Carbapenemases and phenotypic methods for detection

Carbapenemases are beta-lactamases that significantly hydrolyze at least imipenem and/or meropenem. Carbapenemases involved in acquired resistance are of Amber classes A, B and D. They may be plasmid or chromosomally encoded.

Carbapenemases are mainly found in Amber class A, B and D, with KPC, NDM and OXA-48 being the most frequently encountered in *Enterobacteriaceae*.

The emergence of carbapenemases in gram-negative bacteria, including *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species has become a major health crisis worldwide.

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. Rapid detection and identification of the carbapenemase genes is critical for the implementation of health control policies.

Confirmation of carbapenemase activity can be achieved with phenotypic tests such as combined disc methods and rapid colorimetric based tests.

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.

Adler et al (67) report that carbapenemase-producing *K. pneumoniae* exhibited a marked inoculum effect and were more resistant to the bactericidal effect of meropenem, suggesting that MIC measurement alone may not be sufficient in predicting efficacy.

Potron et al (4) confirms that multidrug resistance is quite common among non-fermentative gram-negative rods, in particular *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The authors give details of the most important mechanisms of resistance in *P. aeruginosa* and *A. baumannii* and their most recent dissemination worlwide.

Logan LK et al (5) report that carbapenem-resistant *Enterobacteriaceae* (CRE) in US children showed a major increase during 1999-2012 (from 0.0% to 5.2 %) and the most substantial increases were in children 1 – 5 years of age. Among CRE isolates, the largest number (36.8%) was *Enterobacter spp*. from urine and from non-ICU patients.

Hargreaves et al (8) report the clonal dissemination of *Enterobacter cloacae* harboring KPC-3 in the upper Midwestern of USA. All strains possessed identical IncFIA-like plasmids and an additional Inc X3 plasmid. The spread of this clone is unprecedent for *E. cloacae* and highlights the importance of continued surveillance.

Humphries et al (9) report that in 2010 the CLSI revised the carbapenems breakpoints for *Enterobacteriaceae*. However, no manufacturer of commercial automated susceptibility testing systems has obtained FDA-clearence for these new standards, despite 5 years having passed. The authors mention that the Check-Point CPE for detection of CRE in rectal surveillance cultures had an unacceptable number of false-positives.

Harbarth et al (10) report that gene identification is becoming more accessible, but some commercially available tests are unable to discern between gene variants (OXA-48, 163 and 405). False positives can also be a problem as detected resistance genes may be present in non-pathogenic bacteria. Besides genes may be present but expressed poorly or not al all.

Al-Bayssari et al (11) report that several methods have been developed to rapidly detect carbapenemases. One is the use of combined tablets (triple disk from Rosco Diagnostica) which allows the detection of KPC+MBL in the same strain. The Microarrays method, appear to be both time-consuming and expensive, while low-cost approaches like the rapid colorimetric tests (98024, CarbaNP) appear to be highly reliable in detecting carbapenemases.

Chea et al (6) report that when they used the CDC and Prevention CRE definition in their study, 21 % of *K. pneumoniae* carbapenemase-producing were misclassified as non-CP. The new definition requiring resistance to 1 carbapenem resulted in 55% false positive results and when adding the Hodge test (MHT) to the definition decreased false positives to 12 %. The MHT might falsely identify NDM-producing strains as non-CP and may falsely identify non-CP *Enterobacter spp* as CRE. The authors recommend adding resistance-mechanism testing in order to identify real carbapenemase production.

Bush K (7) in a Review reports the beta-lactamase-inhibitor (BLI) combinations, available today and in the near future, that are effective against multidrug- resistant Gram-negative pathogens. It contains the new generation of BLIs such as avibactam, relebactam, RPX7009 and their combinations.

Meletis (12) reports that carbapenem-resistant gram-negative nosocomial pathogens will continue to evolve accumulating more carbapenem-resistance mechanisms or more than one carbapenemase-encoding gene and untreatable infections could emerge.

Oikonomou et al (13) report the rapid dissemination in Central Greece of colistin and carbapenem resistant ST101 clone of *Acinetobacter baumannii*. Colistin resistance gradually increased and reached 21.1% in 2014.

Both the CLSI and EUCAST recommend reporting the susceptibility result against carbapenems ”as found”, independent of the presence of a carbapenemase or not (3). New clinical data would potentially modify these recommendations, because the production of a carbapenemase also affects specific treatment advice. Detecting of carbapenemases is not only for infection control purposes but for the establishment of a suitable antimicrobial therapy (in many cases combining a carbapenem with other antibacterials) that should be guided by breakpoints values (Meropenem/Imipenem MICs <= 8 ug/ml)

<u>Screening</u>

Recently the EUCAST subcommittee for detection of resistance mechanisms proposed screening cut-off values for carbapenems. For bacteria producing OXA-48 beta-lactamases, temocillin was proposed as an indicator antibiotic with high sensitivity. The use of Temocillin 30 ug (zone below 12 mm) and Piperacillin-tazobactam 100/10 ug (zone< 16 mm) can significantly increase the sensitivity of the screening procedure for OXA-48 and similar.

Hartl et al (48) proposed resistance to temocillin combined with the meropenem double disk synergy test as an algorithm that could be introduced into diagnostic laboratories for the identification of carbapenemase production and differentiation of Amber Class A, B and D enzymes

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 >= 0.25 µg/ml) or Ertapenem (zone < 25 mm) on Mueller-Hinton Agar with McFarland 0.5 inoculum should be suspected of possessing carbapenemases. Ertapenem Neo-sensitabs is the most sensitive indicator, for possible carbapenemases. It is important to recognize small resistant colonies growing inside the Ertapenem zone.

P. aeruginosa with inhibition zones to 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.

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-5	S	4-64	≥ 16	0.25-8	± wk	no	yes
	IMI-1 to IMI-6	S	S	≥64	4-32	+	no	yes
	KPC-2 to KPC-19	≥ 32	≥ 64	4→16	4→16	+ or wk	no	yes
	GES-2 to GES-27	≥ 32	16→R	0.25→16	0.5-16	+ or 0	no	yes
B Metallo-beta-lactamases	IMP 1-16-51	≥ 32	S→R	0.5-128	0.25→R	no	yes	no
	VIM 1-12-45	≥ 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
	NDM-1 to 16	R	S	R	R	no	yes	no
	IND-1 to 7							
	AIM, DIM, KHM							
D Oxacillinases	OXA 23-27-49	> 256	> 256	4-64	4-128	± wk	no	no
	OXA 40-143	S→R	S→R	2-64	0.25-64	wk	no	no
	OXA 54-55	S	S	4	0.25	wk	no	no
	OXA-48-162-181-204-232-244-245	S					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.
2) Martinez-Martinez L et al: Carbapenemases in *Enterobacteriaceae*: types and molecular epidemiology. Enfer Infecc Microbiol Clin **32**, (suppl 4), 4-9, 2014.
3) Canton R et al: Breakpoints for carbapenemase-producing *Enterobacteriaceae*: is the problem solved? Enferm Infecc Microbiol Clin **32** (Suppl 4), 33-40, 2014.
4) Potron A et al: Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and Acinetobacter baumannii: mechanisms and epidemiology. Int J Antimicr Agents **45**, 568-585, 2015.
5) Logan LK et al: Carbapenem-resistant *Enterobacteriaceae* in children, US, 1999-2012. Emerg Inf. Dis **21**, 2014-2021, 2015.
6) Chea N et al: Improved phenotype-based definition for identifying carbapenemase producers among carbapenem-resistant *Enterobacteriaceae*. Emerg Infect Dis **21**, 1611-1616, 2015.
7) Bush K: A resurgence of beta-lactamase inhibitor combinations effective against multidrug-resistant Gram-negative pathogens. Int J Antimicr Agents (2015) Aug 11, Epub.
8) Hargreaves M et al: Clonal dissemination of *Enterobacter cloacae* harboring KPC-3 in the upper Midwestern USA. Antimicr Ag Chemother **59**, 7723-7734, 2015.
9) Humphries RM et al: Continuing challenges for the Clinical Laboratory for detection of carbapenem-resistant Enterobacteriaceae. J Clin Microbiol **53**, 3712-3714, 2015.
10) Harbarth S et al: Antimicrobial resistance: one world, one fight. Antimicr Resistance and Infection Control **4**, 49, 2015.
11) Al-Bayssari Ch et al: Detection of expanded-spectrum-beta-lactamases in Gram-negative bacteria in the 21st century. Expert Review of Antiinfective Therapy **13**, 1139-1158, 2015.
12) Meletis G: Carbapenem resistance: overview of the problem and future perspectives. Therapeutic Advances in Infectious Disease, 1 – 7, Dec 2015.
13) Oikonomou O et al: Rapid dissemination of colistin and carbapenem resistant Acinetobacter baumannii in Central Greece: mechanisms of resistance, molecular identification and epidemiological data. BMC Infect Dis, Dec 9th 2015:559.

Detection of acquired carbapenemases Amber classes A and D in *Enterobacteriaceae*

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

Phenylboronic acid is an inhibitor of class A carbapenemases and therefore, 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).

Boyd et al (64) describe the isolation of two *E. coli* and two *Serratia marcescens* harboring GES-5 on plasmids persisting in a hospital in Canada. GES-5 are Class A ESBLs that contains variants that hydrolyze carbapenems and consequently are carbapenemases. It appeared that the GES-5 plasmid had persisted in an environmental niche for at least 2 years in the hospital.

Class D carbapenemases correspond to the enzymes classified as OXA-types (oxacillinase activity). They hydrolyze imipenem and meropenem weakly and do not hydrolyze third generation 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.

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

Poirel et al (60) explain why phenotypic methods are useful for the detection of carbapenemases. The diagnostic techniques must be easy to perform and interpret. Routine laboratories cannot rely on complicated screening strategies that may provide difficult to interpret results (molecular methods).

Opposite to the phenotypic methods, the molecular techniques are expensive, they require significant expertise, correlation between gene identification and resistance is not always observed and by definition they do not identify the novel and emerging resistance genes.
In some occasions, carbapenemase-producers showing low MICs towards carbapenems are difficult to detect, particularly when automatic methods are employed.

Oteo et al (61) describe the clonal dissemination of *K. pneumoniae* ST11 and ST101 producing KPC-2 in Madrid. Two of the isolates coproduced KPC-2+OXA-48.
Prats et al (62) found that the combination of the Rosco KPC/MBL and OXA-48 Confirm kit with the modified Hodge test had a good sensitivity and specificity in the detection of carbapenemases in *Enterobacteriaceae* and non-fermenters.

Lombardi et al (65) describe the emergence of carbapenem-resistant *K. pneumoniae* in a cardiac surgery division. 35% were carbapenemase producers. 89 of 98 of the isolates were positive for KPC-2 and 9 of 98 were positive for VIM-1. The combined disk test: KPC/MBL Confirm kit from Rosco and PCR were used to detect the carbapenemase genes.

Acquired carbapenemases Ambler class A and D

Ambler class.	Enzymes	MICs µg /ml PIP	3rd gen cepha	AZT	IMI	MRP	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-4	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-2-6	16→128	0.1-2	4-8	64	4-32	+	yes	*E. asburiae*	Plasmid
	KPC-1	> 128	≥32	> 64	16	16	+	yes	*K. pneumoniae*	Plasmid
	KPC-2-13	≥64	≥8	> 16	8→16	≥16	+	yes	*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	(+ +)	yes	*K. pneumonia Enterobacter E. coli, P. mirabilis, Citrobacter*	Plasmid
	KPC-4				R> 16	> 16	(+)	yes	*Enterobacter*	
	KPC-5-19							yes	*Serratia*	Plasmid
	FRI-1	R	S	R	R	R		yes		plasmid
	BCK-1	R	R	R	R	R	+	yes	*K. pneumon.*	plasmid
	VCC-1	R	S	R	R	R	(+)		*Vibrio cholerae*	
A	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

Ambler class.	Enzymes	PIP	MICs µg /ml 3rd gen cepha	AZT	IMI	MRP	IMIPENEM + CLAV (synergy)	Mero-penem+ Boronic synergy	Organisms	Genetic Location
	GES-5-6	R	R	R	8-32	8-32	+	yes	*P.aeruginosa, K.pneumoniae*	Integron
	GES-11-14-18-27	R	> 256	> 256	4	8		yes	*A. baumanni P.aeruginosa*	plasmid
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-48-162-181-204-232-244-245.	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. baumannii*	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) in Enterobacteriaceae

Isolates giving negative metallo-beta-lactamase test results, 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 Phenylboronic 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).
Interpretation (Double disk synergy test)

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

a) Negative metallo-beta-lactamases tests.
b) Positive synergy test between Phenylboronic Acid and the carbapenems (one or both).
c) Negative synergy test between Cloxacillin and the Carbapenems (11)
d) 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.
Rosco Diagnostica has introduced kit **98015**: KPC/MBL and OXA-48 confirm kit together with the Triple disk: Meropenem+ Boronic+Dipicolinic acid, permitting the detection in *Enterobacteriaceae* of KPC, MBLs and OXA-48 together with double enzymes KPC+MBL using the triple disk.
For *P. aeruginosa* and *Acinetobacter* the kit **98025**: KPC/MBL in *P. aeruginosa* and *Acineobacter* Version 2, permitting the detection of these enzymes in non-fermenters.

Clinically relevant carbapenemases (49)

	Class A (KPC)	MBL (VIM, IMP)	NDM	Oxacillinases
Klebsiella pneumoniae	+++	+++	++	+++ (OXA-48 or similar)
E. coli	+	+	++	++
P. mirabilis	+/0	+/0	-	+
Providencia spp	-	+/0	+/0	
K oxytoca	+/0	+/0	+/0	
S. marcescens	+/0	+		
Enterobacter spp	+/0	+	+/0	
Citrobacter freundii	+/0	+/0	+/0	
M. morganii		+/0	+/0	
Salmonella spp	+/0	-		
P. aeruginosa	+	+++		+
P putida	+	+/0		
Acinetobacter baumannii	-	++	+/0	+++ (OXA 23-40-58-143)
Acinetobacter spp	-	+	-	+

+++ = high prevalence,
++ = moderate prevalence (1-10%)
+ = low prevalence (< 1 case)
+/0 = isolated cases

Albiger at al (22) report the distribution of carbapenemase-producing *Enterobacteriaceae* (CPE) in Europe (May 2015). They noticed the rapid spread of OXA-48 and NDM producing *Enterobacteriaceae*. 13/38 countries reported inter-regional spread of or an endemic situation for CPE.OXA-48 and NDM were the most commonly found, followed by KPC and at a lower level VIM, while IMP was rare.

Zhao et al (73) report the dissemination of drug-resistant KPC-2 producing *K. pneumoniae* isolated from bloodstream infections in China. Most of the isolates co-carried ESBLs, particularly CTX-M-24 and SHV11.

Tomasso et al (68) report a large nosocomial outbreak of colistin-resistant, carbapenemase producing *K. pneumoniae* in Siena, Italy. The outbreak was attributable to the clonal expansion of a single mgrB deletion mutant from a KPC-3 producing Kl. pneumoniae.
Camargo et al (69) report the successfull treatment of carbapenemase producing (KPC) pandrug-resistant *K. pneumoniae* bacteremia using a combination of an intravenous CAZ-avibactam with ertapenem. Synergism in vitro was observed between a CAZ-avibactam disk and an ertapenem disc (ghost zone).

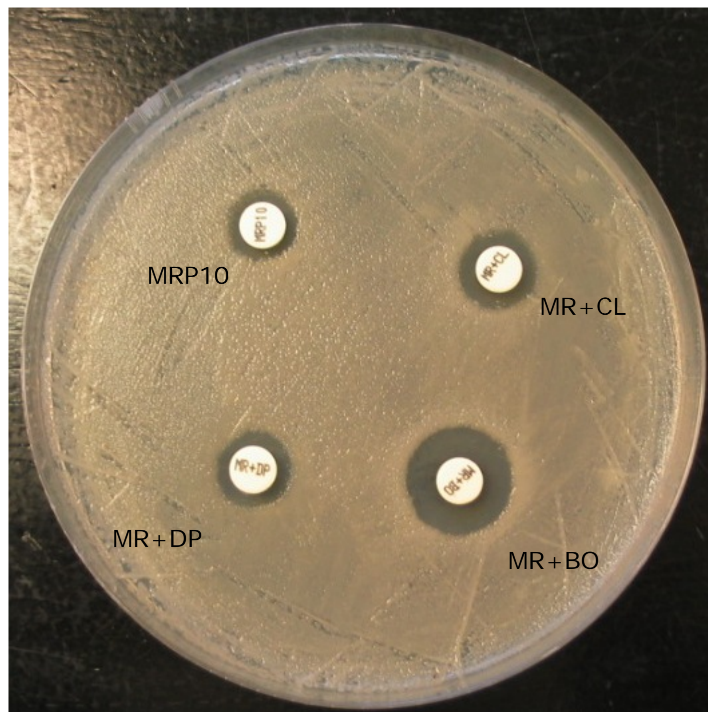
Anchordoqui et al (74) describe 3 cases of intrapatient transfer of the KPC-2 gene. *E. coli* and *K. pneumoniae* isolated from a rectal swab harboured both KPC-2. In a second case, *Enterobacter cloacae* and *K. pneumoniae* from blood cultures harboured both KPC-2. In a third, case *Citrobacter freundii* and *K. oxytoca* isolated from skin possessed the KPC-2 gene. In conclusion, the authors document the horizontal dissemination of the KPC-2 gene from diverse *Enterobacteriaceae* clinical isolates, with different genetic backgrounds.

Fattouh et al (76) in Canada, indicate that the CLSI proposes screening of carbapenemases on isolates showing MIC >= 2 ug/ml against ertapenem or meropenem, while EUCAST recommends screening cutoff MIC >= 0.25 ug/ml for ertapenem and/or meropenem. They found out that 14% of carbapenemase producers may go undetected when using the current CLSI recommendations and conclude that it would be important to detect the presence of carbapenemase genes even in isolates deemed “susceptible” to carbapenems. In their study carbapenemase activily was confirmed in parallel with molecular detection using the KPC/MBL Confirmation kit from Rosco Diagnostica.

Detection of KPC-Beta-Lactamases

Combined disk tests. KPC + MBL Confirm kit (98006) and KPC/MBL and OXA-48 Confirm kit (98015) for *Enterobacteriaceae*

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



K. pneumoniae PHA3 CL5761 KPC positive

Interpretation of 98006 and 98015 (*Enterobacteriaceae*)

A Meropenem + Boronic inhibition zone ≥ 4 mm then Meropenem, Meropenem+DPA and Meropenem+Cloxacillin indicate a presence of a KPC enzyme (or other class A).
Meropenem+Phenyl Boronic and Meropenem+Cloxacillin inhibition zones ≥ 5 mm, then Meropenem and Meropenem+DPA indicates AmpC hyperproduction + porin loss, or efflux (30).
Negative synergy for all tests and Temocillin 30 ug zone < 12 mm indicates the presence of OXA-48.

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

For non-fermenters use kit: **98025**: KPC/MBL in *Pseudomonas/Acinetobacter*, version 2.
Schwensen et al (34) found that the KPC/MBL Confirm kit (98006) could be used for detection of the cfiA metallo-beta-lactamase in *Bacteroides fragilis*. The authors conclude that Meropenem +DPA (included in the 98006 kit) should be preferred for phenotypic detection of MBLs in *Bacteroides fragilis*, and not Imipenem + DPA, included in the E-test.

Hammerum et al (35) report for the first time in DK an outbreak of NDM-1 producing *Citrobacter freundii* and secondary in vivo spread to of an IncA/C2 plasmid with NDM-1 gene to other *Enterobacteriaceae*. NDM-1 was detected using the KPC/MBL and OXA-48 Confirm kit (98015).

Karatuna et al (36) evaluated the Rosco KPC/MBL and OXA-48 kit (98015) and the corresponding Mast kit D70C. The Rosco kit detected correctly 100 % of the beta-lactamases, including strains coproducing OXA-48 and MBL and the Mast kit detected 75 %

Thoms-Rodriguez et al (37) using the Rosco KPC/MBL Confirm kit (98006) identified, in Jamaica, the NDM-1 gene in *Klebsiella pneumoniae*, from a patient coming from India.

Reduced susceptibility to ertapenem, synergy between Phenyl 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.

Al-Bayssari et al (70) describe the methods for detection of expanded-spectrum beta-lactamases in gram negatives in the 21st century. The authors describe the KPC/MBL and OXA-48 kit (98015) from Rosco, including an algorithm proposed by Miriagou for detecting the mentioned enzymes including the detection of co-produced MBL + KPC in the same strain.

Pantel et al (71) evaluated the performance of 2 rapid phenotypic tests for the detection of carbapenemases in *Enterobacteriaceae*: The Rapid CARB Screen with a sensitivity of 97.6% and specificity of 94.4 and the KPC/MBL and OXA-48 (98015) with a sensitivity of 98.8% and a specificity of 93.1%. The authors conclude that the 98015 kit provides a reliable phenotypic detection and results are obtained after 18 hours. Its interpretation is easy and the coexpression of several carbapenemases can be detected.

Willey et al (72) compare Temocillin Neo-Sensitabs (included in the kit 98015) against Temocillin paper discs from MAST as a marker for Class D and B carbapenemase producing *Enterobacteriaceae*. The authors mention that detecting temocillin resistance (TemR) enhance recognition of the hard to detect OXA-48-like or VIM-type carbapenemases.
Only the Temocillin Neo-Sensitabs showed high specificity (100%) while MAST specificity varied by lot of paper discs (71-82%) for excluding carbapenemase-negative isolates. The lower specificity may be explained by a deterioration of the unstable paper discs.

Dortet et al (75) report the evaluation of an algorithm recommended by the CA-SFM (French Society of Microbiology) to screen for carbapenemases in *Enterobacteriaceae*.
Enterobacteriaceae showing the following inhibition zones should be reported as **no-carbapenemase producers.** The algorithm is the following:

	Ticarcillin + Clav	Temocillin 30 ug	Imipenem 10 ug
Inhibition zones	>= 15 mm	>= 15 mm	>= 22 mm

Procedure for Oxacillinase detection (Class D enzymes) in *Enterobacteriaceae*

Strains of *Enterobacteriaceae* producing oxacillinases (OXA-48 or similar) will currently show zones of inhibition < 22 mm with Ertapenem and/or <27 mm with Meropenem Neo-Sensitabs. Most are resistant to Aztreonam.

Interpretation for *Enterobacteriaceae* (**OXA-48 and similar**)

The following results will presumably indicate the presence of oxacillinases in *Enterobacteriaceae*:

a) Negative metallo-beta-lactamase tests.
b) Negative synergy test between Phenyl 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) Resistant to Temocillin 30 ug Neo-Sensitabs (zone < 12 mm) and resistant to Piperacillin + Tazobactam 100+10 ug (zone < 16 mm) or Piperacillin + Tazobactam 30 + 6 ug (Zone < 13 mm) (13).

OXA-48. Oxacillinases (carbapenemases) in *Enterobacteriaceae*. KPC/MBL and OXA-48 Confirm kit (98015)

OXA-48 was discovered in a clinical *K. pneumoniae* isolate from Istanbul in 2001. This OXA-variant was plasmid encoded and had less than 50% aminoacid identity to the other OXA-members.
The first outbreak of carbapenem-resistant *K. pneumonia* isolates from Istanbul, producing OXA-48 was reported by Carrer in 2008. They co-produced various beta-lactamases, particularly ESBLs. The identification of *K. pneumoniae* isolates harboring the worldwide spread CTX-M15 together with the OXA-48 carbapenemase is worrying.
Isolates of *E. coli* and *K. pneumoniae* possessing the OXA-48 carbapenemase are currently resistant to penicillins (including temocillin) and combinations with beta-lactamase inhibitors (including Piperacillin + tazobactam), show reduced susceptibility to carbapenems (particularly ertapenem) and show susceptibility to 3rd gen cephalosporins and aztreonam.
The co-production of an ESBL mask the zone of inhibition around 3rd gen cephalosporins and aztreonam discs, and consequently they may appear as they are resistant to all beta-lactams.

Cubero et al (21) report an infection in a tertiary hospital of *K. pneumoniae* co-producing OXA-48 and CTX-M-15 beta-lactamases. They remark the importance of using Temocillin 30 ug for detecting OXA-48 producing isolates.

An OXA-48 (or similar) producing E. coli or K. pneumoniae may be suspected when:
Zones of inhibition around ertapenem are reduced, while the isolate is susceptible to 3rd generation cephalosporins (zones around ceftazidime and cefotaxime larger than around imipenem/ertapenem) and the isolate is resistant to Temocillin 30 ug Neo-Sensitabs (zone < 12 mm) and resistant to Piperacillin + Tazobactam 100+10 ug (zone < 16 mm), or Piperacillin + Tazobactam 30 + 6 ug: Zone < 13 mm (13).
When the isolate shows multiple resistance to all beta-lactams, one should perform an ESBL test. If the ESBL test is positive, one should compare the zones around the Cefotaxime + Clavulanate against the zone around Imipenem 10 ug. If the zone around the clavulanate combinations is >= 5 mm larger than around

Cefotaxime 30 ug and Imipenem 10 ug, the isolate produce an ESBL and most probably also OXA-48 (if temocillin resistant).

If the ESBL test is negative in the multiple resistant isolate, search for other mechanisms of resistance such as KPC or MBLs.
Diverse OXA-48 like beta lactamases (**11**) have been identified worldwide (OXA-162, OXA-181, OXA-162, OXA-204, OXA-244-OXA-245, OXA-232). OXA-162 and OXA-204 share the same hydrolytic properties as OXA-48.
OXA-181 possesses a higher ability to hydrolyze carbapenems, while OXA-232 hydrolyzes carbapenems less efficiently.
OXA-163 hydrolyzes broad-spectrum cephalosporins and do not possess significant carbapenemase activity. The same is valid for OXA-405.They will show negative results with the carbapenemase colorimetric kits.

Decousser et al (12) report the failure of the Xpert Carba-R assay to detect carbapenem-resistant *E. coli* producing OXA-48-like beta -lactamases. The authors conclude that molecular diagnostic techniques in the current phase of their development should not be considered as Reference Standards for the detection of carbapenemase resistant *Enterobacteriace*ae carriers.

Barragan et al (14) found found in their hospital *K. pneumoniae* producing OXA 48 and CTX-M15 and being resistant to colistin.

Lopez-Urrutia et al (15) detected carbapenemases using the KPC/MBL and OXA48 Confirm kit from Rosco and conclude that it is necessary to use a phenotypic method to detect the different types of carbapenemases.

Ortega et al (16) looking at the results from the Vigilance Program from the Microbiology National Center, conclude that particularly OXA-48 in *E. coli* is increasing in Spain. The use of the Carba NP test showed negative results in 9.3 % of OXA-48 isolates.

Rosario-Quintana C et al (17) found that the OXA-48 has a great capacity of intrahospital dissemination. ESBLs were detected with the Rosco ESBL kit and the carbapenemases including OXA-48 using the KPC/MBL and OXA-48 kit from Rosco.

Branas P et al (19) report a study of the epidemiology of carbapenemase-producing *K. pneumoniae* in a tertiary care facility in Madrid from 2009-2014. Of 97 isolates, 59 harboured OXA-48, 37 harbouring VIM-1 and 1 isolate with KPC-2. There is an increasing trend in carbapenemase-producing isolates and the study highlights the establishment of OXA-48 and CTX-M-15 genes coproducing ST11 clone.

Dortet et al (20) conclude that OXA-48 type beta-lactamases are more diverse than expected. They are not all true carbapenemases as exemplified with OXA-405 and OXA-163 that are ESBLs, but belong to the OXA-48 type.
Temocillin-resistance is a good criterion for differenting OXA-48-type producers with carbapenemase activity (Temocillin resistant) from OXA-48-type without carbapenemase activity (OXA-163 and OXA-405) that are Temocillin susceptible.

Therefore, the first line screening of carbapenemase-producers in *Enterobacteriaceae* must be based on the biochemical detection of carbapenemase activity in clinical settings. The molecular techniques may overreport OXA-48-like producers as being all carbapenemases and conversely, may fail to detect carbapenemase producers related to totally novel or slightly structurally modified carbapenemase genes.

Summary detection of carbapenemases in Enterobacteriaceae: Kit 98015

Meropenem	MRP+DPA	MRP+BOR	MRP+Cloxa	Temocillin
Metallo-β-lactamases	Synergy	No synergy	No synergy	R
KPC	No synergy	Synergy	No synergy	S/V
AmpC impermeability	No synergy	Synergy	Synergy	S
Oxacillinases (OXA-48)	No synergy	No synergy	No synergy	R
OXA-48 + KPC	No synergy	Synergy	No synergy	R

KPC/MBL in *P. aeruginosa/Acinetobacter*, version 2 (kit 98025)

This kit is for the detection of KPC/MBL in non-fermenters. Not to be used for *Enterobacteriaceae*.
It is composed of Imipenem and its combinations with Phenylboronic acid, Cloxacillin High, Dipicolinic acid and EDTA.
Heinrichs et al (78) showed that **Imipenem + DPA** (but not Meropenem +DPA) for detecting MBLs in *P. aeruginosa*, showed a sensitivity of 99 % and a specificity of 95 %. Meropenem + DPA is useful for detecting MBLs in *Enterobacteriaceae*.
Hammerum et al (66) describe a possible outbreak of carbapenem-resistant *Acinetobacter baumannii* in Denmark. Five ST2 isolates producing OXA-23, two ST1 isolates producing OXA-72 and one ST158 producing OXA-23. There was a spread between 2 patients hospitalized in the same ward and between 2 patients living in the same nursing home.

Summary detection of carbapenemases in P. aeruginosa/Acinetobacter: Kit 98025

Imipenem	IMPBO	IMCX4	IMPDP	IMIED	
No Carbapenemases	No syn.	≥ 5 mm	No syn.	No syn.	*P. aeruginosa*
KPC	≥ 4 mm **and**	< 3 mm	No syn.	No syn.	*P. aeruginosa*
MBL	No syn.	No syn.	≥ 5 mm **and/or**	≥ 8 mm	*P. aer./Acinetob.*
Oxacillinases	No syn.	No syn.	No syn. **and**	4-7 mm	*Acinetobacter.*

Carbapenemase or not in *Pseudomonas aeruginosa* (Kit 98030)

In order to improve the detection of carbapenemase-producing *Pseudomonas aeruginosa*, Fournier et al (41) evaluated the performance of a very simple, inexpensive detection method that Rosco Diagnostica has now commercialized.
It consists of an Imipenem 10 ug and a combination Imipenem + Cloxacillin high(IMCX4) tablets.
If added to the standard disc diffusion antibiogram, the kit 98030, may allow the detection of potentially epidemic carbapenemase producers with 100 % specificity and sensitivity, the same day the susceptibility data are obtained.
Crystalline imipenem in the tablets means increased stability and longer shelf-life, compared to all other methods. Kit 98030 has a shelf-life of 3 years, even from cartridges that have been opened many times.

References for OXA-48:

1) Yilmaz M et al: Warning! Oxacillinase-mediated carbapenem resistance spreading in *Enterobacteriaceae*. Presentation P-1517, ECCMID 2008
2) Gulmez D et al: Carbapenem-resistant *E. coli* and *K. pneumoniae* isolates from Turket with OXA-48 like carbapenemases and outer membrane protein loss. Int J Antimicr Agents **31**,523-526, 2008.
3) Carrer A et al: Spread of OXA-48 positive carbapenem-resistant *K. pneumoniae* isolates in Istanbul. Antimicr Ag Chemother **52**, 2950-2954, 2008.
4) Cuzon G et al: Plasmid-encoded carbapenem-hydrolyzing OXA-48 in an imipenem-susceptible *K. pneumoniae* strain from Belgium. Antimicr Ag Chemother **52**, 3463-3464, 2008.
5) Poirel L et al: Cross-border transmission of OXA-48 producing *Enterobacter cloacae* from Morocco to France. J Antimicr Chemother **66**, 1181-1182, 2011,
6) Potron A et al: European dissemination of a single OXA-49 producing *Klebsiella pneumoniae* clone. Clin Microbiol Infect, accepted 03-09-2011.
7) van Dijk Kv et al: A novel phenotypic detection strategy for class A, B and OXA-48 carbapenemases in *Enterobacteriaceae* using temocillin. Presentation at ECCMID 2013.
8) Woodford N et al: In vitro activity of temocillin against multidrug-resistant clinical isolates of *E. coli*, *Klebsiella spp* and *Enterobacter spp* and evaluation of high-level temocillin resistance as a diagnostic marker for OXA-48 carbapenemase. J Antimicr Chemother **69**, 564-567, 2014.
9) Te-Din-Huang et al: Temocillin and Piperacillin/tazobactam resistance by disc diffusion as antimicrobial surrogate markers for the detection of carbapenemase-producing *Enterobacteriaceae* in geographical areas with high-prevalence of OXA-48 producers. J Antimicr Chemother **69**, 445-450, 2014.
10) Fernandez J et al: Cluster of *E. coli* isolates producing a plasmid-mediated OXA-48 beta-lactamase in a Spanish Hospital. J Clin Microbiol **52**, 3414-3417, 2014.
11) Oueslati S et al: Heterogeneous hydrolytic features for OXA-48-like beta-lactamases. J Antimicr Chemother **70**, 1059-1063, 2015.
12) Decousser JW et al: Failure to detect carbapenem-resistant *E. coli* producing OXA-48-like using Xpert Carba-R assay. Clin Microbiol Infect **21,** e9-e10, 2015.
13) Edquist P et al: Usefulness of amoxycillin-clavulanic acid and piperacillin-tazobactam as phenotypic markers of *Enterobacteriaceae* producing OXA-48 like carbapenemases. ECCMID 2015.Presentation P0155.
14) Barragan H et al: Rapid hospital appearance of *K. pneumoniae* producing OXA-48 and CTX-M-15, resistant to colistin, with an unusual way of epidemiological dispersion. SEIMC 2015, Presentation 228. (spanish)
15) Lopez-Urrutia et al: Measures adopted at the Microbiology Laboratory in the presence of a cluster of *K. pneumoniae* OXA-48 like in a terciary hospital. SEIMC 2015, presentation 232. (spanish)
16) Ortega A et al: *E. coli* producing carbapenemases: Results from Program of Vigilance from the Microbiology National Center. SEIMC 2015, Presentation 235. (spanish)
17) Rosario-Quintana C et al: Description of the first isolates of *Enterobacteriaceae* producing OXA-48 carbapenemase in the South of Gran Canaria. SEIMC 2015, presentation 633 (Spanish).
18) Demir Y et al: Investigation of VIM, IMP, NDM-1, KPC and OXA-48 enzymes in *Enterobacteriaceae* strains. Pak J Pharm Sci **28**, (Suppl 3) 1127-1133, 2015.
19) Branas P et al: Molecular epidemiology of carbapenemase-producing *K. pneumoniae* in a hospital in Madrid. Successful establishment of an OXA-48 ST11 clone. Int J Antimicr Agents **46**, 111-116, 2015.

20) Dortet L et al: Genetic and biochemical characterization of OXA-405, an OXA-48-type ESBL without significant carbapenemase activity. Antimicr Ag Chemother **59**, 3823-3829, 2015.
21) Cubero M et al: Carbapenem-resistant and carbapenem-susceptible isogenic isolates of *K. pneumoniae* ST101 causing infection in a tertiary hospital. BCM Microbiology**15**, 177, 2015.
22) Albiger B et al: Carbapenemase-producing *Enterobacteriaceae* in EUROPE: assessment by National Experts from 38 countries, May 2015. Eurosurveillance, **20**, 12 november 2015.

RAPID Detection of CARBAPENEMASE activity

Rapid identification of carbapenemase producers in gram-negatives is critical to limit their spread.
The optimal phenotypic methods are based on the hydrolysis of imipenem by bacterial colonies, which is detected by changes in pH value by indicators (phenol red or bromothymol blue).
These methods are described below.

Mirande et al (32) conclude that the detection of enzymatic carbapenem degradation by Maldi-tof MS lacks well-standardized protocols. KPC degrades antibiotics ver quickly, while it takes 90 min for other enzymes. This enzymatic constraint limits the implementation of a standard protocol in routine microbiology laboratories.

Lutgring et al (36) report that reliable detection of carbapenemases in *Enterobacteriaceae* is the first step in comatting the emergence and spread of carbapenemase producers. The authors mention the Rosco Rapid CARB Screen and others as representatives of tests that can be used.

Pires et al (37) mention the causes of error that have been identificated in rapid colorimetric tests: the lack of standardization of inoculum and improper storage of test reagents (especially imipenem). The last problem does not exist when using the very stable Rosco Rapid colorimetric kits.

Rapid colorimetric (15 min-60 min) kits for detection of Carbapenemases.

No Lysis Buffer needed!!!

Neo-Rapid CARB (98024). (*Enterobacteriaceae, Pseudomonas, Acinetobacter*)

Add one 10 ul loop of the strain to be tested into 200 ul of 0.9 % NaCl, adjusted with 0.01N NaOH to pH 8.5. (8.3-8.7).
Dilute 1.07 g Triton X-100 in 100 ml water and add 20 ul of this solution to the bacterial suspension.
Vortex for 1 minute and maintain at room temperature for 30 min.
Add 1 Imipenem (x2)+ Indicator Diatabs and close the tube.Vortex for 1-2 seconds to disintegrate the tablet.
Incubate the test at 35-37 degrees Celsius for 15 min, 30 min and 1 hour respectively.
The same process is repeated using the CARB negative Control Diatab.

An alternative (to avoid the pH regulation): Add One 10 ul loop of bacteria to a mixture of 150 ul of 0.9% NaCl sol + 50 ul TRIS-HCL lysis buffer and proceed as above (no pH regulation necessary).

Interpretation

A change of color from red to yellow (or orange-yellowish or light yellow) indicates a positive reaction, when the negative control remains red: carbapenemase positive.
If the reaction is positive after 15 min or 30 min, it is not necessary to incubate further. Longer incubation may result in false reactions.

Pasteran et al (35) found that the use of Triton X-100 at 0.1% (instead of lysis buffer) gave an enhanced detection of carbapenemase producers. Thefore we recommend this improved procedure now. This procedure is also useful to detect oxacillinases in *Acinetobacter spp*, in which case the use of 2x 10 ul loop of bacteria is recommended. Qun Yan et al (38) confirm the results from Pasteran using 0.1% Triton X-100, instead of extraction buffer.

In the ECCMID 2015 in Copenhagen, they were several presentations concerning the use of the Rapid CARB Screen kit.

Haldorson et al (16) compared the Carba NP and the Rosco Rapid CARB Screen using a collection of 99 *Enterobacteriaceae* and 39 *P. aeruginosa* isolates identified by PCR. Both tests showed a sensitivity of 96 % and a specificity of 100%.

Willey et al (18) compared the Rapidec Carba-NP, with Rosco Rapid CARB Screen, a modified Rosco CARB Screen and the Rosco CARBA Blue against 206 isolates that previously have shown to be difficult to detect. The Rapidec Carba detected only 23% of the OXA enzymes and was associated with 12.9% false positive. Most OXA-enzymes were detected with the 3 Rosco kits. The modified CARB Screen did not contain lysis buffer and performed well having the smallest amount of false positives.

Hombach et al (33) evaluated the Rapidec CARBA NP test (Bio-Merieux) and arrived at the following conclusions: when reading after 30 min incubation sensitivity was 49%. The test should strictly be read after 120 min incubation and the inoculum should be higher than recommended by the manufacturer.

Boran et al (22) tested 133 isolates the Rapid CARB Screen and found 98.7% sensitivity and 87.7% specificity and recommend the use of the Rapid CARB Screen in a carbapenemase detection algorithm for use in the routine laboratory.

De Sloovere et al (21) found that the combined positivity/negativity of both the Rapid CARB Screen and the Hodge test had a positive predictive value of 100% and a negative predictive value of 92%.

Hernandez-Cabeza et al (19) evaluated the Rosco Rapid CARB Screen kit for detecting carbapenemases in *P. aeruginosa*. The kit had a good performance, although they were some orange-yellow results that should be interpreted as positive. With the use of the new Neo-Rapid CARB Screen kit, the problem with orange color will be much less.

Morton et al (17) found that when combining the results of the Rosco Rapid CARB Screen kit and the modified Hodge test will detect the organisms with possible carbapenemases.

Lopez-Quintana B et al (25) used the Rosco Rapid CARB Screen to detect carbapenemases directly from blood cultures. They conclude that the Rosco kit is a rapid and reliable for the detection of gram-negative bacilli directly from positive hemocultures, with a sensitivity of 92% and specificity of 100%. It detected 24 out of 25 OXA-48 from *E. coli, K. pneumoniae, E. aerogenes and S. marcescens*.

Pitout (27) indicates the the ideal phenotypic confirmatory test should be able to **rapidly** detect different carbapenemases in *Enterobacteriaceae*, *Pseudomonas spp* and *Acinetobacter spp*. A commercial version of Carba NP test is available from Rosco Diagnostica (Neo-Rapid CARB 98024) and can be combined with temocillin resistance to ensure that all isolates with OXA-48 enzymes are detected.

Abdel Ghani S et al (29) tested 87 Class isolates, 40 Class B, 12 Class D (OXA-48 and OXA-181) and 50 non-carbapenemase producers comparing the Rosco Neo-Rapid CARB kit (98024) with the Carba NP and a modified Carba NP. The Rosco kit showed a **sensitivity of 99% and a specificity of 100%**. The authors conclude that the Rosco kit is the most convenient test to perform and its greater reagent stability is an advantage compared to Carba NP and modified Carba NP.

Abdel Ghani et al (31) recommend that when results are difficult to interpret with kit 98024 do as follows: 1) holding the tube vertical inspecting the bottom of the tablet for yellow color (positive) and 2) Compare test and Control tubes titled gently to horizontal and examine in bright light on a white background. If the result is unclear, repeat the test using a higher inoculum.

Bou Casals (34) critizises the comparative study of Dortet et al. on rapid colorimetric tests. Dortet et al have a patent for NPCarba transferred to Bio-Merieux. Dortet et al have used an obsolete kit (98021) in their comparative study, while kit 98024: Neo-Rapid CARB kit has substituted kit 98021 more than 8 months ago. Bou Casals reports that kit 98024 contains twice as much imipenem as 98021 and uses much less lysis buffer than its predecessor and the kit has a shelf life of 3 years.

Paulussen (39) in a comparative study of Neo-Rapid CARB kit against Rapidec Carba NP and others found a sensitivity of 98 % and a specificity of 100 % with the Neo-Rapid CARB kit, while the Rapidec showed a specificity of 84.2 %.

Fernandez et al (40) took 8 drops (- 200 ul) of the positive blood culture and inoculated 2 Mueller-Hinton plates and incubated **4 hours at 37 degrees** in a CO2 atmosphere. The Carba NP test was performed on all Enterobacteriaceae, using as much inoculum as possible from the 2 MH plates. This rapid procedure could be used with kit 98024 as well.

Rapid CARB Blue (98023) (*Enterobacteriaceae, Pseudomonas; Acinetobacter*)

No lysis buffer is needed for the tests
Add 1 x 10 ul loop of the strain to be tested to 200 ul of 0.9% NaCl sol adjusted at pH 8.5 (8.3-8.7) with 0.01 NaOH.In case of Acinetobacter use 2x10 ul of bacteria
Vortex the suspension for 1 minute and maintain at room temperature for 30 min.
Add 1 Imipenem (x2)+ Brthymol Blue Diatabs and close the tube. Vortex for 1-2 seconds to disintegrate the tablet.
Incubate the test tube at 35-37 degrees for 15 min, 30 min and 1 hour respectively.
The same process is repeated with using the CARB Blue Negative Control Diatab.

Interpretation

A change of color in the test tube, from blue to yellow indicates a positive reaction (carbapenemase present).
If the test is green/yellowish and the negative control is blue, means Carbapenemase positive.
If the test is yellow and the negative control is green, the result is carbapenemase positive.

At the ECCMID 2015 there were several presentations about the use of the Rapid CARB Blue kit.

Novais et al (12) compared the Rapid CARB Blue kit with the in-house Blue Carba test (developed by Peixe and Novais). The authors report very good sensitivity 94.5% and specificity (91.7%) in the detection of carbapenemase producers by the Rapid CARB Blue kit in a representative collection of carbapenemase and non-carbapenemase producing gram-negative isolates.

Novais et al (29) in a new evaluation of the recently launched Rapid CARB Blue kit, report a high sensitivity (93.3%) and specificity (100%).

Pasteran et al (13) tested both the Rapid CARB Blue and the in-house Blue Carba test. Pasteran concludes that both products had equivalent performances for carbapenemase detection. For labs concerned with the widely disseminated KPC and NDM producers, the Rapid CARB Blue kit could be an accurate and cost-effective method to rapidly identify these carbapenemases.
Pasteran (22) studied the possibility of detecting carbapenemase from blood cultures using the Blue Carba test. He concludes that the Blue Carba test could be a rapid and cost-effective for detecting KPC and NDM producers directly from blood cultures.

Pires et al (23) compared Carba NP and Blue Carba in the detection of carbapenemases in *Enterobacteriaceae*. Both methods showed high sensitivity and specificity, but particularly the OXA-48 producers yielded stronger results with Blue Carba. The authors conclude also that the use of more experienced operators yield fewer mistakes (human errors cannot be excluded).

Pasteran et al (27) conclude that the Blue-Carba Test could be an accurate and cost-effective way to rapidly identify KPC, MBL producers and Class D OXA carbapenemase-producing *Acinetobacter*. For some mechanisms, such as OXA-48-like producing *Enterobacteriaceae*, a negative result could require additional tests such as the Modified Hodge Test in combination with Temocillin or Piperacillin+Tazobactam susceptibility results.

Nastro et al (38) describes a variation of the method, which allows the detection of carbapenemases (*Enterobacteriaceae, P.aeruginosa, Acinetobacter*) **after 4 hours incubation,** from a haze of bacterial growth obtained from a positive blood culture, with a sensitivity of 98.1 % and a specificity of 100 %.

Paulussen (39) in a comparative study of Rapid CARB Blue against Rapidec Carba NP found a sensitivity of 97 % and a specificity of 100 % with the Rapid CARB Blue kit, while the Rapidec showed a specificity of 84.2 %

KITS References

1. Giske CG et al: A sensitive and specific phenotypic assay for detection of MBL and KPC in *K. pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin Microbiol Infect 2010.
2. Grundmann H et al: Carbapenem non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Eurosurveillance 18 nov 2010.
3. Cohen Stuart J et al: Guideline for phenotypic screening of carbapenemases in *Enterobacteriaceae*. Int j Antimicr Agents **36**, 205-210, 2010.
4. Cohen-Stuart J et al: Screening and confirmation methods for carbapenemases in *Enterobacteriaceae*. Presented at the ECCMID 2011.
5. Bou Casals J et al: Phenotypic method for the detection of MBL and KPC carbapenemases in the same isolate of *Enterobacteriaceae*. Poster P-697, ECCMID 2011.
6. Hartl R et al: Carbapenemases in *Enterobacteriaceae* - Report from the Austrian National Reference Center.Presented at the 21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2011).
7. Dongeun Yong et al: Evaluation of double-disk potentiation and disk potentiation tests using Dipicolinic acid for detection of MBL-producing *Pseudomonas spp* and *Acinetobacter spp*. J Clin Microbiol **50**, 3227-3232, 2012.
8. Hansen F et al: Evaluation of Rosco Neo-Sensitabs for phenotypic detection and subgrouping of ESBL, AmpC and carbapenemase-producing *Enterobacteriaceae*. APMIS**120**, 724-732, 2012.
9. Evaluation of the modified KPC + MBL Confirm kit for the phenotypic detection of class A and B carbapenemases in *K. pneumonia* isolates. Presented at the ECCMID 2012.
10. Bou Casals J: Stable Meropenem/Inhibitor combinations for phenotypic detection of KPC, MBL and KPC+MBL in *Klebsiella pneumoniae*. Presentation D-674, ECCMID 2012.
11. Hartl R et al: Temocillin and Meropenem to discriminate resistance mechanisms leading to decreased carbapenem susceptibility with focus on OXA-48 in *Enterobacteriaceae*. Clin Microbiol Infect 2013 (accepted publ.)
12. Miriagou V et al: Combined disc methods for the detection of KPC and/or VIM-positive *Klebsiella pneumonia*: improving reliability for the double carbapenemase producers. Clin Microbiol Infect **19**, E412-E414, 2013.
13. Van Dijk K et al: A novel phenotypic detection strategy for class A, B and OXA-48 carbapenemases in *Enterobacteriaceae* using Temocillin. ECCMID presentation 2013.
14. Van Dijk K et al: A disc diffusion assay for detection of class A, B and OXA-48 carbapenemasess in *Enterobacteriaceae* using phenylboronic acid, dipicolinic acid and temocillin. Clin Microbiol Infect **20** ,345-349, 2014
15. EUCAST: EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Version 1.0, December 2013.
16. Ambrettii S et al: Evaluation of phenotypic and genotypic approaches for the detection of Class A and Class B carbapenemases in *Enterobacteriaceae*. Microbial Drug Resistance **19**, 212-215, 2013.
17. Kerschner H et al: Carbapenemases in *Enterobacteriaceae* in Austria 2010-2012. Presentation P 1243, ECCMID 2013.

18. Vanegas JM et al: Similar frequency of *Pseudomonas aeruginosa* producing KPC and VIM carbapenemases in tertiary care hospitals in Medellin, Colombia. Presentation C-083, ICAAC 2014.
19. Bartolini A et al: Comparison of phenotypic methods for the detection of carbapenem-non-susceptible *Enterobacteriaceae*. Gut Pathogens **6,** 1-7, 2014.
20. Woodford N: Detecting carbapenemase-producing *Enterobacteriaceae*. Presentation at the BSMT, May 16th, 2014.
21. Morosini MI: Microbiological diagnostic of microorganisms producing carbapenemases (Spanish), presented on June 5th 2014.
22. Hrabak J et al: Detection of carbapenemases in *Enterobacteriaceae*: a challenge for diagnostic microbiological laboratories. (Review). Clin Microbiol Infect **20**, 839-853, 2014.
23. Kemble S et al: Validation of Rosco Diagnostica diffusion discs for identification of carbapenem resistance mechanisms in a Clinical Laboratory. Presentation 1394, IDWeek. Philadelphia, USA, 2014.
24. Fournier D et al: Dissemination of GES-1 and GES-5-producing strains of *P. aeruginosa* in France. Presentation P1121, ECCMID 2014.
25. A) Poirel L et al: Rapid tests for detection of carbapenemase producers in *P. aeruginosa*: what do we really need? Enferm Infecc Microbiol Clin **32**, 623-624, 2014
26. B) Hartl R et al: Carbapenemase-producing *Enterobacteriaceae* in Austria 2010-2013. Presentation eP 592, ECCMID 2014.
27. Vanstone G et al: Improving screening for carbapenemase-producing organisms: evaluation of 2 chromogenic media and confirmatory disc diffusion assay. Presentation eP316, ECCMID 2014.
28. Foschi C. et al: Use of temocillin for identification of class D carbapenemases. Presentation eP325, ECCMID 2014.
29. Accogli M et al: Emergence of *E. coli* ST131 subclone H30 producing VIM-1 and KPC3 carbapenemases, Italy. J Antimicr Chemother. May 2014.
30. Pantel A et al: Evaluation de deux utils de detection rapide des Enterobacteries productrices de carbapenemases. Presentation 563, RICAI 2014. (J Clin Microbiol **53**,3359-3362, 2015).
31. Heinrichs A et al: Evaluation of several phenotypic methods for the detection of carbapenemase-producing *Pseudomonas aeruginosa*. Eur J Clin Microbiol Infect Dis, april 21, 2015
32. Moraitu H et al: Direct test for rapid identification of carbapenemase-producing bloodstream isolates. ECCMID 2015, Presentation P0154.
33. Matros L et al: Phenotypic detection of *P. aeruginosa* producing carbapenemases. ECCMID 2015, Presentation EV0584.
34. Schwensen SA et al: Phenotypic detection of the cfiA metallo-beta-lactamase in *Bacteroides fragilis* with the meropenem-EDTA double-ended Etest and the Rosco KPC/MBL Confirm kit. J. Antimcr Chemother **72**,437-440, 2017.
35. Hammerum AM et al: Use of WGS data for investigation of a long term NDM-1 producing *C. freundi*i outbreak and secondary in vivo spread of NDM-1 gene to *E. coli*, *K. pneumoniae* and *K. oxytoca*. J Antimicr Chemother **71**,3117-3124, 2016.
36. Karatuna O et al: Evaluation of the performance of Mast and Rosco phenotypic carbapenemase detection kits for *E. coli* and *K pneuminae* isolates carrying carbapenemase genes. ECCMID 2016, presentation EV0433.
37. Thoms-Rodriguez CA et al: New Delhi metallo-beta-lactamase in Jamaica.J of Infect in Develop Countries **10,** 183-187, 2016.

References. Rapid colorimetric kits for detection of carbapenemases

1) Te-Din Huang et al: Comparative evaluation of Carba NP and ROSCO Rapid CARB Screen kit for the detection of carbapenemase-producing *Enterobacteriaceae*. Poster 427, 2013.
2) Pires J et al: Blue-Carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. J Clin Microbiol **51**, 4281-4283, 2013.
3) Bharatan B et al: Evaluation of the Rapid CARB Screen Diatabs test for detection of carbapenemase production in gram-negative bacteria. Presentation eP335, ECCMID 2014
4) Hopkins KL et al: Evaluation of Rosco Diagnostica Rapid Screen kit for detection of carbapenemase activity. PHE, London 2013.
5) Williams A et al: Direct detection of carbapenemase-producing Enterobacteriaceae and *Pseudomonas spp* from positive blood cultures. BSAC Spring Meeting 20/03/2014.
6) Te-Din Huang et al: Comparative evaluation of 2 Chromogenic tests for Rapid detection of carbapenemase in *Enterobacteriaceae* and in *P. aeruginosa* isolates. J Clin Microbiol **52**, 3060-3063, 2014
7) Simner PJ et al: Evaluation of 5 chromogenic agar media and the Rosco Rapid CARB Screen kit for the detection and confirmation of carbapenemase production in Gram-negative bacilli. J. Clin Microbiol 29 october 2014 (published ahead of print).
8) Degandt S et al: The Rapid CARB Screen as a standardized alternative for the Carba NP test in detecting carbapenemase-producing strains. Presentation eP339, ECCMID 2014.
9) Te-Din Daniel Huang et al: Comparative evaluation of Carba NP and the Rosco Rapid CARB Screen for the detection of carbapenemases in *P. aeruginosa*. Presentation eP331, ECCMID 2014.
10) Pires J et al: Further validation of Blue-CARBA, a recently described quick and reliable method for carbapenemase detection. Presentation eP327, ECCMID 2014.
11) Pantel A et al: Evaluation de deux outils de detection rapide des Enterobacteries productrices de carbapenemases. Presentation 563, RICAI 2014.
12) Novais A et al: Evaluation of the recently launched Rapid CARB Blue kit for detection of carbapenemase-producing Gram-negative bacteria. Presentation O042 at the ECCMID 2015, Copenhagen.
13) Pasteran F et al: Comparison of the in-house Blue Carba Test (BCT) with the Rapid CARB Blue kit for the detection of carbapenemase-producing gram-negative bacilli. ECCMID P0147, 25th ECCMID Copenhagen 2015.
14) Walkty A et al: Isolation of multiple carbapenemase-producing gram-negative bacilli from a patient recently hospitalized in Nigeria. Diagn Microbiol Infect Dis **81**, 296-298, 2015.
15) Haldorsen B et al: Comparison of the CARBA NP test and Rapid CARB Screen for the phenotypic detection of carbapenemases in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. ECCMID 2015, Presentation EV0553.
16) Morton H et al: Combined use of the Rosco Rapid CARB Screen and modified Hodge test to identify carbapenemase production in gram-negative bacteria with resistance to carbapenems. ECCMID 20115, Presentation EV0504.
17) Willey BM et al: Comparative evaluations of Rosco CARBA Blue, Rapid CARB Screen, and a modified Rapid CARB Screen protocol for phenotypic detection of carbapenemase-producing organisms. ECCMID 2015. Presentation P0156.

18) Hernandez-Cabezas A et al: Comparative evaluation of Rapid CARB Screen test and IP/IPI test to detect carbapenemase producing *Pseudomonas aeruginosa*. ECCMID 2015, Presentation P0145.
19) Horner C et al: Detecting carbapenemase producing *Enterobacteriaceae*: all media and methods are not created equal. ECCMID 2015, Presentation P0172
20) De Sloovere M et al: The challenge of carbapenemase-producing *Enterobacteriaceae*: the 87-strain-experience. ECCMID 2015, Presentation P0149.
21) Boran N et al: Formation of a carbapenemase resistance detection algorithm for use in the routine laboratory. Br J Biomedical Science **27**, 12-22, 2015.
22) Pasteran F et al: Rapid detection of carbapenemase-producing gram-negative bacilli from blood cultures using the Blue Carba test. ECCMID 2015, Presentation P0148.
23) Pires J et al: Detection of carbapenemase-producing *Enterobacteriaceae*: comparison between Carba NP abd Blue Carba tests. ECCMID 2015, Presentation P0144.
24) Lopez-Quintana et al: Evaluation of the Rosco: Rapid CARB Screen for the detection of gram-negative bacilli, carbapenemase-producers directly from positive hemocultures and other samples. SEIMC 2015, Presentation 047. (spanish)
25) Hoyos-Mallecot et al: Comparative evaluation of 3 biochemical methods for the rapid detection of Carbapenemases in *Enterobacteriaceae* and *P. aeruginosa*. SEIMC 2015, Presentation 610 (spanish)
26) Pitout JDD: *Enterobacteriaceae* with carbapenemases: implications for Clinical Diagnostic Laboratories. Canadian J Pathol **7**, 4-8, 2015.
27) Pasteran F et al: Evaluation of the Blue-Carba Test for rapid detection of carbapenemases in gram-negative bacilli. J Clin Microbiol **53,** 1996-1998, 2015.
28) AbdelGhani S et al: Rosco Neo-Rapid CARB kit, an accurate and convenient alternative to the Carba NP Test for carbapenemase detection. ASM 2015, presentation 547.
29) Novais A et al: Evaluation of the recently launched Rapid CARB Blue kit for detection of carbapenemase-producing gram-negative bacteria. J Clin Microbiol 53, 3105-3107, 2015.
30) AbdelGhani S et al: Comparison of the carba NP, modified Carba NP and Updated Rosco Neo-Rapid CARB kit tests for carbapenemase detection. J Clin Microbiol **53**, 3539-3542, 2015
31) Mirande C et al: Rapid detection of carbapenemase activity: benefits and weaknesses of Maldi-tof MS. Eur J Clin Microbiol Infect 34, 2225-2234, 2015.
32) Hombach M et al: Evaluation of the Rapidec CARBA NP test for the detection of carbapenemases in *Enterobacteriaceae*. J Clin Microbiol Sept 30, 2015 (ahead of print).
33) Bou Casals: Comment on “Evaluation of Rapidec Carba NP, the Rapid CARB Screen and the Carba NP test for biochemical detection of carbapenemase-producing *Enterobacteriaceae*. J Antimicr Chemother, October 22, 2015 (ahead of print)
34) Pasteran F et al: Simplified method for CarbaNP test enhanced detection of carbapenemase producers directly from bacterial cultures. J Clin Microbiol **53**, 3908-3911, 2015.
35) The problem of carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* detection. J Clin Microbiol **54**, 529-534, 2016.
36) Comparison of the in-house made Carba NP and Blue-Carba tests: considerations for better detection of carbapenemase-producing *Enterobacteriaceae*. J Microbiol Methods **122,** 33 – 37, 2016.
37) Qun Yan et al: Detection of carbapenemase-producing gram-negative bacteria using a simplified Carba NP test. J. Microbiological Methods **123**, 1 – 3, 2016.

38) Nastro M et al: Rapid Blue Carba Test: reduction of the detection time of carbapenemases performed from a 4-hour bacterial lawn. J Chemotherapy, June 27, 2016 (ahead of print).
39) Paulussen G: Rapid detection of carbapenemases in Gram-negative bacteria (Dutch). U.C. Leuven, Limburg (Holland) 2015-2016.
40) Fernandez J et al: J Microbiological Methods **129**, 133-135, 2016.
41) Fournier D et al: A convenient method to screen for carbapenemase-producing *P. aeruginosa*. J Clin Microbiol **51**, 3846-3848, 2013.

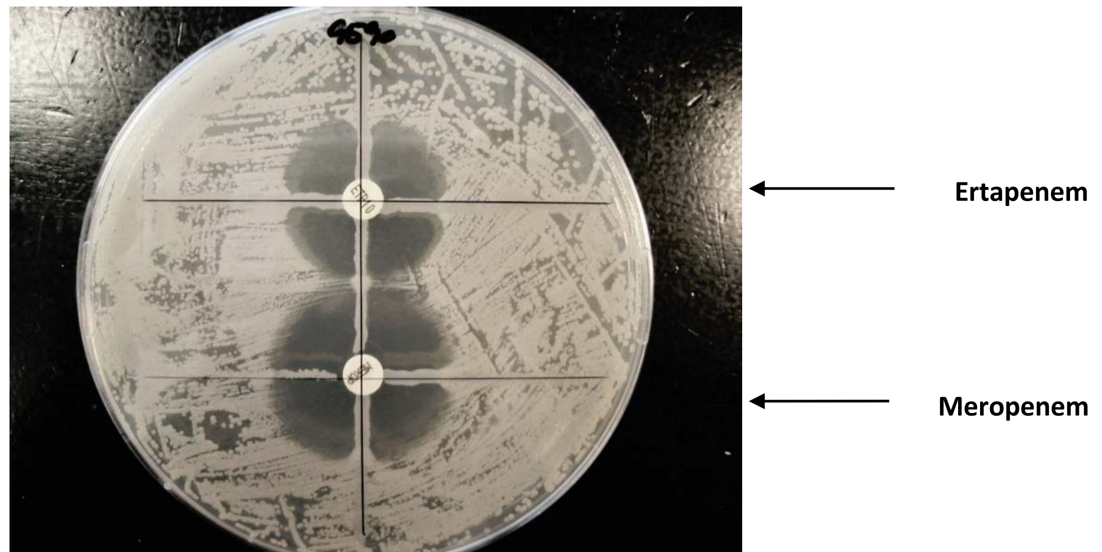
Modified Hodge Test

The 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. Instead of Mac Conkey, MH agar supplemented with 20 mg/ml of bile can be used.
Pasteran et al found, when testing against *P. aeruginosa*, that replacing the indicator strain with *K. pneumoniae* ATCC 700603 led to an improved performance with 100% sensitivity and 97% specificity.
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 indicative of the presence of a carbapenemase (figure).

Takayama et al (63) found that in order to avoid false positives, the addition of 200 ug/ml cloxacillin to the MHA improves the reliability of the method. In fact, 5 isolates of *Enterobacter cloacae* that showed false positive results with MHA alone were negative when using MHA+cloxacillin. Rosco Diagnostica has a Diagnostic Tablet: Imipenem + cloxacillin 4 mg (IMCX4) that can be used for the same purpose (kit 98030).

One can use current MHA and perform the modified Hodge Test **using the IMCX4 Diagnostic tablet**, instead of Imipenem, Ertapenem or Meropenem. Imipenem 10 ug can be used in parallel as a control. The indentation will be clear around the IMCX4 tablet for the organisms producing carbapenemase and false positives (high production of AmpC) will be avoided. Indicator strain is *E. coli* ATCC 25922.
The same procedure may be used for *P. aeruginosa*, where the strain recommended by Pasteran (*K. pneumoniae* ATCC 700603) is used as indicator strain.

Pasteran et al (77) found that the addition of Triton X-100 during the test improves the sensitivity of the test for the detection of NDM and other carbapenemases. The test is performed flooding the MH Agar plate with 50 ul of pure Triton-X-100 reagent (0.2 % in the MHA plate) and quickly distributed by streaking a swab over the entire plate. Meropenem is the best substrate, while the use of ertapenem may increase false positive results.



K.pneumoniae KPC positive

Limitations:

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

References General:

1) Pottumarthy S et al: NmcA carbapenem-hydrolyzing enzyme in *E. cloacae* in North America. Emerg Infect Dis **9**, 999-1002, 2003.
2) Yigit H et al: Novel carbapenem-hydrolyzing beta-lactamase KPC-1 from a carbapenem-resistant strain of *K. pneumoniae*. Antimicr. Ag. Chemother. **45**, 1151-61, 2001.
3) Smith-Moland E et al: Plasmid-mediated carbapenem-hydrolyzing beta-lactamase KPC-2 in *K. pneumoniae* isolates. J. Antimicr. Chemother. **51**, 711-14, 2003.
4) Aubron C et al: Carbapenemase-producing *Enterobacteriaceae*, U.S. rivers. Emerg.Infect.Dis. **11**, 260-4, 2005.
5) Bratu S. et al: Detection of KPC carbapenem-hydrolyzing enzymes in *Enterobacter spp* from Brooklyn, N.Y. Antimicr. Ag. Chemother. **49**, 776-8, 2005.
6) Lopez-Otsoa F et al: Endemic carbapenem resistance associated with OXA-40 carbapenemase among *A. baumanni* isolates from a hospital in Northern Spain. J. Clin. Microbiol **40**, 4741-3, 2002.
7) Bratu S. et al: Emergence of KPC-possessing *K. pneumoniae* in Brooklyn, N.Y.: epidemiology and recommendations for detection. Antimicr. Ag. Chemother., **49**, 3018-20, 2005.
8) Brown S., Amyes S.: OXA beta-lactamases in *Acinetobacter*: the story so far. JAC, **57**, 1-3, 2006.
9) Dong-Min K. et al: Treatment failure due to emergence of resistance to carbapenem during therapy for *Shewanella alga bacteremia.* J.C.M., **44**, 1172-4, 2006.
10) Schneider I. et al: Novel carbapenem-hydrolyzing oxacillinase OXA-62 from *Pandoraea pnomenusa*. Antimicr. Ag. Chemother., **50**, 1330-35, 2006.

11) Villegas M.V. et al: First identification of *P. aeruginosa* isolates producing a KPC-type carbapenem hydrolysing ß-bactamase. Antimicr. Ag. Chemother., **51**, 1553-55, 2007.
12) Tenover F.C. et al: Carbapenem resistance in *K. pneumoniae* not detected by automated susceptibility testing. Emerg. Inf. Dis., **12**, 1209-1213, 2006.
13) Sundin D.R.: Rapid screening for KPCs. Presentation D 1555, ICAAC September 2007, Chigago, USA.
14) Queenan A.M. et al: Carbapenemases: the versatile ß-lactamases. Clin. Microbiol. Reviews, **20**, 440-458, 2007.
15) Lee K. et al: Effect of Oxgall on the Imipenem disk Hodge test in screening MBL- producing gram-negative bacilli. ECCMID, poster P-891, Barcelona 2008.
16) Yilmaz M. et al: Warning! Oxallinase-mediated carbapenem-resistance spreading in *Enterobacteriaceae*. Poster P1517, ECCMID. Barcelona, April 2008.
17) Fernandez F. et al: Association between heteroresistance to carbapenems in *A. baumannii*, production of beta-lactamases (AmpC and OXA). XIII Congress SEIMC presentation 12, Madrid, May 2008.
18) Marchaim D. et al: Isolation of Imipenem-R *Enterobacter spp*: emergence of KPC-2 carbapenemase, molecular characterisation, epidemiology and outcomes. Antimicr. Ag. Chemother **52**, 1413-18, 2008.
19) Urban C. et al: Carbapenem-resistant *E.coli* harboring *K. pneumoniae* carbapenemase beta-lactamases associated with long-term care facilities. CID **46** e127-e130, 2008.
20) Pasteran F. et al: Detection of carbapenemases of group 2f. Promising additive uses of the Boronic acid disk test. Poster 371-20836 XI, Argentinian Congress of Microbiology 2007.
21) Kohlerschmidt D.J. et al: *Klebsiella pneumoniae* KPC detection may require screening multiple colonies for accurate carbapenem resistance assessment. ASM, presentation C-028, June 2008.
22) Pasteran F. G. et al: *Klebsiella pneumoniae* KPC-2, Buenos Aires, Argentina. Emerg. Infect. Dis. **14**, 1178-1180, 2008.
23) Ya-Gang Chen et al: In vivo development of carbapenem resistance in clinical isolates of *E. aerogenes*, producing multiple beta-lactamases. Int. J. Antimicr. Ag, **32**, 302-307, 2008.
24) Cuzon G. et al: Plasmid encoded carbapenem hydrolyzing β-lactamase OXA-48 in an Imipenem susceptible strain from Belgium. Antimicr. Ag. Chemother, **52**, 3463-4, 2008.
25) Castanheira M. et al: Emergence and clonal dissemination of OXA-24 and OXA-48 producing *A.baumannii* strains in Houston, Texas: report from the SENTRY-ASP. J. Clin. Microbiol. **46**, 3179-80, 2008.
26) Doi Y. et al: Simple disk based method for detection of *K.pneumoniae* carbapenemase by use of Boronic acid compound. J. Clin Microbiol. **46**, 4076-83. 2008.
27) Giske C. et al: Personal communication 2009
28) Tsakris A. et al: First occurrence of KPC-2 possessing *K. pneumoniae* in a Greek hospital and recommendation for detection with Boronic acid disk tests. J. Antimicr. Chemother 62, 1257-60, 2008.
29) Pournaras S. et al: Clonal spread of KPC-2 carbapenemase producing *K. pneumoniae* in Greece. JAC, **64**. 348-52, 2009.
30) Smith Moland E. et al: Concerns about KPC screening and confirmatory tests. Presentation D-729, 49th ICAAC 2009.
31) Endimiani A. et al: Evaluation of the 2009 CLSI criteria for determining carbapenem susceptibility of *K. pneumoniae*. Presentation D-722, 49th ICAAC, 2009.
32) Anderson K. et al: Characterization of *Enterobacteriaceae* with a false positive Modified Hodge Test. Presentation D-719, 49th ICAAC, 2009.
33) Landman D. et al: Contribution of OmpK 36 to carbapenem susceptibility in KPC-producing *K. pneumoniae*. J. Med. Microbiol. **58**, 1303-1308, 2009.

34) Kristo et al: Investigation of meropenem heteroresistance in KPC-2 producing *K. pneumoniae*. Presentation E-183. 49th ICAAC. 2009.
35) Tsakris A. et al: Evaluation of Boronic acid disk tests for differentiating KPC-possessing *K. pneumoniae* isolates in the clinical laboratory. J. Clin. Microbiol. **47**, 362-67, 2009.
36) Pasteran F. et al: Sensitive screening tests for suspected class A carbapenemase production in species of *Enterobacteriaceae*. J. Clin. Microbiol. **47**, 1631-39, 2009.
37) Weisenberg S.A. et al: Clinical outcomes of patients with *K. pneumoniae* KPC producing, after treatment with imipenem or meropenem. Diagn. Micr. Infect. Dis. **64**, 233-35, 2009.
38) Nicoletti A.G. et al: *K. pneumoniae* non-susceptible to carbapenems in Brazil. What are the mechanisms behind? Presentation C2-663, 49th ICAAC, 2009.
39) Castanheira M et al: First descriptions of KPC in *Raoultella spp* (*R. planticola* and *R. ornitholytica*): Report from the Sentry Antimicrobial Surveillance program. J. Clin. Microbiol. **47**, 4129-30, 2009.
40) Thomson KS et al: Thomson KS: ESBL, AmpC and Carbapenemase issues. J Clin Microbiol **48**, 1019-1025, 2010.
41) Miriagou V et al: Acquired carbapenemases in gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect **16,** 112-122, 2010.
42) Nicolau CJ et al: Carbapenemases in genus Pseudomonas. Enferm Infecc Microbiol Clin **28,** Suppl1, 19-28, 2010.
43) Lolans K et al: Direct Ertapenem disk screening method for identification of KPC-producing *K. pneumoniae* and *E. coli* in surveillance swab specimens. J Clin Microbiol **48,** 836-841, 2010.
44) Hammerum AM et al: Detection of the first 2 *K. pneumonia* isolates with sequence type 258 producing KPC-2 carbapenemase in Denmark. Int J Antimicr Agents **35**, 610-611, 2010.
45) Pournaras S et al: Inhibitor-based methods for detection of KPC carbapenemase producing *Enterobacteriaceae* in clinical practice by using boronic acid compounds. J Antimicr Chemother **65**, 1319-1321, 2010.
46) Pasteran F et al: Controlling false-positive results obtained with the Hodge and Masuda assays for detection of Class A carbapenemases in *Enterobacteriaceae* by incorporating boronic acid. J Clin Microbiol **48**, 1323-1332, 2010.
47) Carvalhaes CG et al: Clover-leaf (modified Hodge-test) for detecting carbapenemase production in *K pneumoniae*: be aware of false positive results. J Antimicr Chemother **65**,249-251, 2010.
48) Cohen Stuart J et al: Guideline for phenotypic screening and confirmation of carbapenemases in *Enterobacteriaceae*.Int J Antimicr Agents.**36**, 205-210, 2010.
49) Walsh T: Emerging carbapenemases: a global perspective. Int J Antimicr Agents **36**, Suppl 3, S8-S14, 2010.
50) Hocquet D et al: Natiowide investigation of ESBLs, MBLs and extended-spectrum oxacillinases produced by ceftazidime-resistant *P. aeruginosa* strains in France. Antimicr Ag Chemother **54,** 3512-3515, 2010.
51) Nordmann P et al: Identification and screening of carbapenemase-producing *Enterobacteriaceae*. Accepted paper Clinical Microbiology and Infection, feb 2012.
52) Girlich D et al: Strategy of detection of carbapenemase-producing *Enterobacteriaceae*. Presentation at ECCMID 2012.
53) Maurer FP et al: Evaluation of carbapenemase screening and confirmation tests in *Enterobacteriaceae* and development of a practical diagnostic algorithm. J Clin Microbiol, published ahead of print October 2014.
54) New PHE guidance: carbapenemase producers. AMRHAI News, Issue 04, Winter 2014.

55) Diene SM et al: Carbapenemase genes and genetic platforms in Gram-negative bacilli: *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species. Review. Clin Microbiol Infect 20, 831-838, 2014.
56) Hammoudi D et al: How to detect carbapenemase producers? A literature review of phenotypic and molecular methods. J Microbiol Methods **107**, 106-118, 2014.
57) Martinez-Martinez L et al: Carbapenemases in *Enterobacteriaceae*: types and molecular epidemiology. Enferm Infecc Microbiol Clin, **32** (suppl 4) 4-9, 2014.
58) Oteo J et al: Evolution of carbapenemase-producing *Enterobacteriaceae* at the global and national level: what should be expected in the future? Enferm Infecc Microbiol Clin **32** (suppl 4) 17-23, 2014.
59) Canton R et al: Breakpoints for carbapenemase-producing *Enterobacteriaceae*: is the problem solved? Enferm Infecc Microbiol Clin **32** (suppl 4) 33-40, 2014.
60) Poirel L et al: Rapid tests for detection of carbapenemase producers in *P. aeruginosa*: what do we really need? Enferm Infecc Microbiol Clin **32**, 623-624, 2014.
61) Oteo J et al: *Enterobacteriaceae* carbapenemase KPC producers: clonal dissemination of *K. pneumoniae* belonging to ST11 and ST101 producing KPC-2. SEIMC 2015, Presentation 083. (Spanish)
62) Prats I et al: Evaluation of phenotypic methods for detecting isolates producing carbapenemases. SEIMC 2015, Presentation 609. (spanish).
63) Takayama Y et al: Modified Hodge test, using MHA supplemented with cloxacillin improves screening for carbapenemase-producing clinical isolates of *Enterobacteriaceae*. J Med Microbiol May 2015.
64) Boyd D et al: Complete sequence of 4 multidrug-resistant MOB-Q1 plasmids harboring GES-5 isolated from *E. coli* and *Serratia marcescens* presisting in a Hospital in Canada. Microbial Drug Resistance **21**, 253-260, June 2015.
65) Lombardi F et al: Emergence of carbapenem-resistant *K. pneumoniae*: progressive spread and 4-year period of observation in a cardiac surgery division. BioMed Research Int, article ID871947, 2015.
66) Hammerum AM et al: Investigation of a possible outbreak of carbapenem-resistant *Acinetobacter baumannii* in Odense, DK using PFGE, MLST and whole genome-based SNPs. J Antimicrob Chemother **70**, 1965-1968, 2015.
67) Adler A et al: The effect of resistance mechanisms on the inoculum effect of carbapenems in carbapenem-resistant *K. pneumoniæ*. Antimicr Agents Chemother may 18, 2015 (Epub ahead of print).
68) Tomasso G et al: Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *K. pneumoniae* traced to clonal expansionof an mgrB deletion mutant. J Clin Microbiol **53**, 3341-3344, 2015.
69) Camargo JF et al: Successful treatment of carbapenemase-producing pandrug resistant *K. pneumoniae* bacteremia. Antimicr Ag Chemother **59**, 5903-5908, 2015.
70) Al-Bayssari CH et al: Detection of expanded-spectrum beta-lactamases in gram-negative bacteria in the 21st century. Exp Rev Anti Infect Ther **13**, 1139-1158, 2015.
71) Pantel A et al: Evaluation of 2 phenotypic screening tests for carbapenemase-producing *Enterobacteriaceae*. J Clin Microbiol **53**, 3359-3362, 2015.
72)Willey BM et al: Comparison of Rosco tablets and MAST disks for detecting Temocillin resistance as a marker for Class D and B carbapenemase-producing organisms. ICACC 2015, Presentatiom D-184.
73) Zhao F et al: Dissemination of extensively drug-resistant and KPC-2 producing *K. pneumoniae* isolated from bloodstream infections. J Inf Dis Dev Countries **9**, 1016-1021, 2015.
74) Anchordoqui MS et al: In vivo horizontal dissemination of the KPC-2 gene carried on diverse genetic platforms among clinical isolates of *Enterobacteriaceae*. J Global Antimicr Resistance **3**, 210-213, 2015.
75) Dortet L et al: Prospective evaluation of an algorithm for the phenotypic screening of carbapenemase-producing *Enterobacteriaceae*. J Antimic Chemother Oct 12, 2015.

76) Fattouh R et al: What is the appropriate meropenem MIC for screening of carbapenemase-producing *Enterobacteriaceae* in low-prevalence settings?. Antimicr Ag Chemother **60**, 1556-1559, 2016.
77) Pasteran F et al: Triton Hodge Test: improved protocol for modified Hodge Test for enhanced detection of NDM and other carbapenemase producers. J Clin Microbiol **54**, 640-649, 2016.
78) Heinrichs A et al: Evaluation of several phenotypic methods for the detection of carbapenemase-producing *Ps aeruginosa*. Eur J Clin Microbiol Infect Dis **34**, 1467-1474, 2015.

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 6 major types: IMP, VIM, SPM, GIM, SIM and NDM type enzymes. In Enterobacteriaceae: IMP, VIM, GIM and NDM enzymes have been found 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 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).

Schrøder Hansen et al (40) report for the first time an NDM-5 outbreak in Europe. None of the patients had been travelling recently. The isolates belonged to a single clone.

Ruiz et al (41) describes the intrahospitalary dissemination of *K. pneumoniae* ST 437, producing carbapenemase NDM-7 in Madrid.

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.

Pesesky et al (62) sequenced 78 *Enterobacteriaceae* from Pakistan and the USA in order to characterize the genomic context of NDM-1 and *K. pneumoniae* carbapenemase (KPC). High similarities of the results indicate rapid spread of carbapenema resistance between strains.

Neves Andrade et al (63) conclude that efforts should be made for rapid microbiology detection and clinical and epidemiological measures to control infections caused by NDM producers and NDM-gene dissemination (for ex using simple tests to detect carbapenemases)

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.

Ameen et al (66) determined the frequency of imipenem-resistant MBL producing *Pseudomonas* isolates collected from clinical samples in a tertiary hospital of Karachi, Pakistan. They found that 35% of the isolates were resistant to imipenem and MBL production was confirmed in 64.9% of the resistant isolates of *Pseudomonas aeruginosa*.

Pobiega et al (97) examined 21 imipenem-resistant *P. aeruginosa* from urine in Poland.42.8% were MBL-positive and VIM-2 was present in 14.2% of isolates.

Lange et al (98) out of 192 carbapenemase-producing *E. coli* isolates in Germany, found OXA-48 in 44.8%, VIM in 18.8 %, NDM-1 in 11.5 % and KPC-2 in 6.8 %

Seara et al (67) report the first global outbreak of NDM-7 producing *K. pneumoniae* in 3 different hospitals in Madrid. 7 patients were involved. Frequently transfer of aged or chronically ill patients between the facilities may have favoured the spread of the NDM-7 outbreak.

Hissong et al (68) studies different phenotypic methods for the detection of carbapenemases (MBL) in *Bacteroides fragilis* group. 5 isolates were cfiA positive. The authors recommend a combination of phenotypic methods (KPC/MBL in *Pseudomonas/Acinetobacter* kit) and the Carba NP.

Ank et al (72) report in Denmark, the identification of a multidrug-resistant *Bacteroides fragilis* recovered from blood of a patient that had been in vacation in Thailand. The isolate was MBL positive (Imipenem/Imipenem + EDTA synergism) and possessed nimE, cfiA and ermF genes corresponding to metronidazole, carbapenem and clindamycin resistance.

Neves Andrade et al (69) describes the detection of NDM in Brazil in *Providencia rettgeri*, NDM-1 in *Morganella morganii*, *E. coli, K. pneumoniae*, *Acinetobacter baumannii* and *Citrobacter freundii*. It seems like a rapid national expansion of NDM-encoding genes and NDM producers in Brazil. Efforts should be made for rapid microbiology detection using simple tests to detect carbapenemases.

Qing Yang et al (71) describes in China that from a total of 186 carbapenem-resistant *Enterobacteriaceae*, 90 isolates were identified as harboring the KPC-2 genes and 5 were NDM-1 positive. The authors report, for the first time, the endemic spread of *Enterobacteriaceae* spp with the NDM-1 gene in their hospital in Zhejiang. The isolates included *E. cloacae*, *K. pneumoniae* and *E. coli*, all of which belonged to the IncX3 -type plasmid.

Huang et al (74) report the presence of NDM-1 producing *Citrobacter freundii*, *E. coli* and *Acinetobacter baumannii* from the urine and stool of a single patient in China. The case the broad host range of NDM-1 gene and its potential to spread between *Enterobacteriaceae* and *A. baumannii*.
Huang TW et al (82) report the transfer of NDM-1 plasmid among *Acinetobacter spp*. and suggest that these non-pathogenic *Acinetobacter* species may serve as a reservoir of this NDM-1 plasmid in the environment.

Giske CG (99) reports that old antimicrobials could be useful for treating infections by carbapenemase-producers. Recent data suggest that NDM and IMP producers are frequently susceptible to mecillinam, while KPC and VIM producers are resistant. Besides mecillinam is highly in vitro active against OXA-48 producers.

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 IMP 12 IMP 13-51	≥128 ≥128 ≥256	≥8/16 32 4-128	≥8 32 ≥64	≥8 128 ≥64	*Pseudomonas spp.* *Alcaligenes spp.* *Acinetobacter baumannii* *Pseudomonas putida* *Pseudomonas aeruginosa*	**Chromosomal** **plasmid** **integron** integron
VIM 1-3 VIM 4-11	R > 256	S →R S →R	2-128 32-256	1-128 32-256	*Achromobacter xylosoxidans* *Pseudomonas aeruginosa* *Pseudomonas putida* (VIM 2 and 4) *Acinetobacter baumannii* *Pseudomonas aeruginosa, A.baumannii*	**Chromosomal** **plasmid** **integron**
VIM 15-16	≥64	16-32	>128	≥128	*Pseudomonas aeruginosa*	integron
VIM 18-45-47	R	S →R	R	R	*Pseudomonas aeruginosa*	integron
SPM-1	≥256	4	R	R	*Pseudomonas aeruginosa*	Plasmid (**not** integron)
GIM-1-2	16 →32	8-16	> 8	> 8	*P.aeruginosa, Enterobacter cloacae*	Integron
SIM-1	≥256	128	8-16	16	*Acinetobacter baumannii*	Integron
IND1-7 DIM-1,2 FIM-1 TMB-1 POM-1 EBR-2	1-32-128	32-128	4-32-128	4-16-128	*Chryseobact indologenes* *P. stutzeri, P putida* *P. aeruginosa* *P. aeruginosa* *Pseud otitidis* *Empedobacter falsenii*	Chromosomal (23) Integron Chrom
AIM-1 NDM 1-17 CPS-1	R		R	R	*Pseudomonas aeruginosa* *Pseud/Acinetobacter* *Chryseobacterium*	Chrom/plasmid

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*	Integron
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,*	
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, K. pneumoniae*	
IMP-6	> 128	128	32	> 128	*Serratia marcescens*	
IMP 8-48	R	S →R	0.5-8	0.25-4	*Enterobacter cloacae, Klebsiella pneumoniae, S.marcescens, Enterobacter*	
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 12-35	≥128	16	8	4	*K. pneumoniae*	Plasmid (16)
VIM-12	> 32	1	1	0.25	*E. coli*	Plasmid (22)
VIM-27					*E. coli*	Integron
GES7						
GIM-1					*Enterobacter, E.coli, C. freundii*	
KHM-1	R	0.25	2	4	*C. freundii*	Plasmid
SMB-1					*Serratia marcescens*	
NDM1-17	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 eitherticarcillin, 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).

Double disk synergy test

a) Enterobacteriaceae

Apply one Dipicolinic Acid Diatabs (DPA) on an inoculated Mueller Hinton (MH) plate. Apply one Meropenem Neo-Sensitabs and one Imipenem 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.

b) Non-fermenters

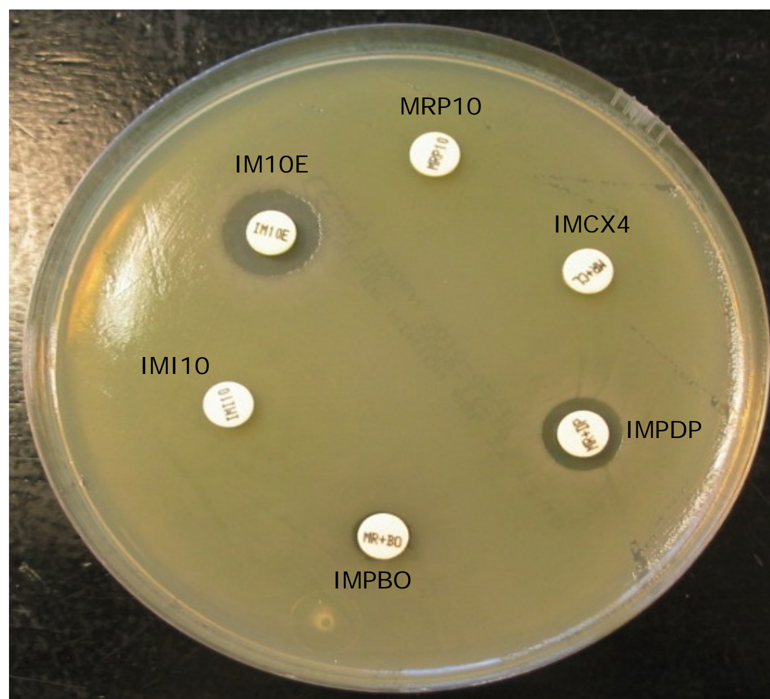
Apply one DPA Diatabs on the MH plate. Apply only one Imipenem Neo-Sensitabs 5 mm from the DPA (edge to edge). Apply Imipenem + EDTA (IM10E) on the inoculated MH plate.

Heinrichs et al (67) evaluated several phenotypic methods for detecting carbapenemases in *P. aeruginosa*. Imipenem + DPA displayed high sensitivities (99%) and specificities (95%) for detecting MBL producing *P. aeruginosa*. Imipenem + Cloxacillin high showed a sensitivity of 97% and a specificity of 96% compared to 88% and 99% respectively for the Carba NP test. Both the Imipenem + DPA and Imipenem + Cloxacillin High are components of the Rosco kit: KPC/MBL in *Pseudomonas* and *Acinetobacter*, section 2. (98025).
Moraitu et al (68) reports a rapid test for identification of carbapenemase-producing bloodstream isolates. After gram-stain, direct susceptibility and phenotypic tests for carbapenemase activity were performed on each blood culture positive for gram-negative bacteria. Imipenem + Phenylboronic and Imipenem+EDTA were used to detect KPCs and MBLs respectively.
Matros et al (69) found that the introduction of routine tests for detection of MBL and other carbapenemases (KPC) is necessary. The authors used the Rosco kit KPC/MBL in *Pseudomonas/Acinetobacter*, version 2 (98025) and detected MBLs in 22 isolates out of 32 of *P. aeruginosa*.
Al-Sultan et al (77) detected carbapenem resistance in 69% of *Acinetobacter baumannii* in Saudi Arabia. The VIM gene was detected in 94% of isolets, while the OXA-23 like, genes were detected in 58%.

Combined disk test.

KPC and MBL Confirm ID kit (98006) *Enterobacteriaceae*
KPC/MBL and OXA-48 Confirm kit (**98015**) *Enterobacteriaceae*
KPC/MBL in *Pseudomonas/Acinetobacter*, Version 2 (**98025)** Non-fermenters
Total MBL Confirm kit (**98016**) Any species.

Apply Imipenem, Imipenem+DPA on an inoculated MH plate. Interpretation: An Imipenem+DPA inhibition zone ≥ 5 mm than Imipenem alone indicates the presence of a metallo-beta-lactamase. (*Enterobacteriaceae*).



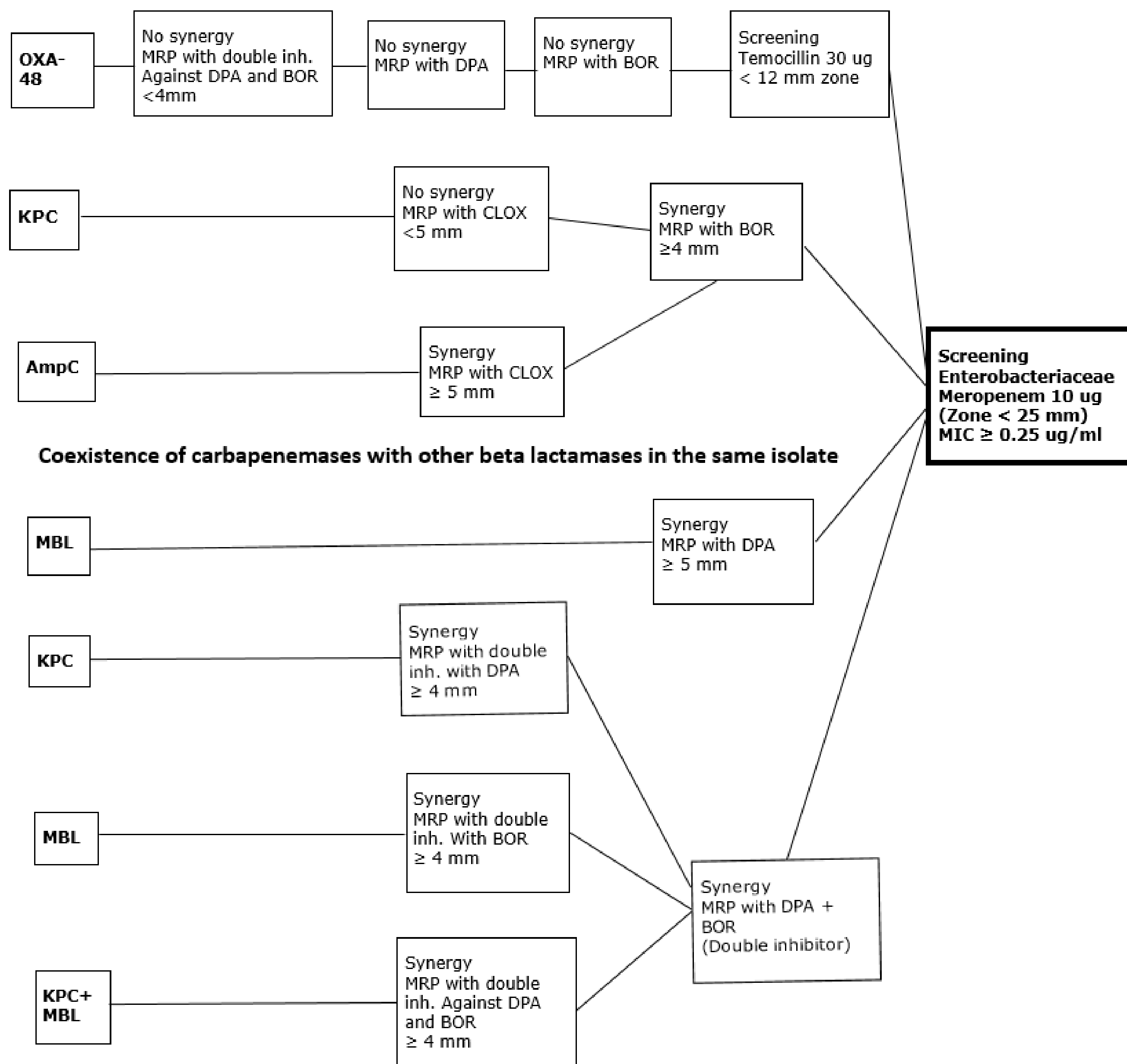
P.aeruginosa FN 8173 metallo-β-lactamase positive

Total MBL Confirm kit (98016)
Composed of:
Meropenem 10 ug (MRP 10)
Dipicolinic acid (DPA)
Imipenem 10 ug (IMI10)
Imipenem + DPA (IM+DP)
Imipenem + EDTA (IM10E)

Interpretation:
Test only Ceftazidime resistant isolates.
1) ***Enterobacteriaceae***: Synergism between MRP10 and DPA
Synergism between IMI10 and DPA and/or IM10E >= 8mm end IMI10
2) ***Pseudomonas /Acinetobacter***: Synergism IMI10 and DPA.

IM+DP >= 5 mm end IMI10 and/or IM10E >= 8mm end IMI10
Acinetobacter and oxacillinases: IM+DP <= 3 mm end IMI10 and IM10E: >=4 -7 mm end IMI10

Algorithm for interpretation of combined disk synergy tests to detect carbapenem-non-susceptible Enterobacteriaceae (38, 39, 40, 49, 59)



Coexistence of carbapenemases with other beta lactamases in the same isolate

Increasing reports show that double-carbapenemase-producing and even multiple carbapenemase-producing bacteria are emerging in some parts of the world, diminishing the already limited treatment options (80).

Detection of KPC and ESBL enzymes in the same strain of *Enterobacteriaceae*

Use kit 98015 and include Ticarcillin + Clavulanate (TCC) Neo-Sensitabs. Synergy between Meropenem and Meropenem + Boronic indicates the presence of a KPC enzyme.

Place TCC at approx. 10 mm from Menopenem + Boronic (edge to edge). Synergy (ghost zone) between the 2 tablets indicates the presence of an ESBL.

Li Wang et al (81) report the coexistence of KPC and CTX-M-55 in *Klebsiella pneumoniae*. Simillar coexsistences have been reported in several countries.

MER10   MER+BOR - 10mm - TCC
Syn   ghost zone
(Syn)

Detection of KPC and MBL enzymes in the same strain of *Enterobacteriaceae* (Triple disk 98010).

The spread of double carbapenemase producers has compromised the performance of the chelator-based and boronate-based tests described previously.

Miriagou et al (49) introduced the double inhibitor disk (triple disk) for detecting double carbapenemase producers.
She described an algorithm, for the detection of KPC, MBL, KPC+MBL and OXA-48 carbapenemases in *Enterobacteriaceae*.This algorithm has been recommended by Hraback et al (59) and it is used in several laboratories worldwide. We present the algorithm in the next page.
Apply Meropenem 10 ug, Meropenem + Phenylboronic, Meropenem + Dipicolinic (kit **98015**) and Meropenem + Phenylboronic+DPA (Triple disk ref **98010**) on an inoculated MH plate.

Interpretation:

Zone around Meropenem+Phenylboronic+DPA disk >= 4 mm larger than zone around Meropenem + DPA and zone around Meropenem + Phenylboronic indicates the presence of a **double enzyme**: MBL and KPC.
Silva et al (44) describes an outbreak caused by KPC-2 and IMP-10-producing *Serratia marcescens* isolates in a Brazilian teaching hospital. This is the first time that a clonal strain with the co-production of IMP-10 and KPC-2 appeared in *S. marcescens* isolates causing an outbreak associated with high mortality in the ICU at a Brazilian teaching hospital.

MER10 ug	MER+BOR	MER+DPA	MER+BOR+DPA
	Syn	Syn	(Triple disc) ≥ 4 mm

Silva Pereira et al (45) showed in their study the co-production of 2 important carbapenemases KPC-2 and NDM-1 associated with mobile genetic elements of worldwide epidemiological importance, in *Enterobacter hormaechei*.
Feng et al (78) describe the coproduction of KPC-2 and NDM-1 in *Citrobacter freundii*.
Wu et al (79) describe the emergence of *Enterobacter cloacae* producing both KPC and NDM carbapenemases.
Kashikar et al (83) describe the presence of KPC-3 carbapenemase in Indiana (USA), with the co-production of either VIM or NDM metallo-beta-lactamases.
Wenjing Wu et al (84) describe an *Enterobacter cloacae* producing both KPC and NDM carbapenemases in China. The coexistence of 2 carbapenemase genes on separate plasmids will probably mediate the spread of antimicrobial resistance genes.
Zheng B et al (89): present the first description of the coexistance of KPC-2 and IMP-4 from the genus *Raoultella*. This study reinforces the idea odf a rapid dissemination of the IMP-4 and KPC-2 genes in clinical isolates of *Enterobacteriaceae* in China.
Feng et al (93) report the coexistence of KPC-2 and NDM-1 in 2 different plasmids in a clinical isolate of *Citrobacter freundii* in a Chinese Teaching Hospital.
Smita (100) reported in Jaipur (India) that 6.6% of *K. pneumoniae* isolates co-produced both MBL and KPC.
Solanki et al (101) reported the presence of NDM-1 in 44 /70 isolates of *K. peumoniae*, while NDM-1 + KPC were present in 14/70 isolates.
Thomson et al (108) describe an *Enterobacter cloacae* strain coproducing KPC-18 and VIM-1 from a patient in Kentucky (USA).
Castanheira et al (109) describe a ST258 *Klebsiella pneumoniae* coproducing KPC and VIM-4 in a patient from a New York Hospital.

Detection of MBL and ESBL on the same strain.

It is not uncommon that some microorganisms co-express both an ESBL and a MBL.
In these cases, ESBL production is masked by the MBL and is difficult to detect directly.
In *Enterobacteriaceae* co-expression of both MBL and ESBL can be detected by a disc approximation test using Aztreonam 30 ug Neo-Sensitabs and Amoxycillin + Clav 20 + 10 ug Neo-Sensitabs. The 2 Neo-sensitabs are placed 10 to 15 mm apart (edge to edge).
Aztreonam is hydrolyzed by the ESBL but not by the MBL. The Clavulanate from the Amox+Clav disk blocks the activity of the ESBL resulting in a ghost zone or keyhole between the 2 disks, in the case of a positive ESBL production.
The MBL is detected as usual using Meropenem 10 ug and Meropenem + DPA Neo-Sensitabs (Zone enlargement >= 5 mm). Use **kit 98006 or kit 98015** for *Enterobacteriaceae.* Chevet et al (46) detected a *Klebsiella pneumoniae* isolate co-producing a VIM metallo-beta-lactamase and an ESBL. They conclude that the presence of synergism between Aztreonam and Amox+Clav, together with multiresistance to beta-lactams must indicate the presence of an ESBL + MBL.

Papagianiannitsis et al (47) report a transmissible plasmid encoding VEB-1 and VIM-1 in an isolate of *Proteus mirabilis*.
Dimude et al (48) tested *Enterobacter cloacae* isolates from Egypt and Edinburgh and found a linked carriage of VIM-4 and CTX-M-14 on the plasmid, resulting in resistance to all beta-lactamas and limited treatment options.
Douka et al (59) describe an outbreak of a pandrug resistant *Providencia stuartii* involving 15 critically ill patients in a Greek intensive care unit. All isolates harboured the VIM-1 as well as the SHV-5 genes.
Toleman et al (60) found NDM-1 in 62% of gram-negative bacteria from environmental waters in Dhaka, Bangladesh. Most co-produced CTX-M-15.
Branas et al (73) report the copresence of VIM-1 and CTX-M-15 in *K. pneumoniae*, in a tertiary hospital in Madrid.
Datta et al (75) describe the use of MH Agar added of 200 mg/L dipicolinic acid in order to neutralize the MBLs of the sample. Using this medium and the CAZ+ Clav+Cloxa compared to CAZ+Cloxa (Rosco kit 98019) it was possible to detect ESBLs and AmpCs in the presence of NDM-1 producing *Enterobacteriaceae* and *Acinetobacter spp*.
Zhou et al (76) describe in a Chinese teaching hospital, an outbreak of multidrug-resistant KPC-2 producing *K pneumoniae* coproducing CTX-M-65, as well as other resistance genes. Both horizontal gene transfer and clonal spread were responsible for this outbreak.
Baraniak et al (85) describe the largest NDM outbreak in a non-endemic country (Poland) being an alarming phenomenon with 374 cases of infection/colonization with NDM-positive *Enterobacteriaceae*. The early isolates also co-produces CTX-M-15.

In non-fermenters (*P. aeruginosa, Acinetobacter*) and *P. mirabilis*: Ticarcillin + Clavulanate 75 + 10 ug is used as the source of clavulanate and Aztreonam 30 ug are used for the ESBL test.
For the MBL test use: Imipenem 10 ug and Imipenem + DPA Neo-Sensitabs. Use **kit 98025**
Glupczynski et al (49) found that BEL enzymes were produced in 80% of *P. aeruginosa* isolates with evidence of ESBL production. BEL or PER ESBLs co-existed with VIM carbapenemases in 15 isolates.
Yakupogullari et al (50) report a multidrug-resistant P. aeruginosa. MICs of imipenem and meropenem were > 128 ug/ml.ESBL detection was performed by a synergy test using aztreonam and ticarcillin+clavulanate.

AZT 10-15 mm AMC	MER10 ug MER+DPA
Syn	≥ 5 mm

Detection of MBL and OXA-48 in the same strain.

In *Enterobacteriaceae* co-expression of MBL and OXA-48 can be detected by disk approximation test using Aztreonam 30 ug and Temocillin 30 ug Neo-Sensitabs. The 2 Neo-Sensitabs are placed approx 10mm apart (edge to edge).
Aztreonam is not hydrolyzed by MBLs, while Temocillin is resistant to both MBLs and OXA-48. Aztreonam protects Temocillin from MBLs and consequently a ghost zone will be formed between the 2 disks, while the Temocillin Neo-Sensitabs will show no zone on the opposite side to the Aztreonam disk. This indicates the possible presence of an OXA-48 enzyme.

The MBL is detected using Imipenem 10 ug and Imipenem + DPA Neo-Sensitabs, or Meropenem 10 ug and Meropenem + DPA (**kit 98015 or kit 98025**).

Kilic et al (43) found the first *Klebsiella pneumoniae* isolate co-producing OXA-48 and NDM-1 in Turkey.
Castanheira et al (51) report the early dissemination of NDM-1 and OXA-181 (OXA-48 like) producing *Enterobacteriaceae* in Indian hospitals in 2006.
Poirel et al (52) report the occurrence of OXA-48 and VIM-1 carbapenemase-producing *Enterobacteriaceae* in Egypt.
Doi et al (53) report the co-production of NDM-1 and OXA-232 (OXA-48 like) by *Klebsiella pneumoniae* in the USA. They conclude that high-level resistance to amikacin and gentamicin can serve as a clue for suspecting potential NDM-1-producing isolates in clinical diagnostic laboratories.
Bousquet et al (54) et al reports the first case in a French hospital of a multidrug-resistant NDM-1 and OXA-232 (OXA-48 like) carrying *Klebsiella pneumoniae*.
Sun Young Cho et al (61) detected *K. pneumoniae* co-producing NDM-5 and oxacillinase OXA-181 (an OXA-48 like) beta-lactamases in South Korea in 2014.
Haciseyitoglu et al (86) describe an interhospital spread of carbapenemase producing *K. pneumoniae* producing OXA-48 and NDM carbapenemases that started in 2011 in Turkey.
Kazi M et al (94) describe the co-presence of NDM-1 and OXA 48 /181 as well as NDM-1 and VIM in *Enterobacteriaceae* in Mumbai, India.
Anandan et al (95) describe the co-production of OXA-48 and NDM in *E. coli* and *K. pneumoniae*, corresponding to 12.5% of the isolates.
Khajuria et al (102) report the emergence of *E. coli* co-producing NDM-1 and OXA-48 in 55% of urinary isolates in atertiary care Center in India.
Anandan et al (103) report 12.5% of *K. pneumoniae*/*E. coli* from blood stream infections co-produced NDM-1+ OXA 181. The Xpert Carba R missed to detect OXA-48 like carbapenemases, because only 4 of 10 variants of OXA-48 like were included in the Xpert Carba R.
Lyman et al (106) mention that CDC received reports of 52 *Enterobacteriaceae* isolates producing OXA-48 from 2010-2015 in the US 12% of the isolates co-produced NDM and OXA-48 carbapenemases. The CDC recommends determining the mechanisms of resistance for any carbapenem-resistant *Enterobacteriaceae*.

AZT – 10 mm – TEMO	MER10 MER+DPA
ghost zone	≥ 5 mm

Detection of MBL, OXA-48 and ESBL in the same strain of *Enterobacteriaceae.*

In *Enterobacteriaceae*, we use the disk approximation test. Aztreonam 30 ug Neo-sensitabs is placed between Temocillin 30 ug and Amoxycillin + Clavulanate 20 + 10 ug Neo-Sensitabs. The distance between Temocillin and Aztreonam is 10 mm (edge to edge) and the distance between Aztreonam and Amoxycillin + Clav is 10 to 15 mm.
Besides use the **kit 98015**: KPC, MBL and OXA-48 confirm kit from Rosco that contains Temocillin 30 ug.
If the isolate shows the following: a ghost zone between Temocillin and Aztreonam (OXA-48) as well as Synergism between Aztreonam and Amoxycillin + Clav (ESBL) and finally a zone of inhibition around Meropenem+DPA >= 5mm larger than Meropenem 10 ug (MBL) it then means that the isolate co-produces MBL+OXA-48+ESBL.

Seifert et al (55) report the emergence of *Klebsiella pneumoniae* co-producing NDM-1, OXA-48, CTX-M-15, CMY-16, QnrA in Switzerland, resistant to all antibiotics except colistin.
Aggoune Khinache et al (56) report the co-expression of NDM-1, OXA-48, CTX-m-15 and SHV 11 lactamases in *Klebsiella pneumoniae* from Algeria.
Meryern Iraz et al (81) report combinations of beta-lactamases including NDM-1, OXA-48 and CTX-M in the same strain of *Klebsiella pneumoniae* in Turkey. Of the 37 isolates tested 76% possessed OXA-48 + ESBL and there was a high prevalende of NDM-1 (19%) being a large increase in Turkey of the NDM-gene.
Bathoorn et al (90) report (august 2015) the isolation of a NDM-5 producing *K. pneumoniae* ST16, isolated from a Dutch patient in a long-term care facility without recent travel history abroad. The NDM-5 gene was accompanied of a CTX-M-15 (ESBL) and was clonally related to isolates detected in 4 patients in Denmark in 2014.
Torres-Gonzalez et al (91) report an outbreak caused by a NDM-1-harboring plasmid spread through different bacterial species (*E. cloacae*, *E. coli*, *K. pneumoniae*), in a tertiary care hospital in Mexico City.
Besides NDM-1, the isolates harbored an ESBL (CTX-M-15).
Dortet et al (92) report the dissemination of carbapenemase-producing *Enterobacteriaceae* and *P. aeruginosa* in Romania. NDM-1 and OXA-48 were most common in *Enterobacteriaceae* and VIM-2 in *P. aeruginosa*. Besides NDM-1 or OXA-48 the isolates harbored ESBLs (CTX-M-15)
Hammerum et al (104) report in Denmark the detection of *K. pneumoniae* co-producing NDM-7 and OXA-181 and CTX-M-15 in a patient that was hospitalized in Goa. Besides, the same patient also carried *E. coli* producing NDM-5 and *A. baumannii* producing OXA-23. GeneXpert did not detect the OXA-181 gene. The study highlights the importance of screening for carbapenemase-producing bacteria from patients travelling to countries with a high occurrence of carbapenemase-producing bacteria.

TEMO – 10 mm – AZT – 10-15 mm – AMC   MER10   MER+DPA
ghost zone   Syn   ≥ 5 mm

Detection of oxacillinases and MBL in the same strain of *Acinetobacter*

Use the **kit 98025**: KPC/MBL in *Pseudomonas/Acinetobacter* as well as Aztreonam 30 ug Neo-Sensitabs.
Apply Imipenem 10 ug Neo-ThiSensitabs and Aztreonam 30 ug Neo-Sensitabs at 10 mm from each other (edge to edge). Besides the remaining tablets from the 98025 kit on the inoculated MH agar plate.
An increase of 4 – 7 mm in the inhibition zone around IMI+EDTA compared to IMP10 ug and no synergy with Imipenem+DPA (compared to Imipenem 10 ug), indicates the presence of an **oxacillinase.**
An increase of >= 8 mm in the zone around IMI+EDTA compared to Imipenem 10 ug and/or an increase of >= 5 mm in the zone around Imipenem+DPA as compared to IMP 10 ug and synergism between Aztreonam and Imipenem indicates the presence of a **MBL**.
An increase >= 8 mm in the zone around IMI+EDTA, and/or an increase >= 5 mm in the zone around Imipenem+DPA as well as no-synergism between Aztreonam and Imipenem indicates the presence of a **MBL and an oxacillinase.**
Bosnjak et al (57) describes for the first time an *Acinetobacter guillouiae* co-producing NDM-1 and OXA-58 in Croatia.
Kumarasamy K et al (58) describes the coexistance of OXA-23 with NDM-1 and armA in clinical isolates of *Acinetobacter baumannii* from India.
Bedenic et al (65) describes 11 isolates of *A. baumannii* co-producing OXA-23 and VIM metallo-beta-lactamase in a nursing home. The E-test and combined disk test using EDTA gave false positive in 7 of 18

isolates. For testing MBLs in *Acinetobacter* is best to use the Imipenem/DPA combination, because the Imipenem/EDTA combination may give false MBLs positives.
Yao Sun et al (96) detected the co-production of OXA-23 and IMP genes in 7.8% of *A. baumannii* in Wenzhou, China.
Jin-Gui Cao et al (105) report for the first time a carbapenem-resistant *Acinetobacter soli* coharboring NDM-1 and OXA-58 genes in China.The authors indicate the importance of accurate epidemiological investigation of non-A. baumannii species.
Yu-Feng et al (107) report the ability of *A. johnsonii* to harbor 9 plasmids (including NDM-1, OXA-58 and PER-1) and suggest that this species could generate various platforms to mediate dissemination of resistance.

IMP – 10 mm – AZT	IM+ED	IM+DPA	Enzymes
No synergy	≥ 4-7 mm	No synergy	Oxacillinase
Synergy	≥ 8 mm **and**	≥ 5 mm	MBL
No synergy	≥ 8 mm **and**	≥ 5 mm	MBL+Oxacillinase

Detection of oxacillinases and Class A carbapenemases in the same strain of *Acinetobacte.*

Hammoudi D et al (64) report the preponderance of OXA-23 and GES-11 co-producing isolates of *A. baumannii* in Lebanon. These resistance traits appear to spread via both bacterial epidemics and horizontal transfer.
To detect them Use the **kit 98025**: KPC/MBL in *Pseudomonas/Acinetobacter* as well as Ticarcillin+Clavulanate Neo-Sensitabs (TTC) and Boronic acid Diatabs (BORON).
Place the Imipenem 10 ug of the kit in between TTC (distance 5-8 mm between edges) and BORON (5 mm distance between edges). Use the remaining of the kit 98025.
Synergy (ghost zone) between Imipenem 10 ug and TTC and between Imipenem 10 ug and BORON indicates the presence of a Class A carbapebnemase (**KPC or GES**).
An increase of 4 – 7 mm in the inhibition zone around IMI+EDTA compared to IMP 10 and no synergy with Imipenem + DPA (compared to IMP 10) indicates the presence of an **oxacillinase.**
Cicek et al (87) report the presence of OXA-23 and GES-11(or GES-22) in extensively drug-resistant *Acinetobacter baumannii* in Turkey.) 6 % of the strains were MDR.
Cherkaoui et al (88) report the co-production of OXA-23 and GES-11 in multidrug-resistant *Acinetobacter baumannii* in Switzerland.

TCC - 5-8 mm – IMP – 5 mm – BORON	IMP10 IMI+ED	IMP+DP	Enzymes
ghost zone ghost zone	Syn	No Syn	Class A (KPC or GES)
	4-7 mm	No Syn	Oxacillinase

Reference MBLs:

1) Walsh T.R. et al: Evaluation of new E-test for detecting metallo-beta-lactamases in routine clinical testing. J. Clin. Microbiol., **40**, 2755-59, 2002.
2) Jong D. et al: Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J. Clin. Microbiol., **40**, 3798-3801, 2002.
3) Larrosa M. N. et al: *E. coli* multi-resistente productora de una metalo-beta-lactamasa (Spanish). SEIMC Congress, Bilbao, 16-19th May, 2004.
4) Lartigue MF et al: First detection of a carbapenem-hydrolyzing metalloenzyme in an *Enterobacteriaceae* isolate in France. Antimicr. Ag. Chemother., **48**, 4929-30, 2004.
5) Castanheira M et al: Molecular characterization of a beta-lactamase gene blaGIM-1, a new subclass of metallo-beta-lactamases. Antimicr. Ag. Chemother., **48**, 4654-61, 2004.
6) Jing-Jou Yan et al: Comparison of the double-disk, combined disk and E-test methods for detecting metallo-beta-lactamases in gram-negative bacilli. Diagn. Microbiol. Infect. Dis., **49**, 5-11, 2004.
7) Walsh T.R. et al: Metallo-beta-lactamases: The quite before the storm? Clin. Microbiol. Reviews, **18**, 306-325, 2005.
8) Tortola M.T. et al: First detection of a carbapenem-hydrolyzing metalloenzyme in two *Enterobacteriaceae* isolates in Spain. Antimicr. Ag. Chemother., **49**, 3492-4, 2005.
9) Chu Y.W. et al: EDTA susceptibility leading to false detection of metallo-beta-lactamase in *P. aeruginosa* by E-test and an Imipenem-EDTA disk method. Intl. J. Antimicr. Agents, **26**, 340-341, 2005.
10) Lincopan N. et al: First isolation of metallo-ß-lactamase producing multiresistant *K. pneumoniae* from a patient in Brazil. J. Clin. Microbiol., **43**, 516-9, 2005.
11) Pitout J.D.D. et al: Detection of *P. aeruginosa* producing metallo-ß-lactamases in a large centralized laboratory. J. Clin. Microbiol., **43**, 3129-35, 2005.
12) Marqué S. et al: Regional occurrence of plasmid-mediated carbapenem-hydrolyzing oxacillinase OXA-58 in Acinetobacter spp. in Europe. J. Clin. Microbiol., **43**, 4885-8, 2005.
13) Choi Y.S. et al: Evaluation of Imipenem Disk Hodge Test and double disk synergy tests to detect SIM-1 type metallo-ß-lactamase-producing *Acinetobacter* isolates. Presentation D-1705, 45th ICAAC, 2005.
14) Lee Kyungwon et al: Novel acquired metallo-ß-lactamase gene blaSIM-1 in class 1 integron from *Ac. baumannii* clinical isolates from Korea. Antimicr. Ag. Chemother., **49**, 4485-91, 2005.
15) Peleg A.Y.: Dissemination of the metallo-↓-lactamase gene blaIMP-4 among gram-negative pathogens in a clinical setting in Australia.CID, **41**, 1549-56, 2005.
16) Pournaras et al: VIM-12 a novel plasmid-mediated metallo-ß-lactamase from *K. pneumoniae* that resembles a VIM-1/VIM-2 hybrid. Antimicr. Ag. Chemother., **49**, 5153-6, 2005.
17) Berges L. et al: Prospective evaluation of Imipenem/EDTA combined disk and E-test for detection of MBL producing *P. aeruginosa*. Clin. Microbiol. Infect., **12**, Suppl. 4, P1451, 2006.
18) Cornaglia G. et al: Metallo-ßlactamases as emerging resistance determinants in Gram-negative pathogens; open issues. Int. J. Antimicr. Ag., **29**, 380-88, 2007.
19) Franklin C. et al: Phenotypic detection of carbapenem-susceptible metallo-ß-lactamase-producing Gram-negative bacilli in the clinical laboratory. J. Clin. Microbiol., **44**, 3139-3144, 2006.
20) Moland E.S. et al: Prevalence of newer ß-lactamases in gram-negative clinical isolates collected in the U.S. from 2001 to 2002. J. Clin. Microbiol., 44, 3318-3324, 2006.

21) Deshpande L.M. et al: Emergence of serine carbapenemases (KPC and SME) among clinical strains of *Enterobacteriaceae* in the U.S. Medical Centers: Report from the MYSTIC Program (1999-2005).
22) Iconomidis A. et al: First occurrence of an *E. coli* clinical isolate producing theVIM1/VIM2 hybrid metallo-ß-lactamase VIM12. Antimicr. Ag. Chemother., **51**, 3038-39, 2007.
23) Perilli M. et al: Identification and characterisation of a new metallo-ß-lactamase from a clinical isolate of *Chryseobacterium indologenes*. Antimicr. Ag. Chemother., **51**, 2988-2990, 2007.
24) Anderson K.F. et al: Evaluation of methods to identify the *K. pneumoniae* carbapenemase in *Enterobacteriaceae*. J. Clin. Microbiol, **45**, 2723-25, 2007.
25) Kim Soo-Young et al: Convenient test using a combination of chelating agents for the detection of metallo-beta-lactamases in the clinical laboratory. J. Clin. Microbio., **45**, 2798-2801, 2007.
26) Giakkoupi P. et al: Supplementation of growth media with Zn2+ facilitates detection of VIM-2 producing *P. aeruginosa*. JCM 46, 1568-9, 2008.
27) Kyeong S.S. et al: Dipicolinic acid and based disk methods for detection of metallo-β-lactamase producing *Pseudomonas spp* and *Acinetobacter spp*. Diagn. Microbiol. Infect Dis **62**, 102-105, 2008.
28) Miriagou V et al: Improved detection of VIM-1 production in *Proteus mirabilis* by an Imipenem-dipicolinic acid double disk synergy test. J Clin Microbiol **48**,667-668,2010
29) Multiresistant hospital bacteria linked to India and Pakistan. HPS Weekly Report News, July 8th, 2009.
30) Carvalho-Assef APD et al: First report of the carbapenem-resistant IMP-16 producing *Pseudomonas stutzeri*. Presentation C2-634, 49th ICAAC, 2009.
31) Pasteran F. et al: Spread of multiple clones of metallo-β-lactamases (MBL) producing *Pseudomonas putida*. Presentation C2-636, 49th ICAAC, 2009.
32) Treviňo M. et al: Carbapenem-resistant *E. cloacae* and the emergence of metallo-β-lactamases producing strains in a third level hospital (Santiago de Compostela, Spain). Eur. J. Clin. Microbiol. Infect.Dis **28**, 1253-58, 2009.
33) Dongeun Yong et al: Characterization of a new metallo-β-lactamase gene NDM-1 and a novel erythromycin esterase gene carried on a unique genetic structure in *K. pneumoniae* sequence type 14 from India**.** Antimicr. Ag. Chemother **53,** 5046-5054, 2009.
34) Samuelsen Ø et al: Molecular epidemiology of MBL-producing *P. aeruginosa* isolates from Norway and Swede shows import of international clones and local clonal expansion. Antimicr Agents Chemother **54,** 346-352, 2010.
35) Poirel L et al: Emergence of MBL NDM-1 producing mulridrug-resistant *E.coli* in Australia. Antimicr Ag Chemother **54,** 4914-4916, 2010.
36) Karthikeyan K et al: Co-existance of OXA-23 with NDM-1 and ArmA in clinical isolates of *A. baumannii* from India. J Antimicr Chemother **65**, 2253-2254, 2010.
37) Miriagou V et al: The ongoing challenge of acquired carbapenemases: simultaneous production og VIM-1 and KPC-2 in *K. pneumoniae*. Poster ECCMID 2010.
38) Lucena A et al: Comparison of phenotypic tests for the detection of metallo-beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. Enferm Infecc Microbiol Clin **32**,625-630, 2014.
39) Dayang Zou et al: A novel NDM variant, NDM-14, isolated in a Chinese hospital possesses increased enzymatic activity against carbapenems. Antimicr Ag Chemother 59, 2450-2453, 2015.
40) Schrøder Hansen D et al: First report of a health-care associated outbreak with NDM-5 producing *K. pneumoniae* in Europe. ECCMID 2015, Presentation P1033.
41) Ruiz G et al: Intrahospitalary dissemination of *K. pneumoniae* ST437 producing Carbapenemase NDM-7. SEIMC 2015, Presentation 084 (spanish)

42) Arroyo A et al: Emerging of *Enterobacteriaceae* with multiple carbapenemase genes. SEIMC 2015.Presentation 080 (spanish).
43) Kilic A et al: The first *Klebsiella pneumoniae* isolate co-producing OXA-48 and NDM-1 in Turkey. Annals of Laboratory medicine **35**, 382-383, 2015.
44) Da Silva et al: Co-production of KPC2 and IMP-10 in carbapenem-resistant *Serratia marcescens* isolated from an outbreak in a Brazilian teaching hospital. J Clin Microbiol, April 2015.
45) Silva Pereira et al: Coproduction of NDM-1 and KPC-2 in *Enterobacter hormaechei* from Brazil.Microbil Drug Resistance **21**, 234-236, 2015.
46) Chevet K et al: Detection phenotypique dune carbapenemasse associee a une beta-lactamase a spectre elargi chez *K. pneumoniae*. Medecine et Maladies Infect **42**, 33-35, 2012.
47) Papagiannitsis CC et al: Characterization of a transmissible plasmid encoding VEB-1 and VIM-1 in *Proteus mirabilis*. Antimicr Ag Chemother **56**, 4024-4025, 2012.
48) Dimude JU et al: Molecular characterization and diversity in *Enterobacter cloacae* from Edinburgh and Egypt carrying CTX-M-14 and VIM-4 beta-lactamase genes. Int J Antimicr Agents **41**, 574-577, 2013.
49) Glupczynski Y et al: Detection and characterization of Class A ESBL-producing *P. aeruginosa* isolates in Belgian Hospitals. J Antimicr Chemother **65**, 866-871, 2010.
50) Yakupogullari Y et al: Multidrug-resistant *P. aeruginosa* isolate co-expressing ESBL PER-1 and metallo-beta-lactamase VIM-2 from Turkey. J Antimicr Chemother Nov 2007.
51) Castanheira M et al: Early dissemination of NDM-1 and OXA-181-producing *Enterobacteriaceae* in Indian hospitals: report from the SENTRY Antimicrobial Surveillance Program 2006-2007. Antimicr Ag Chemother **55**, 1274-1278, 2011.
52) Poirel L et al: Occurrence of OXA-48 and VIM-1 carbapenemase-producing *Enterobacteriaceae* in Egypt Int J Antimicr Agents **41**, 90-95, 2013.
53) Yohei Doi et al: Co-production of NDM-1 and OXA-232 by *Klebsiella pneumoniae*. Emerg Infect Dis, **20**, 163-165, 2014.
54) Bousquet A et al: First case of multidrug-resistant NDM-1 and OXA-232 carrying *Klebsiella pneumoniae* and its probable cross-transmission in a French hospital. Int J Antimicr Agents **44**, 469-470, 2014.
55) Seiffert SN et al: Emergence of *Klebsiella pneumoniae* co-producing NDM-1, OXA-48, CTX-M-15, CMY-16, QnrA and ArmA in Switzerland. Int J Antimicr Agents **44**, 260-262, 2014.
56) Aggoune Khinache et al: Co-expression of NDM-1, OXA-48, CTX-M-15 and SHV111 beta lactamases in *Klebsiella pneumoniae* from Algeria. ECCMID 2015, Presentatiion P1039.
57) Bosnjak Z et al: First report of NDM-1-producing *Acinetobacter guillouiae*. Chemotherapy, **60**, 250-252, 2014.
58) Kumarasamy K et al: Coexistance of OXA-23 with NDM-1 and armA in clinical isolates of *Acinetobacter baumannii* from India. J Antimicr Chemother **65,** 2253-2254, 2010.
59) Douka E et al: Emergence of a pandrug-resistant VIM-1 producing *Providencia stuartii* clonal strain causing an outbreak in a Greek intensive care unit. Int J Antimicr Agents **45**,533-536, 2015**.**
60) Toleman MA et al: Extensively drug-resistant NDM-encoding bacteria in the environment, Dhaka, Bangladesh 2012. Emerging Infect Dis **21**, 1027-1030, 2015.
61) Sun Young Cho et al: *Klebsiella pneumoniae* coproducing NDM-5 and OXA-181 carbapenemases, South Korea. Emerg Infect Dis **21**, 1088-1089, 2015.
62) Pesesky MW et al: KPC and NDM-1 genes in related *Enterobacteriaceae* strains and plasmids from Pakistan and the U.S. Emerg Infect Dis **21**, 1034-1037, 2015.

63) Neves Andrade L et al: Response to detection of NDM-producing bacteria, Brazil. Emerg Infect Dis **21**, 1069-1070, 2015**.**
64) Hammoudi D et al: Spread of imipenem-resistant *Acinetobacter baumannii* co-expressing OXA- 23 and GES-11 carbapenemases in Lebanon. Int J Infect Dis, accepted manuscript may 2015.
65) Bedenic B et al: Nursing home as a reservoir of carbapenem-resistant *Acinetobacter baumannii*. Microbial Drug Resistance **21**, 270-276, 2015.
66) Ameen N et al: Imipenem resistant profile of clinical isolates of *P. aeruginosa*: a cause of public health concern. ASM 2015, Presentation 1876.
67) Seara N et al: Interhospital spread of NDM-7-producing *Klebsiella pneumoniae* belonging to ST437 in Spain. J Antimicr Agents, online april 30, 2015.
68) Hissong E et al: Characterization of carbapenem-resistant *Bacteroides fragilis* group isolates by phenotypic and genotypic methods. ASM 2015, Presentation 536.
69) Neves Andrade L et al: Response to detection of NDM beta lactamase-producing bacteria, Brazil. Emerg Infect Dis **21**, 1069-1071, 2015.
70) Bosnjak Z et al: First report of NDM-1 producing *Acinetobacter guillouiae*. Chemotherapy **60**, 250-252, 2015.
71) Qing Yang et al: Dissemination of NDM-1-producing *Enterobacteriaceae* mediated by the IncX3-type plasmid. PLoS ONE 10(6), june 5th 2015.
72) Ank N et al: Characterization of a multidrug-resistant *Bacteroides fragilis* isolate recovered from blood of a patient in Denmark using whole-genome sequencing. Int J Antimicr Agents **46**, 117-120, 2015.
73) Branas P et al: Molecular epidemiology of carbapenemase-producing *K. pneumoniae* in a hospital in Madrid: successful establishment of an OXA-48 ST11 clone. Int J Antimicr Agents **46,** 111-116, 2015.
74) Huang YM et al: NDM-1-producing *Citrobacter freundii*, *E. coli* and *Acinetobacter baumannii* **i**dentified from a single patient in China**.** Antimicr Agents Chemother, june 2015 (ahead of print).
75) Datta S et al: A reliable phenotypic assay for detection of ESBLs and AmpCs in MBL-producing gram-negative bacteria with the use of aminophenylboronic acid, dipicolinic acid and cloxacillin. J Microbiol Methods **115**, 100-103, 2015.
76) Zhou T et al: An outbreak of infections caused by extensively drug-resistant *K. pneumoniae* strains during a short period in a Chinese teaching hospital: epidemiology study and molecular characteristics. Diagn Microbiol Infect Dis **82**, 240-244, 2015
77) Al-Sultan et al: Dissemination of multiple carbapenem-resistant clones of *Acinetobacter baumannii* in the eastern district of Saudi Arabia. Frontiers in Microbiology **6,** 634, 2015.
78) Feng J et al: Coexistance of a novel KPC-2-encoding MDR plasmid and an NDM-1-encoding pNDM-HN380-like plasmid in a clinical isolate of Citrobacter freundii. J Antimicr Chemother Aug 9, 2015 (ahead of print)
79) Wu W et al: The emergence of *Enterobacter cloacae* producing both KPC and NDM carbapenemases: characterization by whole genome sequencing. Antimicr Ag Chemother, july 27, 2015 (Epub ahead of print).
80) Meletis G et al: Double and multi-carbapenemase producers: the excessively armored bacilli of the current decade. Eur J Clin Microbiol. Infect Dis **34**, 1487-1493, 2015.
81) Meryem Iraz et al: Distribution of beta-lactamase genes among carbapenem-resistant *K. pneumonia* strains isolated in Turkey.Annals Lab Medicine **35**,595-601, 2015.
82) Huang TW et al: Effective transfer of a 47 kb NDM-1-positive plasmid among *Acinetobacter* species. J Antimicr Chemother **70,** 2734-2738, 2015.

83) Kashikar A et al: Occurrence of VIM and NDM carbapenemases in Indiana Health Care centers carbapenem-resistant *Enterobacteriaceae* from 2009 to 2013. ICAAC 2015, presentation C-1095.
84) Wenjing Wu et al: Characterization of *Enterobacter cloacae* strain producing both KPC and NDM carbapenemases by whole genome sequencing. Antimicr Ag Chemother **59**, 6625-6628, 2015.
85) Baraniak A et al: NDM-producing *Enterobacteriaceae* in Poland 2012-14: inter-regional outbreak of *K. pneumoniae* ST11 and sporadic cases. J Antimcr Chemother Epub ahead of print 19 sept. 2015.
86) Haciseyitoglu D et al: The first *Enterobacter cloacae* co-producing NDM and OXA-48 carbapenemases and interhospital spread of OXA-48 and NDM-producing *K. pneumoniae*, Istanbul, Turkey.
87) Cicek AC et al: OXA and GES-type beta-lactamases predominate in extensively drug-resistant *A. baumannii* isolates from a Turkish University Hospital. Clin Microbio Infect 20, 410-415, 2013.
88) Cherkaoui A et al: Characteristics of multidrug resistant *A. baumannii* strains isolated in Geneva during colonization or infection. Ann Clin Microbiol Antimicrob 14:42, 2015.
89) Zheng B et al: Emergence of *Raoultella ornitholytica* coproducing IMP-4 and KPC-2 carbapenemases in China. Antimicr Ag Chemother **59**, 7086-7089, 2015.
90) Bathoorn E et al: Isolation of an NDM-5 producing ST16 *K. pneumoniae* from a Dutch patient without travel history abroad, August 2015. Eurosurveillance **20**, August 2015.
91) Torres-Gonzalez P et al: Outbreak caused by *Enterobacteriaceae* harboring NDM-1 MBL carried in an IncFII plasmid in a tertiary care hospital in Mexico City. Antimicr Ag Chemother **59**, 7080-7083, 2015.
92) Dortet L et al: Dissemination of carbapenemase-producing *Enterobacteriaceae* and *P. aeruginosa* in Romania. Antimicr Ag Chemother **59**, 7100-7103, 2015.
93) Feng J et al: Coexistance of a novel KPC-2-encoding MDR plasmid and an NDM-1-encoding pNDM-HN380-like plasmid in a clinical isolate of *Citrobacter freundii*. J Antimicr. Chemother **70**, 2987-2991, 2015.
94) Kazi M et al: The Carbapenemase menace: Do dual mechanisms code for more resistance? Infect Control & Hospital Epidem. **36**, 116-117, 2015.
95) Anandan S et al: Rapid screening for carbapenem resistant organisms: current results and future approaches. J Clin Diagn Res. Epub 2015, September 1.
96) Yao Sun et al: Prevalence and molecular characterization of carbapenemase-producing gram-negative bacteria from a University Hospital in China. Infect Dis, Epub 2015, October 14.
97) Pobiega M et al: Molecular characterization of carbapenem-resistant *P. aeruginosa* strains isolated from patients with UTIs in Southern Poland. Diagn Microbiol Infect Dis **83**, 295-297, 2015.
98) Lange et al: Molecular epidemiology of VIM-producing *E. coli*, the National Reference Laboratory. Int J Med Microbiol **305**, 784-789, 2015.
99) Giske CG: Contemporary resistance trends and mechanisms for the old antibiotics colistin, temocillin, fosfomycin, mecillinam and nitrofurantoin. Clin Microbiol Infect **21**, 899-905, 2015.
100) Smita Sood: Identification and differentiation of Carbapenemases in *K. pneumoniae*: a phenotypic test evaluation study from Jaipur, India. J Clin Diagn Res, 2014, jul 20.
101) Solanki R et al: Comparative evaluation of multiplex PCR and routine laboratory phenotypic methods for detection of carbapenemases among gram-negative bacilli. J Clin Diagn Res 2014, net.
102) Khajuria A et al: Emergence of *E. coli* co-producing NDM-1 and OXA-48 carbapenemases in urinary isolates at a tertiary care Centre at central India. J Clin Diagn Res 2014, June 20.
103) Anandan S et al: Rapid screening of carbapenem resistant organisms: current results and future approaches. J Clin Diagn Res. 2015.

104) Hammerum AM et al: Detection of *K. pneumoniae* co-producing NDM-7 and OXA-181, *E. coli* producing NDM-5 and *A. baumannii* producing OXA-23 in a single patient. Int J Antimicr Ag **46**, 597-598, 2015.

105) Jin-Gui Cao et al: First report of carbapenem-resistant *Acinetobacter soli* isolates coharboring NDM-1 and OXA-58 genes from China. Diagn Microbiol Infect Dis. **83,** 407-408, 2015.

106) Lyman M et al: Carbapenem-resistant *Enterobacteriaceae* producing OXA-48-like carbapenemases. United States 2010-2015. CDC, December 4, 2015, **64**, 1315-1316, 2015.

107) Yu-Feng et al: Characterization of *Acinetobacter johnsonii* isolate XBB1 carrying 9 plasmids and encoding NDM-1, OXA-58 and PER-1 by genome sequencing. J Antimicr Chemother **71**, 71-75, 2016.

108) Thomson GK et al: Coproduction of KPC-18 and VIM-1 carbapenemases by *Enterobacter cloacae*: implications for newer beta lactam-beta lactamase inhibitor combinations. J Clin Microbiol **54**, 791-794, 2016.

109) Castanheira M et al: *Klebsiella pneumoniae* isolate from a New York City Hospital belonging to sequence type 258 and carrying KPC-2 and VIM-4. Antimicr Ag Chemother 60, 1924-1927, 2016.