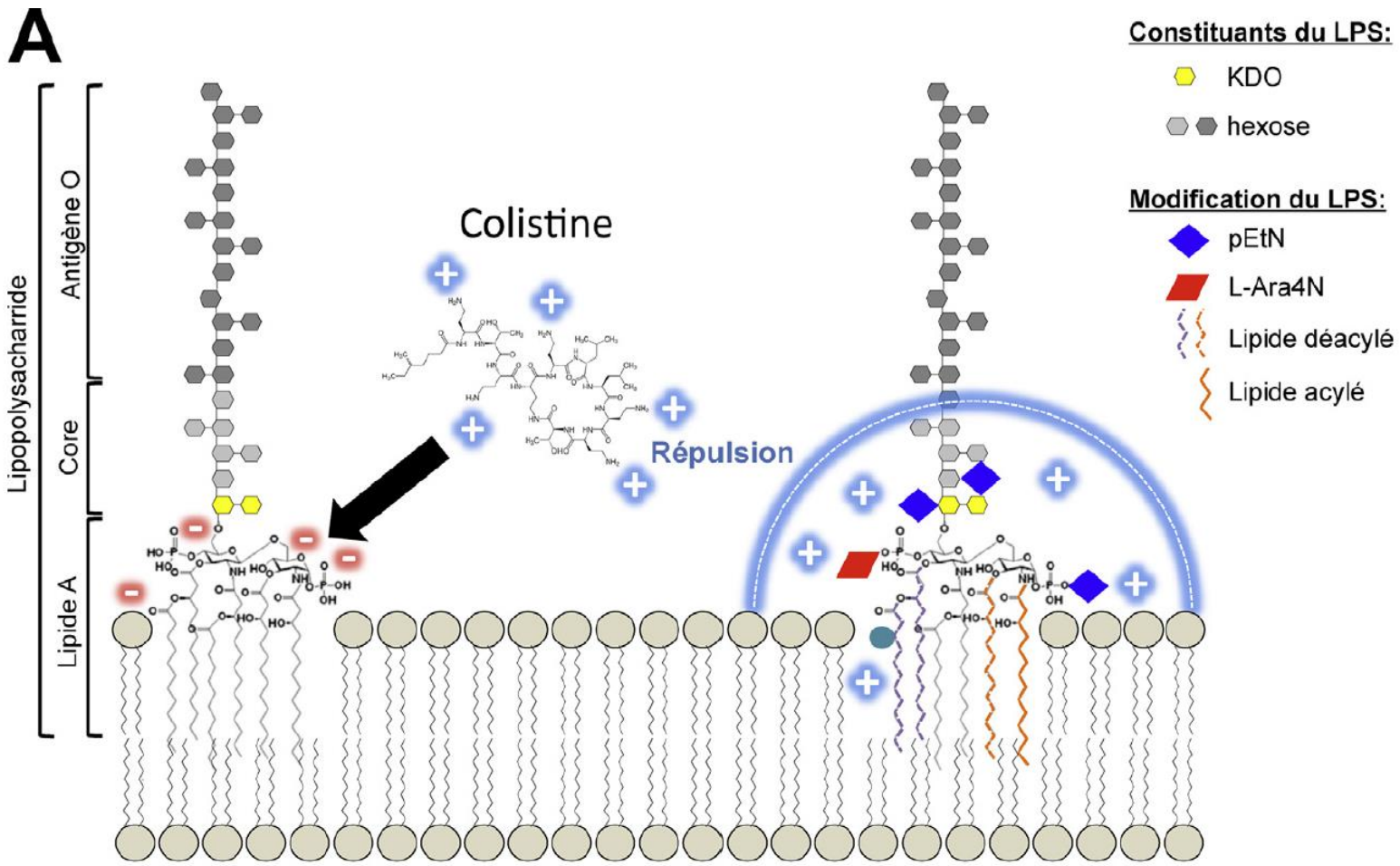
VELKOV, T. et al. Future Microbiol (2013); DERIS, Z. Z. et al. Bioconjugate Chem (2014)

Mecanismo de ação das polimixinas



DERIS, Z. Z. et al. Bioconjugate Chem (2014)

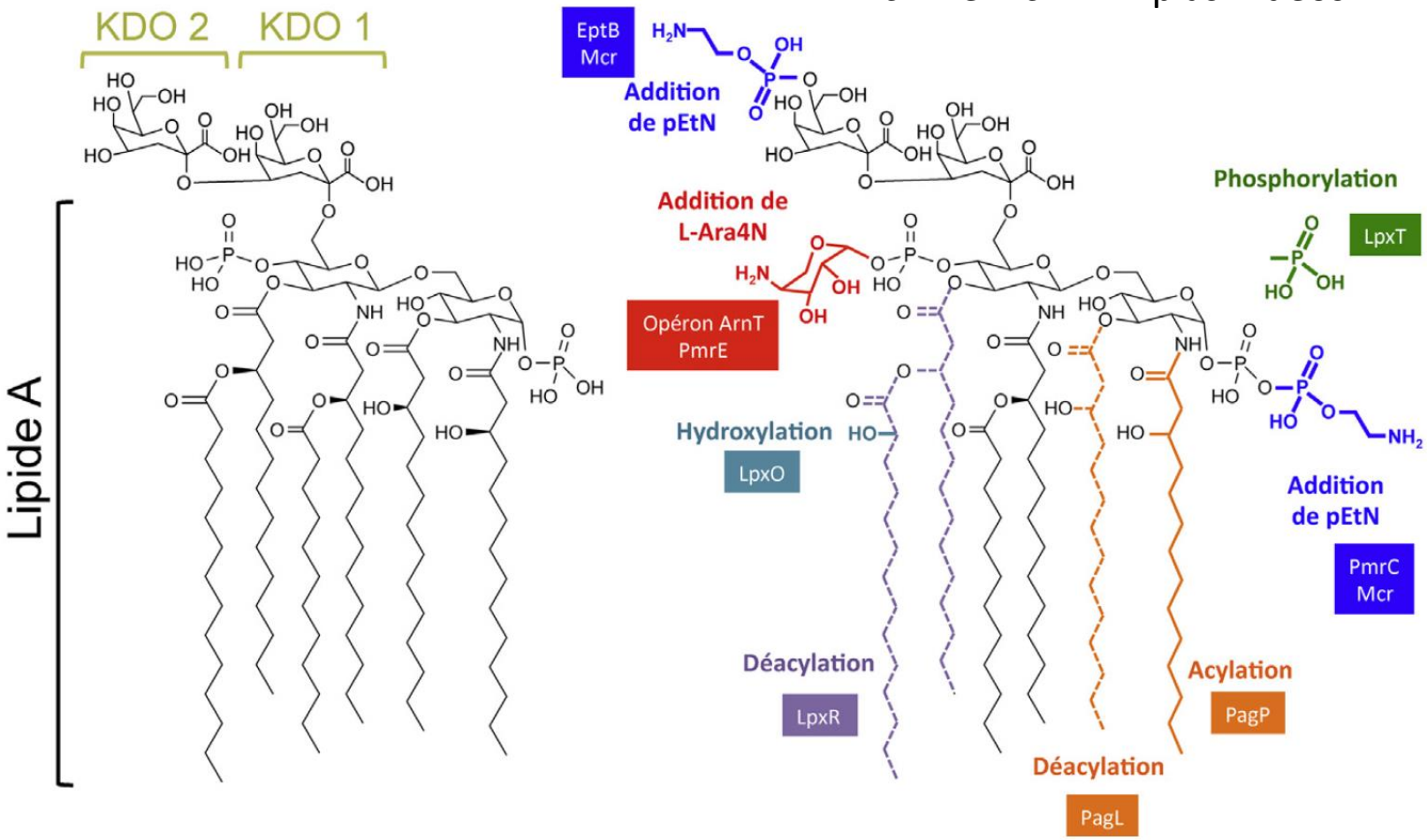
Mecanismos de Resistência às Polimixinas



Dortet et al., 2016

Modificações do lipídeo A que levam à resistência às polimixinas

mcr-1 e *mcr-2* → plasmídeos



Dortet et al., 2016

Brasil

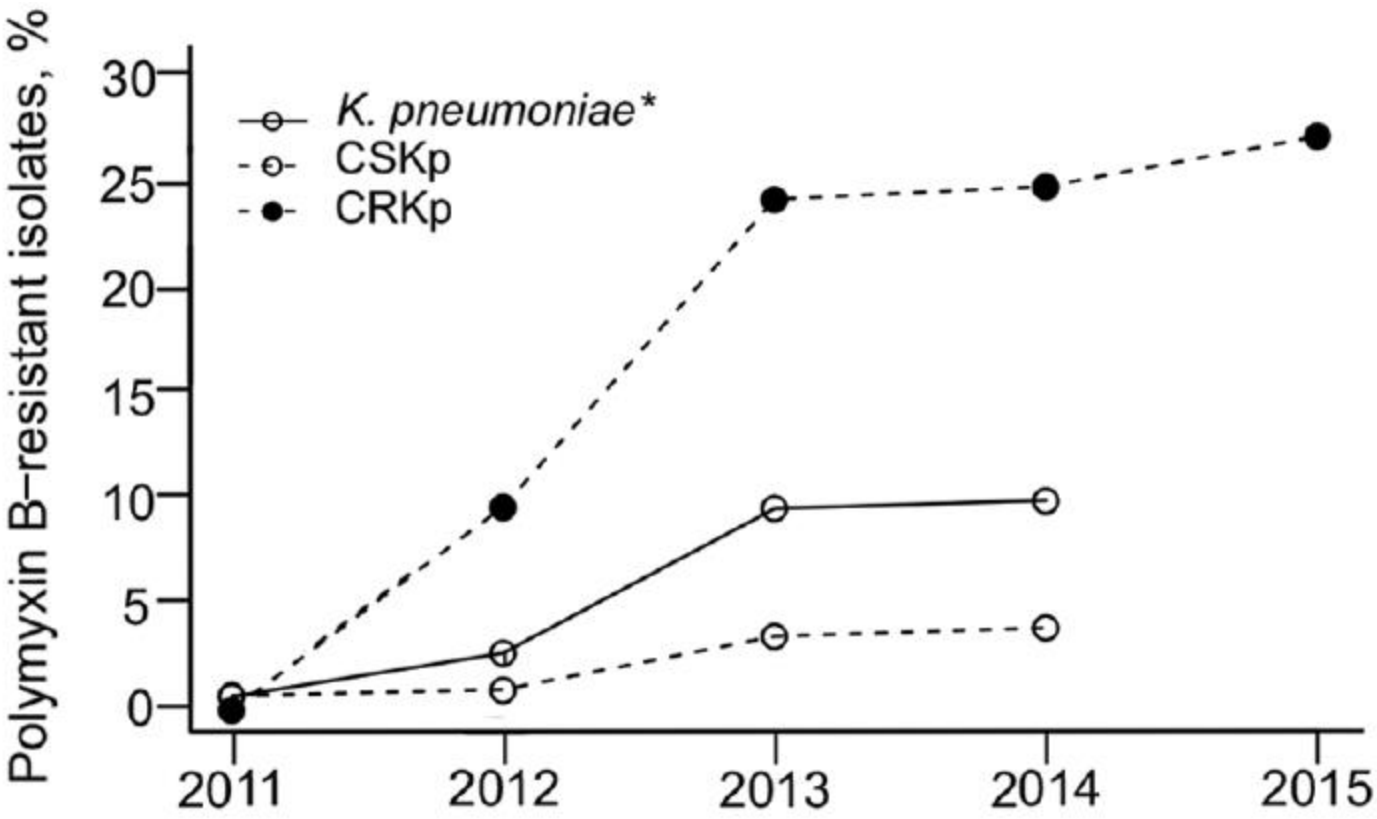
- Principal mecanismo alterações em genes de localização cromossômica
  - Aires CA et al. Antimicrob Agents Chemother. 2016;60(11):6969-6972.
- A disseminação do gene *mcr-1* é um risco relevante

  - Fernandes MR et al. Silent dissemination of colistin-resistant *Escherichia coli* in South America could contribute to the global spread of the *mcr-1* gene. Euro Surveill. 2016;21(17).

  - Dalmolin TV et al. Co-occurrence of *mcr-1* and bla_{KPC-2} in a clinical isolate of *Escherichia coli* in Brazil. J Antimicrob Chemother. 2017.

  - Aires CAM et al. Emergence of plasmid-mediated *mcr-1* gene in clinical KPC-2-producing *Klebsiella pneumoniae* ST392 in Brazil. Antimicrob Agents Chemother. 2017.

São Paulo - Resistência às polimixinas: o problema é maior nas cepas resistentes aos carbapenêmicos



Bartolleti et al., Emerging Infect Dis, 2016

Métodos NÃO recomendados para testar a sensibilidade às polimixinas

- Falsa sensibilidade
  - Disco-difusão
  - Diluição em ágar
  - Gradientes em fitas
    - http://www.eucast.org/ast_of_bacteria/warnings/#c13111

Gradientes de colistina em fita apresentam taxas de erros graves acima do limite aceitável. O método a ser utilizado para a avaliação da sensibilidade às polimixinas é o de microdiluição em caldo

Colistin gradient tests can be obtained from bioMérieux and from Liofilchem. The EUCAST Development Laboratory has evaluated the two available gradient tests. Results were similar for both gradient tests on Mueller-Hinton agar from two manufacturers. Etests were also tested on MH-E (the MH produced by and recommended by bioMérieux). Both gradient tests underestimated MIC values by one or more twofold dilutions, especially for concentrations on or above the breakpoint of 2 mg/L, **leading to false susceptible results (VME)**. Results on Etest were marginally better when the test was performed on the MH recommended by the manufacturer but VMEs were still a significant problem, and even more so with *Pseudomonas* and *Acinetobacter*.

http://www.eucast.org/ast_of_bacteria/war

Evaluation of five commercial MIC methods for colistin antimicrobial susceptibility testing for Gram-negative bacteria

P 161

Erika Matuschek, Jenny Åhman, Catherine Webster and Gunnar Kahlmeter
EUCAST Development Laboratory, Växjö, Sweden

EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING
European Society of Clinical Microbiology and Infectious Diseases

Introduction

An accurate method for antimicrobial susceptibility testing for colistin is crucial in an era of increasing numbers of multi-resistant bacteria and the simultaneous increasing colistin resistance. EUCAST and CLSI have agreed on how to perform broth microdilution (BMD) for colistin (www.eucast.org). EUCAST breakpoints for Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter* spp. is S≤2, R>2 mg/L.

Objective

The objective of this study was to evaluate three commercial BMD methods and two gradient tests for colistin MIC determination using frozen BMD panel MICs as reference.

Methods

Antimicrobial susceptibility testing was performed on an international collection of Gram-negative bacteria (n=75) with colistin MICs of 0.25-128 mg/L: *Escherichia coli* (n=14), *Klebsiella pneumoniae* (n=18), *Pseudomonas aeruginosa* (n=21) and *Acinetobacter* spp. (n=22). Colistin MIC determination was performed according to the manufacturers' instructions on frozen BMD panels (Thermo Scientific), three BMD methods with freeze-dried antibiotics: SEMPA1 (custom Sensititre plate, Thermo Scientific), MICRONAUT-S and MICRONAUT MIC-Strip (MERLIN Diagnostika) and two gradient tests: Etest (bioMérieux) and MIC Test Strip (MTS, Liofilchem). Etest and MTS were tested on Oxoid (Thermo Fisher Scientific) and BBL (BD) Mueller-Hinton agar in parallel, and Etest also on the bioMérieux' MHE medium (as recommended by the manufacturer). Isolates with skipped wells for BMD were retested. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *E. coli* NCTC 13846 (*mcr-1* positive) were used as quality control (QC). Essential and categorical agreements were calculated according to ISO 20776-2 vs. EUCAST Breakpoint Tables v. 7.1 using colistin MICs on frozen panels as reference.

Acknowledgements for contribution with isolates: Paul Rhomberg, JMI Laboratories, USA (isolates from the worldwide SENTRY surveillance program); Sören Gatermann, Bochum, Germany; Rene Henriksen, DTU, Copenhagen, Denmark; Ørjan Samuelsen, Tromso, Norway; Jordi Vila, Barcelona, Spain and Luis Martinez-Martinez, Santander, Spain.

	Organism	*E. coli* and *K. pneumoniae* (n=32)	*P. aeruginosa* (n=21)	*Acinetobacter* spp. (n=22)	All isolates (n=75)
	Colistin MIC range (mg/L)	0.25-32	0.25-128	0.5-32	0.25-128
Essential agreement (EA)	Sensititre custom plate*	27	19	20	66 (96%)
	MICRONAUT-S	31	21	20	72 (96%)
	MICRONAUT MIC-Strip	31	21	22	74 (99%)
	Etest, Oxoid MH	27	13	13	53 (71%)
	Etest, BBL MH	20	11	1	32 (43%)
	Etest, MHE	24	9	2	35 (47%)
	MTS, Oxoid MH	19	12	9	40 (53%)
	MTS, BBL MH	24	12	13	49 (65%)
Major Errors (ME)	Sensititre custom plate	1	1	2	4
	MICRONAUT-S	2	1	3	6
	MICRONAUT MIC-Strip	2	0	3	5
	Etest, Oxoid MH	2	0	0	2
	Etest, BBL MH	1	0	0	1
	Etest, MHE	2	0	0	2
	MTS, Oxoid MH	0	0	0	0
	MTS, BBL MH	0	0	0	0
Very Major Errors (VME)	Sensititre custom plate	0	0	0	0
	MICRONAUT-S	0	2	0	2
	MICRONAUT MIC-Strip	0	2	0	2
	Etest, Oxoid MH	0	6	6	12
	Etest, BBL MH	1	7	7	15
	Etest, MHE	0	5	4	9
	MTS, Oxoid MH	6	6	4	16
	MTS, BBL MH	5	6	7	18

Table 1. Essential and categorical agreements for colistin MIC tests for 75 Gram-negative bacteria with MICs on frozen broth microdilution panels as reference.
Essential agreement (EA) = MICs being within ± 1 dilution of reference MIC

* The total number of tests for calculation of EA was 28 for *E. coli*/*K. pneumoniae* and 19 for *P. aeruginosa* due to truncation at ≤0.25 and >32 mg/L.

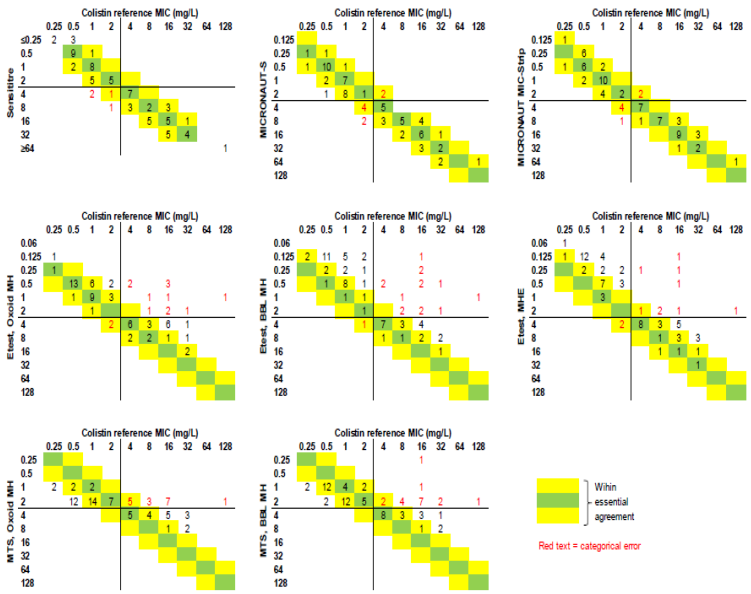


Figure 1. Correlation for colistin MIC values between test methods and reference broth microdilution for three BMD methods (Sensititre custom plate, MICRONAUT-S and MICRONAUT MIC-Strip) and gradient tests from two manufacturers (Etest and MTS). For gradient tests, results are shown per Mueller-Hinton (MH) agar.
MICs identical with reference MICs are highlighted in green. EUCAST breakpoints (S≤2, R>2 mg/L) are shown as lines.

Results

Essential and categorical agreements for the five methods are shown in **Table 1**. The correlation with reference MICs was good for all BMD methods but poor for gradient tests (**Figure 1**). Skipped wells occurred occasionally on all BMD panels and resulted in unreliable results unless retested. The BMD methods tended to overcall resistance to a small extent, resulting in a few major errors. Gradient tests generally underestimated MICs, resulting in a significant number of false susceptible results (very major errors). For Etest, very major errors were more abundant for *P. aeruginosa* and *Acinetobacter* spp. than for *E. coli* and *K. pneumoniae*.

For BMD methods, all QC results were within ranges, except for one reading below the range for MICRONAUT MIC-Strip with *E. coli* ATCC 25922 (**Table 2**). All MICs for MTS were within range for both QC strains. All Etest MICs were out of range for *E. coli* ATCC 25922 on BBL and MHE agar, and below range or in the lower part of the range for *P. aeruginosa* ATCC 27853. For *E. coli* NCTC 13846, all MICs were within ± 1 dilution of the expected 4 mg/L.

Table 2. Quality control results per MIC method and QC strain.
QC target values are highlighted in green and upper and lower limits in yellow.
Results out of range are shown in red text.

Colistin MIC method	Colistin MIC (mg/L)												
	Escherichia coli ATCC 25922					*Pseudomonas aeruginosa* ATCC 27853					*Escherichia coli* NCTC 13846**		
	0.125	0.25	0.5	1	2	0.25	0.5	1	2	4	2	4	8
Reference frozen panel			7	1				8			1	7	
Sensititre custom plate		4*	4				1	7				8	
MICRONAUT-S		5	3					4	4			7	1
MICRONAUT MIC-Strip	1	6	1					8			2	6	
Etest, Oxoid MH		2	5					7				8	
Etest, BBL MH	12						4	8				8	
Etest, MHE	7					3	4				5	3	
MTS, Oxoid MH				6	1			2	5			8	
MTS, BBL MH			1	6				4	3			8	

* ≤0.25 mg/L ** *mcr-1* positive

Conclusions

The commercial BMD methods reliably determined colistin MICs when no skipped wells were present. The correlation between gradient tests and reference MICs was poor, even when QC results were within range. This was probably related to the poor diffusion of colistin in agar. Based on the results of this study, EUCAST recommends laboratories to use BMD methods for colistin MIC determination and advice against the use of gradient tests at this point.

For more information, please contact erika.matuschek@escmid.org

Critérios para Avaliação

Tipo de Concordância	Definição	Critério FDA
EA (Concordância Essencial)	Concordância de ± 1 diluição do sistema testado e a referência	≥90%
CA (concordância categórica)	Concordância entre categorias do sistema testado e a referência	≥90%
VME (erro muito grave)	Sistema testado = S e Referência = R (falsa sensibilidade)	≤ 1,5%
ME (erro grave)	Sistema testado = R e Referência = S (falsa resistência)	≤ 3,0%

Evaluation of five commercial MIC methods for colistin antimicrobial susceptibility testing for Gram-negative bacteria

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EUCAST

Etest, MHE \ Colistin reference MIC (mg/L)	0.25	0.5	1	2	4	8	16	32	64	128
0.06	1									
0.125	1	12	4				1			
0.25		2	2	2	1		1			
0.5			7	3			1			
1			3							
2					1	2	1			1
4				2	8	3	5			
8						1	3	3		
16						1	1	1		
32								1		
64										
128										

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	Etest, Oxoid MH	2	0	0	2
	Etest, BBL MH	1	0	0	1
	Etest, MHE	2 6,4%	0	0	2
	MTS, Oxoid MH	0	0	0	0
	MTS, BBL MH	0	0	0	0
Very Major Errors (VME)	Sensititre custom plate	0	0	0	0
	MICRONAUT-S	0	2	0	2
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	MTS, BBL MH	5	6	7	18

Automação

- A performance da automação na avaliação da sensibilidade às polimixinas ainda não foi discutida no BrCAST
- Dados não publicados – Jorge Sampaio
  - Vitek 2 Compact
    - Polimixina B (microdiluição não automatizada *in house*) x Colistina Vitek 2 – testados em 2017
    - 50 isolados da rotina (*K. pneumoniae* e *Acinetobacter* sp.)
      - 90% de concordância
      - 6% de erros graves (major error)
      - 4% de erros muito graves (very major error)

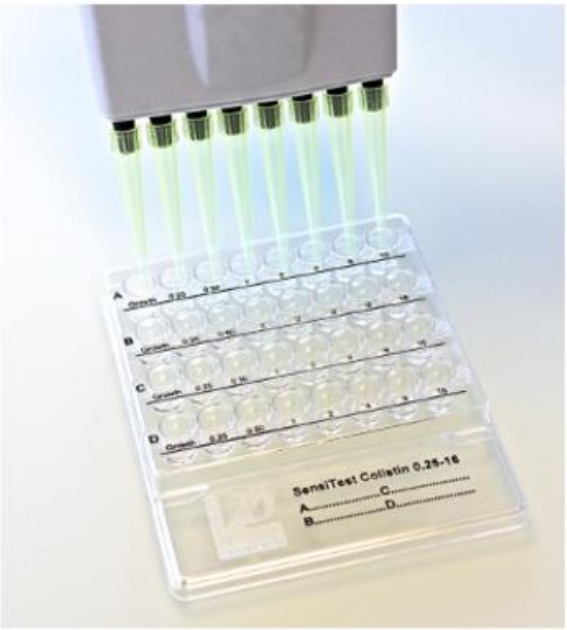
Automação

- A performance da automação na avaliação da sensibilidade às polimixinas ainda não foi discutida no BrCAST
- Dados não publicados – Jorge Sampaio
  - Phoenix com painel NMIC-203
    - Polimixina B (microdiluição não automatizada *in house*) x Colistina (Phoenix) – testes realizados em 2015
    - Coleção de 128 *K. pneumoniae* KPC+ com várias cepas com CIMs de 4 e 8 mg/L, que apresentam menor frequência na rotina, mas são um desafio para a detecção da resistência
      - 86,8% de concordância
      - 10,9% de erros muito graves (very major error)
      - 2,3% de erros graves (major error)

Automação

- A performance da automação na avaliação da sensibilidade às polimixinas ainda não foi discutida no BrCAST
- Dados não publicados – Jorge Sampaio
  - Walkaway
    - Polimixina B (microdiluição não automatizada *in house*) x Colistina (Walkaway)
    - Coleção de 114 *K. pneumoniae* KPC+
      - 2,8% de erros graves (major error)

Como testar: microdiluição em caldo



SensiTest™ Colistin

LIOFILCHEM

Atualmente (25-05-2017) sem registro na ANVISA

POLICIMBAC - PROBAC

- Registrado na ANVISA
- Ainda não avaliado pelo BrCAST

Como testar?

INTERNATIONAL STANDARD

ISO 20776-1

First edition
2006-11-15

Clinical laboratory testing and *in vitro* diagnostic test systems — Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices —

Part 1:
Reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases

Como testar

- Microdiluição em caldo
- Principais variáveis:
  - pH
  - Concentrações de cátions divalentes (Ca++ e Mg++)
    - Girardello R, Bispo PJ, Yamanaka TM, Gales AC. Cation concentration variability of four distinct Mueller-Hinton agar brands influences polymyxin B susceptibility results. J Clin Microbiol. 2012;50(7):2414-8.

Como testar preparando as placas no laboratório

- Placas de poliestireno estéreis com fundo em U, 96 cavidades, sem tratamento, com tampa
  - Corning
  - Sarstedt
  - TPP
- Caldo Mueller-Hinton II cátion ajustado
  - BD
  - Oxoid

Como testar preparando as placas no laboratório

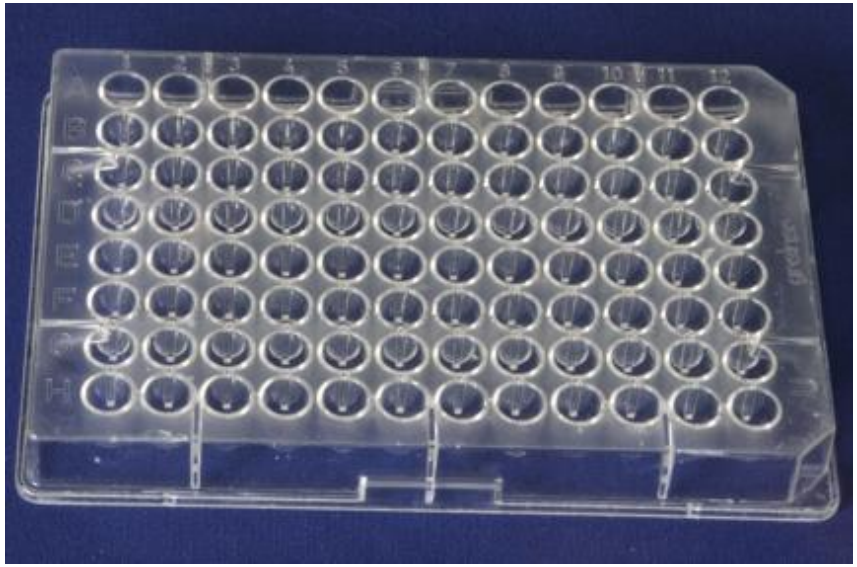
- Sulfato de polimixina B
  - Sigma (ideal)
  - Eurofarma (produto injetável)
- Sulfato de colistina
  - Sigma
  - Produto injetável não funciona pois é uma pró-droga (metanossulfonato)

Preparo da solução de polimixina B

- Cada mg de polimixina B base pura é equivalente a 10.000 UI de polimixina B.
- Utilizar preferencialmente sulfato de polimixina B – Sigma P0972 ou equivalente
- Se for utilizada o sulfato de polimixina B da Eurofarma, cada frasco vem com 500.000 UI (cerca de 50 mg). Dissolver o conteúdo do frasco em 10 ml de água estéril, de modo a obter solução com 5 mg/ml. Diluir em água estéril conforme necessidade.
- Caso seja utilizado sulfato de polimixina B Sigma ou outro fornecedor é necessário obter o certificado de análise para saber quantas UI por mg o produto tem.
- Exemplo:
  - Sulfato de polimixina B com 7.800 UI por mg da Sigma. Fazer regra de três, de modo a justar a quantidade necessária para obter a concentração correta de sulfato de polimixina B puro.
  - 1 mg de sulfato de polimixina B da marca A ---------- 7.800 UI
  - x mg de sulfato de polimixina B da marca A ---------- 10.000 UI
  - Portanto, para obtermos 1 mg de sulfato de polimixina B puro devemos pesar 1,28 mg de sulfato de polimixina B da marca A.

Preparo das placas

- Preencher todos os poços, exceto os da primeira coluna, com 50 µL de CMHCA
- Preencher todos os poços da primeira coluna com 100 µL de CMHCA contendo 128 mg/L de sulfato de polimixina B ou sulfato de colistina
- Realizar diluições sucessivas (50 µL)utilizando pipeta multicanal , exceto na última coluna



Inoculação das placas

- Preparar suspensão em salina estéril com turbidez MF 0,5
- Diluir 1/200 em CMHCA
- Inocular da direita para esquerda, 50 µL em cada um dos poços
- Incubar 16 a 20 horas, ar ambiente, 35 ± 1 °C em câmara úmida

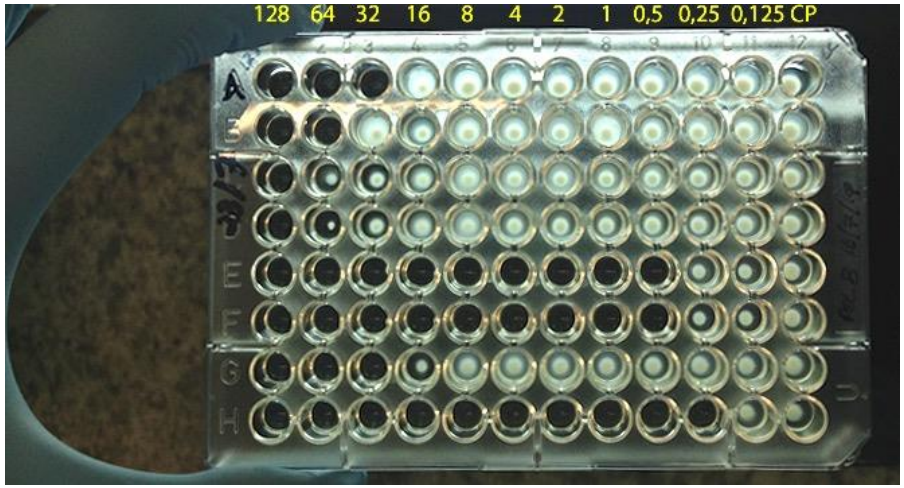
Controle de qualidade

- *P. aeruginosa* ATCC 27853
- *E. coli* ATCC 25922
- Ver tabelas de CQ BrCAST

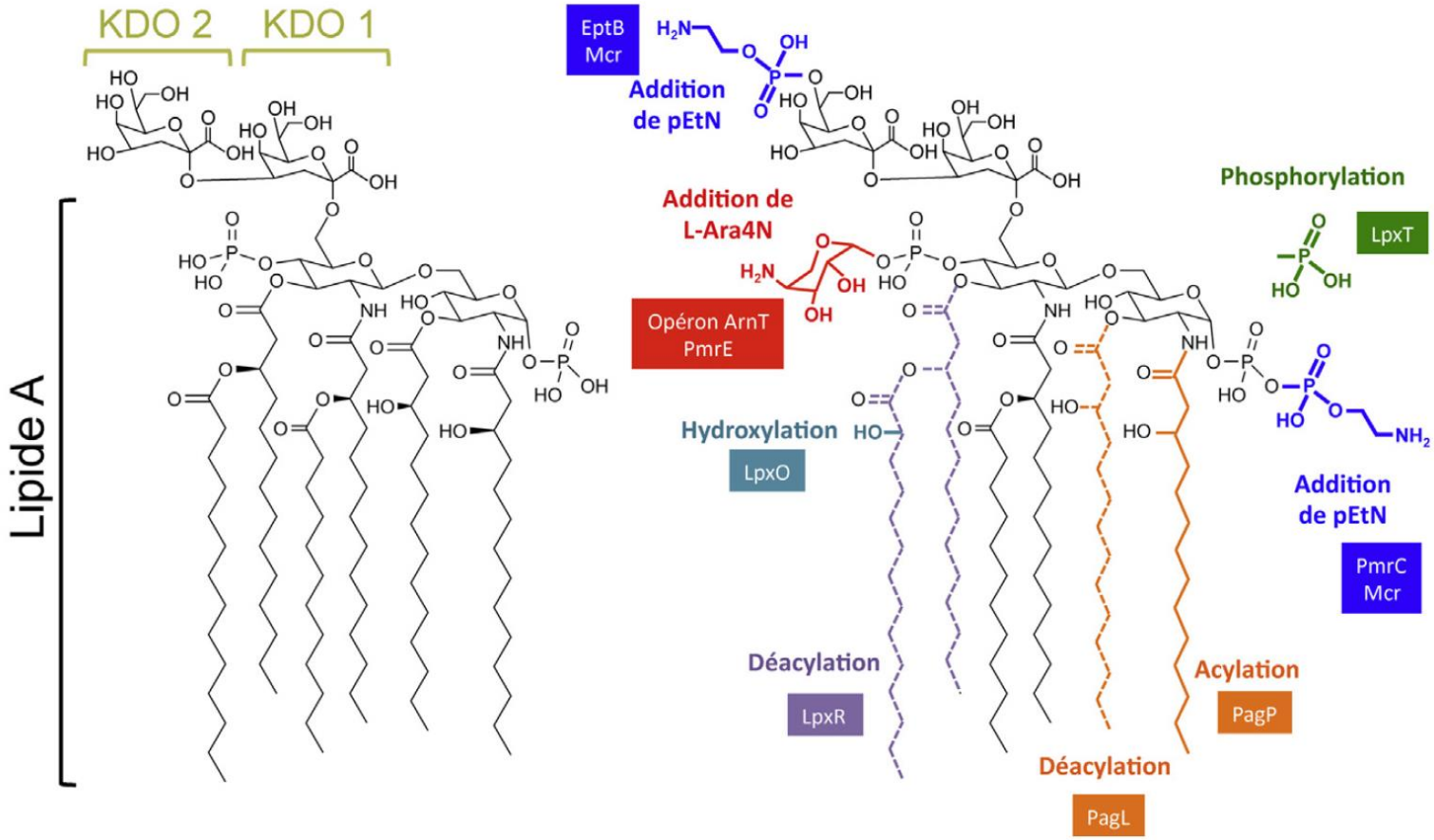
Como interpretar?

- Utilizar os pontos de corte do BrCAST-EUCAST

Agentes diversos	Ponto de corte p/ CIM (mg/L)		
	S ≤	I	R >
Cloranfenicol	8	-	>8
Colistina[1]	2	-	>2
Polimixina B[3]	2	-	>2



O futuro da detecção da resistência às polimixinas é a espectrometria de massas?



Dortet et al., 2016

O futuro da detecção da resistência às polimixinas é a espectrometria de massas?

- ECCMID – 2017
- *Late-breakers in diagnostics* – OS0558D
- Rapid detection and discrimination of plasmid- and chromosome-mediated resistance to polymyxins in Enterobacteriaceae using MALDI-ToF MS
  - Laurent Dortetn Alain Filloux, Gerald Larrouy-Maumus

Considerações finais

- A resistência às polimixinas em *Enterobacteriaceae* é um problema grave e crescente no Brasil e no mundo.
- Todos os métodos de automação e os gradientes em fita apresentam atualmente algum tipo de limitação na avaliação da sensibilidade.
- A microdiluição em caldo não automatizada é o único método atualmente adequado para avaliação da sensibilidade *in vitro* às polimixinas.

Obrigado pela atenção!