Methodological and interpretative problems in antimicrobial susceptiblity tests of *P. aeruginosa*

Y. Glupczynski

Laboratoire de bactériologie
Cliniques Universitaires UCL de Mont-Godinne
Université Catholique de Louvain

Symposium on *P. aeruginosa* resistance
and therapeutic options
(29/03/2006)

Méthodes d'antibiogramme utilisées pour *P. aeruginosa*

Diffusion	Dilution
• Diffusion des disques en gélose • E-test (variante quantitative de diffusion en gélose)	• Dilution en agar • Dilution en bouillon – Macrodilution – Microdilution

Systèmes automatisés: - Croissance à 1-2 [ ] critique, lecture à point fixe (S/I/R)
- détermination de CMI (échelle limitée de concentrations)
analyse cinétique de croissance (techniques rapides)

Factors influencing results of susceptibility tests for *P. aeruginosa*

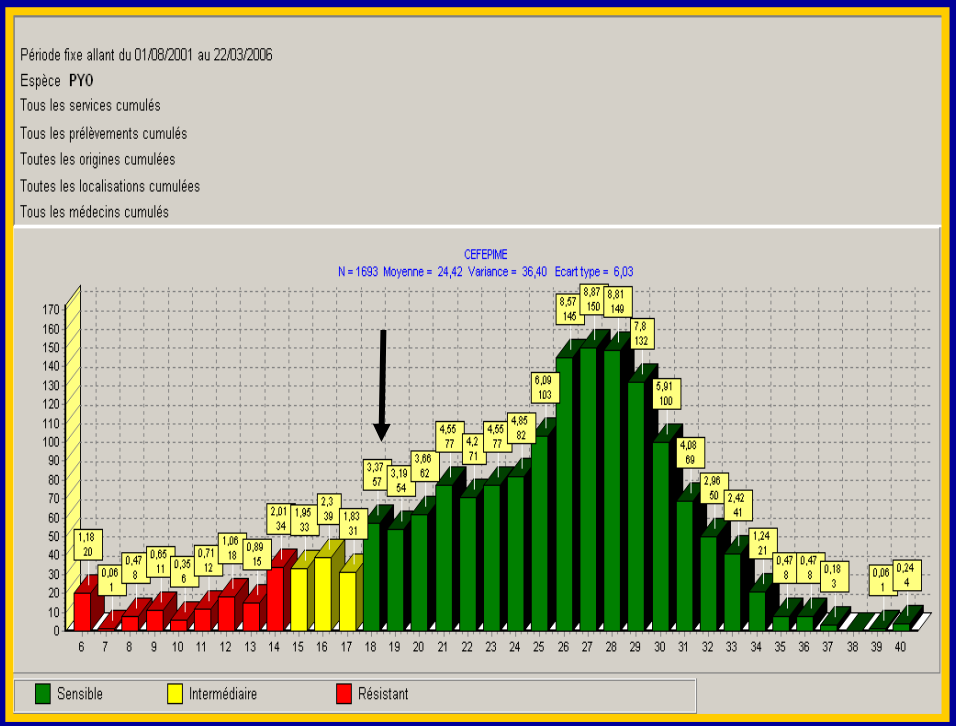
- Increased inoculum (↑of MICs to β-lactams 5x- 500x)
- Culture medium (Mueller-Hinton, Isosensitest)
- pH (aminoglycosides, quinolones)
- Concentration of divalent cations (Ca/Mg/Zn)
  - Quinolones, Aminoglycosides, Carbapenems
- Diffusibility of drug in culture medium
  - (poor diffusion of colistin in solid media)
- Growth rate
- Temperature and duration of incubation

→ ***need for standardization of inoculum and internal QC with reference strain*** (*P. aeruginosa* ATCC 27853)

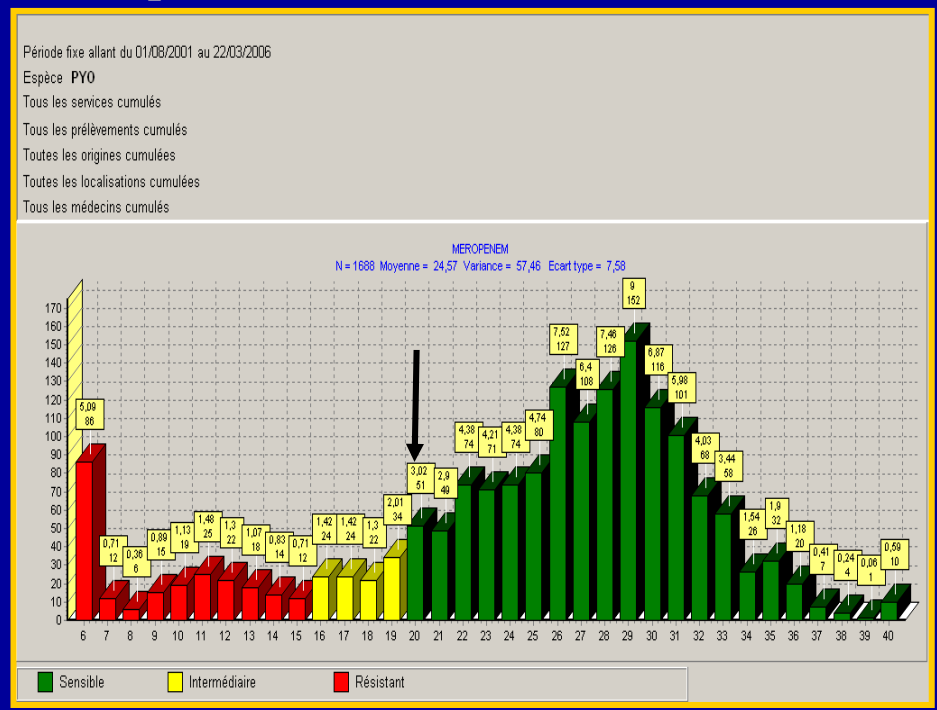
Zone distribution for all *P. aeruginosa* isolates tested at Cliniques UCL Mont-Godinne

(08/2001-03/2006)

Cefepime



Meropenem



Susceptibility and resistance breakpoints for *P. aeruginosa* defined by different reference standards

	CA-SFM			BSAC			CLSI		
	S ≤	I	R≥	S ≤	I	R≥	S ≤	I	R≥
Piperacillin/ tazo	16/4	32-64/4	128/4	16/2	-	32/2	64/4	-	128/4
Ceftazidime	4	8-32	64	8	-	16	8	16	32
Cefepime	4	8-32	64	NA			8	16	32
Imipenem Meropenem	4 8	8 16	16 4	4 4	- -	8 8	4 4	8 8	16 16
Amikacin	**8**	**-**	**16**	4	8	16	16	32	64
Tobramycin	4	8	16	2	4	8	4	8	16
Ciprofloxacin	**0,5**	**1**	**2**	2	-	4	1	2	4

General technical conditions for dilution and diffusion susceptibility testing methods

	CA-SFM	BSAC	CLSI
Inoculum preparation	Direct colony suspension or growth method 0,5 MF (≈10^8 CFU/ml)	Growth in IS broth 0,5 MF (≈10^8 CFU/ml)	Direct colony suspension/growth method
Culture medium	Mueller-Hinton	Iso-sensitest	Mueller-Hinton (CAMHB) MIC
Final inoculum	MIC (1/10 dilution), 2 µl ≈10^4 CFU/spot Diffusion (1/100 dilution) ≈10^6 CFU/ml	Agar dilution (10^4CFU/spot); broth dilution (10^5 CFU/ml) Diffusion (1/100 dilution) ≈10^6 CFU/ml	0,5 McFarland suspension
Incubation	35-37°C, 18-24 h in air	35-37°C, 18-20h in air	35-37°C, 16-18h

Accuracy of disc diffusion tests for susceptibility testing of *P. aeruginosa*

A national survey in the UK (25 sentinel labs)

Antimicrobial agent	Total N° identified as R by central lab*	N° (%) correctly found R/I by sentinel lab	Total N° identified as S by central lab*	N° (%) correctly found S by sentinel lab
Piperacillin	34	29 (85)	263	254 (96.6)
Piperacillin/tazo	28	25 (89)	269	260 (96.7)
Ceftazidime	27	21 (78)	231	226 (97.8)
Imipenem	56	54 (96)	241	228 (94.6)
Meropenem	22	22 (100)	275	254 (92.4)
Amikacin	48	39 (82)	250	234 (93.6)
Gentamicin	94	57 (61)	252	219 (90.8)
Ciprofloxacin	66	50 (76)	231	226 (97.8)

**agar dilution MIC reference*

Henwood, JCM 2001; 47: 789-99

Evaluation of E test for antimicrobial susceptibility testing of *P. aeruginosa*

- 248 *P. aeruginosa* isolates (catheter-associated UTI)
- 88% E-test MICs within ± 1 log dilution of agar dilution MICs (98% within ± 2 log dilutions)
- 92.5% agreement with disk diffusion method
- Mostly minor errors (7%) and major errors (1.2%), no very major errors
- Majority of errors with piperacillin and ticarcillin (MIC close to the breakpoints

Di Bonavantura, JCM 1998; 36: 824-6

What about automated systems for susceptibility testing of *P. aeruginosa* ?

Validation of automated instruments for antimicrobial susceptibility testing

Reference method

Automated system	S	I	R
S	CA	me	VME (<1.5%)
I	me	CA	me
R	ME (<3%)	me	CA

Overall category error < 10% with reference method
Very major errors ≤ 1.5%; Major errors ≤ 3%
Resistant strains with characterized mechanisms (n ≥ 35)
Large number of clinical isolates (>=200)

Doern, JCM 1997
Ferraro & Jorgensen CID1999

Concordance of results between VITEK2 and reference microdilution method for *P. aeruginosa* (n=146)

Antimicrobial agent	EA (±1 MIC)	Category agreement	Minor error	Major error	Very major error
Piperacillin	84.2	93.9	0.0	3.4	2.7
Cefepime	89.0	82.9	14.4	2.0	0.7
Ceftazidime	95.2	94.5	4.8	0.7	0
Imipenem	87.0	91.8	6.8	0.0	1.4
Meropenem	85.0	90.4	9.6	0.0	0.0
Tobramycin	97.3	98.6	1.4	0	0
Ciprofloxacin	98.6	96.6	3.4	0	0
All agents	**90.2**	**90.7**	**7.7**	**0.7**	**0.9**

EA: agreement of MICs within ± 1 dilution;
mE: VITEK2 I; MIC R/S or reverse; **ME**: Phoenix R/Disk: S; **VME**: Phoenix S/Disk R

Joyanes, JCM 2001

Concordance results VITEK2/microdilution for 21 resistant *P. aeruginosa* isolates (Cefta/Imip)

Antimicrobial agent	EA (±1 MIC)	Category agreement	Minor error	Major error	Very major error
Piperacillin	85.7	81.0	0.0	9.5	9.5
Cefepime	80.9	71.4	23.8	4.8	0
Ceftazidime	95.2	85.7	14.3	0	0
Imipenem	90.5	66.7	28.5	0.0	4.8
Meropenem	76.2	47.6	52.4	0	0
Tobramycin	100	95.2	4.8	0	0
Ciprofloxacin	100	95.2	4.8	0	0
All agents	**89.1**	**80.5**	**15.2**	**1.4**	**2.9**

EA: agreement of MICs within ± 1 dilution;
mE: VITEK2 I; MIC R/S or reverse; **ME**: Phoenix R/Disk: S; **VME**: Phoenix S/Disk R

Joyanes, JCM 2001

Percentage of category agreement between agar diffusion method and BD Phoenix for *P. aeruginosa*

Antimicrobial agent	Category Agreement	Minor error	Major error	Very major error
Ticarcillin	56.7	43.3	0	0
Piperacillin	73.6	23.3	3.1	0
Piperacillin/tazo	70.6	20	9.4	0
Cefepime	70	30	0	0
Ceftazidime	70	23.2	3.4	3.4
Aztreonam	36.7	63.3	0	0
Imipenem	100	0	0	0
Amikacin	90	10	0	0
Ciprofloxacin	96.7	3.3	0	0
Total (288)	**75.8**	**21.5**	**2.4**	**0.3**

mE: Disk I/Phoenix R/S or reverse; **ME**: Phoenix R/Disk: S; **VME**: Phoenix S/Disk R

Donay, JCM 2004

Multicenter validity testing study for detection of carbapenem resistance in *P. aeruginosa*

Reference method= broth microdilution (BMD) in single centre

Hospital testing method	N° lab	Total N° isolates tested	N° correct (%)	N° major errors (%)	N° minor errors (%)
Disk diffusion	8	33	24 (72.7)	5 (15.2)	4 (12.1)
Microscan	22	135	75 (55.6)	20 (14.8)	40 (29.6)
Pasco	1	13	5 (38.5)	5 (38.5)	3 (23.0)
Sensititre	1	4	3 (75.0)	1 (25.0)	
Vitek	18	140	63 (45.0)	35 (25.0)	42 (30.0)

Major error: Hospital testing method: R/ BMD :S
Minor error: Hospital testing method R/S and BMD/ I or reverse

Steward, JCM 2003; 41: 351-8

False detection of carbapenem resistance in *P. aeruginosa*

- **High rate of overdetection of carbapenem resistance in many labs**
- **High rate of minor errors** (clustering of MIC values around the breakpoint)
- **No factors associated with false resistance to carbapenems**
  - Results not reproducible using same methodology
  - Problem with instrument's susceptibility interpretation
  - Imipenem degradation in test panel ?
  - Improper plate, card, disk storage conditions ?
  - Technical errors (overinoculation of plates ?)
  - Loss of resistance during storage ?

Steward, JCM 2003; 41: 351-8

Accuracy of automated systems for susceptibility testing of *P. aeruginosa* against β-lactams

- 100 clinical isolates of *P.aeruginosa*
- Assessment of categorical and MIC results of three automated systems (Microscan, Vitek, Vitek2)
- Comparison to consensus results of three reference methods (Agar dilution, BMD, disk diffusion)
- Selection of large number of strains with MIC near to the breakpoint

Sader, JCM 2006; 44:1101-4

Accuracy of automated systems for susceptibility testing of *P. aeruginosa* against β-lactams

- False resistant (major errors) acceptable: 0-3%
- High false susceptibility rate (19-27%) to pipera/tazo by VITEK, VITEK2 and Microscan
- Elevated minor error rates (8-32%) to cefepime (VITEK2, VITEK) and to aztreonam (all)
- Trend to false resistance rate with cefepime/aztreonam

→ ***Potential for serious reporting errors; need for reevaluation of β-lactam interpretative algorithms***

Sader, JCM 2006; 44:1101-4

Comparison of susceptibility tesing of *P. aeruginosa* by VITEK2 and E-test

N=150 *P. aeruginosa* isolates; 3 centres

Antimicrobial agent	Category Agreement	Minor error	Major error	Very major error
Piperacillin/tazo	93.6	-	0.2	6.2
Cefepime	84.6	13.7	0.2	1.5
Ceftazidime	90.4	8.2	1.3	0.2
Meropenem	93.6	4.9	0.4	1.1
Amikacin	91.7	7.3	0.9	0.2
Ciprofloxacin	93.2	6.2	0.4	0.2
Total	**91.2**	**8.1**	**0.6**	**1.6**

mE: S or R with Vitek/I Etest or vice-versa; **ME**: Etest S/Vitek R; **VME**: Etest R/ Vitek S

Saegeman, Acta Clin Belg 2004

Susceptibility testing of *P. aeruginosa* from patients with cystic fibrosis

- Multi-resistant isolates and unique phenotypes
- Mucoid isolates (slow growth rate) in 25-50% of cases

Consensus conference on CF microbiology (Saiman, 1994 & 2000)

-> Agar and microbroth dilution MIC = reference methods

-> Automated systems not recommended for CF isolates !!

-> Disk diffusion and Etest recommended (**full 24 h incubation**)

Lower accuracy of disk tests and of Etest for mucoid isolates (correlation coefficient zone vs MIC < 0.8 for pipera/tazo, meropenem)

-> very major errors (false susceptible) < 0.5%;

major errors (false resistant (1 to 2%)

Interpretative aspects of *P. aeruginosa* antibiogram

Identification of *P.aeruginosa* ß-lactam resistance phenotypes

Phenotype	TIC	TCC	PIP	PTZ	CAZ	FEP	AZT	IMP	MER
Wild type	S	S	S	S	S	S	S	S	S
Penicillinase	R	I/R	I/R	I/R	S	I/S	S	S	S
Cephalosporinase	I/R	R	I/R	I/R	I/R	S/I/R	I/R	S	S
Efflux	I/R	I/R	S	S	S	I/S	I/R	S	I/S
Porin D2 deficiency	S	S	S	S	S	S	S	I/R	S/I
ESBL	R	R	S/I	S/I	R	R	R	S	S
Carbapenemase	R	R	I/R	I/R	R	R	S/I	R	R

G. Vedel, JAC 2005; 56: 657-64
F. Bert, JCM 2003; 41: 3712-8

Identification of *P. aeruginosa* β-lactam resistance phenotypes by the Osiris expert system

- Evaluation of Osiris video reader / extended Expert system for disk diffusion test
- 53 *P.* isolates with characterized resistance mechanisms
- Phenotypes against 13 beta-lactam agents tested
- High proportion of unusual ESBL phenotypes (PER, VEB, OXA derivatives)

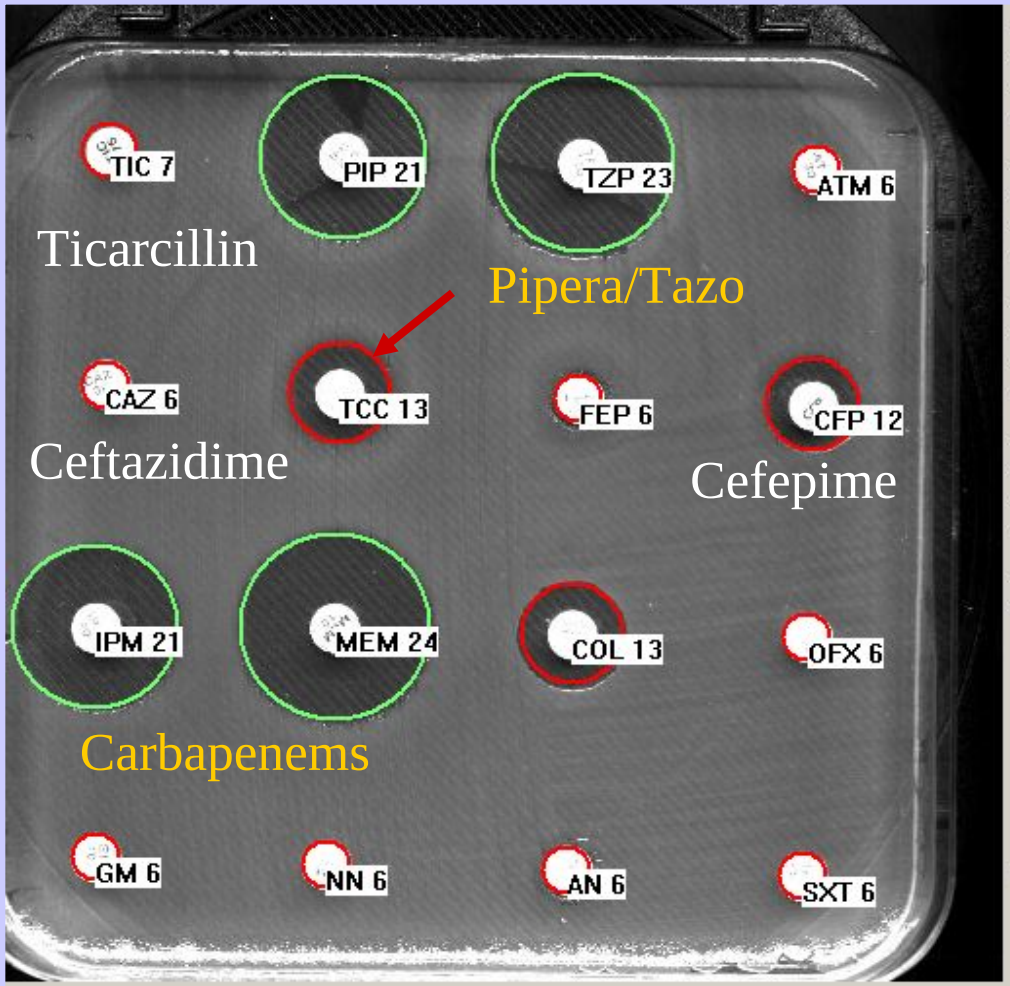
- 88.2% accurate identification of phenotypes; 3.8% association with several mechanims
- Misidentification: - Low level penicillinases
  - partially depressed cephalosporinases
  - efflux system, ESBL

Bert, JCM 2003; 41: 3712-8

Detection of resistance mechanisms in *P. aeruginosa (I)*

- **ESBLs**
  - No standardized procedures for detection
  - Different from those present in *Enterobacteriacae* (PER, VEB, GES, IBC, OXA, >>> TEM, SHV)
  - Less or not inhibited by clavulanate, tazobactam
  - Overexpression of chromosomal cephalosporinases
  - Simultaneous presence of other mechanisms of resistance (impermeability/efflux, other enzymes)

P. aeruginosa : PER-1

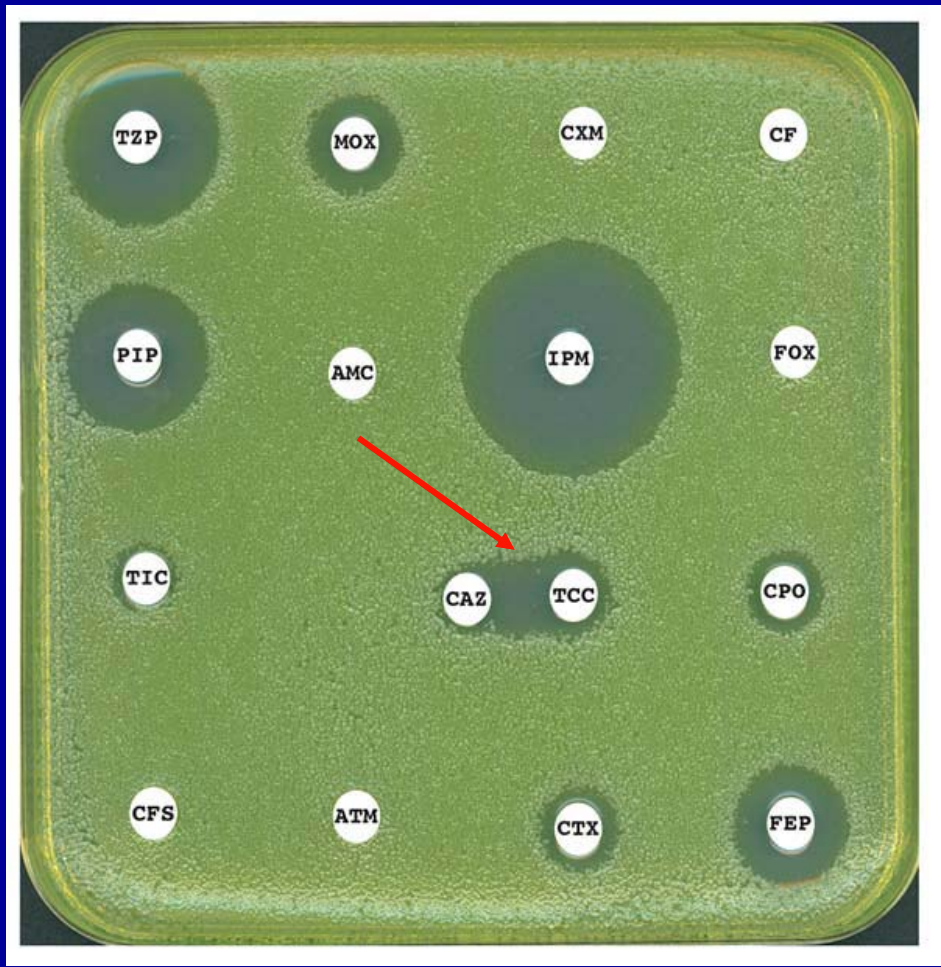


ANTIBIOTIQUE	C.M.I	Resultat	DIAMETRE
COL	>= 2	Résistant	13
AN	> 128	Résistant	6
NN	32	Résistant	6
GM	32	Résistant	6
SXT	> 16	Résistant	6
OFX	64	Résistant	6
ATM	> 128	Résistant	6
FEP	512	Résistant	6
CFP	128	Résistant	12
CAZ	512	Résistant	6
MEM	4	Sensible	24
IPM	0,5	Sensible	21
TZP	2	Sensible	23
PIP	8	Sensible	21
TCC	256	Résistant	13
TIC	> 512	Résistant	7

Activity of Ticar partly restored by Clavulanate Pipera, Pipera/tazo and Carbapenems active

Suspicion of ESBL in *P. aeruginosa* if resistance to ticarcillin, ceftazidime and all other β-lactams except ureidopenicillins (pip/tazo) and carbapenems

P. aeruginosa : PER-1



Positive synergy by DDST between Ticar/Clav. and Ceftazidime by reducing distance between disks to <20 mm

Slide kindly provided by A. Philippon

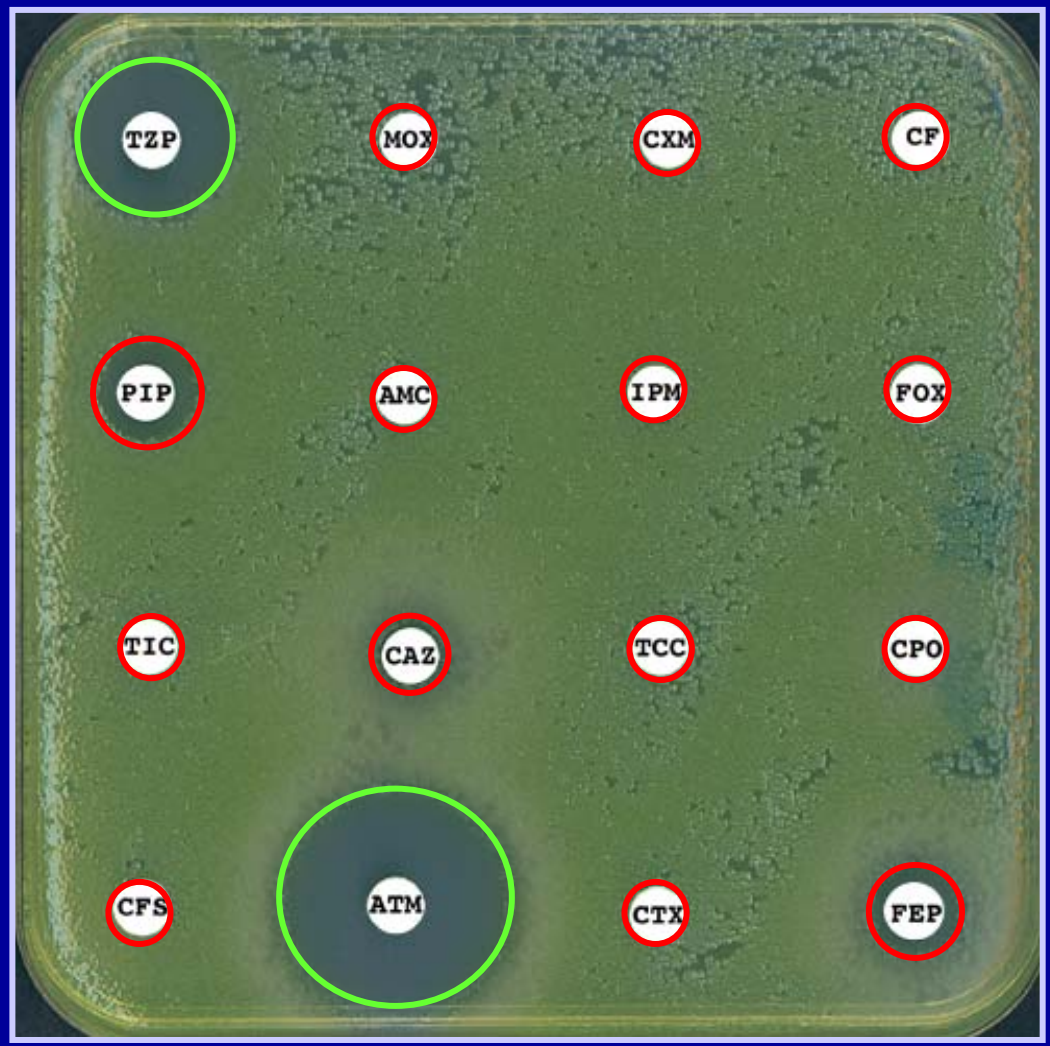
Suggested tools for detection of ESBLs in *P. aeruginosa*

- DDST between ceftazidime/cefepime/aztreonam and ticarcillin-clavulanic acid
- Reduce disk distance ($\leq$ 20 mm) between disks
- Synergy between ceftazidime and imipenem (GES-1/2, PER-1)
- Perform DDST on cloxacillin (250 µg/ml) containing agar to inhibit the activity of AmpC cephalosporinase
- Confirmation and identification of ESBL require molecular tests (PCR and sequencing of *bla* PER, GES, VEB, etc.)

Detection of resistance mechanisms in *P. aeruginosa* (II)

- **Class B Carbapenemases (MBL)**
  - No standardized procedures for detection
  - High level resistance to carbapenems (usually > 32 µg/ml imipenem and meropenem)
  - High-level resistance to ceftazidime (cefepime)
  - Variable susceptibility to aztreonam and ureidopenicillins
  - Associated resistance to aminoglycosides (genes cassettes located on Integrons)

Class B Carbapenemase- producing *P. aeruginosa*: VIM-2



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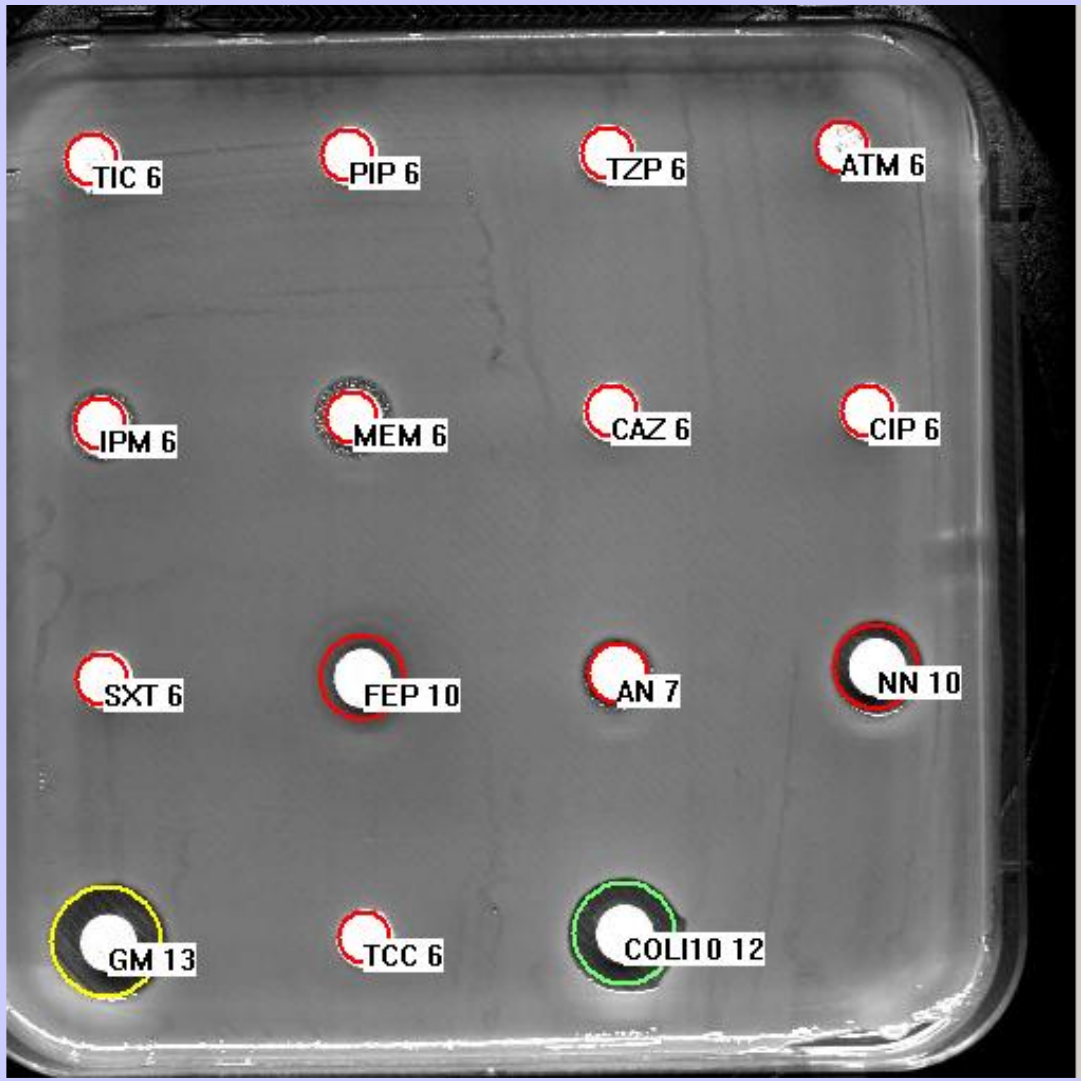
Class B Carbapenemase- producing *P. aeruginosa*: VIM-2



ANTIBIOTIQUE	C.M.I	Resultat	DIAMETRE
AN	> 128	Résistant	7
NN	> 8	Résistant	10
GM	> 4	Intermédiaire	13
SXT	> 16	Résistant	6
CIP	32	Résistant	6
ATM	> 128	Résistant	6
FEP	128	Résistant	10
CAZ	512	Résistant	6
MEM	> 16	Résistant	6
IPM	> 256	Résistant	6
TZP	> 512	Résistant	6
PIP	> 512	Résistant	6
TCC	> 512	Résistant	6
TIC	> 512	Résistant	6
COLI10	<= 0	Sensible	12

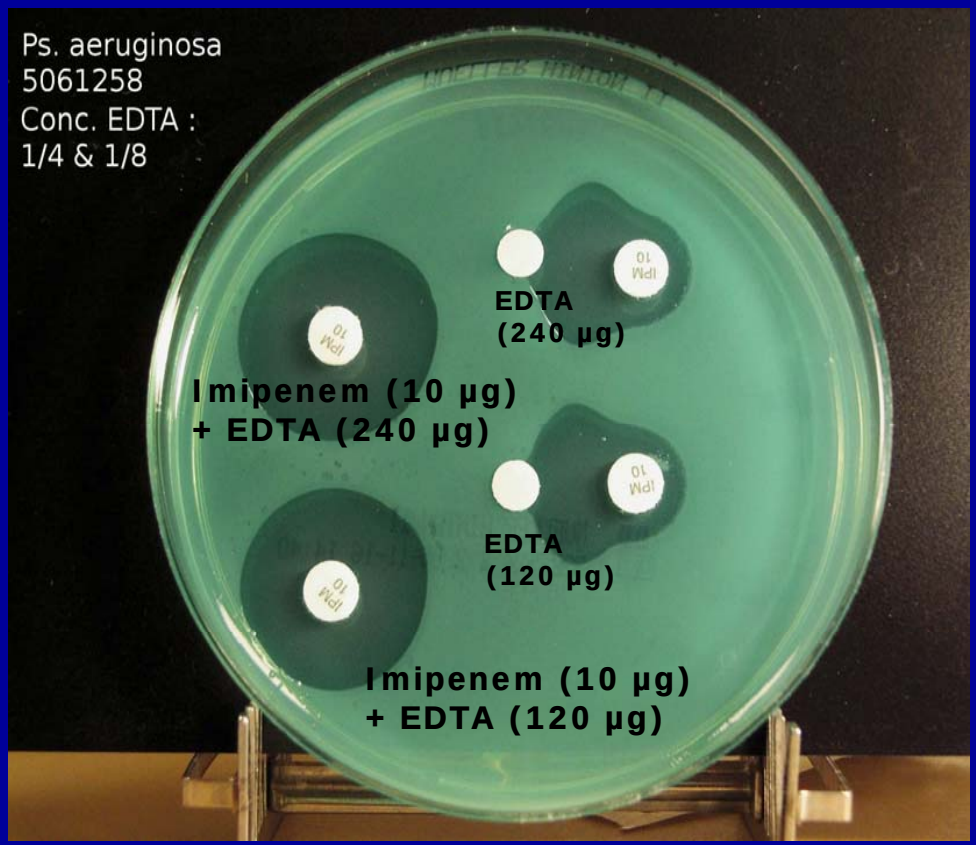
High-level resistance to carbapenems and to all β-lactams including aztreonam

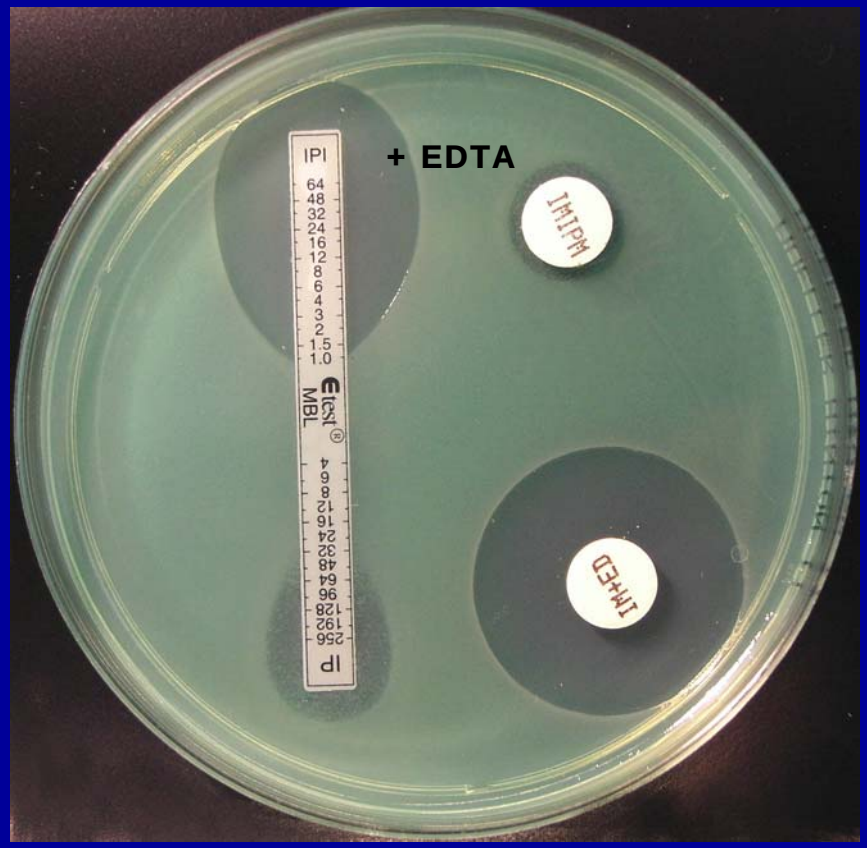
Suggested tools for detection of MBL carbapenemases in *P. aeruginosa*

- Disk approximation DDST between ceftazidime and 2-Mercapto-propionate/EDTA
- Disk diffusion Imipenem / EDTA
- Etest MIC Imipenem/EDTA vs Imipenem
- Carbapenem hydrolysis of crude bacterial extracts by spectrophotometric assay (Meropenem +/- EDTA)
- Molecular detection (PCR for genes for IMP, VIM, etc., sequencing of gene)

Class B beta-lactamases

Metalloenzymes (MBL) - Carbapenemases





Pseudomonas aeruginosa
VIM-2

MBL detection techniques

- Combination Imipenem / EDTA not able to detect all MBLs
- Variable levels of Zn in Mueller-Hinton ; quality control needed when using EDTA
- False positive results: effect of Zn on expression of OprD
  effect of EDTA on membrane permeability
- False negative results : MBL-producing isolates with MICs falling in the intermediate category range (MIC: 4-8 µg/ml)

Conclusion

- Nobody is perfect… (Automatic systems !)
- Importance of adjusted inoculum, internal quality control mandatory
- Use of more than technique is required for susceptibility testing of *P. aeruginosa* (resistant isolates !)
  - β-lactams (carboxy-, ureido-penicillins, cephalosporins, carbapenem)
- Importance of interpretative lecture to detect resistance mechanisms
  - Need to develop tests and algorithms for detection of clinically important resistant mechanisms

Prévention de la grippe aviaire dans un hôpital de campagne
ou Antibiogramme de *Pseudomonas aeruginosa*

« Catch me if you can ? »