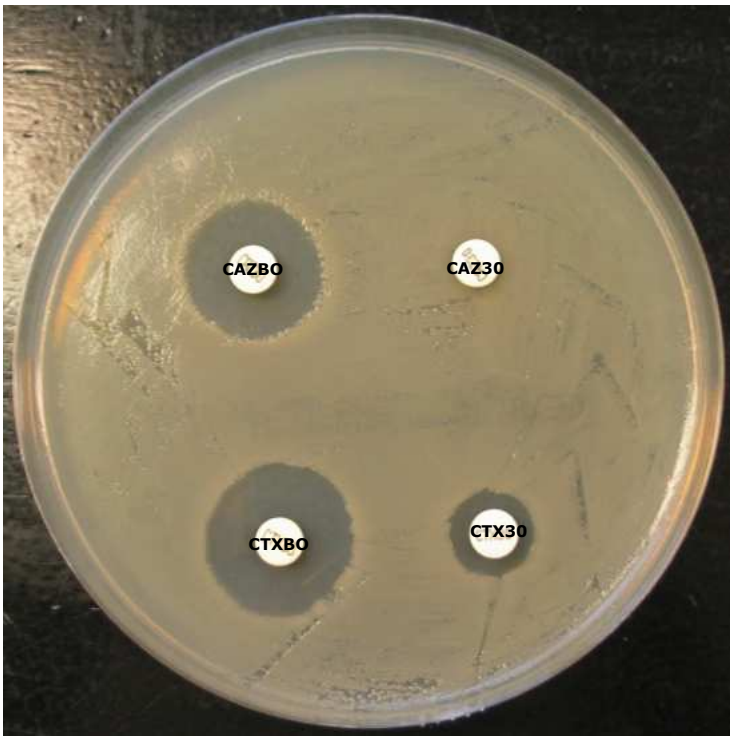
Detection of resistance mechanisms using Neo-Sensitabs™ and Diatabs ™

Main index

Overview of all documents

Detection of resistance mechanisms



E.coli AmpC positive

K. pneumoniae KPC positive

using Neo-Sensitabs™ and Diatabs™

2010

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PREFACE

Detection of Resistance Mechanisms 2010

Laboratories must be able to identify resistant microorganisms, trends in resistance, emerging resistance patterns among clinically relevant bacteria. In order to recognize resistance phenotypes, a suitable number of antimicrobial agents must be used in susceptibility testing, as well as specific agents (indicator drugs) implying mechanisms conferring resistances that may be less obvious in current tests.

With this approach, it would be possible for the microbiologist to:

a) detect mechanisms of resistance,including low level expression
b) modify clinical classifications that are inconsistent with the inferred resistance mechanism and
c) inference of susceptibility results for antimicrobials that are not included in the antibiogram.

The Users Guide for Detection of Resistance Mechanisms includes a series of newly developed tests, particularly useful for the detection of beta-lactamases : ESBL, AmpC, Carbapenemases as well as many other enzymes such as 16S rRNA methylases, Plasmid mediated quinolone resistance, and has been written by J.B.Casals on behalf of Rosco Diagnostica.

Besides, the booklet contains information on the technique used to detect HVISA, VISA strains,using Vancomycin and Teicoplanin Neo-sensitabs by the prediffusion method. Technique for detecting Daptomycin non-susceptible staphylococci and enterococci using Daptomycin Neo-Sensitabs and the prediffusion method.

As well as the detection of colistin resistant P. aeruginosa, A. baumannii and Enterobacteriaceae using Colistin 10 ug Neo-Sensitabs by the prediffusion method.

Tests for detecting Hypermutable strains and Heteroresistant strins,as well as Efflux pump inhibitors are described too.

Finally, a chapter on resistance mechanisms and automated methods, could be interesting lecture for laboratories relying only on automated methods. It is obvious, that these laboratories will have good help in using the tests mentioned in this booklet to complement their results.

The Users Guide is available at our website www.rosco.dk and updated information will continuously be included.

ROSCO DIAGNOSTICA is welcoming any feedback and questions from users directly (info@rosco.dk) or through our representatives.

Neo-Sensitabs™/Diatabs™ Range

Beta-lactamases	Code		Code
ESBL screening			
Cefpodoxime 10 µg	CPD10		
Cefpodoxime+Clavulanate 10+1 µg	CPD+C		
ESBL		**ESBL Confirm ID kit**	
Cefotaxime 5 µg	CTX.5	Cefotaxime 30 µg	CTX30
Ceftazidime 10 µg	CAZ10	Cefotaxime+Clavulanate	CTX+C
Cefotaxime+Clavulanate 5+1 µg	CT5+C	Ceftazdime 30 µg	CAZ30
Ceftazidime+Clavulanate 10+1 µg	CZ10C	Ceftazidime+Clavulanate	CAZ+C
		Cefepime 30 µg	FEP30
		Cefepime+Clavulanate	FEP+C
AmpC		**AmpC Confirm ID kit**	
Cefotaxime 30 µg	CTX30		
Cefotaxime+Boronic acid	CTXBO	Cefotaxime 30 µg	CTX30
Cefotaxime+Cloxacillin	CTXCX	Cefotaxime+Cloxacillin	CTXCX
Ceftazidime 30 µg	CAZ30	Ceftazidime 30 µg	CAZ30
Ceftazidime+Boronic acid	CAZBO	Ceftazidime+Cloxacillin	CAZCX
Ceftazidime+Cloxacillin	CAZCX		
Boronic acid Diatabs	BORON		
Cloxacillin Diatabs	CLOXA		
		ESBL+AmpC Screen kit	
		Cefotaxime 30 µg	CTX30
		Cefotaxime+Clavulanate	CTX+C
		Cefotaxime+Cloxacillin	CTXCX
		Cefotaxime+Clavulanate+Cloxacillin	CTXCC
Carbapenemases		**KPC+MBL Confirm ID kit**	
Meropenem 10 µg	MRP10		
Meropenem + Boronic	MRPBO	Meropenem 10 µg	MRP10
Meropenem + Cloxacillin	MRPCX	Meropenem+Boronic	MRPBO
Meropenem+ Dipicolinic	MRPDP	Meropenem+Cloxacillin	MRPCX
Dipicolinic acid Diatabs	D.P.A	Meropenem+Dipicolinic	MRPDP
Cloxacillin Diatabs	CLOXA		
Boronic and Diatabs	BORON		
Metallo-β-lactamases (MBL)			
Meropenem 10 µg	MRP10		
Meropenem+Dipicolinic	MRPDP		
Imipenem 10 µg	IMI10		
Imipenem+EDTA	IM10E		

Others	Code
Screening 16S rRNA methylases Amikacin 30 µg	 AMI30 or AMIKA
Screening of plasmid-mediated quinolone resistance Nalidixic acid 30 µg Ciprofloxacin 1 µg Norfloxacin 10 µg Ceftazidime 30 µg Tobramycin 10 µg	 NAL30 CIPR1 NORFX CAZ30 TOB10

	Code
Detection of hypermutable strains Fosfomycin Rifampicin 30 µg Ceftazidime 30 µg Ciprofloxacin 1 µg Imipenem 10 µg Meropenem 10 µg Tobramycin 10 µg	 FO200 or FOSFO RIFAM CAZ30 CIPR1 IMI10 MRP10 TOB10
Efflux pumps inhibitors NMP Diatabs	 NMP

Prediffusion method	Code
hVISA/VISA Vancomycin 30 µg Teicoplanin 30 µg	 VAN30 TPN30
Vancomycin resistant enterococci Vancomycin 30 µg Teicoplanin 30 µg	 VAN30 TPN30
Daptomycin non-susceptible staphylococci/enterococci Daptomycin (+Ca) 30 µg	 DAPCa
Colistin resistance in P.aeruginosa, A. baumannii, Enterobacteriaceae Colistin 10 µg	 CO.10

Extended-Spectrum Beta-Lactamases (ESBL)

Screening and Confirmatory Tests for Extended-Spectrum Beta-Lactamases (ESBL)

Transferable plasmid-mediated beta-lactamases that produce resistance towards third generation cephalosporins and monobactams (e.g. aztreonam) have been described in strains of *Klebsiella pneumoniae*, *K. oxytoca*, *E. coli* and other Enterobacteriaceae. These enzymes are classified as extended-spectrum beta-lactamases (ESBL) and they have been implicated in clinical resistance to monobactams and broad-spectrum cephalosporins such as ceftazidime (CAZ), cefotaxime (CTX), and ceftriaxone (CTR).

Some ESBLs confer high-level resistance to these beta-lactams and are easily detected as resistant (or intermediate) by disk (tablet) diffusion testing. But the ESBL may provide low levels of resistance (MIC 1-2 µg/ml) to monobactams and third generation cephalosporins that can be easily overlooked by routine susceptibility methods and current interpretative criteria (1). These latter isolates may not reach current CLSI breakpoints for resistance, yet can be clinically resistant to beta-lactam therapy (2).

Since some ESBLs are more active on CAZ, while others are more active on CTX, the choice of cephalosporins tested can also affect the ability of laboratories to detect resistant strains (3).
Most ESBLs are inhibited by clavulanic acid, tazobactam or sulbactam and can be readily detected by the double-disk (tablet) synergy test (4).

Double disk (tablet) synergy test

Inoculate a Mueller-Hinton plate as for susceptibility testing and apply Ceftriaxone (CTR) Neo-Sensitabs, Cefotaxime (CTX) Neo-Sensitabs, Ceftazidime (CAZ) Neo-Sensitabs, Cefepime (FEP) Neo-Sensitabs and Aztreonam (AZT) Neo-Sensitabs **at approximately 20 mm** (30 mm from tablet center to tablet center) from a tablet containing Amoxycillin+Clavulanate Neo-Sensitabs (AMC) using a dispenser. Incubate overnight at 35 °C.

Extension of the zone of inhibition (synergism) towards the tablet containing AM+CL, indicates the presence of an extended spectrum beta-lactamase (ESBL).

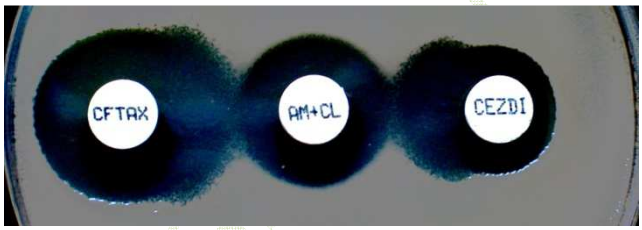

Plate 1.1.0-a. *Klebsiella pneumoniae* (ATCC 700603) producing extended-spectrum beta-lactamases (ESBL). Note the synergy between cefotaxime Neo-Sensitabs (CTX), ceftazidim Neo-Sensitabs (CAZ) and Amoxycillin+Clavulanate Neo-Sensitabs (AMC).

Another possibility of screening for ESBL is the use of lower MIC break-points for ceftazidime and aztreonam. Livermore et al (5) showed that most ESBL producers were resistant to CAZ at 2 µg/ml and AZTRM at 1 µg/ml. The corresponding zones with Neo-Sensitabs using McFarland 0.5 inoculum are 24 mm (CAZ and CTR) and 27 mm (AZT). As a consequence, *Klebsiella* spp, *E. coli* and *Salmonella* spp. showing zones < 24 mm with Ceftazidime, Cefepime and/or Ceftriaxone Neo-Sensitabs and/or ≤ 27 mm with Aztreonam and/or Cefotaxime Neo-Sensitabs, should be suspected of ESBL production. The CLSI has adopted practically all the same MIC breakpoints.

Cefpodoxime 10 µg may also be used in the screening of ESBL. Zones < 17 mm should be suspected of strains with ESBL production (18). Recently, the CLSI changed their Cefpodoxime screening breakpoints for ESBL from ≥ 2 to ≥ 8 µg/ml (18).

In a study comparing several ESBL screening methods, Vercauteren et al. (10) found that the double tablet synergy test using Neo-Sensitabs detected 96.9 % of ESBL producers while the E-test ESBL Screen detected 81.2 %.

De Gheldre et al showed that synergism between ceftazidime and cefepime with clavulanate (Neo-Sensitabs) was very useful to detect ESBL in *Enterobacter aerogenes* from Belgian hospitals (13).

Rodriguez-Villalobos et al. (20) and Fluit et al. (21) showed that the double disk (Neo-Sensitabs) synergy test has a higher sensitivity for the detection of ESBL than all combination disks (Oxoid, E-test).

Florijn et al (16) conclude that the use of ceftazidime, ceftriaxone and amoxycillin+clavulanate as Neo-Sensitabs is a cheap and reliable method for detection of *E. coli*, *Klebsiella* spp. and *P. mirabilis* isolates suspected of carrying ESBL. It performs better in a routine setting than the E-test, which often yields a result that cannot be interpreted.

Pitout et al. (22) recommend the use of cefepime and piperacillin+tazobactam when testing against strains with high level expression of AmpC beta-lactamases (*E. cloacae*, *E. aerogenes*, *C. freundii*, *S. marcescens)*. Synergism between cefepime and tazobactam indicates presence of an ESBL.

Synergism between Ticarcillin + Clavulanate and aztreonam/ceftazidime/cefepime permit the detection of ESBL producing strains of *Ps. aeruginosa* (SFM 2001). These strains show currently no zone around Ceftazidime Neo-Sensitabs (14).

Confirmatory Tests for ESBL (combination disks)

The CLSI (9) recommends the use of Ceftazidime in combination with Clavulanic acid: Ceftazidime+Clavulanate Neo-Sensitabs, as a phenotypic confirmatory test for the presence of ESBL. Perform the antibiogram using Mueller Hinton Agar and McFarland 0.5 inoculum. Test both Ceftazidime+Clavulanate, Cefepime+Clavulanate and Ceftazidime/ Cefepime Neo-Sensitabs.
An increase in zone diameter of ≥ 5 mm for the combination Ceftazidime+Clavulanate or Cefepime+Clavulanate compared to Ceftazidime/ Cefepime alone is confirmatory of the presence of an ESBL.

Steward et al (12) showed that synergism between cefepime and clavulanate (Cefepime + Clavulanate Neo-Sensitabs) is very useful to detect ESBL in *Klebsiella pneumoniae*, differentiating strains producing ESBL (synergy between cefepime and clavulanate) from strains producing Amp C or hyperproducers of beta-lactamase.

Enterobacter, Serratia, *Morganella morganii*, Providencia, *Citrobacter freundii* and *Pseudomonas aeruginosa* produce chromosomally encoded inducible **Amp C** beta-lactamase. High level expression of Amp C may prevent the recognition of ESBL. Cefepime is practically not affected by Amp C and consequently Cefepime Neo-Sensitabs should be included as an ESBL screening agent when testing Enterobacter, Serratia ect. Synergism between Amox-Clav and Cefepime will indicate ESBL production (11,12,13,17,19). Strains with Cefepime zones < 24 mm should be suspected of ESBL production.

Recently Schwaber et al (32) found that the Vitek 2, Advanced Expert System identified the ESBL phenotype in only 62.5 % isolates of *Enterobacter* spp. and erroneously reported cephalosporin susceptibility in 28 %.

Cefepime+Clavulanate (and cefepime) Neo-Sensitabs should be used in the confirmatory tests for ESBL, because they are effective in detecting ESBL in strains of Klebsiella, *E. coli* etc. that may produce Amp C or are hyperproducers of beta-lactamase (31).

ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa* (30). Cloxacillin 500 µg or Boronic acid Diatabs can be used to inhibit AmpC, for example by prediffusing (1 hour) one of these compounds on the agar before inoculation and before adding the antibiotic tablets (Neo-Sensitabs), placed on the same spots.

With *Klebsiella oxytoca*, synergism between Amoxycillin+Clavulanate (AMC) and Aztreonam or Ceftriaxone but not with ceftazidime indicates the presence of hyperproduction of K-1 chromosomal beta-lactamase (but **negative** for ESBL). Strains producing ESBL show synergism between AMC and ceftazidime (Use Ceftazidime+Clavulanate).
The use of cefotaxime, ceftriaxone, cefepime, aztreonam with AMC may result in **false positive** results for ESBL in *Klebsiella oxytoca* (Vitek,41).

The emergence of ESBL in Salmonellae merits concern. They cause frequently neonatal meningitis in many developing countries and are often already resistant to ampicillin and chloramphenicol (7).
Karas et al (8) reports clinical failure due to ESBL, in spite of the organism being susceptible with disk diffusion and MIC test (CTX MIC 0.75 µg/ml). The double disk diffusion test indicated the presence of an ESBL, but the test was first performed when therapy with cefotaxime was stopped, due to treatment failure.
The laboratory report should indicate that ESBL-producing strains may be resistant clinically to all penicillins, cephalosporins and aztreonam (9).

For serious systemic infections, even if the isolate appears susceptible to Amoxycillin+Clavulanate, Ticarcillin+ Clavulanate or Piperacillin+Tazobactam, do not report it as susceptible, because resistant mutants may be selected during therapy.

For Q.C. use *Klebsiella pneumoniae* ATCC 700603: zone of Ceftazidime+Clavulanate and Cefepime+Clavulanate is ≥ 5 mm larger than Ceftazidime/Cefepime Neo-Sensitabs (see document **1.1.1**)

Detection of ESBLs using Neo-Sensitabs™

ENTEROBACTERIACEAE

At present there are no CLSI guidelines available for detection of ESBL in other species than E.coli, K.pneumoniae, P.mirabilis and Salmonella spp.

ESBLs are also found in other Enterobacteriaceae such as Enterobacter spp. etc (22)

Strains showing cefotaxime and/or ceftazidime MICs ≥ 1 µg/ml, showing reduced susceptibility to amoxicillin + clavulanate should be tested further for the presence of ESBLs.

Procedure 1 (Double disk synergy test)

Mueller Hinton agar plates are inoculated with the strain to be tested and Neo-Sensitabs applied onto the agar. Cefotaxime, Ceftazidime and Cefepime Neo-Sensitabs at a distance of 15-20 mm (edge to edge) from Amoxycillin + Clavulanate Neo-Sensitabs.

Procedure 2 (Combination disks synergy test). ESBL Confirm ID kit

K. pneumoniae ATCC 700603, ESBL positive

Use Cefotaxime, Ceftazidime and Cefepime and their combinations with Clavulanate (ESBL Confirm ID kit).

Interpretation

A key hole or ghost zone between Amoxycillin + Clavulanate and any of Cefotaxime, Ceftazidime or Cefepime Neo-Sensitabs indicates the presence of an ESBL.
When using the combination disks, a ≥ 5 mm larger zone for any of the combinations compared to the corresponding single antimicrobial indicates the presence of an ESBL.

Cefpodoxime and Cefpodoxime + Clavulanate may be used for screening purposes.
Klebsiella oxytoca hyperproducing K-1 beta-lactamase may show a false positive result (potentiation of cofotaxime and /or cefepime). Only when the strain is resistant to ceftazidime and shows synergism between ceftazidime and clavulanate should it be reported as ESBL positive.

ESBL + AmpC beta-lactamases

Current susceptibility tests are not accurate enough for surveillance of ESBL and AmpC producing pathogens. Laboratories testing for ESBL and AmpC need to be aware that some of these organisms may test false positive for ESBL, using CLSI methodology (40).

Combined disk test (ESBL+AmpC). ESBL+AmpC Screen kit

Apply one of each: Cefotaxime (CTX30), Cefotaxime+Clavulanate (CTX+C), Cefotaxime+Cloxacillin (CTXCX) and Cefotaxime+Clavulanate+Cloxacillin (CTXCC) Neo-Sensitabs on a MH plate inoculated (McFarland 0.5) with the strain to be tested.

Interpretation

		Cefotaxime CTX30	Cefotaxime+Clav. CTX+C	Cefotaxime+Cloxa. CTXCX
ESBL	CTX+C or CTXCC	≥ 5 mm -	- <4 mm	- ≥ 5 mm
AmpC	CTXCX or CTXCC	≥ 5 mm -	- ≥ 5 mm	- <4 mm
ESBL+AmpC	CTX+C and CTXCC	<4 mm -	- ≥ 5 mm	- ≥ 5 mm

In strains possessing both chromosomal (Enterobacter, Cit. Freundii etc. (44) or plasmidic AmpC beta lactamases and ESBLs, Boronic acid or Cloxacillin are used as inhibitors of the AmpC beta lactamase.

Combined disk test (KPC+ESBL)

For detection of ESBLs in clinical isolates of KPC carbapenemase-possessing Enterobacteriaceae, the following method is used (43):

Inoculate (McF 0.5) the MH agar plate with the strain to be tested and add:

1 Cefotaxime+Boronic acid and 1 Cefotaxime+Clavulanate+Boronic acid, 1 Ceftazidime+Boronic acid and 1 Ceftazidime+Clavulanate+Boronic acid.

Interpretation

Cefotaxime+Boronic+Clav. zone ≥ 5 mm than Cefotaxime+Boronic and/or Ceftazidime+Boronic+Clav. zone ≥ 5 mm than Ceftazidime+Boronic indicates the presence of an ESBL.

Detection of ESBLs in different strains

The presence of ESBLs may be masked by the overexpression of AmpC beta-lactamases or by the induction of AmpC beta-lactamase by clavulanate used in synergy tests. ESBLs may be confused with enzymes such as *K. oxytoca* chromosomal ß-lactamase (K1). Laboratory staff must be aware of the increasing array of different resistance mechanisms and phenotypes.

NON-FERMENTERS

Here are particularly *P. aeruginosa* and *A. baumannii* that may possess several types of beta-lactamases. Non-fermenters showing reduced susceptibility to ceftazidime and/or cefepime and/or aztreonam should be tested for the presence of ESBLs.

Procedure

Apply Ceftazidime, Cefepime and Aztreonam Neo-Sensitabs. At a distance of approx. 15 mm (edge to edge) apply Ticarcillin + Clavulanate Neo-Sensitabs. Separately apply Ceftazidime + Clavulanate and Cefepime + Clavulanate Neo-Sensitabs.

Interpretation

A key-hole zone or ghost zone between Ticarcillin + Clavulanate and any of Ceftazidime, Cefepime or Aztreonam Neo-Sensitabs indicates the presence of an ESBL.
With the combination disks a ≥ 5 mm larger zone for Ceftazidime + Clavulanate and/or Cefepime + Clavulanate compared to the single antimicrobials indicates the presence of an ESBL.
The prediffusion procedure with Boronic acid may also be used (30) when ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa*.

1) E. coli, Klebsiella spp., Salmonella spp., Proteus mirabilis, Shigella sonnei:

ESBL	Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Cefoxitin S.
VEB-1 (ESBL) (25)	Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Synergism Imipenem and Ceftazidime and/or Cefoxitin and Ceftazidime.
AmpC plasmid (no ESBL)	No synergism Ceftazidime+Clavulanate and Cefepime+Clavulanate. Cefoxitin R, Ceftazidime R. Synergism Cefotaxime/Ceftazidime and Cloxacillin and Cefotaxime or Cefotaxime and Boronic acid.
DHA (Induc. plasmid AmpC)	Antagonism Clavulanate (AMC) and 3rd generation. Synergism Cefotaxime/Ceftazidime and Cloxacillin or Boronic acid.
DHA+ ESBL (33)	Antagonism Clavulanate (AMC) and 3rd gen. cephalosporins (DHA). Synergism Tazobactam (Piperacillin+Tazobactam) and Ceftazidime/Cefepime.
Amp C + ESBL	Synergism Cefepime+Clavulanate: ESBL (31).Cefoxitin R, Ceftazidime R. Synergism Cefotaxime or Ceftazidime and Cloxacillin or Boronic acid: AmpC.
DHA + ESBL	Synergism Ceftazidime + Clavulanate and/or Cefepime + Clavulanate: ESBL. Antagonism Clavulanate (AMC) and 3rd gen. cephalosporins: DHA/ACT-1.
ESBL + Metallo-beta-lactamases (24,28)	Synergism Aztreonam + Clavulanate (Amoxicillin+Clavulanate): ESBL. Synergism Imipenem+EDTA: metallo-beta-lactamases. Synergism Meropenem and DPA: metallo-beta-lactamases.

ESBL + 16S rRNA methylases (26)	Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Cefoxitin S. No zone with Amikacin, Gentamicin, Tobramycin Neo-Sensitabs.
ESBL+carbapenemases (not MBL)	Synergism Aztreonam +Clav. (Amox+Clav) Ertapenem I/R, metallo-β-lactamases neg. Positive Hodge test.

2) High level K-1 (*Klebsiella oxytoca*):

(no ESBL)	No synergism Ceftazidime+Clavulanate. Ceftazidime S. Synergy is currently observed with third gen. cephalosporins and Clavulanate as well as with Cefepime+Clavulanate.
K-1 + ESBL (23)	Synergism Ceftazidime+Clavulanate. Ceftazidime I/R.

3a) Enterobacter spp., Serratia spp., Providencia rettgeri, Citrobacter freundii:

ESBL	Synergism Cefepime and Clavulanate (Amoxycyllin+Clavulanate) and/or Ceftazidime and Clavulanate.
ESBL + 16S rRNA methylases	Synergism Cefepime and Clavulanate and/or Ceftazidime +Clavulanate. No zone with Amicacin, Gentamicin, Tobramycin Neo-Sensitabs.
ESBL + Metallo-beta-lactamases	Synergism Aztreonam (or Cefepime)+Clavulanate: ESBL Synergism Imipenem + EDTA and/or Meropenem + DPA: Metallo-beta-lactamases.

3b) Morganella morganii

ESBL	Synergism Cefepime and Tazobactam (Piperazillin+Tazobactam). Synergism Sulbactam (Ampicillin+Sulbactam) and Ceftazidime or Cefotaxime.

4) Pseudomonas aeruginosa:

ESBL	Ceftazidime S or R. Ticarcillin resistant. Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Synergism Aztreonam/Ceftazidime or Cefepime with Ticarcillin+Clavulanate
ESBL (PER-1)	Piperacillin S, Ceftazidime R. Synergy as above.
VEB-1 (ESBL)	Synergism between Imipenem and Ceftazidime (or Cefepime) in the presence of Cloxacillin. Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate.
OXA-18 (ESBL)	Ceftazidime R, Ticarcillin R, Aztreonam R, Meropenem I. Synergism Ceftazidime/Cefepime with Ticarcillin+Clavulanate.
ESBL + Metallo-beta-	Synergism Aztreonam (or Cefepime)+Clavulanate: ESBL lactamases Synergism Imipenem + EDTA and/or Meropenem + DPA: Metallo-beta-lactamases.

5) Acinetobacter spp. Use Double Disk Synergy Test

ESBL	Synergism Ceftazidime/Cefepime and Amoxycillin/Clavulanate. Synergism Ceftazidime/Cefepime and Ticarcillin+Clavulanate
PER-1 (ESBL)	Cephalosporins R, Aminoglycosides R. Synergy TIC+Clav, Synergy PIP+TAZO.
VEB-1 (ESBL)	Synergism Cefepime and Ticarcillin+Clavulanate (distance 15 mm). Best at 30 °C in the presence of Cloxacillin or Boronic acid.

Beceiro et al (37) have shown that the double disk synergy test gives the best results with Acinetobacter spp. due to Acinetobacter's intrinsic susceptibility to clavulanic acid.

6) Achromobacter xylosoxidans

VEB-1 (ESBL)	Synergy between Ceftazidime and Clavulanate.

7) Haemophilus influenzae:

ESBL (27)	Compare Cefpodoxime and Cefpodoxime+Clavulanate. Zones: ≥ 5 mm larger with the combination.

Note:

Although the lower cephalosporins MIC breakpoints of EUCAST and those forcoming from CLSI are better able to separate ESBL pos/neg populations of E.coli and Klebsiella spp, significant numbers of ESBLpositive isolates remain below these breakpoints. Confirmation testing of ESBL is necessary (42).

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ESBL Quality Control

***Klebsiella pneumoniae* ATCC 700603**

Original

NEO-SENSITABS	POTENCY	CODE	Zone diameter in mm	MIC µg/ml (15)
Aztreonam	30 µg	AZTRM	11-19	64
Cefepime	30 µg	CFEPM	21-26	1
Cefepime+Clavulanate	30+10 µg	CP+CL	≥5 mm larger	≤ 0.12
Cefotaxime	30 µg	CFTAX	20-26	8
Ceftazidime	30 µg	CEZDI	11-19	32
Ceftazidime-Clavulanate	30+10 µg	CZ+CL	≥5 mm larger	1
Ceftriaxone	30 µg	CETRX	16-24	16

CLSI/EUCAST potency Neo-Sensitabs™

CLSI/EUCAST potency NEO-SENSITABS™	POTENCY	CODE	Zone diameter in mm	MIC µg/ml (15)
Cefotaxime	30 µg	CTX30	17-25	8
Cefotaxime-Clavulanate	30+10 µg	CTX+C	≥5 mm larger	-
Ceftazidime	30 µg	CAZ30	10-18	32
Ceftazidime- Clavulanate	30+10 µg	CAZ+C	≥5 mm larger	1
Cefepime	30 µg	FEP30	19-25	1
Cefepime- Clavulanate	30+10 µg	FEP+C	≥5 mm larger	≤0.12
Cefpodoxime	10 µg	CPD10	9-16	-
Cefpodoxime-Clavulanate	10+1 µg	CPD+C	≥5 mm larger	-

Inducible Cephalosporinases or AmpC Beta-lactamases

Inducible cephalosporinases or AmpC beta-lactamases are produced by *Enterobacter cloacae*, *E. aerogenes*, *Serratia marcescens*, *Citrobacter freundii*, *Hafnia alvei*, *Providencia stuartii* and *Morganelli morganii*, and they are inhibited by aztreonam, but not by clavulanic acid, sulbactam or tazobactam.
Resistant mutants with high beta-lactamase activity are present at a high frequency. As a result therapy with cephalosporins (except fourth generation agents) and monobactams may fail because of selection of such mutants.

The tablet approximation test is useful to demonstrate the presence of inducible cephalosporinases, during routine antibiogram testing.
Neo-Sensitabs containing an inducer, e.g. cefoxitin (or imipenem) and indicators such as piperacillin+tazobactam, cefotaxime or ceftazidime are placed approx. 20-25 mm apart center to center. A wider spacing (30 mm) may be preferable for e.g. *M. morganii* and *Providencia* spp.
Following overnight incubation at 35 °C in air, the presence of an inducible beta-lactamase is indicated by the blunting of the zone of inhibition around the indicator drug (piperacillin+tazobactam, cefotaxime/ceftazidime) adjacent to the inducer (cefoxitin/imipenem).

Dunne et al (1) have shown that the combination Imipenem and Piperacillin + Tazobactam has the highest sensitivity (97.1 %) followed by Imipenem and Ceftazidime (94.2 %).

The result should be reported as R (resistant) for penicillins (except temocillin), penicillin/inhibitor combinations, cephalosporins (except cefpirome and cefepime), cephamycins and monobactams, irrespective of the size of the inhibition zone.

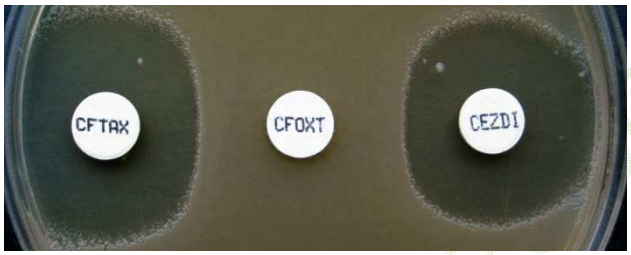

Plate 1.2.0-a

Plate 1.2.0-b

Demonstration of the presence of inducible beta-lactamases in *Enterobacter cloacae* (ATCC 13047). Note the flattened edges of Cefotaxime Neo-Sensitabs (CFTAX) and Ceftazidime Neo-Sensitabs (CEZDI) zones adjacent to Cefoxitin Neo-Sensitabs (CFOXT, Plate 1.2.0-a) and Imipenem Neo-Sensitabs (IMIPM, Plate 1.2.0-b), respectively.

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1) Dunne W.M. et al: Use of several inducer and substrate antibiotic combinations in a disk approximation assay format to screen for AmpC induction in patient isolates of P. aeruginosa, Enterobacter spp., Citrobacter spp. and Serratia spp. J.C.M., **43**, 5945-9, 2005.

Testing / Reporting of Susceptibility to Beta-lactams against Enterobacteriaceae and Non-fermenters

Use the table below for testing/reporting of susceptibility to beta-lactams against Enterobacteriaceae and non-fermenters causing serious infections when inducible beta-lactamases are present [a]:

	AMP	AMC	CXM	CTX	CTR	CAZ	FEP	CFO	AZT	IMI	MRP	TIM	PI+TZ
E. aerogenes/cloacae *C. freundii /* *S. marcescens*	R	R	R	R	R	R	T	R	R	T	T	R	R
Prov. stuartii/rettgeri *Morg. morganii*	R	R	R	R	R	R	T	R	T	T	T	R	T
P. vulgaris/penneri	R	T	R	R	R	T	T	T	R	T	T	T	T
Klebsiella oxytoca [e]	R	T	R	R	R	T	T	T	R	T	T	T	T
Hafnia alvei	R	R	R	R	R	R	T	R	R	T	T	R	R
Enterobacteriaceae with ESBL (**no** inducible ß-lactamases)	R	T [b]	R	R	R	R	R	R	R	T	T	T [b]	T [b]
Enterobacteriaceae with inducible ß-lactamases and ESBL	R	R	R	R	R	R	R	R	R	T	T	R	R
Aeromonas with A2 [c] (most *A. sobria*)	R	R	T	T	T	T	T	T	T	R	R	R	R
Aeromonas with A 1 and A 2	R	R	R	R	R	R	T	R	T	R	R	R	R
Ps. aeruginosa *Burkholderia* spp.	R	R	R	R	R	T	T	R	T	T	T	T	T
S. maltophilia	R	R	R	R	R	T	R	R	R	R	R	T	R
A. baumannii	R	T [d]	R	R	R	T	T	R	R	T	T	T	T

AMC, Amoxycillin+Clavulanate; AMP, Ampicillin; AZT, Aztreonam; CXM, Cefuroxime; CTR, Ceftriaxone; CAZ, Ceftazidime; FEP, Cefepime; CFO, Cefoxitin; CTX, Cefotaxime, IMI, Imipenem; MRP, Meropenem; PI+TZ, Piperacillin+Tazobactam; TIM, Ticarcillin+Clavulanate.

A1 Inducible cephalosporinase, the enzyme is usually found in *A. hydrophila* and *A. caviae*. These species are considered resistant to cephalosporins and cephamycins.
A2 Penicillinase/carbapenemase that hydrolyses imipenem and meropenem. The expression may be heterogeneous.

a) R, the microorganism is resistant and may possess a resistance mechanism not always detected by the diffusion method.
T, these antimicrobials may be used for testing.
b) Test isolates from urine only. Isolates from other sites are considered resistant.
c) Test also for Cefazolin. *A. caviae* does not posses a carbapenemase (A2) and can be tested against imipenem and meropenem.
d) Use Ampicillin+Sulbactam.
e) *K. oxytoca* producing a K-1 enzyme are susceptible to ceftazidime. They may show synergism between other third gen. cephalosporins and amoxicillin+clavulanate and may be mistaken as ESBL producers.

Adopted from CDS 2005 with modifications.

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Plasmid-mediated AmpC Beta-lactamases

Plasmid-mediated beta-lactamases represent a new threat, since they confer resistance to aminopenicillins, carboxypenicillins, ureidopenicillins, although they are generally susceptible *in vitro* to mecillinam and/or temocillin.

The enzymes provide resistance to third generation cephalosporins and cefoxitin. The enzymes are also active against aztreonam although for some strains the aztreonam MICs are in the susceptible range. Susceptibility to cefepime is little affected (inoculum effect) and the carbapenems are not affected. The enzymes are not affected by beta-lactamase-inhibitors, except for CMY-8 and CMY-9 that are inactivated by tazobactam.
Their expression is generally constitutive, nevertheless inducible plasmid AmpC (ACT-1, DHA-1, DHA-2, CFE-1, CMY-13) have been reported (6).

Plasmid-mediated AmpC beta-lactamases have been found most frequently in species naturally negative for AmpC, such as *K. pneumoniae*, *E. coli*, *K. oxytoca*, Salmonella and *P. mirabilis*. Recently they were also found in *Enterobacter* spp. (2).

The strains with plasmid-mediated AmpC show resistance to cefoxitin (MIC > 16 µg/ml) and ceftazidime (MIC > 32 µg/ml) corresponding to zones of inhibition < 16 mm (McF. 0.5).

Strains with plasmid-mediated AmpC do not show antagonism between cefoxitin and 3rd generation cephalosporins (are not inducible), while inducible plasmid-mediated AmpC (ACT-1, DHA-1, DHA-2, CMY-13) show antagonism between cefoxitin (or imipenem) and third generation cephalosporins.

Isolated that coproduce an ESBL and a plasmid mediated AmpC beta-lactamase may yield a positive confirmatory test for ESBL using cefepime and cefepime+clavulanate (synergism).

Characteristics of AmpC beta-lactamases:

	Chromosonally mediated AmpC (partially derepressed AmpC mutants)	Plasmid-mediated AmpC (derepressed AmpC mutants)	Inducible plasmid-mediated AmpC ACT-1, DHA-1, DHA-2, CFE-1, CMY-13	ESAC in P. aeruginosa (21)
Cefepime+Clavulanate and/or Ceftazidime+Clavula-nate	No synergism	No synergism (except MOX-1, MOX-2)	No synergism	No synergism
Cefoxitin, Imipenem or Amoxycillin+Clavula-nate (7)	Cefoxitin R (zone < 16 mm) **Antagonism** with 3rd gen. cepha.	Cefoxitin R (zone < 16 mm) **No antagonism** with 3rd gen cepha.	Cefoxitin R (zone < 16 mm) **Antagonism** with 3rd gen. cepha.	**No antagonism** Imipenem / 3rd gen cepha.
Ceftazidime Cefepime	S → R S	R (zone < 20 mm) S	S → R S	Ceftazidime R Cefepime I/R
Cloxacillin	Synergism Cloxacillin-cefotaxime	Synergism Cloxacillin+cefotaxi-me Cloxacillin+ceftazidi-me	Synergism Cloxacillin+cefotaxi-me Cloxacillin+ceftazidi-me	Synergism Cloxacillin-cefepime Cloxacillin-Carbapenems
Boronic acid synergy	Cefotaxime-ceftazidime	Cefotaxime-ceftazidime	Cefotaxime-ceftazidime	Carbapenems

Enterobacter spp., *C. freundii, M. morganii, Hafnia alvei, Providencia* spp., Proteus indole positive *and Serratia marcescens*, all produce an inducible chromosomal AmpC beta lactamase, which is not inhibited by clavulanate. There may be seen an antagonism between amoxycillin and clavulanate (smaller zone with the combination that with amoxycillin alone) due to the presence of the inducible beta-lactamase.

All these strains should be reported as resistant to ampicillin/amoxycillin and to amoxycillin+clavulanate (except P. vulgaris).
Using an Amoxycillin+Clavulanate disc (Neo-Sensitabs) better performance is obtained due to the dual action of clavulanic acid: 1) induces expression of inducible plasmid mediated AmpC beta-lactamases (antagonism with 3rd gen. cephalosporins) and 2) permits the detection of an ESBL by enlarging inhibition zones of 3rd gen. cephalosporins (synergism) (7). In the presence of an ESBL + an inducible plasmid-mediated AmpC, both antagonism and synergism can be detected in the same plate (7).

Differentiation of AmpC beta-lactamases in E. coli

Mirelis et al (9) and Aragon (15) found a simple phenotypic method for the differentiation between plasmid-mediated and chromosomal AmpC-ß-lactamases in E. coli and P.mirabilis using Cloxacillin Diatabs and by visual examination of the antibiogram plates. The presence of scattered colonies located near the edge of the zone of inhibiton of cefoxitin, cefotaxime, ceftazidime and aztreonam indicated the presence of plasmid-mediated AmpC beta-lactamases.
Cloxacillin or Boronic acid alone do not distinguish between chromosomal or plasmidic AmpC beta-lactamases.

E. coli (AMC I/R, Cefotaxime I/R, Ceftazidime I/R Cefoxitin: most I/R)

	Plasmid AmpC	**Inducible plasmid AmpC**	**Chromosomal AmpC hyperprod.**	**Chromosomal ESAC (14, 18)**
Cloxacillin	Synergy with ceftazidime or cefotaxime	Synergy with ceftazidime or cefotaxime	Synergy with ceftazidime or cefotaxime	Synergy
Boronic acid	Synergy with Ceftazidime and/or Cefotaxime	Synergy with Ceftazidime and/or Cefotaxime	Synergy with Ceftazidime. and/or Cefotaxime	Synergy
Cefoxitin Imipenem	No antagonism with 3rd gen. cephalosporins	Antagonism with 3rd gen. cephalosporins	No antagonism	No antagonism
Antibiogram	Scattered colonies (resistant mutants) near the edge of the zone of cefoxitin, cefotaxime, ceftazidime and aztreonam	Scattered colonies	Well defined edge of zone	
Cefepime MIC µg/ml	Cefepime MIC ≤1 µg/ml	Cefepime MIC ≤1 µg/ml	Cefepime MIC ≤1 µg/ml	Cefepime MIC 1-8 µg/ml (zone<26mm) Ceftazidime R Cefoxitin R

- ESAC=extended spectrum AmpC (14)
- The same procedure will be appropriate for K. pneumoniae and P. mirabilis strains.
- Chromosomal AmpC's of E.coli are not inducible.

Plasmid-mediated AmpC beta-lactamases MIC µg/ml

Beta-lactamases	FOX	CAZ	AZT	FEP	IMP	MRP	Microorganisms
AAC-1	4-8	≥32	1	0.25	0.125-0.8	0.03	E. coli, K. pneumoniae, P. mirabilis, Salmonella, C. freundii
ACT-1	> 256	4 - > 128	4 → > 128	≤ 0.06-8	1	·	E. coli, K. pneumoniae, E. cloacae (inducible)
BIL-1 (CMY-2)	R	> 16	4-16	1	0.5	0.06	E. coli
CMY-1	256	4-128	32	0.25-4	≤ 0.5	0.06	K. pneumoniae
CMY-2	32-256	32-128	16-64	0.5-4	≤ 0.5	0.06	E. coli, Salmonella, K. pneumoniae
CMY-3	128	64	32-256	1	0.25	0.03	P. mirabilis
CMY-4	8 - > 256	8-256	0.5-32	0.06-4	0.25	0.125	E. coli, Salmonella, P. mirabilis
CMY-5	R	256	64	-	0.5-1	-	K. oxytoca
CMY-6	256	256	64	0.5	0.25	0.06	E. coli
CMY-7	R	> 32	·	I	0.25	< 2	E. coli, Salmonella
CMY-8	> 256	32-64	·	·	0.25-0.5	-	K. pneumoniae
CMY-9	> 128	128	8	0.25	0.5	0.06	E. coli
CMY-10	> 128	8-64	4-128	0.12-0.5	0.25-0.5	≤ 0.125	E. coli, E. aerogenes, K. pneumoniae
CMY-11	> 256	256	128	·	·	·	E. coli
CMY-12	256	128	8-32	16	0.25-4	0.5	P. mirabilis
CMY-13	512	256	64	1	0.25	≤ 0.03	E. coli (inducible)
CMY-14	128	128	16-32	0.5-32	0.25-2	0.06	P. mirabilis
CMY-15	512	128	8-32	0.25-8	0.25-16	4	P. mirabilis
CMY-16	≥ 32	≥ 32	1	2	2	0.05	P. mirabilis / Synergy TAZO-FEP
CMY-19	≥ 128	> 128	16	4	0.25	≤ 0.06	K. pneumoniae (8)
CMY-20	≥ 128	> 128	>64	4	-	1	E. coli
CMY-21	> 64	64	32	0.5	0.25	0.06	E. coli
CMY-29	≥ 256	128	·	1	0.5	0.12	E.coli
CMY-30	128	32	128	5	0.5	0.12	E.coli
ESAC (14)				1-8			E.coli
ESAC (18)	> 256	> 256	≥32	32			E.coli
CFE-1	R	64	8	0.25	0.25	·	E. coli (inducible)
DHA-1	128-512	8-64	1-16	≤ 0.125-2	≤ 0.125 - 0.5	·	E. coli, K. pneumoniae, Salmonella, P. mirabilis (inducible)
DHA-2	16	8	2	0.03	0.25	·	K. pneumoniae (inducible)
FOX-1	128	8	1	1	0.25	≤ 0.03	E. coli, K. pneumoniae
FOX-2	256	32	2	0.13	0.5	0.03	E. coli
FOX-3	64	16	1	≤ 0.06	0.12	-	E. coli, K. oxytoca
FOX-4	> 512	> 128	64	2	0.5	0.12	E. coli
FOX-5	512	128	8-16	0.5	0.5	-	K. pneumoniae, E. coli
FOX-7	-	-	-	-	-	-	E. coli, K. pneumoniae, E. cloacae
LAT-1	64-256	> 128	64	1	0.25-2	0.06	K. pneumoniae
LAT-2 (CMY-2)	256	> 256	64-256	-	-	-	E. coli, K. pneumoniae, E. aerogenes
LAT-3 (CMY-6)	256	128	64	0.5	0.25	0.06	E. coli
LAT-4 (CMY-1)	64-256	8-256	8-128	0.125-1	0.25	0.125	E. coli
MIR-1	≥ 256	128	128	1	1	0.125	E. cloacae, K. pneumoniae
MOX-1	R	16 → R	16	-	0.5	-	K. pneumoniae,
MOX-2	≥ 128	4-256	·	0.25-4	≤ 0.125	-	K. pneumoniae

Gupta et al. (3) describes isolation of multiresistant Salmonella, with plasmid-mediated AmpC beta-lactamase, from cattle and humans in the USA.
Three cases of invasive infections caused by Salmonella enterica serotype cholerasuis found in Taiwan (5). The strains were resistant to ciprofloxacin (mutations gyrA and ParC) and to ceftriaxone (presence of plasmid-mediated CMY-2 beta-lactamase).

Recent studies (4) show that the outcome of cephalosporin treatment in serious infections due to AmpC beta-lactamase producing K. pneumonia isolates was poor. A standard test for detection of plasmid-mediated AmpC beta-lactamases is needed. Emergence of cefepime-hydrolyzing CMY-19 in Japan (8).

Detection of Plasmid-mediated-AmpC beta-lactamases

The capability to detect AmpC is important to improve the clinical management of infections and provide sound epidemiological data. Reduced susceptibility to cefoxitin in the Enterobacteriaceae may be an indicator of AmpC activity, but it should be confirmed by other tests. Laboratories should be able to recognize AmpC derepressed strains and those with plasmid AmpC. Guidelines from the CLSI are not yet available for detection of bacteria with AmpC beta-lactamases.

Screening

Derepressed/plasmid AmpC should be suspected when we see:

- Resistance to 3rd generation cephalosporins – NOT Cefepime.
- Resistance to Cefoxitin (inhibition zone < 16 mm).
- No cephalosporin / Clav. synergism.
- I / R to Amoxycillin + Clav.
- AmpC derepressed Serratia are S to ceftazidime.
- Providencia, Morganella and Serratia inducible & derepressed may appear S /I to cefoxitin.
- Strains producing AAC-1 beta-lactamase are susceptible to cefoxitin

Confirmation

1.Combined disk test. AmpC Confirm ID kit

Apply Cefotaxime, Cefotaxime+Boronic, Ceftazidime, Ceftazidime+Boronic, Cefotaxime+Cloxacillin and Ceftazidime+ Cloxacillin in an inoculated plate.

E.coli ATCC FN9414 AmpC positive

Interpretation

A Cefotaxime+Cloxacillin inhibition zone ≥ 5 mm than Cefotaxime alone and/or a Ceftazidime+Cloxacillin zone ≥ 5 mm than Ceftazidime alone indicates the presence of an AmpC.

A Cefotaxime+Boronic inhibition zone ≥5 mm than Cefotaxime alone and/or a Ceftazidime+Boronic inhibition zone ≥5 mm than Ceftazidime alone indicates the presence of an AmpC.

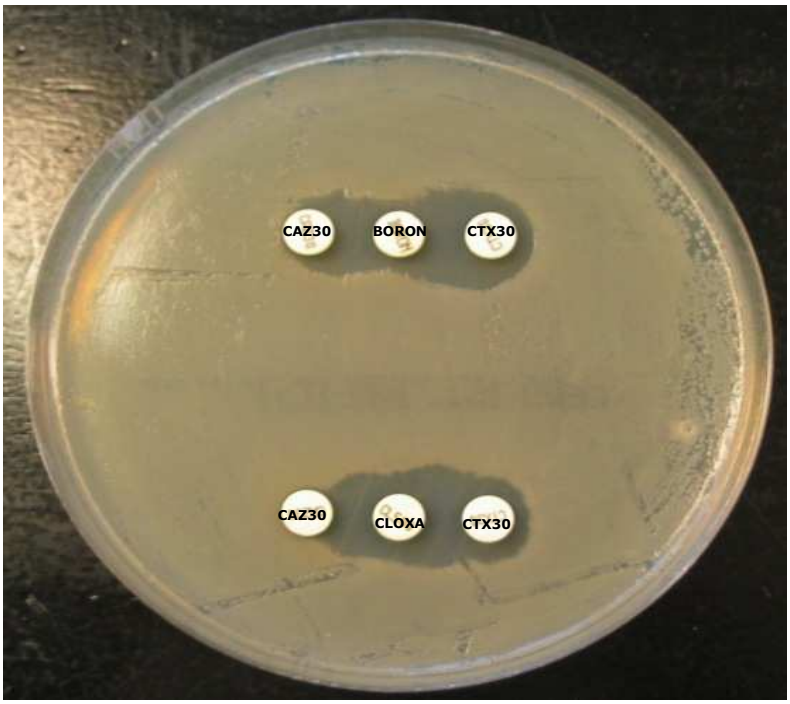
Inhibition zone ≥ 5 mm than Ceftazidime alone, indicates the presence of AmpC.

2. Double disk synergy test

Apply one Cefotaxime (CTX) and one Ceftazidime (CAZ) Neo-Sensitabs on an inoculated MH agar plate. In between apply one Boronic Acid Diatabs (BOR) at a distance of approx. 10 mm (edge to edge). If the strain is totally resistant to the Cefalosporins combination, the distance should be reduced to 5 mm.

Apply one Ceftazidime (CAZ) and one Cefotaxime (CTX) Neo-Sensitabs. In between at a distance of 5-10 mm edge to edge, apply one Cloxacillin Neo-Sensitabs.

Instead of Cefotaxime and Ceftazidime their combinations with clavulanate may be used for the synergy test (19).



E.coli ATCC FN9414 AmpC positive

Interpretation

A keyhole or ghost zone (synergism) between Boronic Acid and any of Cefotaxime or Ceftazidime indicates the presence of an AmpC beta-lactamase.

A keyhole or ghost zone between Cloxacillin and Ceftazidime and/or Cefotaxime indicates the presence of an AmpC beta-lactamase.

Plasmid mediated AmpC differ from chromosomal AmpC in being uninducible (few exceptions).Strains producing inducible plasmid AmpC beta-lactamases (ACT-1, DHA-1, DHA-2, CFE-1, CMY-13) will show antagonism (distorted zone) between Cefoxitin or Imipenem and 3rd generation cephalosporins.

Strains of Klebsiella spp, Salmonella spp and P. mirabilis showing synergism with Boronic Acid and/or Cloxacillin possess presumptively plasmid mediated AmpC beta-lactamases.

The method cannot distinguish between chromosomal and plasmid mediated AmpC beta-lactamases in E. coli, but the test is useful to select strains for further analysis. Plasmid mediated are often multiresistant and may show scattered colonies near the edge of the zone of third gen. cephalosporins and aztreonam disks.

Inducible phenotype

The inducible phenotype is identified by a tablet approximation test, using Imipenem or Cefoxitin against 3rd generation cephalosporins (distance 15 mm from edge to edge).

Distorted zones indicate the presence of an inducible AmpC beta-lactamase.

Treatment with 3rd generation cephalosporins should be avoided in severe Enterobacter, C. freundii, Serratia and Morganella infections except in UTI, because of risk for selection of cephalosporin-resistance during therapy.

AmpC + ESBL. ESBL +AmpC Confirm ID kit

Screening criterion for ESBL presence among AmpC-producing Enterobacter, C. freundii and Serratia marcescens is Cefepime MIC > 1 ug/ml (inhibition zone< 26 mm).

High level expression of AmpC may prevent recognition of an ESBL. Use of Cefepime is more reliable to detect these strains because high AmpC production has little effect on cefepime activity.

Combined disk test (ESBL+AmpC)

Apply one of each:

A) Cefotaxime (CTX 30)
B) Cefotaxime+Clavulanate (CTX+C)
C) Cefotaxime+Cloxacillin (CTXCX)
D) Cefotaxime+Clavulanate+Cloxacillin (CTXCX) on the inoculated MH plate.

Interpretation

		Cefotaxime CTX30	**Cefotaxime+Clav. CTX+C**	**Cefotaxime+Cloxa. CTXCX**
ESBL	CTX+C or CTXCC	≥ 5 mm -	- <4 mm	- ≥ 5 mm
AmpC	CTXCX or CTXCC	≥ 5 mm -	- ≥ 5 mm	- <4 mm
ESBL+AmpC	CTX+C and CTXCC	<4 mm -	- ≥ 5 mm	- ≥ 5 mm

Neither ESBL or AmpC: All zones within 2 mm of each other CTXCC= Cefotaxime+Clavulanate+Cloxacillin.

Detection of ESAC in P.aeruginosa (10)

ESAC in P. aeruginosa constitutes a favorable background for the selection of carbapenem-resistant strains. P. aeruginosa isolates being I/R to Imipenem and Ceftazidime should be tested for ESAC.

Place 1 Cloxacillin Diatabs between Imipenem and Cefepime Neo-Sensitabs (distance 8 mm from edge to edge). Synergy between Cloxacillin and Imipenem and/or Cefepime indicates the presence of and ESAC (extended spectrum AmpC)

Please note:
KPC and other class A carbapenemases (Sme, IMI, GES, NmcA) may give a positive synergy test with Boronic acid. They do not show synergism when using Cloxacillin (differentiation from AmpC).
K. oxytoca hyperproducing K-1 enzyme may give a positive synergy test with Boronic acid, but they show susceptibility to Ceftazidime and are therefore easily detected.

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Inhibitor Resistant TEM Beta-lactamases (IRT)

Strains with this phenotype give patterns of antibiotic resistance similar to TEM 1 or 2 or SHV 1 beta-lactamases, but they are resistant to amoxicillin + clavulanate. IRT are found mainly in *E. coli* and *Klebsiella pneumoniae*.
Are R (resistant) to amoxicillin + clavulanate. Zone diameter for Amoxicillin+Clavulanate Neo-Sensitabs < 17 mm. Are S (generally susceptible) to cephalosporins: cefazolin, cefoxitin, cefotaxime.

References:

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Carbapenemases

Carbapenemases are beta-lactamases that significantly hydrolyze at least imipenem and/or meropenem. Carbapenemases involved in acquired resistance are of Ambler classes A, B and D. They may be plasmid or chromosomally encoded.
Because several of these carbapenemases confer only reduced susceptibility to carbapenems in Enterobacteriaceae, they may remain underestimated, because they are not detected in the laboratory.
Acquired carbapenemases are increasingly reported worldwide and consequently it is important to be able to detect them in the laboratory.

For many isolates with carbapenemases, the MICs of carbapenems are around the susceptible breakpoint making resistance difficult to detect - particularly with automated systems. Therefore special zone breakpoints are needed in first line screening

Enterobacteriaceae with reduced susceptibility to Imipenem 10 µg (zone < 23 mm or MIC > 1 µg/ml) or Meropenem 10 µg (inhibition zone < 25 mm or MIC > 0.5 µg/ml) or Ertapenem (zone ≤ 22 mm) on Mueller-Hinton Agar with McFarland 0.5 inoculum, should be suspected of possessing carbapenemases

P. aeruginosa with inhibition zones Imipenem 10 µg (< 22 mm) or Meropenem 10 µg (< 26 mm) should be suspected of possessing carbapenemase. Most isolates with KPC and GES enzymes are highly resistant to Ceftazidime. Ertapenem Neo-Sensitabs is the most sensitive indicator for possible carbapenemase, but in approximately 20% of cases other resistance mechanisms are involved (confirmation of carbapenemase with Modified Hodge Test is necessary). It is important to recognize small resistant colonies growing inside the Ertapenem disk zone.

Carbapenemases classification (1)

Ambler classification	Enzymes	MICs µg / ml				Inhibited by		
		3rd gen cepha	AZT	IMP	MRP	CLAV	EDTA	Boronic acid
A	NmcA	S	4	≥ 16	2-8	± wk	no	yes
	Sme-1 to Sme-3	S	4-64	≥ 16	0.25-8	± wk	no	yes
	IMI-1 to IMI-2	S	S	≥ 64	4-32	+	no	yes
	KPC-1 to KPC-4	≥ 32	≥ 64	4→16	4→16	+ or wk	no	yes
	GES-2 to GES-5	≥ 32	16→R	0.25→16	0.5-16	+ or 0	no	yes
B Metallo-beta-lactamases	IMP 1-16	≥ 32	S→R	0.5-128	0.25→R	no	yes	no
	VIM 1-12	≥ 64	S→R	1→R	0.5→R	no	yes	no
	SPM-1	≥ 256	4	R	R	no	yes	no
	GIM-1	16-32	8-16	> 8	> 8	no	yes	no
	SIM-1	≥ 256	128	8-16	16	no	yes	no
D Oxacillinases	OXA 23-27	> 256	> 256	4-64	4-128	± wk	no	no
	OXA 40-48	S→R	S→R	2-64	0.25-64	wk	no	no
	OXA 54-55	S	S	4	0.25	wk	no	no
	OXA-60	S	R	0.5	2	no	no	no
	OXA-58	4-128	≥ 32	3-32	2→64	no	no	no

wk = weak

References:

1) Nordmann P et al: Emerging carbapenemases in gram-negative aerobes. Clin Microbiol. Infect **8**, 321-331, 2002.

Detection of acquired carbapenemases Ambler classes A and D

Class A carbapenemases are penicillinases with greater activity against imipenem than meropenem and they also give resistance to penicillins, cephalosporins and aztreonam.

Boronic acid in an inhibitor of class A carbapenemases and consequently synergy with meropenem or imipenem, is the best method to detect these enzymes (26,27,28,22).

Clavulanate is an inhibitor of class A carbapenemases and therefore synergy with imipenem may be useful to detect these enzymes (1,2,3,4,5).

The KPC family of enzymes confer greater resistance to third gen cephalosporins than to carbapenems (3,5).

KPC possessing *Enterobacter* spp. and *K. pneumoniae* were reported as falsely susceptible to carbapenems using automated systems (Vitek). MIC microdilution using standard inocula of 10^4 or 10^5 CFU/ml did not detect carbapenem resistance, while diffusion methods (E-test) using inocula of 10^8 CFU/ml detected resistance (5,7,12,18).

K. pneumoniae intermediate or resistant to ertapenem or meropenem should be considered resistant to all carbapenems (7). KPC possessing E.coli was identified in nine patients in New York. Three of the isolates possessed also ESBL: CTX M15 (19).

Pasteran et al (20) found that Boronic acid disks could be used to detect carbapenemases of type 2f (Class A) in Enterobacteriaceae. Class A producing strains showed synergy between Imipenem and Boronic acid disks (distance from edge to edge 6 mm). Strains showing zones of inhibition ≤21 mm with Imipenem 10 µg disks were screened with this test.

Carbapenemase IMI-2 is the first inducible and plasmid-encoded carbapenemase.

Please note that KPC detection may require screening multiple colonies, because carbapenemase susceptible strains may co-exist with resistant (21).

Class D carbapenemases correspond to the enzymes classified as OXA-types (oxacillinase activity). They hydrolyze imipenem and meropenem weakly and do not hydrolyze third gen cephalosporins and aztreonam (although MICs against the later drugs are often increased due to the presence of other beta-lactamases).
Clavulanate is a progressive inhibitor of most OXA carbapenemases, but not all. The synergy test (clavulanate and imipenem) may have value for the detection of these enzymes.

Clinicians should be aware of the potential for clinical failure (Class D, OXA-55 carbapenemase) when imipenem is used for treatment of serious infections caused by *S. algae* (9).

Fernandez et al report heteroresistance carbapenems in Ac. baumannii associated with Imipenems MIC 4-16 µg and the presence of OXA-58 (17).

Yilmaz et al (16) report oxacllinases (OXA-48) in 21 Enterobacteriaceae, mainly K.pneumoniae, but also in E.coli and Enterobacter cloacae/aerogenes in Turkey, and warns that oxacillinases (carbapenemases) are spreading in Enterobacteriaceae.

Castanheira et al (25) report the clonal dissemination of OXA-24 and OXA-58 producing A. baumannii in Houston, Texas.

Acquired carbapenemases Ambler class A and D

Ambler class.	Enzymes	MICs µg/ml PIPER	3rd gen cepha	AZTRM	IMI	MEROP	IMIPENEM + CLAV (synergy)	Mero-penem+ Boronic synergy	Organisms	Genetic Location
A	NmcA	S	S (0.25-2)	4	≥ 16	2-8	± wk	yes	*E. cloacae*	Chromosomal
	Sme-1 to Sme-3	S	0.25-0.5	4-64	≥ 26	0.25-8	± wk	yes	*S.marcescens*	Chromosomal
	IMI-1	> 256	1-2	8	> 64	4	+	yes	*E. cloacae*	Chromosomal
	IMI-2	16→128	0.1-2	4-8	64	4-32	+	yes	*E. asburiae*	Plasmid
	KPC-1	> 128	≥ 32	> 64	16	16	+		*K. pneumoniae*	Plasmid
	KPC-2	≥ 64	≥ 8	> 16	8→16	≥ 16	+		*K. pneumoniae* /*oxytoca. Raoultella(39) Salmonella Enterobacter*	Plasmid
(11)	**KPC-2**	.	≥ 256	> 256	256	256	(+)	yes	*P. aeruginosa*	Plasmid/Chrom.
	KPC-3	256	256	> 256	> 4	> 4	(+ +)		*K.pneumoniae Enterobacter E. coli, P. mirabilis, Citrobacter*	Plasmid
	KPC-4 KPC-5/6				> 16	> 16	(+)		*Enterobacter*	
	GES-2	128	≥ 32	16	4→16	4-16	+	yes	*E. cloacae P. aeruginosa*	Plasmid, integron
	GES-3	128	64→256	64	0.25	0.5	+	yes	*K. pneumoniae*	Plasmid
	GES-4	128	R	R	8	8	(+)	yes	*K. pneumoniae*	CEFOX R
	GES-5	R	R	R	8-32	8-32	+	yes	*P. aeruginosa, K.pneumoniae*	Integron
	GES-11	R	> 256	> 256	4	8		yes	*A. baumanni*	
D	**OXA-23** to **OXA-27**	> 256	> 256	> 256	4-64	4→128	± wk	no	*A. baumanni*	Chromosomal ± integron
	OXA-40	R	4→128	4→128	> 32	≥ 32	wk	no	*Ac.haemolyticus K. pneumoniae P. aeruginosa*	Plasmid
	OXA-48	8→R	S→R	S→R	2→64	0.25→64	wk	no	*K. pneumoniae* E.coli	Plasmid
	OXA-54	32	S	S	1	0.12	wk	no	*Sh. putrefaciens*	Not integron
	OXA-55	S	S	S	1-4	0.25	no wk	no	*Sh. algae* (9)	Chromosomal
	OXA-58	256	4-128	≥ 32	2-32	2→64	no	no	*A. baumanii*	Plasmid
	OXA-60	S	S	R	0.5	2	no	no	*R. pickettii*	Chromosomal
	OXA-62	S→R	S→R	S→R	2→64	64 →128	no	no	*Pandorea* (10) *pnomenusa*	Chromosomal
	OXA-23, **27**, 49 (subgroup 1)			>16	8→32	8→>32		no	*Ac. baumannii*	Plasmid (only 23)
D (8)	**OXA-24**, **25, 26, 40** (subgroup 2)	-	> 256		> 128	> 128	-	no	*Ac. baumannii*	Chromosomal
	OXA-51 + OXA-64-66, 68-71, 78-82-107 OXA-51-like (subgroup 3)		> 32		≥ 1	≥ 1		no	*Ac. baumannii*	Chromosomal plasmid
	OXA-58 (subgroup 4)	R	R	> 16	4/16			no	*Ac. baumannii*	Plasmid (only 58)
	OXA-143	R	FEP4	.	32	32	-	no	*Ac. baumannii*	Plasmid

Bold = involved in outbreaks

Procedure for KPC carbapenemases detection (Class A enzymes)

Isolates giving negative metallo-beta-lactamase tests, may produce other carbapenemases. The most current are KPC enzymes isolated from *Enterobacteriaceae (K. pneumoniae, E. coli, Enterobacter spp., P.mirabilis)* particularly *K. pneumoniae*, but also Sme, IMI, GES and Nunc A are found.
To detect these strains in rectal swab screening samples, direct plating on McConkey agar in the presence of Ertapenem Neo-Sensitabs and Imipenem Neo-Sensitabs may be useful.

Place one Boronic Acid Diatabs between one Ertapenem and one Imipenem Neo-Sensitabs (distance 6 mm from edge to edge).

Place one Cloxacillin Diatabs between Ertapenem and Imipenem Neo-Sensitabs - (6 mm from edge to edge).Perform Modified Hodge Test (MHT) with Ertapenem and Meropenem Neo-Sensitabs.

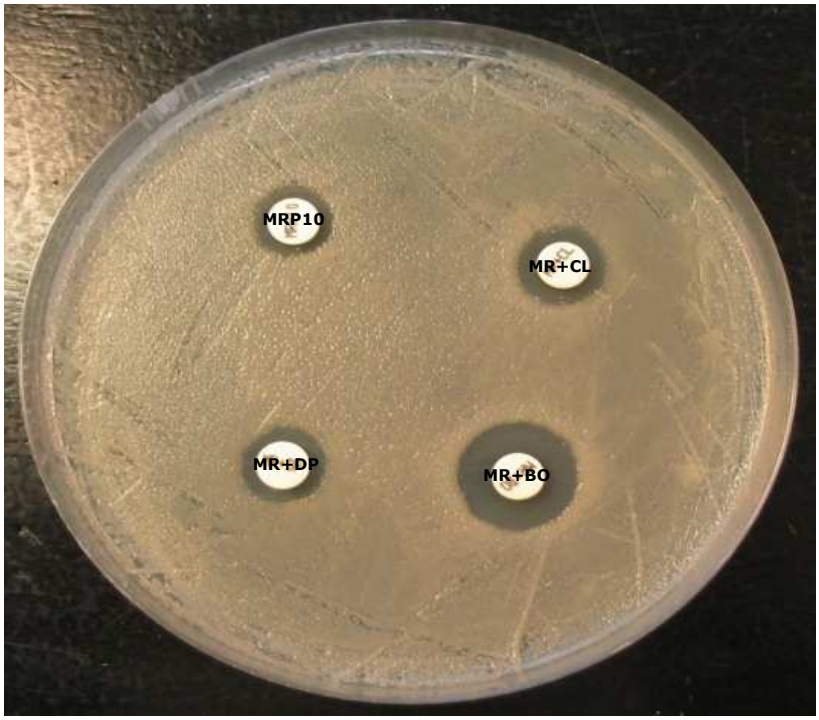
Interpretation (Double disk synergy test)

The following results will presumably indicate the presence of a KPC beta-lactamase:

a) Negative metallo-beta-lactamases tests.
b) Positive synergy test between Boronic Acid and the carbapenems (one or both).
c) Negative synergy test between Cloxacillin and the Carbapenems (11)
d) Positive synergy test between clavulanate (AMC) and carbapenems (one or both). Not always easy to see. Although isolates with ESBL + impermeability may give false positive results.
e) Positive Modified Hodge Test.
f) Sme, IMI, GES and Nunc A will show the same results as KPC, but the mentioned enzymes result in smaller zones around Imipenem compared to Ertapenem. With KPC enzymes zones around Imipenem and Ertapenem are similar.

Combined disk test. KPC + MBL Confirm ID kit

Apply Meropenem, Meropenem+DPA, Meropenem+Boronic, Meropenem+Cloxacillin on an inoculated plate.



K.pneumoniae PHA3 CL5761 KPC positive

Interpretation (combined test)

A Meropenem + Boronic inhibition zone ≥ 5 mm then Meropenem, Meropenem+DPA and Meropenem+Cloxacillin indicates a presence of a KPC enzyme (or other class A). Meropenem+Boronic and Meropenem+Cloxacillin inhibition zones ≥ 5 mm, than Meropenem and Meropenem+DPA indicates AmpC hyperproduction + porin loss, or efflux (30).

A Meropenem +DPA inhibition zone ≥ 5 mm than Meropenem, indicates the presence of a metallo-β-lactamases (MBL).

Please note

Test only ertapenem-resistant strains. Ertapenem susceptible strains may provide a false positive result with Boronic Acid.

Conclusion

Reduced susceptibility to ertapenem, synergy between Boronic Acid and the carbapenems, and no synergy between Cloxacillin and the carbapenems is clearly indicative of KPC enzyme being present (or other class A enzymes). Isolates producing high level AmpC + impermeability can be detected by synergy between Cloxacillin and the carbapenems (11). Isolates producing ESBL + impermeability will show synergy between AMC and the carbapenems or cephalosporins.

Procedure for Oxacillinase detection (Class D enzymes)

Strains producing oxacillinases will currently show zones of inhibition < 22 mm with Ertapenem and/or <25 mm with Meropenem Neo-Sensitabs. Most are resistant to Aztreonam. These enzymes are mainly found in Acinetobacter baumannii but also in Enterobacteriaceae (K. pneumoniae, Enterobacter) and P. aeruginosa although these are rare.

Interpretation

The following results will presumably indicate the presence of oxacillinases:

a) Negative metallo-beta-lactamase tests.
b) Negative synergy test between Boronic acid/Cloxacillin and the carbapenems. (one or both).
c) Negative (or weak positive) synergy test between clavulanate (AMC) and carbapenems (one or both)
d) Positive Modified Hodge Test.

Summary detection of carbapenemases

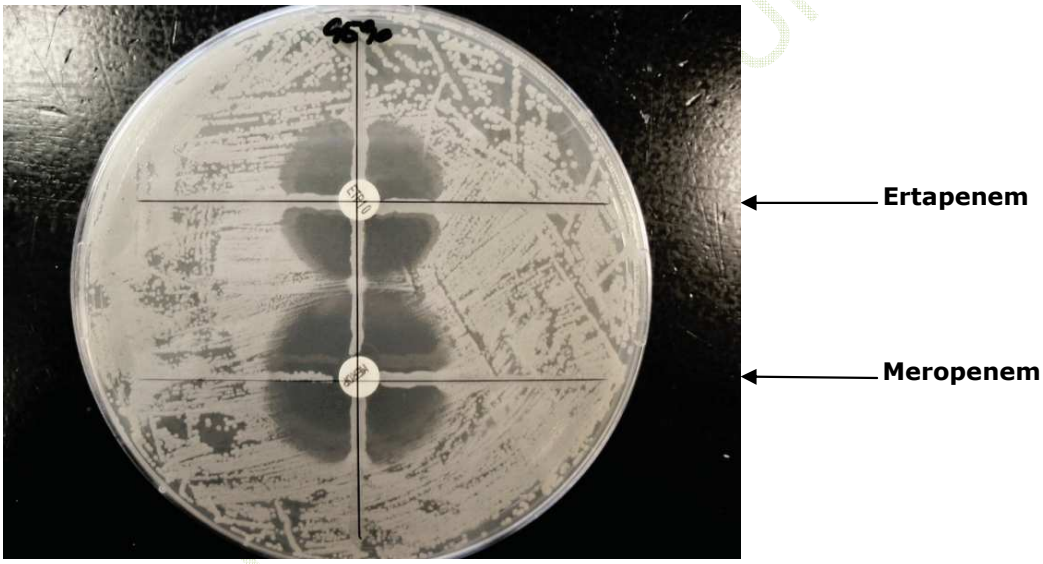
Meropenem	MRP+DPA	MRP+BOR	MRP+Cloxa
Metallo-β-lactamases	Synergy	No synergy	No synergy
KPC	No synergy	Synergy	No synergy
AmpC impermeability	No synergy	Synergy	Synergy
Oxacillinases	No synergy	No synergy	No synergy

Modified Hodge Test

Is used to determine if resistance to carbapenems is caused by a carbapenemase. A MH agar plate (or a McConkey plate) is inoculated with the susceptible strain E. coli ATCC 25922 (Mc Farland 0.5, diluted 1/10) as for disk diffusion.
When testing Enterobacteriaceae, one Ertapenem Neo-Sensitabs and one Meropenem Neo-Sensitabs are applied onto the plate approx.30 mm apart from each other. For non-fermenters one Imipenem Neo-Sensitabs and one Meropenem Neo-Sensitabs are applied.

A suspension of the microorganism to be tested for carbapenemase is adjusted to Mc Farland 0.5 standard and a loop is used to make a heavy streak passing through the two carbapenem disks. Two more streaks are placed perpendicularly making a cross.

Thereafter incubation for 18-24 hours at 35-37 C. Alteration in the shape (indentation) of the zones of inhibition around the test organism is considered indicitive of the presence of a carbapenemase (figure).



K.pneumoniae KPC positive

Limitations:

- Not reliable for detection of SME from S. marcescens
- P. mirabilis swarming may give lecture problems.

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Detection of acquired Metallo-beta-lactamases (MBL)

The worldwide spread of acquired metallo-beta-lactamases (MBL) in gram-negative aerobes is of great concern. MBL production in clinical isolates of key gram-negatives: *P. aeruginosa, E. cloacae, S. marcescens* and *K. pneumoniae* should be carefully monitored (5).

MLBs are classified into 5 major types: IMP, VIM, SPM, and GIM and SIM type enzymes. In Enterobacteriaceae only IMP and VIM enzymes have been found as yet.
MBLs hydrolyze most beta-lactams (carbapenems and large expectrum cephalosporins), except aztreonam. This phenotype of multiple beta-lactam resistance and aztreonam susceptibility may be helpful for identification of these strains in the laboratory. If the strain is resistant to aztreonam it may be due to additional resistance mechanisms (efflux, other beta-lactamases, ESBL etc.). Their expression is not inducible.

The MBL enzymes are resistant to beta-lactamase inhibitors and susceptible to chelating agents like EDTA (2-MPA) and Dipicolinic acid (DPA).

Early detection of MBL-producing microorganisms is essential to prevent dissemination of these organisms. The enclosed tables, including strains of Enterobacteriaceae and Non-fermenters producing MBLs, show that MPL-producers (particularly in Enterobacteriaceae) may show low MIC values against carbapenems making it difficult for the laboratory to detect MBL-positive isolates.

Suspicious isolates (resistant to ceftazidime showing no synergy between clavulanate and third gen. cephalosporins and possibly showing reduced susceptibility to carbapenems) should be tested for carbapenemase activity using Imipenem, Meropenem and EDTA and Dipicolinic acid tests.

The first metallo-beta-lactamase producing strain of *E. coli* (in Spain) has been detected in Barcelona, using Imipenem+EDTA Neo-Sensitabs and E-test (3,8). The first metallo-beta-lactamase producing strain of *K. pneumoniae* was found in France (4).

MBL- producing gram-negatives have now emerged in Australia (15).The resistance gene bla-IMP4 appears highly mobile, this outbreak involved 5 different gram-negative genera. Diagnostic laboratories in Australia and other countries must be now in high alert, because early detection may limit the wide dispersal of MBL-genes.

Kyegong (27) and Miriagou (28) showed the efficiency of Dipicolinic acid (DPA) to detect metallo-β-lactamases in Enterobacteriaceae and non-fermenters. Miriagou found that the DPA/Imipenem synergy test was positive for all VIM-producing isolates of Klebsiella/Enterobacter and P. mirabilis, while EDTA based tests could not identify VIM-producing P. mirabilis

Acquired Metallo-beta-lactamases
NON-FERMENTERS

MBL	3rd gen. Cepha MIC	AZT MIC µg/ml	IMP MIC µg/ml	MRP MIC µg/ml	Microorganisms	Genetic location
IMP 1-11	≥ 128	≤ 8/16	≥ 8	≥ 8	*Pseudomonas* spp. *Alcaligenes* spp. *Acinetobacter baumannii*	Chromosomal plasmid integron
IMP 12	≥ 128	32	32	128	*Pseudomonas putida*	
IMP 13-16	≥ 256	4-128	≥ 64	≥ 64	*Pseudomonas aeruginosa*	integron
VIM 1-3	R	S → R	2-128	1-128	*Achromobacter xylosoxidans* *Pseudomonas aeruginosa* *Pseudomonas putida* (VIM 2 and 4) *Acinetobacter baumannii*	Chromosomal plasmid integron
VIM 4-11	> 256	S → R	32-256	32-256	*Pseudomonas aeruginosa, A.baumannii*	
VIM 15-16	≥ 64	16-32	>128	≥128	*Pseudomonas aeruginosa*	integron
VIM-18	R	S → R	R	R	*Pseudomonas aeruginosa*	integron
SPM-1	≥ 256	4	R	R	*Pseudomonas aeruginosa*	Plasmid (**not** integron)
GIM-1	16 → 32	8-16	> 8	> 8	*Pseudomonas aeruginosa*	Integron
SIM-1	≥ 256	128	8-16	16	*Acinetobacter baumannii*	Integron
IND1-6	1-32-128	32-128	4-32-128	4-16-128	*Chryseobact indologenes*	Chromosomal (23)
AIM-1					*Pseudomonas aeruginosa*	

MBL are not inhibited by clavulanate, but are inhibited by EDTA or DPA

Acquired Metallo-beta-lactamases
ENTEROBACTERIACEAE

MBL	3rd gen. Cepha MIC	AZT MIC µg/ml	IMP MIC µg/ml	MRP MIC µg/ml	Microorganisms	Genetic location
IMP-1	≥ 32	< 0.5	2	0.5	*E. coli*	
IMP-1	≥ 32	0.5 → R	4-128	4-128	*S. marcescens, K. pneumoniae, K. oxytoca, E. cloacae / E. aerogenes, Cit. freundii, P. rettgeri, M. morganii,*	Integron
IMP-3	64	0.5	1	·	*Shigella flexneri*	
IMP-4	256	·	3	6	*Citrobacter youngae*	Plasmid
IMP-6	> 128	0.25	2-8	64	*E. coli*	
IMP-6	> 128	128	32	> 128	*Serratia marcescens*	
IMP-8	R	S → R	0.5-8	0.25-4	*Enterobacter cloacae, Klebsiella pneumoniae, S.marcescens*	
VIM-1	R	8-128	8-32	2-32	*E. coli, P. mirabilis (integron) C. koseri, K. oxytoca*	Plasmid
VIM-1	16-128	S → R	1-64	1-32	*Klebsiella pneumoniae, E. cloacae*	Plasmid (integron)
VIM-2	≥ 32	S → R	≥ 1	0.5 → > 2	*Citrobacter freundii / E.cloacae*	Plasmid
VIM-2	≥ 128	32	16-64	8-64	*Serratia marcescens, P. rettgerii*	Integron
VIM-2	8	16	4	0.1 (S)	*Klebsiella oxytoca*	Plasmid (integron)
VIM-4	≥ 32	4 → R	2-4	0.5-1	*K. pneumoniae / E. cloacae*	Plasmid
VIM-12	≥ 128	16	8	4	*K. pneumoniae*	Plasmid (16)
VIM-12	> 32	1	1	0.25	*E. coli*	Plasmid (22)
VIM-2 + GES7					*E. coli*	Integron
KHM-1	R	0.25	2	4	*C. freundii*	Plasmid
NDM-1	R				*K. pneumoniae, E.coli, C.freundii (31,33)*	Plasmid

MBL are not inhibited by clavulanate, but are inhibited by EDTA or DPA.

Procedure for metallo-beta-lactamase (MBL) detection

Some resistance profiles may suggest MBL production, for example:

a) *Pseudomonas aeruginosa*, *Pseudomonas*. spp. and *Acinetobacter* spp.
All isolates non-susceptible to carbapenems and resistant to either ticarcillin, ticarcillin+clavulanate or ceftazidime should be tested for MBL production.

b) *Enterobacteriaceae*
For *E. coli*, *Klebsiella* spp., *P. mirabilis*, *Salmonella* spp. and *Shigella* spp.: All carbapenem S-I-R isolates that are resistant to cefoxitin and amoxicillin+clavulanate and are non-susceptible to ceftazidime (inhibition zone < 18 mm) should be tested for MBL production. In all other cases all isolates are non-susceptible to carbepenems (18).

Enterobacteriaceae

Apply one Dipicolinic Acid Diatabs (DPA) on an inoculated Mueller Hinton (MH) plate. Apply one Meropenem Neo-Sensitabs and one Ertapenem Neo-Sensitabs onto the plate on either side of the DPA, 5mm from the DPA (edge to edge). Apply Imipenem 10 µg + EDTA (IM10E) on an inoculated MH plate. Apply one Imipenem 10 µg Neo-Sensitabs.

Non-fermenters

Apply one DPA Diatabs on the MH plate. Apply one Imipenem Neo-Sensitabs and one Meropenem Neo-Sensitabs at either side of the DPA, 5 mm from the DPA (edge to edge). Apply Imipenem + EDTA (IM10E) on the inoculated MH plate. Apply one Imipenem Neo-Sensitabs.

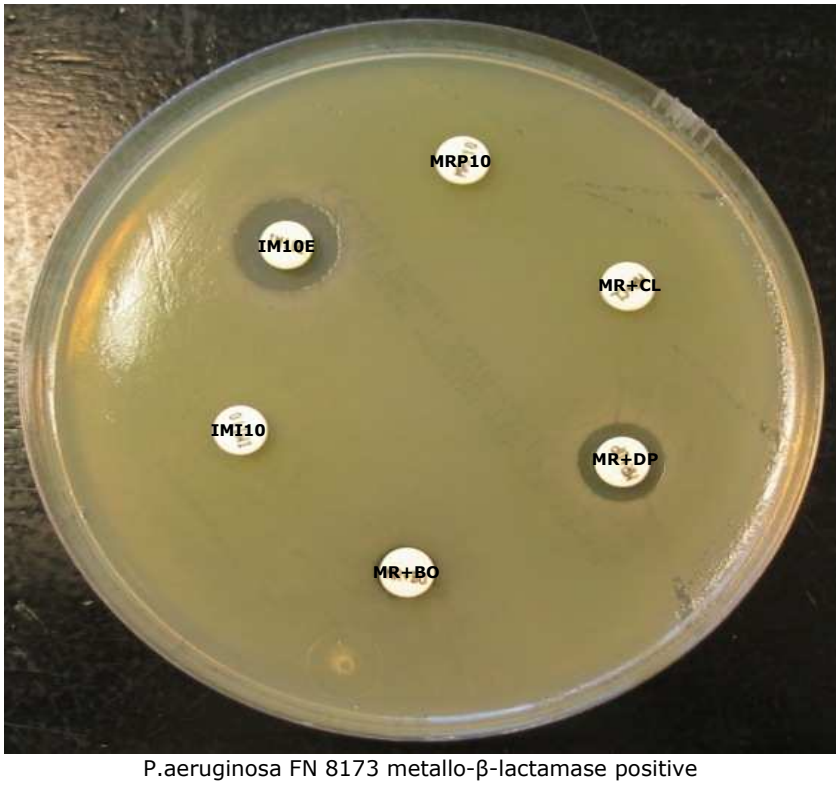
Interpretation (Double disk synergy test)

The use of two chelating agents EDTA and DPA will enhance the detection of metallo-ß-lactamases (MBL) in the clinical laboratory. A key hole or ghost zone between carbapenems (one or more) and Dipicolinic Acid indicates the presence of an MBL.
• An Imipenem + EDTA (10+750 µg) zone 7mm larger than Imipenem 10 µg indicates the presence of a Metallo-Beta-Lactamase.

A key hole or ghost zone between carbapenems (one or more) and Dipicolinic Acid indicates the presence of a MBL.

Combined disk test. KPC and MBL Confirm ID kit

Apply Meropenem, Meropenem+DPA on an inoculated MH plate. Interpretation: A Meropenem+DPA inhibition zone ≥ 5 mm than Meropenem alone indicates the presence of a metallo-beta-lactamase.



P.aeruginosa FN 8173 metallo-β-lactamase positive

Please note:

Most MH agar brands contain physiological levels of Zn++ions and should be used for carbapenem testing. Iso-Sensitest agar has low levels of zinc ions and may give false susceptibility results for carbapenems in the presence of MBL. Strains of Acinetobacter baumannii producing certain oxacillinases may give a false positive metallo-ß-lactamase test result.

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Detection of multiple beta-lactamases in one strain

Diagnostic problems posed by coexistance of different classes of beta-lactamases in a single bacterial isolate could be solved by the combined use of various phenotypic detection methods. See below example with multiresistant *K. pneumoniae* from Taiwan and USA.

	Neo-Sensitabs				
K. pneumoniae producing:	Cefoxitin	Cefepime	Ceftazidime+ Clavulanate or Cefepime+ Clavulanate synergy	D.P.A. + Meropenem or Imipenem+ EDTA synergy	Boronic acid Cefotaxime/ Ceftazidime or Cloxacillin Cefotaxime/Ceftazidime synergy
AmpC	R	S	negative	negative	POSITIVE
ESBL	S (V)	I / R	POSITIVE	negative	negative
Metallo-β-lactamase	R	I / R	negative	POSITIVE	negative (V)
KPC*	R	I / R	negative	negative	Negative (V)
AmpC + ESBL	R	I / R	POSITIVE	negative	POSITIVE
AmpC + metallo-ß-lactamases	R	I / R	negative	POSITIVE	POSITIVE
AmpC + ESBL + metallo-ß-lactamases	R	I / R	POSITIVE	POSITIVE	POSITIVE

*KPC shows synergism between Boronic acid and Imipenem/Meropenem, but **no synergism** between Cloxacillin and Imipenem/Meropenem.

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Detection of ß-lactam Resistance Phenotypes in Enterobacteriaceae

	AMP	AMC	CLOT	CAZ	FEP	FOX	CAZ+CLAV FEP+CLAV	IMI	IMI+ EDTA MRP+DPA	CLOXA Boronic	Comments
1) E. coli / P. mirabilis / Salmonella spp. / Shigella spp. / Klebsiella spp., /C. diversus											
Penicillinase low (E. coli)	I/R	S/I	S	S	S	S		S			
Penicillinase high	R	R	R	S	S	S		S			
Cephalosporinase low	S/I	S/I	I/R	S	S	S		S			
AmpC high / plasmid	R	R	R	I/R	S	I/R		S		synergy CAZ or CTX	FOX antagonism with CAZ or FEP indicates inducible
AmpC + ESBL	R	R	R	R	S/R	R	Synergy	S		synergy CAZ or CTX	
IRT	R	R	S/I	S	> CAZ (zone)			S			
Oxacillinase	R	R	S/I	S	≤ CAZ (zone)			S			
Penicillinase + Cephalosporinase	R	R	R	S/I	S	I/R		S			
Chromosomal K-1 high (Klebsiella oxytoca)	R	I/R	R	S	S/I	S	false synergy FEP+CLAV	S			
ESBL	R	V/R	I/R	V/R	V/R	S/R	synergy	S			
Metallo-ß-lactamase	R	R	R	I/R	I/R	R	-	S/R	Synergy		AZT S^R
Carbapenemase class A KPC	R	S/R	R	I/R	I/R	R		I/R (syn CLAV)		No synergy CLOXA with MRP	Synergy: IMI OR MRP with BORONIC
2) Enterobacter spp. / C. freundii											
Penicillinase	R	R	R	S	S	R		S			
AmpC derepressed	R	R	R	R	S	R		S		syn CTX syn CAZ	
AmpC + ESBL	R	R	R	R	S/R	R	Synergy	S		syn CTX syn CAZ	
ESBL, Metallo-ß-lactamase and Carbapenemases	As for *E. coli* etc.										
3) M. morganii / Providencia spp. / Serratia spp.											
Penicillinase	R	R	R	S	S	R		S			
Amp C derepressed	R	R	R	R	S	I/R		S		syn CTX syn CAZ	
ESBL, Metallo-ß-lactamase and Carbapenemases	As for *E. coli* etc.										
4) P. vulgaris / P. penneri											
Penicillinase	R	S	R	S	S	S		S			

Chromosomal ß-lactamase derepressed	R	S	R	R	S	S		S			

AMP; Ampicillin, AMC; Amoxicillin – clavulanic acid, CLOT; Cephalothin, CAZ; Ceftazidime, FEP; Cefepime, FOX; Cefoxitin, CAZ+CLAV; Ceftazidime + clavulanic acid, FEP+CLAV; Cefepime + clavulanic acid, IMI; Imipenem, IMI+EDTA; Imipenem+EDTA, Cloxa ; Cloxacillin, AZT; Aztreonam; CTX=Cefotaxime;MRP=Meropenem.

Detection of ß-lactam resistance phenotypes in non-fermenters

	TIC	TCC	PIP	CAZ	FEP	IMI	MRP	CAZ+CLAV FEP+CLAV	IMI+EDTA MRP+DPA	Remarks
1) *Pseudomonas aeruginosa*										
Penicillinase (TEM 1-2, PSE 1-4)	R	S/I	I/R	S	S	S	S			
Amp C partial derep.	S/I	any	I	I	S/I	S	S			
Amp C derepressed	I/R	R	I/R	R	I/R	S	S			CAZ < AZT TIC > CTAX
Oxacillinase	I/R	I/R	any	S	S/R (≤CAZ)	S	S			FEP ≤ CAZ TIC > CTAX
OXA-31, OXA 1, 4.	I/R	I/R	any	S	I/R	S	S			
Mex XY-OprM Efflux I/R (1)				S	I/R	S/R	S			CAZ ≥ FEP zone
PER 1-2, VEB-1 (ESBL)	R	S	S/I	R	R	S (PER-1) I/R (VEB-1)	S (PER-1) I/R (VEB-1)	Synergy		Phenotype PIP S, CAZ R indicates PER-1 enz.
ESBL	I/R	any	R	I/R	R	S	S	Synergy		
Metallo-ß-lactamase	R	R	R	R	R	I/R	I/R		Synergy	AZT, S^R
Carbapenemase Class A KPC	R	S/I	R	I/R	I/R	I/R	I/R			AZT, S^R Syn. MRP+BOR
Increased efflux	I/R	I/R	I/R	I/R	I/R	S^R	I			CAZ > AZT
Loss of Opr porin	S	S	S	S	S	I/R	S/I			
2) *Stenotrophomonas maltophilia*										
Beta-lactamase L-1	R	R	R	R	R	R	R	-	Synergy	AZT, S
Beta-lactamase L-2	-	S	I	R	R	S	-	-	-	AZT, R
Beta-lactamase L-1+L-2	R	R	R	R	R	R	R	-	Synergy	AZT, R
3) *Acinetobacter baumannii*										
	TIC	**PIP**	**PTZ**	**CAZ**	**FEP**	**IMI**	**MERO**			
Penicillinase (TEM 1-2)	R	R	S	S	S	S	S			
Oxacillinase (OXA 21, 37)	R	R	S	S	S	S	S			FEP ≤ CAZ
Amp C partial derep.	I	I	S/I	I	S/I	S	S			
Amp C derepressed	R	R	I/R	R	I/R	S	S			
PER-1, VEB-1 (ESBL)	R	any	S	R	R	S	S	Synergy		Syn. TIC+CLAV Syn. PIP+TAZO
ESBL	R	R	any	R	R	S	S	Synergy		
Metallo-ß-lactamase	R	R	R	R	R	I/R	I/R		Synergy	AZT, S^R

Carbapenemase Class D (Oxa 23-27, 40, 51, 58)	R	R	I/R	I/R	I/R	S/I/R	S/I/R			AZT, S^R
Loss of porins	S	S	S	S	S	I/R	S/I			

TIC; Ticarcillin, TCC; Ticarcillin+clavulanic acid, PIP; Piperacillin, PTZ; Piperacillin+tazobactam, CAZ; Ceftazidime, FEP; Cefepime, IMI; Imipenem, MRP; Meropenem, CAZ+CLAV; Ceftazidime + clavulanic acid, FEP+CLAV; Cefepime + clavulanic acid, IMI+EDTA; Imipenem + EDTA, AZT; Aztreonam; BOR=Boronic acid.

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Screening of 16S rRNA Methylases (HLR to Aminoglycosides)

Unlike aminoglycoside-modifying enzymes that vary in their substrate profile, the acquired 16S rRNA methylases confer high level resistance (HLR) to almost all clinically important aminoglycosides.
They have been identified in several nosocomial pathogens, including P, aeruginosa, Serratia marcescens, E. coli, P. mirabilis, K. pneumoniae and Acinetobacter spp., Enterobacter cloacae, Citrobacter freundii (8,9,11).
These enzymes (RmtA, RmtB, RmtC, ArmA) are capable of conferring very high levels of resistance (MIC > 512 µg/ml) against amikacin, gentamicin, isepamicin, netilmicn and tobramycin, while apramycin, neomycin and streptomycin are not affected. The responsible genes armA, rmtA, rmtB, rmtC and rmtD are located in self-tramsmissible plasmids (7).

Screening method

A high-level amikacin resistance (MIC > 512 µg/ml) corresponding to no-zone of inhibition around Amikacin 40 µg Neo-Sensitabs may be used as a marker for screening the 16S rRNA methylase producing strains.
The diffusion test is performed on MH-agar using a 0.5 McF inoculum and incubation at 35-37 °C overnight.
Strains of Enterobacteriaceae and non-fermenters (*P. aeruginosa* and *Acinetobacter* spp.) showing no-zone of inhibition around Amikacin 40 µg Neo-Sensitabs should be suspected of possessing 16S rRNA methylases.

a) **Enterobacteriaceae**

		NpmA-enzyme
16S rRNA methylase **positive** strains will show:		
Amikacin:	No zone of inhibition	Resistant
Gentamicin:	No zone of inhibition	Resistant
Netilmicin:	No zone of inhibition	Resistant
Tobramycin:	No zone of inhibition	Resistant
Neomycin 120 µg:	Zone of inhibition ≤ 20 mm or no zone	Resistant
Apramycin100 µg:	Zone of inhibition ≥ 20 mm (6) (S)	Resistant

b) **Non-fermenters**

16S rRNA methylase **positive** strains will show:	
Amikacin:	No zone of inhibition
Gentamicin:	No zone of inhibition
Netilmicin:	No zone of inhibition
Tobramycin:	No zone of inhibition
Neomycin 120 µg:	No zone or small zone
Streptomycin 100 µg:	Small zone in most cases

Galimand et al (5) found in 12 clinical isolates of Enterobacteriaceae the armA gene associated with ESBL beta-lactamase CTXM-3 (cefotaxime zone < ceftazidime zone) on a conjugative plasmid.

Bogaerts et al (9) investigated the presence of 16 SrRNA methylase mediated high level resistance to aminoglycosides in clinical isolates of Enterobacteriaceae from 2 University Hospitals in Belgium. They screened for HLR to gentamicin, tobramycin and amikacin resistance and deleted by PCR, armA genes in 18 *K. pneumoniae*, *E. coli*, *E. aerogenes*, *E. cloacae*, and *C. amalonaticus*, whereas rmtB was detected in a single *E. coli* isolate. These strains were susceptible to Apramycin and Neomycin Neo-Sensitabs (except 2 strains). All 16 SrRNA methylase positive strains produced ESBL's predominantly type CTX-M3 (13).

The concomitant presence of 16S rRNA methylase genes (armA or rmtB) and beta lactamase CTX-M among amikacin-resistant ESBL-producing K. pneumoniae isolates are widely spread in Taiwan (12).

The emergence of 16S rRNA methylases in Enterobactericaceae and non-fermenters (*P. aeruginosa*, *Acinetobacter* spp.) in strains that already are ESBL positive, may result in the spread of multidrug-resistant isolates producing both ESBLs and 16S rRNA methylases becoming an important clinical problem.

Wachino et al (10) describes a new plasmid-mediated 16S rRNA methyltransferase NpmA isolated from E.coli and providing total aminoglycoside resistance (including apramycin, neomycin and streptomycin).

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Screening for plasmid-mediated quinolone resistance

The plasmid gene responsible for quinolone resistance (q*nrA, qnrB, qnrC, qnrD qnrS,aac(6´)*Ib-cr and QepA) is carried on class 1 integrons of the In 4 family, an efficient mechanism for rapid horizontal and vertical dissemination og antibiotic resistance determinants among bacteria.
The plasmid mediated mechanisms have led to resistance to almost all clinical important antimicrobials, such as β-lactams, aminoglycosides, macrolides, phenicols, sulphonamides and trimethoprim.

The identification in the US of *qnr* in clinical strains of *K. pneumoniae* isolates besides producing plasmidic β-lactamases and ESBL's (7) and its discovery in strains of *E. coli* from Southeast Asia and Salmonella in Hong Kong indicates the emergence of this new mechanism of quinolones resistance in clinical strains.
It is important to indicate that a significant relation exists between quinolone resistance and resistance to 3. gen. cephalosporins (co-resistance): ESBL and/or plasmid mediated AmpC (6,14).

Poirel et al (10) have shown in vivo selection of fluoroquinolone-resistant E. coli isolates expressing plasmid mediated quinolone resistance and ESBL and physical linkage between ESBL and qnrA-encoding genes in the same integron.

Although *QnrA, QnrB, QnrS* produce low levels of quinolone resistance, it facilitates selection for a high level of quinolone resistance.

QnrB, another plasmid-mediated gene for quinolone resistance has been discovered in plasmids encoding the ESBL: CTA-15 from a K. pneumoniae. These strains show low-level resistance to quinolones and MIC of 16 µg/ml towards nalidixic acid, and show similar multiresistance phenotypes as qnr A containing strains (11).

Lavigne et al (12) screened for qnr genes 112 clinical isolates of ESBL-producing *E. coli* from French hospitals in 2004. 7.7 % of CTX-M-producing *E. coli* presented a plasmid-mediated resistance to quinolones. All strains harboured a qnrA gene located on a class 1 integron.

Poirel et al (13) listed 186 ESBL positive Enterobacteriaceae. From them 2.2 % and 1.6 % carried a QnrA1 and a QnrS1 determinant respectively. The association of the qnrA gene with class 1 integrons was confirmed.

Hyunjoo Pai et al (14) screened *E. coli* and *K. pneumoniae* producing ESBLs or plasmid mediated AmpC beta-lactamases for the presence of qnrA and qnrB genes. QnrB was present in 54 of 54 DHA-1 producing *K. pneumoniae* isolates and 10 of 45 SHV-12 producing isolates. It is possible that qnrB contributes to the widespread sistribution of DHA-1 (plasmid mediated AmpC) in areas, where 3rd generation cephalosporins and fluoroquinolones are widely used.

According to Lavilla (15) and Jones (16): the presence of aac(6´)Ib-cr were associated with quinolone resistance and aminoglycoside resistance (tobramycin is the best indicator).

Pitout et al (18) and Ruiz (20)found that isolates with aac(6´)Ib-cr were often associated with CTX-M-15.

Screening procedure
Perform antibiogram as usual (standard procedure): MH agar, inoculum McF 0.5, incubation at 35-37 °C for 18-24 hours.
Strains of Enterobacteriaceae should be suspected of plasmid-mediated quinolone resistance when showing unusual multiresistance phenotypes such as:

Neo-Sensitabs

Ampicillin	- no zone (HLR)
Sulphonamides	- no zone (HLR)
Trimethroprim	- no zone (HLR)
Trimethroprim+Sulfa	- no zone (HLR)
Streptomycins	- no zone (HLR)
Nalidixan 130 µg	- no zone or zone < 23 mm
Nalidixan 30 µg	- zone < 15 mm (MIC ≥16 µg/ml)
Norfloxacin 10 µg	- zone ≤ 23 mm
Ciprofloxacin 1 µg	- zone < 25 mm (MIC ≥0.125 µg/ml)
Ciprofloxacin 5 µg	- zone < 28 mm (MIC ≥0.125 µg/ml)
Ceftazidime	- zone < 20 mm
Chloramphenicol	- may show resistance
Tetracyclines	- may show resistance
aac(6`)Ib-cr	- Tobra R (20), Kana R
QepA	- Genta R, Tobra R (20), Cipro R, Norflox R (15-17)

Suspected strains can be tested for the presence of the q*nr* gen by PCR.
It should be noted that strains showing the above-mentioned resistance phenotypes are most probably integron-carrying. Enterobacteriaceae and barrier precaution should be established to prevent further spread. A new chromosomal gen,qnrM from S.maltophilia, produce quinolone-resistance in E.coli (21).

In a selected group of ciprofloxacin and ceftazidime-resistant Enterobacteriaceae (mainly *K. pneumoniae* and *E. cloacae*), carriage of qnrA gene was 32 % (9). From those 73 % were ESBL-positive.

Cavaco et al (22) found that the combination of ciprofloxacin, norfloxacin and nalidixic acid is the best option for detecting qnr and aac(6`)Ib-cr resistance mechanisms in Enterobacteriaceae.

Detection of qnr and aac(6`)Ib-cr and differentation from mutations

Screening with nalidixic acid is efficient for the detection of mutants, but it is not efficient for the detection of some isolates carrying qnr and aac(6`)Ib-cr.
Transferable genes will be best detected using ciprofloxacin and norfloxacin, because these fluoroquinolones due to their chemical structures are attacked by strains carrying aac(6`)Ib-cr, reducing their antimicrobial activity.
Combining the use of Nalidixic acid 30 µg, Norfloxacin 10 µg and Ciprofloxacin 1 µg Neo-Sensitabs it is possible to screen for the new resistance mechanisms qnrA, qnrS and aac(6`)Ib-cr in strains of E.coli and Salmonella spp.

Susceptible strains E.coli and Salmonella spp. show zones of inhibition ≤ 25 mm with Nalidixic acid 30 µg Neo-Sensitabs (MIC ≤4-8 µg/ml).
With Ciprofloxacin 1 µg Neo-Sensitabs, susceptible strains show zones of inhibition ≥ 29 mm (MIC ≤0.016-0-03 µg/ml).
With Norfloxacin 10 µg Neo-Sensitabs, susceptible strains show zones of inhibition ≥ 33 mm (MIC ≤0.06 µg/ml).

Strains with **1 or 2 mutations** will show zones of inhibition ≤ 12 mm with Nalidixic acid 30 µg Neo-Sensitabs, while Ciprofloxacin will show zones ≤ 28 mm and Norfloxacin zones ≤ 32 mm.

Strains with **qnr or aac(6`)Ib-cr** will show zones of inhibition ≥ 13 mm with Nalidixic acid 30 µg Neo-Sensitabs, zones of 16-25 mm (MIC 0.125-0.5 µg/ml) with Ciprofloxacin 1 µg Neo-Sensitabs and zones of ≤ 30 mm (MIC 0.25-2 µg/ml) with Norfloxacin 10 µg/ml Neo-Sensitabs.

Conclusion:

Strains with mutations are best detected with Nalidixic acid 30 µg Neo-Sensitabs. Strains with aac(6`)Ib-cr are best detected using Ciprofloxacin 1 µg and Norfloxacin 10 µg Neo-Sensitabs, showing reduzed zones of inhibition compared to fully susceptible strains. Strains possessing aac(6`)Ib-cr, show also resistance to Tobramycin and Kanamycin, because these aminoglycosides are attacked by the mentioned enzyme. This is useful to differentiate them from the qnr enzymes.

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Detection of hypermutable strains

Hypermutable or mutator strains are those that have an increased spontaneous mutation rate due to defects in genes involved in DNA repair or error avoidance systems.
Hypermutable strains have been described in natural populations of E. coli,Shigella spp,Salmonella spp. P. aeruginosa,H. influenzae,Helicobacter pylori,N. meningitidis and S. aureus among others. Hypermutable strains could have an important role in the emergence and spread of antibiotic resistance within bacterial populations,because they are substantially more resistant than nonmutators.

Detection of E.coli hypermutable strains

Galan et al(1) and Conejo et al (2) described a diffusion method for detecting hypermutable strains in E. coli.A similar technique may be used with Neo-sensitabs.
MH-Blood agar plates are seeded with 100ul of an overnight Brain Heart Infusion broth culture. Fosfomycin Neo-sensitabs and Rifampicin 30 ug Neo-sensitabs are placed on the surface of the agar plate.Incubation takes place during 24 hours at 35-37 degress Celsius and the inhibition zones are examined for the presence of colonies inside the inhibition zones.

Interpretation is as follows:

Strong mutators:	Strains with > 70 colonies in the Fosfomycin zone and > 10 colonies in the Rifampicin zone.
Weak mutators:	Strains with > 30 but <70 colonies in the Fosfomycin zone or > 70 colonies in the Fosfomycin zone but < 10 colonies in the Rifampicin zone.
Nonmutators:	Strains with < 30 colonies in the Fosfomycin zone and < 10 colonies in the rifampicin zone.

The same technique should be useful for Shigella and salmonella strains.

Denamur et al (3)found a high frequency of hypermutable strains in uripathogenic E. coli isolates, using nalidixic acid,fosfomycin,spectinomycin and streptomycin.The majority of hypermutable strains yielded colonies inside the inhibition zone(squatered colonies)
Baquero et al (4) found that hypermutable E. coli strains were found more frequently in a collection of ESBL-producing isolates,than in non-ESBL E. coli.
Ellington et al (5) found that hypermutator phenotypes were found among E. coli expressing ESBL CTX-M beta lactamases and they had an increased propensity to fosfomycin resistance.

Detection of P. aeruginosa hypermutable strains

A technique similar to the the one described for E. coli may be used for P. aeruginosa (6,7,8).
MH agar is inoculated with a 0.5 McFarland inoculum or McF 1.0(mucoid colonies).
The following Neo-Sensitabs are added to the agar plate : Ceftazidime 30 ug,Ciprofloxacin 5 or 10 ug,Imipenem 10 or 15 ug,Meropenem 10 ug and Tobramycin 10 or 40 ug Neo-sensitabs.
Incubate for 24 hours(36 hours for slow growers) at 35-27 degrees.
The zones of inhibition are examined for the presence of resistant mutants (squatted colonies) inside the zones.When the diameter of the inhibition zones is reduced by > 5 mm (squatted colonies zone compared to the largest zone) the test is positive for hypermutability.With Tobramycin any resistant mutant inside the zone indicates a positive result.
Hypermutable strains will show the presence of resistant mutants inside the zone for 3 or more antibiotics.

Oliver et al(9)found that chronic infections caused by hypermutable P. aeruginosa of the sort frequently found in cystic fibrosis patients,will almost invariably be present in a high proportion at the onset of treatment,and consequently these strains should be considered resistant to all agents when they are used as monotherapy.
Henrichfreise et al(10)characterized a multiresistant hypermutable strain of P. aeruginosa from an ICU patient using Ciprofloxacin,Tobramycin,Ceftazidime,Imipenem and Meropenem by disk diffusion.

Wayne et al(11)found that the presence of hypermutator P. aeruginosa was associated with poorer lung function in cystic fibrosis patients.Mucoid isolates were more likely to be hypermutators.
Macia et al (12)found that hypermutable P. aeruginosa strains from chronic respiratory infections had an increased susceptibility to colistin.

Hypermutable Haemophilus influenzae

Watson et al (13)found that hypermutable H. influenzae with mutations in mutS were prevalent in the cystic fibrosis lung environment.Rifampicin,Nalidixic acid and Spectinomycin were used to detect the hypermutable strains.Of 14 hypermutable H influenzae isolates,12 were from cystic fibrosis sputum.
Perez-Vazquez et al (14)found that 39.3 % of 28 ciprofloxacin resistant H.influenzae were hypermutable and conclude that resistance to fluoroquinolones in H. influenzae is strongly associated to hypermutability.

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Detection of heteroresistant strains

Introduction

Heteroresistance refers to populations containing a majority of bacteria inhibited by concentrations below the susceptibility breakpoint,together with a small number of microorganisms
(1 to 1000 to 1 10.000.000) that are resistant.

Heteroresistance has been described in S. aureus and coagulase negative staphylococci, particularly in MRSA,hVISA,Besides,in Enterococcus faecium (teicoplanin),pneumococci (penicillin), Acinetobacter baumannii (carbapenems and colistin),Helicobacter pylori (metronidazole,clarithromycin) Klebsiella pneumoniae (colistin),Clostridium difficile (metronidazole),Ps aeruginosa (carbapenems, piperacillin + tazobactam) Cryptococcus neoformans (fluconazole) among others.

The most common method used to detect heteroresistance is to see colonies inside the inhibition zones of disks (Neo-sensitabs),containing different antimicrobials.
There is no doubt that the phenomenon of heteroresistance may be clinically relevant. In S. aureus , heteroresistance to oxacillin/methicillin is clinically relevant.
The clinical importance of S. aureus isolates heteroresistant to glycopeptides, is under discussion, while the amount of hVISA strains is increasing worldwide.
The use of antimicrobials in vivo may select heteroresistant mutants,that later on result in a population stable resistant .

Isolates of A. baumanni heteroresistant to colistin may show stable MICs of more than 8 – 16 ug/ml. These strains can be selected in vitro and probably can be selected in patients treated with colistin. Ac baumanni isolates from patients previously treated with colistin, show a higher degree of heteroresistance.

Carbapenem heteroresistance in A. baumannii

Fernandez-Cuenca et al (1) indicates that A. baumanni heteroresistant to carbapenems should be detected in the laboratory,because it is probable that the resistant subpopulations, will be selected in the presence of imipenem or meropenem,resulting in a therapeutic failure. Microdilution and automatic methods (Wider,Vitek etc) cannot detect heteroresistance, because they are using too small inocula. To detect heteroresistance methods of diffusion on agar should be used and the inoculum should never be below to that corresponding to a 0.5 Mc Farland.

Pournaras et al(2) in 2005 describes the spread of isolates heteroresistant to carbapenems, they showed subcolonies present in the clear zone of inhibition.Resistant colonies were retested and again a subpopulation of resistant isolates was grown inside the zone of inhibition.
Del Rosario Quintana et al (3) conclude that the the automatic system
Wider cannot detect the carbapenem heteroresistant strains of A. baumanni.
Fernandez F et al (4) tested 30 clinical isolates of A. baumannii and heteroresistance was defined as presence of colonies inside the zones around imipenem disks.Heteroresistant strains showed MICs of 4-16 ug/ml towards imipenem and were associated to the presence of the bela-lactamase OXA-58 gen.

Gomez MC et al (5) tested 44 isolates of A. baumannii and concluded that 84 % of the isolates showing colonies inside the zone of imipenem,also possessed the OXA-58 gen, while none of the isolates without colonies inside the zone,possessed the gen OXA-58.
Neou et al (6) tested 142 non-repetitive isolates of A. baumannii.Agar MICs for imipenem were 0.25 to 4 ug/ml.Colonies grown at 8 ug/ml did not show resitance stability when subcultured in drug-free medium.
Agar MICs for meropenem were 0.25 to 4 ug/ml.Colonies grown at 8 to 32 ug/ml showed stability to meropenem resistance after 1 week subculture in drug-free medium, but they were susceptible to imipenem.The authors suggest that apparently carbapenem susceptible A. baumanni populations contain an amount of resistant meropenem subpopulations.
The implementation of screening techniques to identify heteroresistant isolates is of significant

importance.

Observe the presence of subcolonies inside the zone of inhibition of Imipenem and Meropenem Neo-sensitabs

Colistin heteroresistance in A. baumannii

Jian Li et al (7) tested 16 colistin susceptible clinical isolates of A. baumannii by population analysis profiles and by serial passaging with or without exposure to colistin.They demonstrated the presence of heterogeneous colistin-resistant A. baumannii in "colistin susceptible" isolates.
The authors conclude that colistin heteroresistant A. baumannii isolates cannot be discriminated from colistin susceptible by MIC measurements alone.
Colistin heteroresistant A. baumannii may be a preliminary stage that leads to the proliferation of resistant subpopulations upon exposure to colistin.
Hawley et al (8)conclude that the isolates exhibiting heteroresistance is significantly higher among isolates recovered from patients previously treated with colistin
Park et al(9)indicates that high colistin resistance rates in Acinetobacter have been reported from Korean hospitals and that was not due to clonal dissemination,but they arose independently.
Hawley et al (10)identified one colistin-dependent A. baumannii isolate.When plated on Mueller Hinton agar with a Colistin 10 ug disk,the isolate grew heavily immediately around the disk.
The Colistin 10 ug Neo-sensitabs prediffusion method (2 hours + 18/22 hours) will detect both colistin heteroresistant and resistant strains.

Carbapenem and PIP + TAZO Heteroresistance in P. aeruginosa.

The presence of subcolonies inside the carbapenems disk zone indicates that heterogeneous subpopulations with redudced susceptibility to carbapenems may exist in a number of P. aeruginosa strains that appear to be carbapenem susceptible by conventional automated susceptibility methods.
Pournaras et al (11)tested 14 non-repetitive isolates of P. aeruginosa in which a few subcolonies appeared within the zone of inhibition of imipenem and meropenem disks.These isolates represented 27.5 % of the apparently carbapenem susceptible isolates.
Population analysis showed distinct subpopulations that grew in concentrations up to 18 ug/ml imipenem and 12 ug/ml meropenem.The heterogeneous subpopulations retained their resistance levels implying a rather stable expression of resistance.Conventional MIC dilution methods,using the standard 10,000 CFU per spot inoculum may miss carbapenem resistant mutants.
Pournaras et al (12) describe an isolate showing heteroresistance to Piperacillin + tazobactam.The isolate was reported as susceptible by automatic methods and by agar dilution.Nevertheless,the isolate exhibited distinct colonies within the inhibition zone around the piperacillin + tazobactam disk.

Colistin resistant P. aeruginosa

Brannon et al (13) tested 19 colistin-resistant P. aeruginosa isolated from colistin-treated cystic fibrosis patients.
75 % of the colistin resistant CF strains were highly resistant to colistin (MIC > 200 ug/ml) and the remaining were moderately resistant (MIC > 2 ug/ml).
Montero et al (14)tested colistin-resistant P. aeruginosa (CORPA)isolated from 10 patients.In all cases,multidrug-resistant P. aeruginosa susceptible only to colistin and amikacin were isolated,before the emergence of CORPA.9 of the patients had previously received prolonged courses of colistin (mean 40 days).
Detection using Colistin 10 ug Neo-sensitabs and the prediffusion method (2hours + 18/22 hours prediffusion)

Colistin heteroresistant /resistant Klebsiella pneumoniae and carbapenem heteroresistant E. aerogenes

Poudyal et al (15)tested 22 multidrug-resistant clinical isolates of K. pnemoniae.6 isolates were colistin-resistant with MICs >= 32 ug/ml.Colistin heteroresistance was observed in 15 of 16 isolates considered colistin-susceptible.Similar to our recent finding of colistin heteroresistance in A. baumannii,the MIC alone may not provide information to guide treatment,
because heteroresistance is not detected by an MIC method.
Antoniadou et al (16)tested 18 colistin-resistant K. pneumoniae isolates from 13 patients over a 16 months period.Most of the isolates possessed ESBLs or metallo-beta-lactamases or both.Selective pressure due to extensive or inadequate colistin use may lead to the emergence of colistin resistance.
Papaioannou et al (17) in a study over 3 years (2005-2008)and almost 5000 isolates, found that 1.5 % of A. baumanni, 16 % of Klebsiella pneumoniae and 4.8 % of P. aeruginosa were colistin resistant.
Gordon et al (19)showed that the Microscan failed to detect heteroresistance to carbapenems in a patient with E. aerogenes bacteremia,while disk diffusion and E-test detected it.
Detection using Colistin 10 ug Neo-sensitabs and the prediffusion method (2hours + 18/22 hours prediffusion)and Imipenem/Meropenem Neo-sensitabs by the diffusion method

Metronidazole heteroresistance in Clostridium difficile

Pelaez et al (18)found that initially metronidazole-resistant C. difficile isolates became susceptible after thawing.;
However they presented slow-growing subpopulations within the inhibition zones of the metronidazole disk.The authors conclude that: resistance to metronidazole in toxigenic C difficile is heterogeneous,and prolonged exposure to metronidazole can select for in vitro resistance.We recommend routine performance of the disk diffusion method,with primary fresh C. difficile isolates in order to ensure that metronidazole heteroresistant populations do not go undetected.
Similar heterogeneous metronidazole resistance has been observed in Bacteroides spp and Helicobacter pylori.
Detection using Metronidazole Neo-sensitabs.Observe the presence of colonies inside the zone of inhibition

Vancomycin/Teicoplanin heteroresistance in S. aureus and coagulase negative staphylococci

Is treated separately under the prediffusion method (2 hours + 18/22 hours) prediffusion

Vancomycin/Teicoplanin heteroresistance/resistance in Enterococcus

Is treated separately under the prediffusion method (2 hours + 18/22 hours) prediffusion

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Efflux pump inhibitors and NMP Diatabs

Introduction

The accelerated evolution of antibiotic resistance to important human pathogens and the scarcity of new antiinfective drug families under development,makes that other ways are being tried.
Efflux is a general mechanism responsible for bacterial resistance to antibiotics.This active drug transport is involved in low intrinsic susceptibility,cross resistance to chemically unrelated classes of molecules and selection/acquisition of additional mechanisms of resistance.
As a consequence, inhibition of bacterial efflux mechanisms appears to be promising in order to a) increase the intracellular concentration of antibiotics,that are expelled by efflux pumps b) restore the drug susceptibility of resistant clinical strains and c)reduce the capability for acquired additional resistance(1).
The resultant efflux pump inhibitor/antibiotic combination drug should exhibit increased potency,enhanced spectrum of activity and reduced propensity for acquired resistance(2)

Enterobacteriaceae

Kim et al(3)found that Shigella flexneri isolated in Korea and resistant to fluoroquinolones,showed an increased susceptibility
to ciprofloxacin,norfloxacin and ofloxacin in the presence of an efflux pump inhibitor (CCCP).
Freyre et al(4)studied the effect of an inhibitor of efflux pumps(PABN)on the MICs to different fluoroquinolones in clinical isolates of E.coli.Strains resistant to the fluoroquinolones showed an increased reduction of the MICs for levofloxacin and moxifloxacin.
Tran QT et al(5)studied the effect of the efflux pump inhibitor PABN on the resistance of Enterobacter aerogenes and K. pneumoniae.
Efflux was involved in resistance(chloramphenicol,sparfloxacin) in E. aerogenes isolates more frequently than in K. pneumoniae.
Kern WV et al (6)studied the efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP)on clinical isolates of E. coli.
NMP was moderate active in reversing multidrug resistance in clinical isolates of E. coli and can partially restore fluoroquinolone susceptibility through inhibition of efflux pumps.
Schumacher A et al (7)studied the effect of NMP on drug susceptibility of Enterobacteriacea (other than E.coli).NMP has shown to reverse multidrug resistance in E. coli overexpressing RND type efflux pumps.On other Enterobacteriaceae,NMP consistently reduced the MIC of linezolid in C. freundii,Enterobacter aerogenes and K. pneumoniae clinical isolates.Significant effects were also seen for levofloxacin,tetracycline and chloramphenicol in E aerogenes,and for levofloxacin and tetracycline for K. pneumoniae.
Effect of NMP was more likely in isolates with decreased susceptibility to fluoroquinolones.

Non-fermenters and Vibrio

Bina XR et al(8)studied the effect of NMP on antimicrobial susceptibility and virulence factor production in Vibrio cholera.
NMP potentiated antimicrobial compounds that were substrates for the V. cholerae RND efflux systems.NMP inhibited the production of virulence factors cholera toxin and the toxin coregulated pilus.
Bean D et al(9)found that A. baumannii belonging to the multidrug resistant OXA-23 clone1 appeared to decrease in susceptibility to tigecycline in the presence of NMP. The converse was seen when NMP was combined with doxycycline,tetracycline or minocycline.The synergy seen between NMP and the tetracyclines must be due to NMP effect against the AdeABC resistance-nodulation division efflux pump.
Vila J et al (10)give a review of porins,efflux pumps and multidrug resistance in Acinetobacter baumannii.The efflux pump AdeABC of the family RND affects the following antimicrobials : aminoglycosides,betalactams,chloramphenicol,erythromycin,tetracyclines and reduced susceptibility to fluoroquinolones.
Lomovskaya O et al (11)identify and charaterize the inhibitors of multidrug resistance efflux pumps in P. aeruginosa.They conclude that inhibition of efflux pumps in P aeruginosa may significantly improve the

clinical performance of fluoroquinolones.It would suppress the emergence of S. aureus and pneumococci mutants resistant to ciprofloxacin.

NMP Diatabs

Rosco has developed a test (NMP Diatabs) for detecting efflux pumps, particularly of the RND family.
The test is performed on Mueller Hinton Agar using Mc farland 0.5 inoculum and the NMP Diatabs (9 mm)is placed near the corresponding Neo-sensitabs that should be tested for synergy.
The distance between NMP Diatabs and Neo-sensitabs, will depend on the size of the inhibition zone of the corresponding Neo-sensitabs with the particular bacteria.If the zone is <= 20 mm,the NMP tablet should be placed 6-8 mm apart (edge to edge).If the zone is >=30 mm the distance should be 10-12 mm apart.
Synergy (enlargement of the zone of inhibition,keyhole zone or phantom zone) indicates the presence of an Efflux Pump,probably of the RND family.

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Introduction

High molecular weight antimicrobials (vancomycin, teicoplanin, daptomycin, colistin) diffuse poorly on agar media, resulting in difficulties in the interpretation of results when using the current disc diffusion method.

As a consequence, CLSI do not recommend the current diffusion method for vancomycin with staphylococci, daptomycin with staphylococci and enterococci and colistin with gram negative rods.

Teicoplanin has not yet been evaluated, but we expect it will perform like vancomycin.ROSCO Diagnostica has taken on the investigation of this problem and has developed a 2 + 18 hours (or 2 + 22 hours) prediffusion technique, permitting an easier differentiation between susceptible and resistant strains when testing against these antimicrobials.

Principle

The principle of the prediffusion technique, was developed by a Danish microbiologist Frølund-Thomsen, several decades ago.The idea is to give the high molecular weight antimicrobial a longer period of time to diffuse into the agar before bacterial growth takes place.

In a current disc diffusion minute colonies of growth are visible after approximately 8 hours incubation at 35 degrees. As a consequence the antimicrobial has only approximately 8 hours to diffuse into the agar, because when minute colonies are formed, further antimicrobial diffusion will not affect the size of the inhibition zone. When using the 2 + 22 hours prediffusion the antimicrobial has 8 + 24 = 32 hours to diffuse into the agar, i. e. 4 times more than with the current diffusion method.

This results in a much larger zone size difference between 2 consecutive MIC values approximately 5 mm with the prediffusion method compared to 1.0-1.5 mm with the current disc diffusion method (see enclosed regression lines)

Another important point is that with the prediffusion method, the antimicrobial depot is eliminated after 2 hours. From this moment no further antimicrobial is added to the agar and the antimicrobial that has diffused during the 2 hours will continue further diffusion without any pressure from the depot.

DETECTION OF VISA, GISA AND hVISA USING THE NEO-SENSITABS PREDIFFUSION METHOD

1. One Neo-Sensitabs of each Vancomycin 30 µg and Teicoplanin 30 µg are placed on an uninoculated plate containing the susceptibility test medium Mueller-Hinton Agar.

2. After 2 hours at room temperature, the tablet (disc) is removed (by knocking the plate against the table), but prior to this a short name (VAN or TEI) is written on the back of the plate in order to make it possible to identify the antimicrobial.

3. Now the plate is maintained at room temperature for a further 18 to 22 hours (overnight).

4. The plate is now inoculated with the strain to be tested using a McFarland 0.5 inoculum. Additional antimicrobial discs (Neo-Sensitabs) may be added now, using a dispenser and thereafter the plate is incubated overnight at 35 degrees.

5. The zones of inhibition are then measured and compared with the corresponding zone breakpoints

S. aureus CB 182, Susceptible strain. V = Vancomycin, T = Teicoplanin, D = Daptomycin

Please note:

In the laboratory the prediffusion plate can be prepared the day before it is inoculated to avoid loss of time and results are available within 24 hours.Surplus of prediffused plates may be kept in the refrigerator for another 24 hours.

Interpretation

DETECTION of VISA, GISA, hVISA and hGISA

VISA, GISA and hVISA strains will show the following zones of inhibition using Mueller Hinton Agar and McFarland 0.5 inoculum and the prediffusion method:

hVISA, hGISA	VISA,GISA
Teicoplanin 30 ug inhibition zone < 20 mm **OR** Vancomycin 30 ug inhibition zone ≤ 22 mm	Teicoplanin 30 ug inhibition zone < 20 mm **AND** Vancomycin 30 ug inhibition zone ≤ 22 mm

Please notice that Teicoplanin in general is the most sensitive drug to detect these isolates.
The current MIC methods and automatic systems are unable to detect hVISA strains, because they use too small inocula and consequently cannot detect heteroresistant isolates.

In the laboratory it may be useful to test vancomycin, teicoplanin and daptomycin together in order to be able to detect hVISA/VISA strains first and thereafter in order to find the best drug for treatment of MRSA and hVISA/VISA infections.

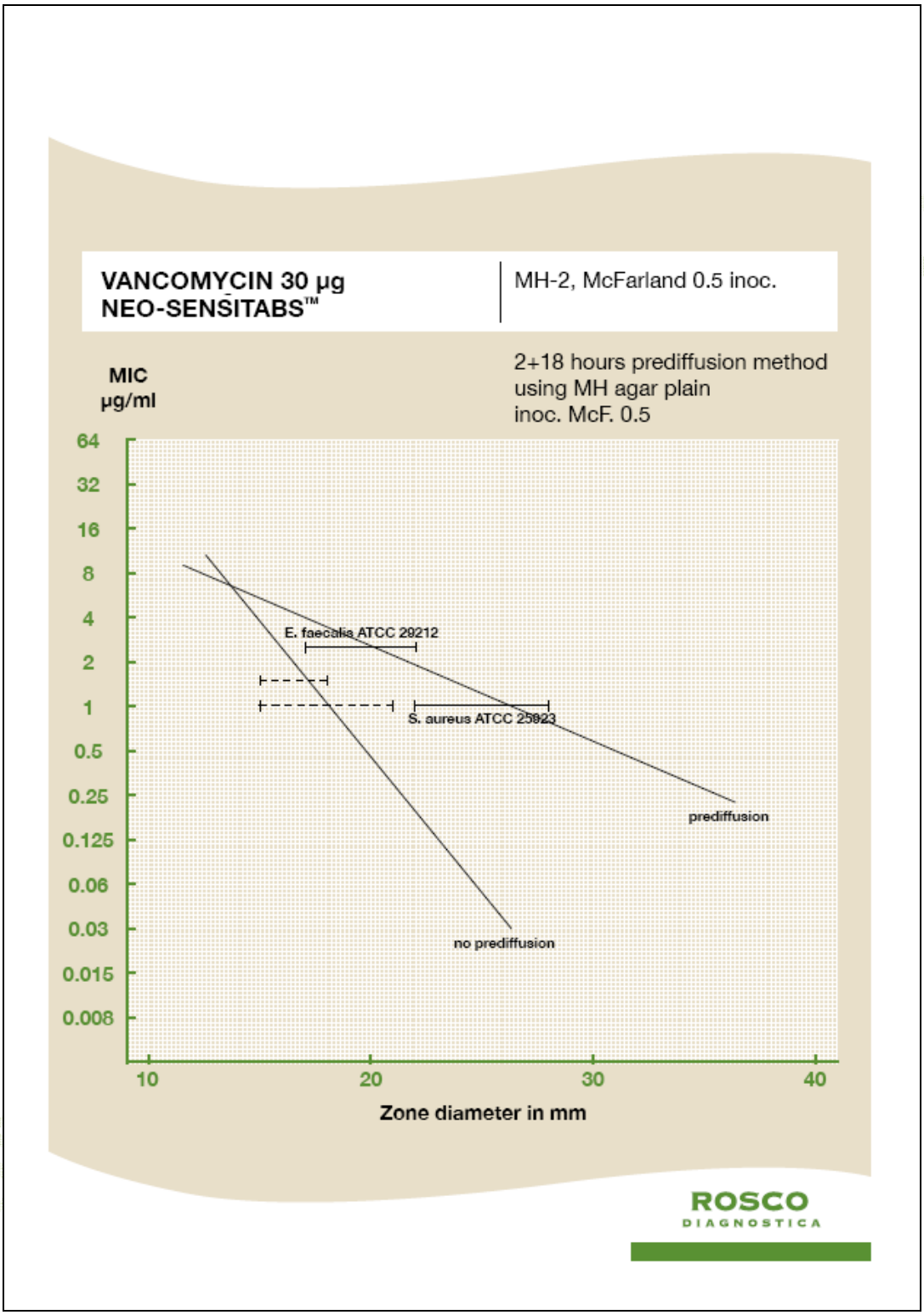
An 9 cm Mueller-Hinton Agar plate will be adequate for testing the 3 antimicrobials by the prediffusion method.

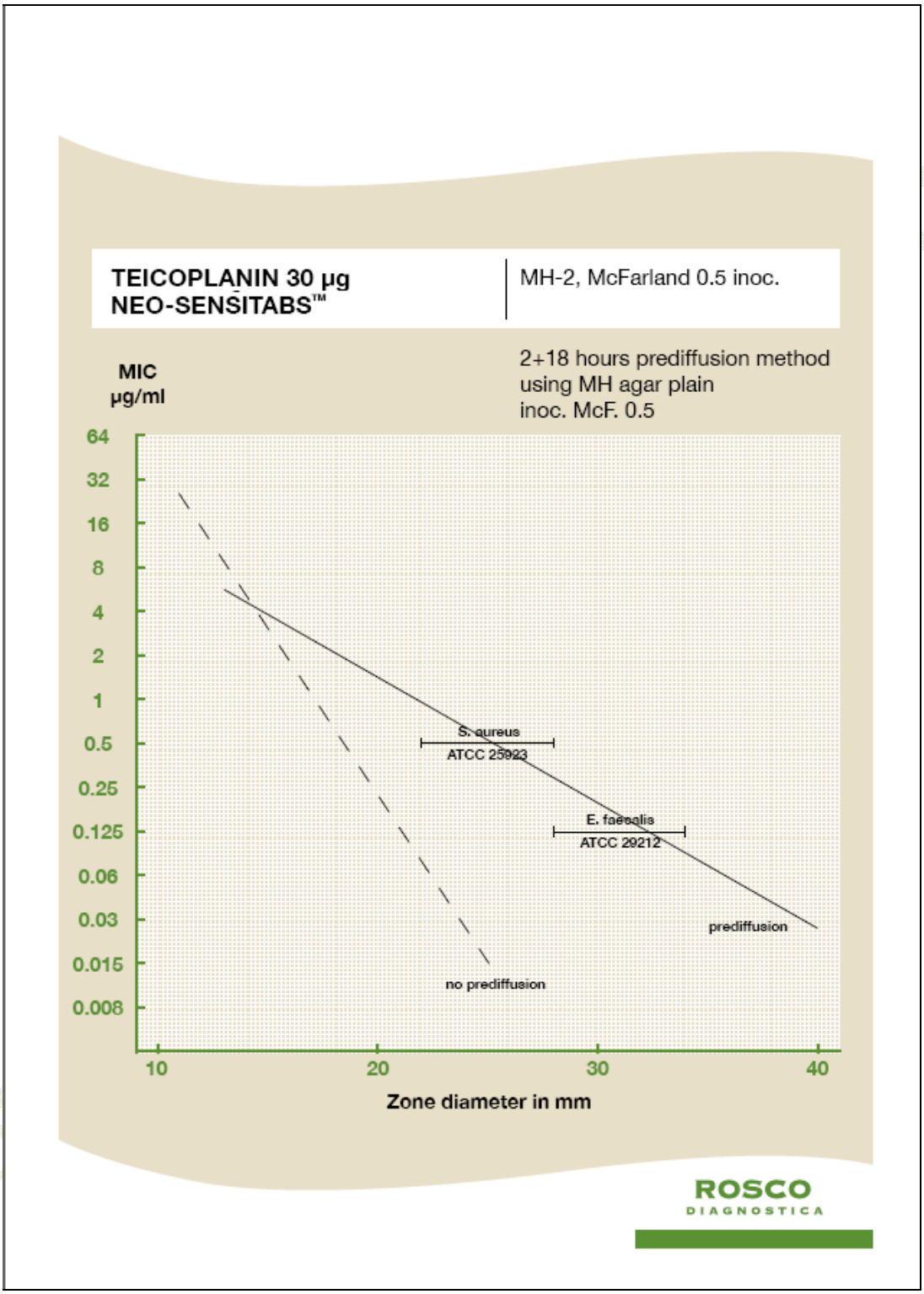
S. aureus ATCC 700698 Daptomycin, susceptible hVISA strain.

DETECTION OF COAGULASE NEGATIVE GLYCOPEPTIDE INTERMEDIATE STAPHYLOCOCCI

The same procedure as above is used for Teicoplanin 30 ug. Isolates showing zones of inhibition < 20 mm with Teicoplanin 30 ug (prediffusion method) should be reported as resistant to teicoplanin and possibly heteroresistant to vancomycin.

S. aureus ATS 403, GISA strain, Daptomycin susceptible.


VANCOMYCIN 30 µg
NEO-SENSITABS™
MH-2, McFarland 0.5 inoc.
2+18 hours prediffusion method
using MH agar plain
inoc. McF. 0.5
MIC
µg/ml
64
32
16
8
4
2
1
0.5
0.25
0.125
0.06
0.03
0.015
0.008
E. faecalis ATCC 29212
S. aureus ATCC 25923
prediffusion
no prediffusion
10
20
30
40
Zone diameter in mm
ROSCO
DIAGNOSTICA


TEICOPLANIN 30 µg
NEO-SENSITABS™
MH-2, McFarland 0.5 inoc.
2+18 hours prediffusion method
using MH agar plain
inoc. McF. 0.5
MIC
µg/ml
64
32
16
8
4
2
1
0.5
0.25
0.125
0.06
0.03
0.015
0.008
S. aureus
ATCC 25923
E. faecalis
ATCC 29212
prediffusion
no prediffusion
10
20
30
40
Zone diameter in mm
ROSCO
DIAGNOSTICA

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Detection of vancomycin resistant enterococci (VRE)

Although clinical laboratories can reliably detect high level resistance to vancomycin,there are reports of poor proficiency in the detection of low-level inducible resistance to vancomycin.

Pendle et al (8) found that the Vitek 2 correctly identified only 2 of 16 isolates of E. faecium vancomycin-resistant isolates(VRE) with low-level vancomycin resistance.The remaining were not identified or identified as aerococcus viridans.Disk testing using the CLSI method,was unreliable for detection of vancomycin resistance upon primary isolation.

Heteroresistance of E. faecium to vancomycin,was reported by Alam et al(1) in 2001.The heterogeinicity of the VRE isolate was observed only by Etest (colonies inside the zone),automated Microscan or microdilution were not adequate for detecting heteroresistance.
In some European countries up to 5 % of non-hospitalised persons could be asymptomatically colonised with VRE (2).Hospital outbreaks of VRE are almost exclusively caused by a specific genogroup of vancomycin-resistant E.faecium caracterised by co-resistance to ampicillin (and ciprofloxacin) and the presence of the variant esp gene (3, 7).

VanB phenotype and vanA genotype Enterococcus faecium with heterogeneous expression of glycopeptide resistance.are reported form Korea(5) and China (14).
Outbreaks of vancomycin resistant E. faecium expressing VanD-like phenotype associated with a vanA genotype are reported from France (4) and Korea (9).Interpretation of MICs for vancomycin was difficult since most of the isolates presented heterogeneous expression of resistance,with colony growth in the E-test zone.Was better visible after 48 hours incubation.Colonies growing inside the zone of inhibition once retested,showed a homogeneous phenotype of resistance to vancomycin.Teicoplanin was not effective against vanD-vanA VRE strains both in vitro and in vivo.

Neves (11) in Brazil reports the emergence of vanA genotype among E. gallinarum from patients in a University Hospital in Rio de Janeiro.Resistance to teicoplanin,when testing motile enterococci,may be a clue to the presence of the vanA gene.

Dobbs et al (6) report the nosocomial spread of Enterococcus faecium resistant to both vancomycin and linezolid in a tertiary care Medical Center.Exposure to fluoroquinolones may be important for the emergence of linezolid resistance.

Souli et al (13) in a prevalence survey of faecal carriage in a tertiary care university hospital in Athens(Greece) found 6 isolates of vancomycin and linezolid resistant vanA positive E. faecium.

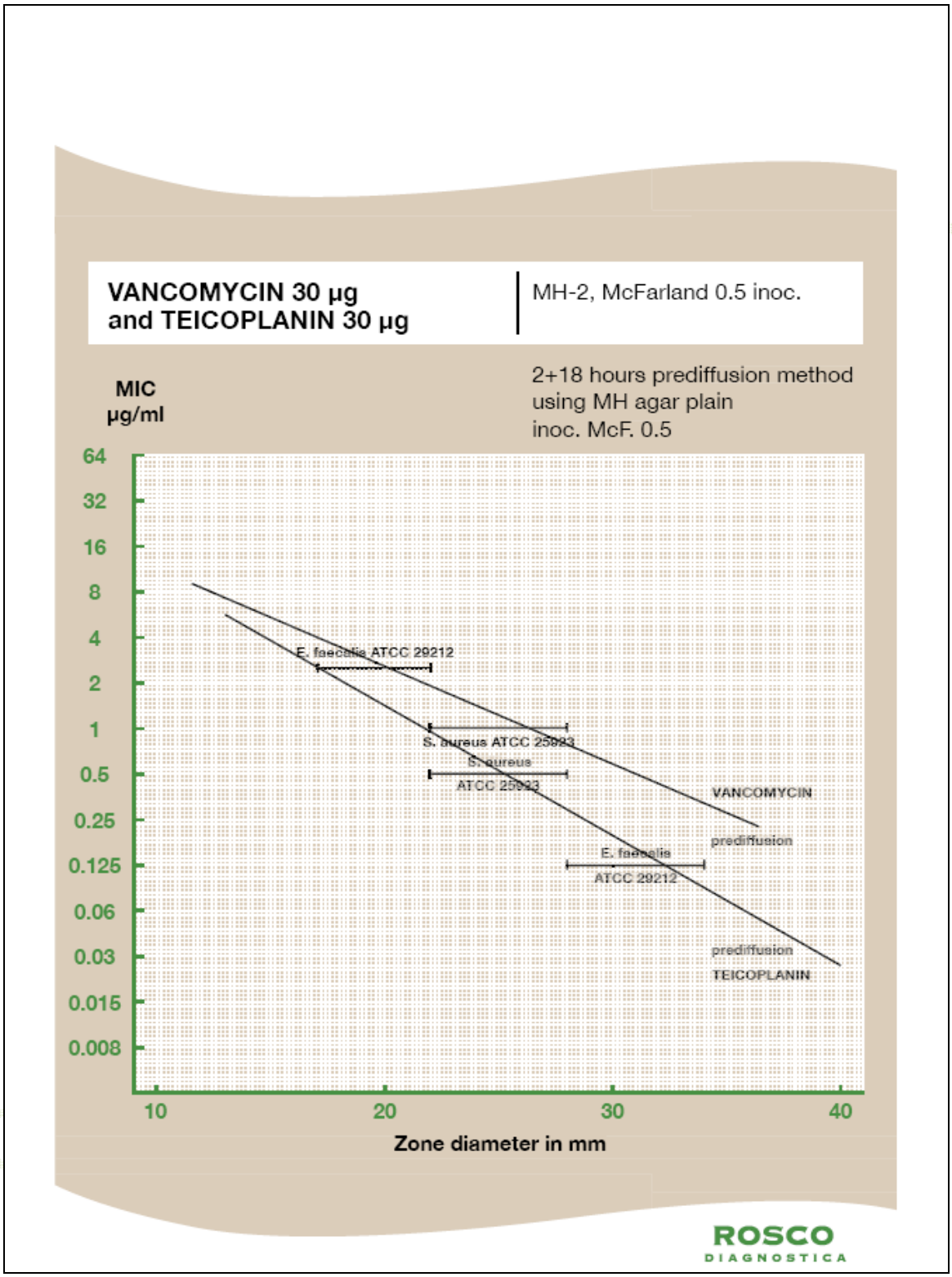
Procedure

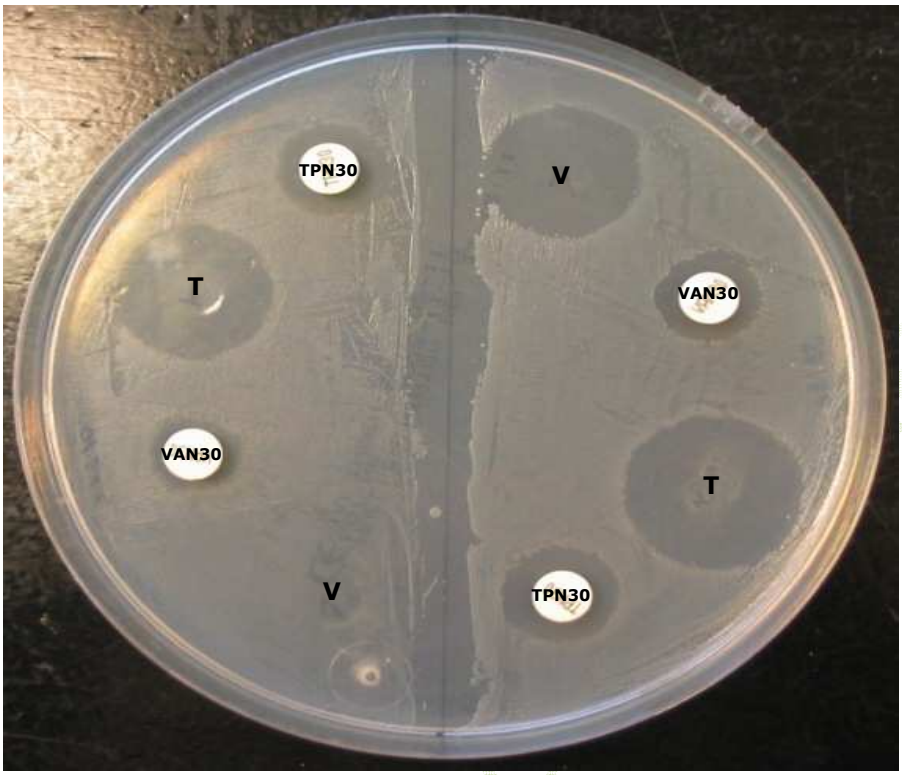
1) One Teicoplanin 30 ug and one Vancomycin 30 ug Neo-sensitabs are placed on an uninoculated plate containing plain MH agar.
2) After 2 hours at room temperature,the tablets are removed(knocking the plate against the table) and the shortnames (VAN and TEI) are written on the back of the plate for identification purposes.
3) The plate is maintained at room temperature for further 18-22 hours (overnight)
4) The plate is now inoculated with the strain to be tested using McFarland 0,5 inoculum and additional antimicrobial disks(Neo-sensitabs) may be added using a dispenser. Thereafter, incubate at 35 degrees overnight.
5) The zones of inhibition are measured and compared with the corresponding zone breakpoints.

Interpretation

VanA:	Vancomycin R and Teicoplanin R : no zone of inhibition
Van B:	Vancomycin zone < 16 mm(hazy edge) and Teicoplanin zone >20 mm.
VanC:	Vancomycin zone < 12 mm (sharp edge). Teicoplanin zone >20 mm.

VanB Phenotype and vanA genotype : Vancomycin no zone,Teicoplanin zone < 6 mm(R).
VanC Phenotype and vanA genotype : Vancomycin no zone,Teicoplanin zone <12 mm (R)
VanD Phenotype and vanA genotype : Vancomycin no zone,Teicoplanin zone < 16mm (R)


VANCOMYCIN 30 µg
and TEICOPLANIN 30 µg
MH-2, McFarland 0.5 inoc.
2+18 hours prediffusion method
using MH agar plain
inoc. McF. 0.5
MIC
µg/ml
64
32
16
8
4
2
1
0.5
0.25
0.125
0.06
0.03
0.015
0.008
E. faecalis ATCC 29212
S. aureus ATCC 25923
S. aureus
ATCC 25923
VANCOMYCIN
prediffusion
E. faecalis
ATCC 29212
prediffusion
TEICOPLANIN
10
20
30
40
Zone diameter in mm
ROSCO
DIAGNOSTICA



E.faecalis ATCC 51299 (van B) compared with susceptible E.faecalis

V=Vancomycin, T=Teicoplanin

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Detection of staphylococci and enterococci non-susceptible to daptomycin using the Neo-Sensitabs prediffusion method

Daptomycin, due to it`s large molecular weight, diffuses slowly into agar, resulting in small differences in the size of inhibition zones between susceptible and non-susceptible isolates.

Extending the diffusion time by a prediffusion method and eliminating the daptomycin depot after 2 hours, allow for better discrimination of non-susceptible isolates showing MICs near the susceptible breakpoint.

Data from a recent study (Katz et al 1) suggests that the 2+18 hours prediffusion method using Daptomycin (DAPCa) Neo-Sensitabs is a promising method for discriminating between daptomycin susceptible and non-susceptible isolates.

Principle

When using the 2+18/22 hours prediffusion, daptomycin has 4 times longer time to diffuse into the agar, before bacterial colonies are formed, than with the current diffusion method. This results in a much larger zone size difference between 2 consecutive MIC, as it can be seen in the enclosed regression line.

Another important feature is that with the prediffusion method the antimicrobial depot is eliminated after 2 hours, avoiding further antibiotic diffusion from the depot into the agar.

Procedure

1) One Daptomycin 30 µg Neo-Sensitabs (DAPCa) is placed on an uninoculated plate, containing plain MH agar.
2) After 2 hours at room temperature, the tablet is removed (knocking the plate against the table) and a short name (DAP) is written on the back of the plate for identification purposes.
3) The plate is maintained at room temperature for further 18-22 hours (overnight)
4) The plate is now inoculated with the strains to be tested using a McFarland 0.5 inoculum. Additional antimicrobial disks (Neo-Sensitabs) may be added now using a dispenser. Thereafter incubate at 35 degrees, overnight.
5) The zones of inhibition are measured and compared with the corresponding zone breakpoints.

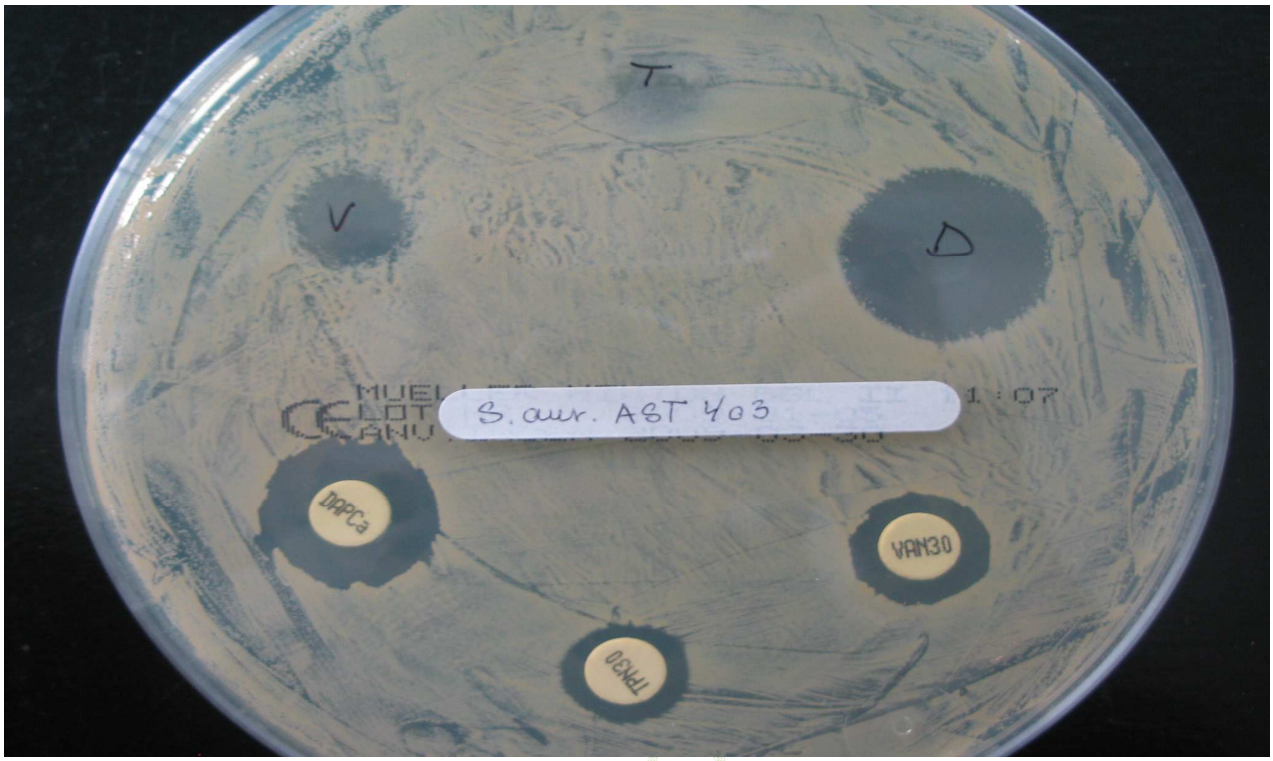
Interpretation

a) Staphylococci

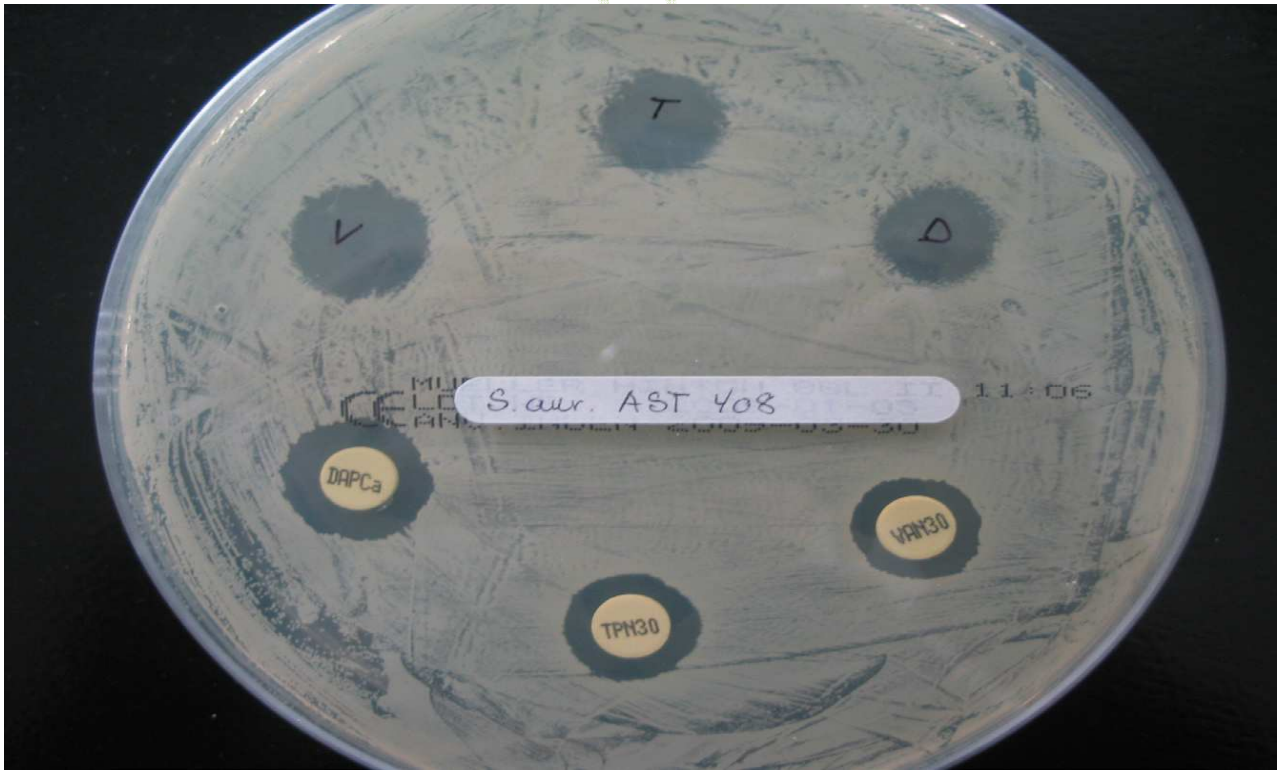
Susceptible:	zone of inhibition ≥ 22 mm (corresponding to MIC ≤ 1 µg/ml)
Non-susceptible:	zone of inhibition < 20 mm (corresponding to MIC ≥ 2 µg/ml)
Indeterminate:	zone 20-21 mm.

b) Enterococci

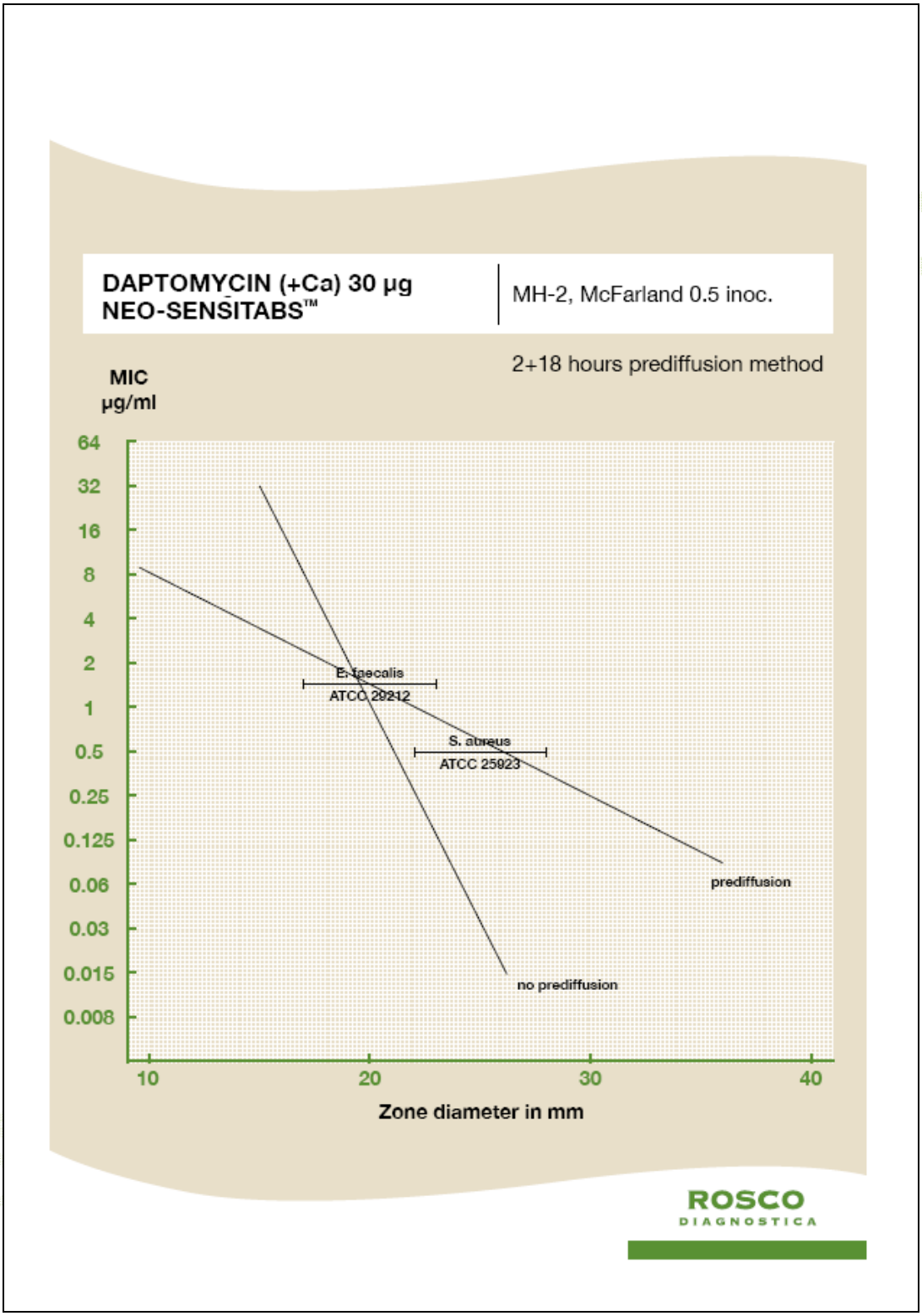
Susceptible:	zone of inhibition ≥ 12 mm (corresponding to MIC ≤ 4 µg/ml)
Resistant:	no zone of inhibition



S. aureus ATS 403, GISA strain, Daptomycin susceptible



S. aureus AST 408 GISA strain Daptomycin non-susceptible.


DAPTOMYCIN (+Ca) 30 µg
NEO-SENSITABS™
MH-2, McFarland 0.5 inoc.
2+18 hours prediffusion method
MIC
µg/ml
64
32
16
8
4
2
1
0.5
0.25
0.125
0.06
0.03
0.015
0.008
E. faecalis
ATCC 29212
S. aureus
ATCC 25923
prediffusion
no prediffusion
10
20
30
40
Zone diameter in mm
ROSCO
DIAGNOSTICA

References

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Detection of Colistin resistant P. aeruginosa, A. baumannii and Enterobacteriaceae

It is well-known the detection of P. aeruginosa showing colistin MICs of 4-16 ug/ml (R)cannot be accurately detected using the current disk diffusion method.Only high level resistance (MIC >= 128 ug/ml),will show no zone of inhibition around a 10 ug disk, and detected.

Testing of Acinetobacter, B. cepacia and Stenotrophomonas is unreliable by the current disk diffusion method.

Testing of one compound (colistin or polymyxin B) predict the results for the other.Colistin is preferred for testing,because it is likely to be used clinically and it is also slightly more sensitive in detecting resistance.

Siqueira et al (3) studied the importance of medium,calcium concentration and inoculum when testing polymyxin B against Acinetobacter spp and P. aeruginosa.Higher calcium content resulted in higher MICs

Lo-Ten-Foe (17),vann der Heijden (5) compared different methods : disc diffusion,E-test,broth microdilution,Vitek 2 and agar dilution for colistin susceptibility testing against P. aeruginosa,A baumannii, and heteroresistant E. cloacae.

The Vitek 2 displayed low sensitivity in the detection of heteroresistant subpopulations of E. cloacae.The current disk diffusion was an unreliable method for testing colistin.

Hiramatsu et al (6) compared disk diffusion,Etest and agar dilution for testing the susceptibility of metallo-beta-lactamases producing P. aeruginosa to polymyxins.The autors found poor concordance between disk diffusion and Etest methods compared to agar dilution

Goi-Barisic (7)and Reis (1) found that the current disk diffusion test with colistin was not adequate for routine testing of A. baumannii.

Ko et al (13) report the emergence of colistin-resistant K. pneumoniae isolates from blood in Korean hospitals.

Landman et al (2) report tha citywide emergence of P. aeruginosa strains with reduced susceptibility to polymyxin B.61 % of the isolates were susceptible to imipenem and 5% of the isolates had reduced susceptibility to polymyxin B (MIC 4 – 8 ug/ml).

Kitzis et al (8) report very high frequency of mutation to colistin resistance in gram negative bacteria, and resistance could not be detected by the current disc-diffusion for most resistant mutants.

Savini et al (15) report the isolation of a colistin-resistant Hafnia alvei,representing a serious clinical and microbiological concern.

Halaby et al (14) report the emergence of colistin resistance during use of selective decontamination of the digestive tract in an ICU,masked by the use of inappropriate laboratory methods.Colistin resistance among ESBL producing K pneumoniae isolates emerged after the introduction of selective decontamination and the routinely used disc diffusion method failed to detect colistin resistance.

Rosco Diagnostica has developed the 2+18 (22) hours prediffusion method, permitting the detection of colistin resistant strains.

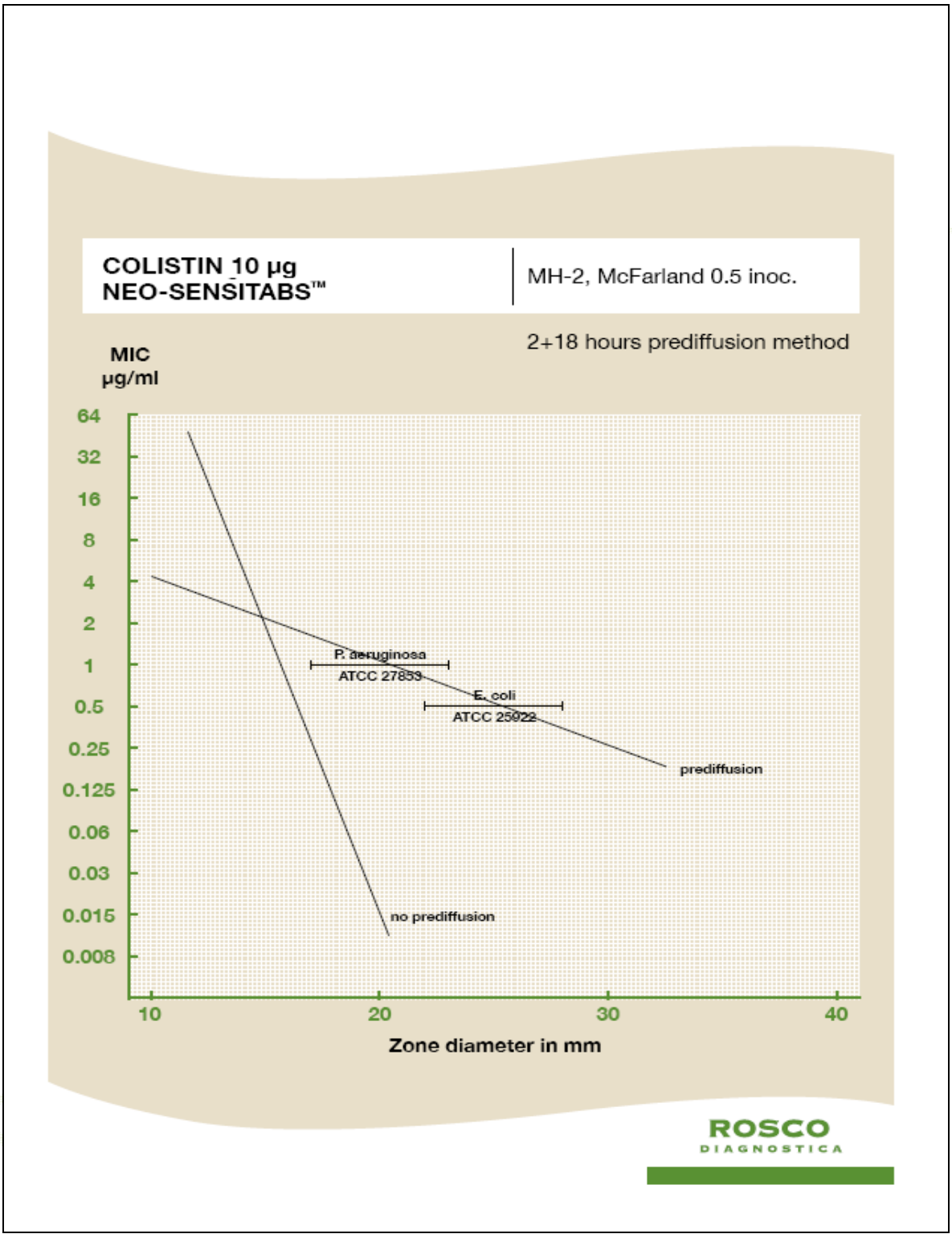
Procedure

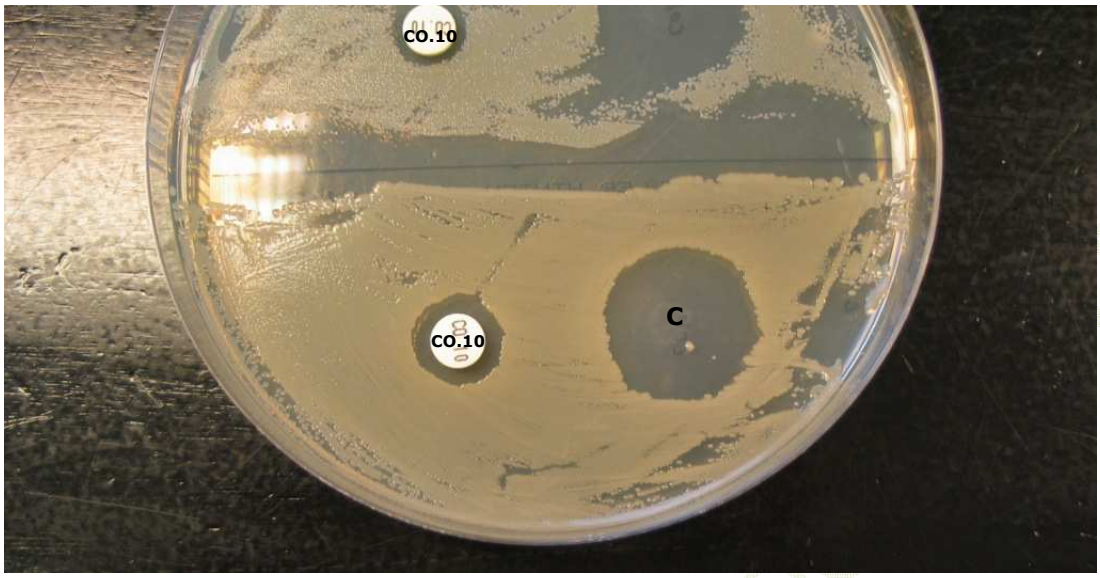
1) One Colistin 10 ug Neo-sensitabs is placed on an uninoculated plate,containing plain MH agar.
2) After 2 hours at room temperature,the tablet is removed (by knocking the plate against the table) and the shortname COL is written on the back of the plate,for identification purposes.
3) The plate is maintained at room temperature for further 18-22 hours (overnight).
4) The plate is now inoculated by the strain to be tested,using Mc Farland 0.5 inoculum and additional antimicrobial disks (Neo-sensitabs) may be added using a dispenser.Thereafter incubate at 35 degrees overnight.
5) The zones of inhibition are measured and compared with the corresponding zone breakpoints.

Detection of resistance mechanisms using Neo-Sensitabs™ and Diatabs ™

Prediffusion method (2+18 or 2+22 hours) for antimicrobials diffusing poorly on agar

Detection of colistin resistant P.aeruginosa, A. baumannii and Enterobacteriaceae





Colistin susceptible E.coli ATCC 25922

C=Colistin

Interpretation

Heteroresistant strains will currently show colonies inside the inhibition zone.

Susceptible strains :	inhibition zone > = 15 mm (MIC <= 2 ug/ml).
Intermediate	(indeterminate) : 14- 11 mm
Resistant :	≤ 10 mm (MIC > 4 ug/ml)

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Resistance mechanisms and automated methods

It is well-known that automated methods, due to their use of small inocula have difficulties in detecting heteroresistant populations,leading to false susceptible results and consequently very major errors. Also well-known is the difficulties of automated methods in testing beta-lactam antimicrobials and detection of beta-lactamases.

We have screened papers and presentations during 2005 to 2009,including only well recognised authors and magazines, such as Clinical Microbiology & Infection,J.Clinical Microbiology, J. Medical Microbiology, Int. J. Antimicrobial Agents and presentations at the ICAAC and ECCMID.

MicroScan

Jones et al(1) report from CAP: false automated system results for VRSA,VISA and piperacillin/tazobactam with P. aeruginosa.Suboptimal MRSA and ESBL detection in commercial automated systems.

Sader et al (2) report an unacceptable rate (21 – 32 %) of Very Major errors for piperacillin/tazobactam with P. aeruginosa.

Unda et al (4) report false resistance to amikacin with the MicroScan WalkAway 96 system as well as major errors with gentamicin and tobramycin;,concluding that the system was not reliable for susceptibility testing of P. aeruginosa to amikacin.

Sader et al (7) studied the accuracy of MicroScan , Vitek and Vitek 2 for susceptibility testing of P. aeruginosa to 5 broad spectrum beta-lactams.All systems tested exhibited a high,unacceptable level of very major (false susceptible) errors for piperacillin/tazobactam (19-27%)and minor error rates were elevated for cefepime and aztreonam (8-32 %),leading to consistent trends towards false resistance.

Jones et al (9) detected errors by automated systems in CAP surveys.Numerous false susceptible and false resistant were noted for each antimicrobial.False positive ESBL results for a CMY-2 AmpC producing strain.Besides, false susceptibility to linezolid and false resistance to cefepime(2005),false susceptibility to amox+ clav and piperacillin/tazobactam (2006).

Juretschko et al (10) reported unacceptable levels of errors (minor,major and very major) with false susceptibility to piperacillin-tazobactam and piperacillin ans false resistance to aztreonam,cefepime and ceftazidime in P. aeruginosa.

Kulah et al (11) studied imipenem resistance in A. baumannii and MicroScan showed the worst performance with 25 % very major errors and 44.6 % minor errors.

Juretschko et al (15) found that 25 % of all cystic fibrosis isolates identified as MSSA by automated methods were mecA positive and therefore MRSA (false susceptibility,very major error).

Gordon et al (20) describe the failure of MicroScan to detect heteroresistance to carbapenems in a patient with E. aerogenes bacteremia and conclude : reliance on automated susceptibility testing,while is more rapid than disc diffusion methods,may result in a delay in detecting or inability to detect the development of resistance.

Phoenix

Juretschko et al (10) reported false resistance to aztreonam and cefepime for P. aeruginosa
Kehl et al (12) found that the Phoenix automated system made false positive identification of vancomycin resistant enterococci(VRE).

Arslan U et al(14) studied automated systems to detect HLR to gentamicin in enterococci.4 of 5 isolates with gentamicin MICs 256 ug/ml,were found susceptible by Phoenix.Strains with aac-aph gene were reported false susceptible .

Juretschko et al (15) found that 25 % of all cystic fibrosis isolates identified as MSSA by Phoenix were mecA positive and therefore MRSA (very major error).

Boyd et al(16) report misidentification of K. pneumoniae carbapenemase (KPC) as ESBL by the Phoenix instrument and conclude that KPC can remain undetected or misinterpreted as ESBL by automated systems.Routine phenotypic testing should be performed.
Boyd et al (18) mention the erroneous reporting of ESBL producers by Phoenix and Vitek 2.
9 of 22 non-ESBL were reported as ESBL by Phoenix (specificity 59 %).The authors conclude that false positive ESBL may be reported by Phoenix and that implementation of phenotypic testing should be considered in ESBL "positive" isolates by automated methods.

Fisher et al (19) compared Phoenix with disc diffusion for identification of ESBL,AmpC and KPC producers.The Phoenix system misclassified nearly 50% of the isolates as ESBL positive.Besides a high false positive detection rate for AmpC. Of 8 KPC producing isolates the Phoenix system reported 50% as meropenem and imipenem susceptible (very major error).The authors conclude that we need reliable methods to detect these important resistance mechanisms in the clinical laboratory.

Vitek 2

Jones et al (1) report from CAP 30 % false susceptibility with piperacillin/tazobactam and P. aeruginosa.

Sader et al (2) report an unacceptable rate (21-32 %) of Very major errors for piperacillin/tazobactam with P. aeruginosa..

Ceyssens et al (3) repot that VITEK-2 overstimates the number of ESBLs among Enterobacteriaceae,that may lead to unnecessary use of carbapenems.

Karatuna et al (5)studied the reliability of carbapenem resistance in P. aeruginosa,by automated systems (Vitek2)and concluded that laboratories using automated systems should consider using at least a second method to validate intermediate or resistant results for carbapenems tested against Pseudomonas spp.

Tokatlidou D et al (6) observed the overdetection of imipenem resistance by Vitek 2 in VIM 12 producing K. pneumoniae with Etest low level carbapenem MICs .

Sader et al (7) found an unacceptable level of very major(false susceptible) errors for piperacillin/tazobactam(19-27 %)as well elevated minor error rates for cefepime and aztreonam,when testing against P. aeruginosa.

Navon-Venezia et al (8) found that when testing cefepime susceptibility of ESBL producing E. coli and K. pneumoniae the Vitek 2 results, were major errors.The high proportion errors and major errors negate the use of Vitek 2 for cefepime MIC determination when ESBL positive strains are considered.

Jones et al (9) studied errors of automated methods according to CAP surveys.False susceptibility to imipenem when testing a SME-1 producing S. marcescens.Besides false susceptibility to meropenem and false resistance to cefoxitin (2003).False susceptibility to piper/tazo , amikacin
imipenem and piperacillin (2004). False susceptibility to linezolid (2005).False resistance to cefoxitin (2006).
Juretschko et al (10) reported unacceptable levels of error with false susceptibility to piperacillin/tazobactam and imipenem and false resistance to aztreonam,cefepime and ceftazidime.

Pitout et al (13) report that Vitek Legacy and Vitek 2 failed to detect piperacillin/tazobactam resistance in 74 % of 101 pipera/tazo resistant,ESBL producing strains,especially CTX-M-15 producing isolates that co-produced OXA-1.

Arslan et al (14) found that Vitek 2 failed to detect HLR gentamicin resistance in E. faecium isolates with aac-aph gene (false susceptible,very major error).

Juretschko et al (15) conclude that the VITEK 2 misidentified 25 % of MRSA isolated from cystic fibrosis patients as MSSA (very major error).

Boyd et al (16) report misidentification of K. pneumoniae carbapenemases (KPC) as ESBL by the Vitek 2 and conclude that KPC isolates can evade detection with automated methods and be misinterpreted as ESBL.

Bulik et al (17) compared meropenem MICs for KPC producing K. pneumoniae by Etest,broth microdilution and Vitek2.When comparing Vitek2 MICs to broth microdilution,only 22 % of isolates were in agreement.

Boyd et al (18) indicates the erroneous reporting of ESBL producers by Vitek 2. 13 of 22 non-ESBLs were reported as ESBL positive by the Vitek2 (specificity 40.9%).Implementation of phenotypic testing should be considered.

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Detection of Resistance Mechanisms (General)

Mechanisms of resistance include production of inactivating enzymes, alteration of drug targets, and altered drug uptake or efflux. Find enclosed in the table below the antibiotics recommended to detect certain resistance mechanisms (1).

Antibiotic (Neo-Sensitabs)	Phenotype	Mechanism of resistance	Bacteria
1) Beta-lactams			
Penicillin + pH indicator (Beta-lactamase - D.T.)	Penicillin resistance	Penicillinase	Staphylococci, Haemophilus, Gonococci
Oxacillin 1 µg res.	Resistance to all beta-lactams	Additional PBP	Staphylococci
Cefoxitin res.	Resistance to all beta-lactams	mecA	Staphylococcus aureus
Ampicillin res.	a)Resistance to penicillins and beta-lactams inhibitor comb.	Altered PBPs	Enterococci
Oxacillin 1 µg (zone < 20 mm) (zone < 14 mm) (zone < 12 mm)	Penicillin resistance	PBP alteration	Pneumococci, Streptococci, Gonococci
Ceftizoxime res.	Resistance to third generation cephalosporins	PBP alteration	Pneumococci
Ampicillin 2.5 µg (zone < 20 mm)	Resistance to AMP,AMX, AM+CL, CCLOR, CEFUR (BLNAR strains)	PBP alteration	Haemophilus
Amoxycillin and Amoxycillin+Clavulanate	b) Penicillin resistance AM+CL synergy	Beta-lactamase (BRO-1, BRO-2)	Moraxella catarrhalis
Ceftazidime, Ceftriaxone (zone < 24 mm)		Screening ESBL	Klebsiella spp, E. coli, Salmonella
Cefpodoxime I/R		Screening ESBL	E. coli, Klebsiella Salmonella
Cefotaxime, Ceftriaxone Ceftazidime/Cefepime and Amoxycillin+Clavulanate	c) Synergy between CTX, CTR, CAZ and AMC, CP+CL, (double disk synergy)	Extended spectrum beta-lactamase (ESBL)	Enterobacteriaceae d)
Ceftazidime+Clavulanate	CAZ+CL zone ≥ 5 mm than CAZ alone	ESBL	Enterobacteriaceae
Cefepime and Amoxycillin + Clavulanate	Synergy between FEP and AMC	ESBL	Enterobacter, Serratia, Citrobacter freundii
Cefepime+Clavulanate	FEP+CL zone ≥ 5 mm than FEP alone	ESBL (confirmatory)	Enterobacteriaceae
Cefotaxime+Clavulanate	CTX+CL zone ≥5 mm than CTX alone	ESBL	Enterobacteriaceae
Cefoxitin + Cephalosporins Imipenem + Cephalosporins	Antagonism, indicates cephalosporin resistance	Inducible cephalosporinase AmpC	Enterobacteriaceae
Cefepime+Clavulanate Cefoxitin/3rd gen. cepha.	No synergy No antagonism Cefoxitin R, Ceftazidime R	Plasmid mediated AmpC	Enterobacteriaceae
Amoxycillin+Clavulanate I/R Cefazolin	Amoxycillin+Clavulanate R Cefazolin S	Inhibitor resistant TEM ß-lactamase	E. coli Klebsiella
Cefotaxime+Boronic Ceftazidime+Boronic	zone ≥5 mm than CTX or CAZ alone	AmpC beta lactamase	Enterobacteriaceae

Antibiotic (Neo-Sensitabs)	Phenotype	Mechanism of resistance	Bacteria
Cefoxitin res.	Antibiotic resistance	Porin alteration	E. coli Klebsiella
Aztreonam, Ceftazidime, Cefepime and Ticarcillin + Clavulanate	Synergy between TC+Cl and AZT, FEP, CAZ.	ESBL	Ps. aeruginosa
Imipenem+EDTA Meropenem+DPA	Synergy between Imipenem and EDTA Synergy MRP and DPA	Metallo-ß-lactamase	Ps. aeruginosa Acinetobacter E. coli
Cloxacillin	Synergy between cefotaxime, ceftazidime and cloxacillin	AmpC beta-lactamase	Enterobacteriaceae
Dipicolinic acid	Synergism with Meropenem and/or Imipenem	Metallo-ß-lactamase	Enterobacteriaceae Non-fermenters
Boronic acid	Synergism with Cefotaxime and/or Ceftazidime	AmpC beta-lactamase	Enterobacteriaceae
Meropenem+Boronic Meropenem+Cloxa	Synergy (≥5mm) No synergy	KPC beta lactamase	K.pneumoniae P.aeruginosa
Meropenem+Boronic Meropenem+Cloxa	Synergy synergy	AmpC+porin loss	Klebsiella Enterobacter
2) Aminoglycosides			
Kanamycin res.	Amikacin and Isepamicin resistance	APH(3'), ANT(4')	Staphylococci
Gentamicin res.	Resistance to aminoglycosides except streptomycin	APH(2")-AAC(6')	Staphylococci
Kanamycin 500 µg (zone < 14 mm)	HLR to amikacin (no synergy with penicillins)	APH(3'), ANT(4')	Enterococci (HLR)
Gentamicin 250 µg (zone < 14 mm)	HLR to all aminoglycosides	APH(2")-AAC(6')	Enterococci (HLR)
Streptomycin 500 µg (zone < 14 mm)	Streptomycin resistance		Enterococci (HLR)
Amikacin + Tobramycin res.	Resistance to aminoglycosides	APH(3')-VI	Acinetobacter
Netilmicin + Tobramycin res.	Resistance to aminoglycosides	AAC(3)	Pseudomonas
3) Others			
Erythromycin + Clindamycin	Inducible MLS resistance (antagonism)	Ribosomal methylation	Staphylococci, Streptococci,
Nalidixan I/R	Reduced sensitivity to quinolones	DNA gyrase	Enterobacteriaceae Vibrio cholerae Haemophilus/Moraxella
Nalidixan I/R	Reduced sensitivity to quinolones	DNA gyrase	Gonococci Meningococci
Ciprofloxacin 0.5 µg (zone < 20 mm)	Quinolone resistance	DNA-gyrase	Gonococci, Haemophilus
Vancomycin Teicoplanin	Vancomycin resistance 2+18 hours' prediffusion	Van A, Van B VISA, hVISA	Enterococci, Staphylococci
Metronidazole	Imidazole resistance	Reductase	Anaerobes

a) for <u>non</u> beta-lactamase producing enterococci.

b) Synergy: AM+CL zone > 5 mm larger than AMOXY (resistance to penicillin, amoxycillin and ampicillin, susceptible to amoxycillin+clavulanate).

c) Beta-lactam resistance (except cephamycins and carbapenems).

d) Except Proteus penneri and P. vulgaris.

Note: The mentioned zone sizes are valid for McFarland 0.5 inoculum.

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Intrinsic (Natural) Resistance

Antimicrobial resistance can be classified as either intrinsic or acquired. Intrinsic resistance may be related to inherent or natural characteristics in a bacteria and may be used for recognition of a bacterial species, and results of in vitro susceptibility testing is not relevant to report as treatment options. The most relevant drug related natural resistance in a group or species is listed below:

BACTERIA	NATURAL RESISTANCE
Enterobacteriaceae	
Enterobacteriaceae in general	Penicillinase stable penicillins, Macrolides, Fucidin, Rifampicin, Glycopeptides
Enterobacteria, group 2 K. pneumoniae, K oxytoca, C. diversus, Esch. hermannii	Aminopenicillins, Carboxypenicillins
Enterobacteria, group 3 E. cloacae, E. aerogenes, C. freundii, S. marcescens, M. morganii, Prov. rettgeri, P stuartii, Hafnia alvei (except *P. vulgaris* AMC S)	Aminopenicillins, Amoxicillin+Clavulanate, 1st gen Cephalosporins
Citrobacter freundii	Aminopenicillins, Amoxicillin+Clavulanate, 1st gen Cephalosporins, Cefoxitin,
Citrobacter koseri (diversus)	Aminopenicillins, Carboxypenicillins
Enterobacter aerogenes, E cloacae	Aminopenicillins, Amoxicillin+Clavulanate, Cefoxitin, 1st gen Cephalosporins, Nitrofurantoin
Klebsiella pneumoniae, K. oxytoca	Aminopenicillins, Carboxypenicillins
Morganella morganii	Aminopenicillins, Amoxicillin+Clavulanate, 1st and 2nd gen Cephalosporins, Cefoxitin, Polymyxins, Tetracyclines, Nitrofurantoin, Fosfomycin
Proteus mirabilis	Polymyxins, Tetracyclines, Nitrofurantoin
Proteus vulgaris, P. penneri	Aminopenicillins, Carboxypenicillins Cefuroxime, Polymyxins, Tetracyclines, Nitrofurantoin
Providencia rettgeri	Aminopenicillins, Polymyxins, Tetracyclines, Nitrofurantoin, Amoxycillin+Clavulanate.
Providencia stuartii	Aminopenicillins, Amoxicillin+Clavulanate, Polymyxins, Tetracyclines, Nitrofurantoin, Gentamicin, Tobramycin, Netilmicin
Salmonella spp.	1st and 2nd gen Cephalosporins, Cefuroxime (active in vitro, not active in vivo) Aminoglycosides (in vivo)
Serratia marcescens	Aminopenicillins, Amoxicillin+Clavulanate, 1st and 2nd gen Cephalosporins Polymyxins
Shigella spp.	1st and 2nd gen Cephalosporins, Aminoglycosides (in vivo)
Yersinia enterocolitica	Aminopenicillins, Carboxypenicillins, Amoxicillin+Clavulanate, 1st and 2nd gen Cephalosporins, Cefoxitin
Gram positive cocci in general	Aztreonam, Nalidixic acid, Polymyxins
Staphylococci	
Staphylococcus spp. in generel	Nalidixic acid, Polymyxins
S. saprophyticus	Novobiocin, Fosfomycin
Penicillin resistant staphylococci (Oxa S)	Penicillin, Aminopenicillins, Ureidopenicillins, Carboxypenicillins
Methicillin resistent staphylococci	All beta-lactams
Micrococcus spp.	Nitrofurantoin, Mupirocin
Streptococci/enterococci	
Streptococcus spp.	Polymyxins, Nalidixic acid, Aminoglycosides (low level)
Enterococcus faecalis	Cephalosporins, Clindamycin, Mupirocin, Aminoglycosides (low level – HLR test), Novobiocin, Trim+Sulfa (in vivo)
E. faecium	Cephalosporins, Aminoglycosides (low level – HLR test), Nitrofurantoin, Trim+Sulfa (in vivo)
E. gallinarum/casseliflavus	Vancomycin (MIC 4-16 µg/ml)
Arcanobacterium spp.	Bacitracin, Mupirocin, Optochin
Pediococcus/Leuconostoc Lactobacillus/Erysipelothrix	Glycopeptides

BACTERIA	NATURAL RESISTANCE
Non fermenters	
Acinetobacter baumanii/calcoaceticus	Aminopenicillins, 1st and 2nd gen Cephalosporins, Chloramphenicol, Trimethoprim, Fosfomycin, Nitrofurantoin
Achromobacter xylosoxidans	Aminopenicillins, 1st, 2nd and 3rd gen Cephalosporins, Aminoglycosides, Aztreonam
Alc denitrificans	Cefotaxime
Burkholderia cepacia	Aminopenicillins, Ureidopenicillins, Carboxypenicillins, Amoxicillin+Clavulanate, 1st and 2nd gen Cephalosporins, Quinolones, Aminoglycosides, Polymyxins, Nitrofurantoin, Fosfomycin, Chloramphenicol, Imipenem, Trimethoprim
Elisabethkingia meningoseptica	Aminoglycosides, Carboxypenicillins, 1st, 2nd and 3rd gen. Cephalosporins, Polymyxins, Tetracyclines, Chloramfenicol, Ticarcillin+Clavulanate, Quinolones, Imipenem
Ochrobactrum anthropi	Ureidopenicillins, Carboxypenicillins, Ticarcillin+Clavulanate, 3rd gen. Cephalosporins, Aztreonam, Ertapenem
Pseudomonas aeruginosa	Aminopenicillins, Amoxicillin+Clavulanate, 1st and 2nd gen. Cephalosporins, Cefotaxime, Ceftriaxone, Chloramfenicol, Nalidixic acid, Trim+Sulfa, Tetracyclines, Nitrofurantoin
Stenotrophomonas maltophilia	Ureidopenicillins, Carboxypenicillins, 1st and 2nd gen Cephalosporins, Imipenem, Cefotaxime, Aztreonam, Aminoglycosides, Tetracyclines (except Minocycline) Fosfomycin
Listeria	Oxacillin, Cephalosporins, Aztreonam, Polymyxins, Nalidixic acid, Clindamycin, Fosfomycin
Neisseria/Branhamella	
Branhamella catarrhalis	Lincomycin, Clindamycin, Trimethoprim
Gonococci, meningococci	Lincomycin, Clindamycin, Polymyxins, Trimethoprim, Vancomycin
Campylobacter/Helicobacter	
Campylobacter spp.	Vancomycin, Trimethroprim, Polymyxins, Lincomycin, Novobiocin, Aztreonam
Helicobacter pylori	Vancomycin, Polymyxins, Nalidixic acid, Trimetoprim, Sulfonamides
Corynebacterium in general	Fosfomycin, Mupirocin, Polymyxins, Nalidixic acid
C. jeikeium/urealyticum	All Penicillins, 1st, 2nd and 3rd gen. Cephalosporins, Amoxicillin+Clavulanate., Imipenem, Meropenem, Aminoglycosides, Chloramphenicol, Nalidixic acid, Trim+Sulfa, Polymyxins, Fosfomycin, Mupirocin, Macrolides
Anaerobes in generel	Aminoglycosides, Aztreonam (exept Fusobacteria), Trimethoprim, Nalidixic acid
Bacteroides fragilis group	Aminoglycosides, Vancomycin, Aminopenicillins, 1st, and 2nd gen. Cephalosporins, Polymyxins, Glycopeptides, Fosfomycin, Aztreonam, Oxgall
Clostridium spp.	Kanamycin, Trimethrprim, Aztreonam, Polymyxins, Fosfomycin
Fusobacteria spp.	Nalidixic acid, Vancomycin, Macrolides (low level)
Porphyromonas spp	Polymyxins, Fosfomycin, Aminoglycosides
Prevotella spp.	Glycopeptides, Fosfomycin, Aminoglycosides
Actinomyces/Propionibacterium	1st, and 2nd gen. Cephalosporins, Polymyxins, Metronidazole
Mobiluncus spp.	Metronidazole
Peptostreptococcus/Eubacterium	Polymyxins, Fosfomycin
Veillonella spp.	Macrolides (low level), Glycopeptides

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