Phenotypic and genotypic methods for detecting carbapenemases

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Resistance trends for resistance to 3GC in Europe

E. coli

K. pneumoniae

2002
- I: 1.12%
- R: 0.76%

2005
- I: 0.97%
- R: 4.13%

2010
- I: 1.53%
- R: 7.25%

Explanation: ESBLs!

2010
- I: 1.43%
- R: 17.83%
Emergence of ESBL-producing isolates

- Limited therapeutic choices
- Overuse of carbapenems
- Increased risks to select for carbapenem resistant strains
Carbapenemases
Carbapenemases in Enterobacteriaceae

- OXA-48
- IMP
- VIM-1
- NDM-1
- KPC-2,3

ESCMID Online Lecture Library © by author
Broad-spectrum β-lactamases in Gram negatives

- **Penicillins**
- **Cephalosporins**
- **Carbapenems**

Extended-spectrum β-lactamases (ESBL); **CTX-M**

Carbapenemases: **NDM, KPC, OXA-48**
Carbapenemases in Enterobacteriaceae

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>Penicillins</th>
<th>1G, 2G cephalosporins</th>
<th>3G, 4G cephalosporins</th>
<th>ß-lactam / clavulanic acid</th>
<th>Carbapenems</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td><strong>KPC, IMI, GES</strong> ...</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td><strong>Metallo-ß-lactamases : VIM, IMP, NDM-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
<td><strong>Oxacillinases : OXA-48, OXA-181, OXA-204</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Carbapenem susceptibility of OXA-48 producers*

N=101 isolates (67 K. pneumoniae, 24 E. coli, 10 E. cloacae)

* CLSI breakpoints
Antimicrobial susceptibility of OXA-48 producers

N=107 isolates (68 Klebsiella spp., 24 E. coli, 10 E. cloacae, 3 Citrobacter spp., 1 P. rettgeri, 1 S. marcescens)

* CLSI breakpoints (EUCAST for tigecycline)

75% of ESBL producers

Potron et al. - Eurosurv 2013
OXA-48-like carbapenemases: the phantom menace

Laurent Poirel*, Anaïs Potron and Patrice Nordmann
OXA-48 + CTX-M-15

K. pneumoniae
Spread of carbapenemase producers in *Enterobacteriaceae*
Europe–2013

Glasner et al., Eurosurveillance 2013
Clinical breakpoints and screening cut-off values for carbapenemase-producing Enterobacteriaceae

<table>
<thead>
<tr>
<th>Carbapenem</th>
<th>MIC (mg/L)</th>
<th>Disk diffusion zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S/I breakpoint</td>
<td>Screening cut-off</td>
</tr>
<tr>
<td>Meropenem¹</td>
<td>≤2</td>
<td>&gt;0.125</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤2</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Ertapenem³</td>
<td>≤0.5</td>
<td>&gt;0.125</td>
</tr>
</tbody>
</table>

¹Best balance of sensitivity and specificity.
²In rare cases OXA-48-producers have zone diameters of 24-26 mm, so 27 mm may be used as a screening cut-off during outbreaks, but with significant reduction in specificity.
³High sensitivity, but low specificity and therefore not recommended.
Phenotypic or Genotypic Test?
Main properties of carbapenemases that may help identifying them phenotypically
Class A carbapenemases

- Are inhibited by clavulanic acid and tazobactam (not that well for KPC)
- Are inhibited in-vitro by boronic acid
- Hydrolyse penicillins, broad-spectrum cephalosporins, carbapenems, and monobactams, but NOT cephemycins (except the GES-14 variant)
Metallo-β-lactamases (class B)

• Requires zinc ions to be functional
• Not inhibited by clavulanic acid nor tazobactam
• Inhibited in vitro by EDTA and dipicolinic acid
• Hydrolyse penicillins, broad-spectrum cephalosporins, cephamycins, carbapenems, but NOT monobactams
• Hydrolyse carbapenems at a high level
Class B carbapenemases

- **Chromosome- or plasmid-encoded**

<table>
<thead>
<tr>
<th>Type</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP-like</td>
<td>Mostly <em>P. aeruginosa</em>, but also <em>Enterobacteriaceae</em></td>
</tr>
<tr>
<td>VIM-like</td>
<td>Mostly <em>P. aeruginosa</em>, but also <em>Enterobacteriaceae</em></td>
</tr>
<tr>
<td>SPM-1</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>GIM-1</td>
<td><em>P. aeruginosa</em>, and some <em>Enterobacteriaceae</em></td>
</tr>
<tr>
<td>NDM-1</td>
<td><em>K. pneumoniae</em>, <em>E. coli</em>, <em>E. cloacae</em></td>
</tr>
<tr>
<td>DIM-1</td>
<td><em>Pseudomonas stutzeri</em></td>
</tr>
<tr>
<td>KHM-1</td>
<td><em>Citrobacter freundii</em></td>
</tr>
<tr>
<td>SIM-1</td>
<td><em>A. baumannii</em></td>
</tr>
<tr>
<td>AIM-1</td>
<td><em>P. aeruginosa</em></td>
</tr>
</tbody>
</table>
Carbapenem-hydrolyzing class D β-lactamases (OXA)

Not inhibited by clavulanic acid nor tazobactam

- Not inhibited by EDTA
- Inhibited in vitro by NaCl
- Hydrolyse penicillins and carbapenems, but NOT broad-spectrum cephalosporins
- Hydrolyse carbapenems at a low level
- Hydrolyse temocillin
Class D carbapenemases

- **OXA-23**  
  *A. baumannii (+ Proteus mirabilis)*

- **OXA-40**  
  *A. baumannii + P. aeruginosa*

- **OXA-58**  
  *Acinetobacter sp.*

- **OXA-143**  
  *A. baumannii*

- **OXA-48 (and derivatives)**  
  *Enterobacteriaceae*
Detection of carbapenemase producers in infected samples

- **Susceptibility testing**: imipenem, ertapenem, meropenem: CLSI, EUCAST guidelines

- **Phenotypic detection**
  - Hodge test; modified Hodge test
  - Inhibition: EDTA, clavulanic acid, boronic acid...

- **Carbapenem hydrolysis** (UV spectrophotometry, Mass spectro)

- **Molecular biology**
  - Specific PCR, multiplex PCR +/- sequencing
  - Real time PCR
  - DNA Microarray
Inhibition tests

**Principal**
- KPC inhibited by boronic acid or clavulanic acid
- MBL inhibited by EDTA or dipicolinic acid

**Tests available**
- Combined Test (ROSCO): meropenem +/- cloxacillin or ac. dicolinic acide or boronic ac.
- E-test MBL
- Inhibition by EDTA (« home-made technique »)

![Images showing inhibition tests](https://example.com/images/inhibition-tests.png)
Detection

IMP + EDTA

IMP (16 µg/ml)
Test +

IMP (≤ 4 µg/ml)
Test ?
Identification of carbapenemase producers using the Modified Hodge Test

1: *K. pneumoniae* wild-type
2: *K. pneumoniae* NDM-1
3: *K. pneumoniae* KPC-2
4: carbapenem-resistant *K. pneumoniae* due to porin-loss
5: *K. pneumoniae* OXA-48
6: *E. coli* DH10B
How to detect carbapenemase production and identify the carbapenemase type in the meantime
Detection of class A carbapenemases (KPC)
KPC detection

*K. pneumoniae*  

*E. coli*  

(Nordmann, Cuzon, Naas Lancet Inf. Dis. 2009)
Evaluation of Boronic Acid Disk Tests for Differentiating KPC-Possessing *Klebsiella pneumoniae* Isolates in the Clinical Laboratory

Athanassios Tsakris, Ioulia Kristo, Aggeliki Poulou, Katerina Themeli-Digalaki, Alexandros Ikonomidis, Dimitra Petropoulou, Spyros Pournaras, and Danai Sofianou

Department of Microbiology, Medical School, University of Athens, Athens, Greece; Department of Microbiology, Medical School, University of Thessaly, Larissa, Greece; Department of Microbiology, Serres General Hospital, Serres, Greece; Department of Microbiology, Tzanio General Hospital, Pireas, Greece; Department of Microbiology, Saint Panteleimon General Hospital, Nicosia, and Department of Microbiology, Hippokration University Hospital, Thessaloniki, Greece.

Received 5 October 2008/Returned for modification 17 November 2008/Accepted 4 December 2008.

The worldwide increase in the occurrence and dissemination of KPC β-lactamases among gram-negative pathogens makes critical the early detection of these enzymes. Boronic acid disk tests using different antibiotic substrates were evaluated for detection of KPC-possessing *Klebsiella pneumoniae* isolates. A total of 57 genotypically confirmed KPC-possessing *K. pneumoniae* isolates with varying carbapenem MICs were examined. To measure the specificity of the tests, 106 non-KPC-possessing isolates (89 *K. pneumoniae* and 17 *Escherichia coli* isolates) were randomly selected among those exhibiting reduced susceptibility to ceftazidime, expanded-spectrum cephalosporins, or carbapenems. As many as 56, 53, and 30 of the non-KPC-possessing isolates harbored extended-spectrum β-lactamases, metallo-β-lactamases, and plasmid-mediated AmpC β-lactamases, respectively. By use of CLSI methodology and disks containing imipenem, meropenem, or ceftazidime, either alone or in combination with 400 μg of boronic acid, all 57 KPC producers gave positive results (sensitivity, 100%) whereas all 106 non-KPC producers were negative (specificity, 100%). The meropenem duplicate disk with or without boronic acid demonstrated the largest differences in inhibition zone diameters between KPC producers and non-KPC producers. By use of disks containing ceftepime, all isolates were correctly differentiated except for five AmpC producers that gave false-positive results (sensitivity, 100%; specificity, 95.3%). These practical and simple boronic acid disk tests promise to be very helpful for the accurate differentiation of KPC-possessing *K. pneumoniae* isolates, even in regions where broad-spectrum β-lactamases are widespread.
KPC: synergy with boronic acid

Disk diffusion synergy test:
IMP + boronic ac.

Disk combination test:
carbapenem + boronic ac.
Metallo-carbapenemase; detection

Walsh et al. JCM, 2002, 2755-9
Detection

Test +
Phenotypic tests: combination disks

MER: meropenem alone
DPA: +dipicolinic acid 1,000 µg
BOR: + boronic acid 600 µg
CLX: + cloxacillin 750 µg
Specific detection of OXA-48 producers
K. pneumoniae OXA-48
OXA-48 and resistance to temocillin
Mass spectrometry: MALDI-TOF

Protocol:
1) Broth culture with the strain to be tested + carbapenem: 3-6h
2) Mass spectrometry
3) if carbapenemase +:
   hydrolysis of the carbapenem molecule leading to a degradation product

Advantages:
Specific / sensitive
Fastness +
Cheap if you dispose from the machine!

Disadvantages:
Material price
Expertise

References:
Hrabák et al. JCM. 2011
Burckhardt et al. JCM. 2011
Hrabák et al. JCM. 2012
Another basic and useful method
(for expert labs)

- Measurement of carbapenem hydrolysis by UV spectrophotometry

- 10 µl of bacterial crude extract + 100 µM of imipenem
- wavelength: 297 nm

Bernabeu, Poirel & Nordmann, Diag Microbiol Infect Dis 2012
Molecular biology: PCR-based techniques

- **Real-Time PCR**: 
  - Check-MDR Real-Time PCR
  - Detect the presence of the carbapenemase gene
  - 4-5 h
  - Cost +++

- **Specific PCR +/- sequencing**: 
  - OXA-48-like / KPC / VIM / IMP / NDM
  - 3 to 5 h
  - Expertise ++
  - Cost +
High-throughput sequencing

Theorically, will allow:
- accurate identification
- obtention of a « virtual » antibiogram
- typing (criteria to be defined)

Preparation of the sample is critical
Gene expression level
Cost

<table>
<thead>
<tr>
<th>Generation</th>
<th>Chemistry</th>
<th>Platform</th>
<th>Throughput per run (bases)</th>
<th>Error rate (expressed as a Phred score)</th>
<th>Read length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second</td>
<td>Pyrosequencing</td>
<td>Life Sciences 454, e.g., GS FLX Titanium XL R70</td>
<td>450 Mb</td>
<td>Q30b</td>
<td>450</td>
</tr>
<tr>
<td>Second</td>
<td>Dye termination/synthesis</td>
<td>Illumina/Solexa, e.g., HiSeq 2500</td>
<td>120,000 Mb</td>
<td>Q15b</td>
<td>2×150 paired</td>
</tr>
<tr>
<td>Second</td>
<td>Ligation</td>
<td>AB SOLiD, e.g., 5500x1</td>
<td>20,000 Mb</td>
<td>Q27b</td>
<td>50</td>
</tr>
<tr>
<td>Third</td>
<td>Semiconductor</td>
<td>Ion Torrent, e.g., Ion PGM</td>
<td>60–100 Mb</td>
<td>Q15–Q20 est</td>
<td>100</td>
</tr>
<tr>
<td>Third</td>
<td>Direct detection</td>
<td>Pacific Biosciences RS</td>
<td>75–150 Mb, estimate</td>
<td>Unknown</td>
<td>1,500 est.</td>
</tr>
</tbody>
</table>

Dune et al., Eur J Clin Microbiol Infect Dis 2012;31:1719-26
NEXT GENERATION SEQUENCING

1. Detection by sequencing
2. Identification and resistance by sequencing
3. Pathogen and host response by sequencing
The Carba NP test

Carbapenems
- Imipenem
- Meropenem
- Ertapenem
- Doripenem

Carbapenemase

Acid production

Colorimetric detection - Optical reading

pH

Nordmann, Poirel, Dortet, EID 2011
Results

*K. pneumoniae CTX-M-15* + impermeability

*K. pneumoniae OXA-48*

*K. pneumoniae KPC-2*

*E. coli VIM-1*

*E. coli IMP-1*

*E. coli NDM-1*

Yellow = carbapenem hydrolysis
Question: any carbapenemase here?

K. pneumoniae

K. pneumoniae

E. coli

E. coli

E. coli
Question: any carbapenemase here?

*K. pneumoniae* CTX-M15 + impermeability

*E. coli VIM-1*

(-) 2 hours

*K. pneumoniae OXA-48*

(+) 30 min

*K. pneumoniae KPC-2*

(+) 5 min

*E. coli VIM-1*

(+30 min

*E. coli IMP-1*

(+25 min

*E. coli NDM-1*

(+20 min
Infections

J 0

Carba NP test

Carriage

Feaces, rectal swabs

J 1

Screening media

J 2

Carba NP test

Molecular Biology

J 3

Molecular identification of the carbapenemases (sequencing)

Urine

Other specimens

Blood cultures
The Carba NP test

1- Rapid; less than 2 h
2- Sensitive; 98% (1,600 tested strains, French National Reference Center)
3- Specific: 100%
4- Detection of any type carbapenemase activity
5- Cheap : 0.5 euro
6- Easy-to-handle
7- Implementable worldwide
This test is now commercially available

RAPIDEC® CARBA NP
Leading the charge on Carbapenemases
Therefore OK for clinical specimens...

But what about the screening of patients for colonization?
Selective media for screening purpose

Drigalski + ertapenem disk

**Advantages**: Easy, low cost

**Disadvantages**: Carba NP test impossible
Weak inoculum
Selective media for screening purpose

Drigalski + ertapenem disk

Media containing 3GC

Ex: ChomID ESBL
Media supplemented with cephalosporins

Lack of detection of OXA-48+ but ESBL- isolates

*K. pneumoniae* OXA-48

ESBL +

*K. pneumoniae* OXA-48

ESBL -
Selective media for screening purpose

- Drigalski + ertapenem disk
- Media containing 3GC
- Media supplemented with a carbapenem

CHROMAGAR KPC
ChromID Carba
Brilliance CRE
SUPERCARBA
Selective media containing carbapenem

- **CHROMAGAR KPC** (Chromagar): Meropenem + chromogenic molecules
  - High concentration of carbapenem → lack of detection if weak hydrolysis of carbapenems (ex: OXA-48) (susceptibility 43%, specificity 67.8%)
  - Necessity to prepare home-made plates

- **ChromID Carba** (bioMérieux): Carbapenem (?) + chromogenic
  - Sensitivity 97.4% [93.4-99.3] \{ according to the manufacturer
  - Specificity 99.7% [98.9-100.0]
  - Problem of detection of OXA-48 (personal data)

- **Brillance CRE agar** (Oxoid): Carbapenem (?) + chromogenic
  - Sensitivity 76.3%
  - Specificity 57.1%

---

New screening medium

SUPERCARBA medium

- Drigalski for selection of Gram negative rods
- Medium supplemented with a carbapenem
  (Inhibition of ESBL+ isolates susceptible to carbapenems)
- Medium supplemented with cloxacillin
  (Inhibition of isolates being resistant to carbapenems because of AmpC overexpression)
- Medium supplemented with ZnSO₄

New screening medium
(SUPER CARBA medium ©)

Combine several advantages

- Excellent sensitivity (96.5%)
- Also for OXA-48 producers thanks to low concentrations of the carbapenem
- Also for MBL producers (including NDM) thanks to the addition of zinc ions

<table>
<thead>
<tr>
<th></th>
<th>SUPERCARBA</th>
<th>Brillance CRE</th>
<th>CHROMagar KPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>96.5</td>
<td>76.3</td>
<td>43</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>60.7</td>
<td>57.1</td>
<td>67.8</td>
</tr>
<tr>
<td>Sensitivity class A</td>
<td>100</td>
<td>85</td>
<td>70</td>
</tr>
<tr>
<td>Sensitivity class B</td>
<td>92</td>
<td>78.4</td>
<td>58.8</td>
</tr>
<tr>
<td>Sensitivity class D</td>
<td>100</td>
<td>69.8</td>
<td>11.6</td>
</tr>
</tbody>
</table>

Conclusion

- Increased prevalence of reported carbapenemases in *Enterobacteriaceae* worldwide.

- Carbapenemases producers are spreading in hospitals and now in the community. This is the cases for NDM and OXA-48 producers. They are already out of control in many areas in the world.

- Microbiology: diagnostic techniques are now available for an accurate diagnostic. They should be implemented NOW.

- Infectious Diseases and Hygiene:
  - Carbapenem stewardship
  - Outbreak prevention and control

- Fundamental research; identification of novel targets and development of novel antibiotics, in particularly carbapenemase inhibitors
GRACIAS

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