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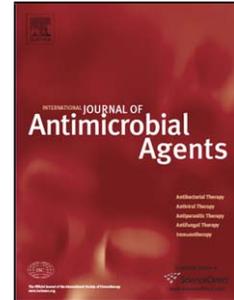
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Guideline for phenotypic screening and confirmation of carbapenemases in Enterobacteriaceae

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ABSTRACT

Adequate detection of carbapenemase-producing Enterobacteriaceae is crucial for infection control measures and appropriate choice of antimicrobial therapy. This guideline aims to improve the detection of carbapenemase-producing Enterobacteriaceae in the routine setting of clinical microbiology laboratories. Detection of carbapenemases in Enterobacteriaceae includes a screening step followed by a genotypic and optional phenotypic confirmatory step. For all Enterobacteriaceae, the meropenem screening breakpoint to detect carbapenemases is set at ≥ 0.5 mg/L or a zone diameter of ≤ 23 mm (10 μ g disk loading). For *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp. and *Citrobacter* spp., the imipenem screening breakpoint is set at ≥ 2 mg/L or a zone diameter ≤ 21 mm. Ertapenem is not advised as an indicator carbapenem as it has a lower specificity compared with imipenem and meropenem. On the first isolate from a patient with a positive carbapenemase screen test, a polymerase chain reaction (PCR)-based test should be performed to detect carbapenemase genes. However, if genotypic confirmation is not immediately available, phenotypic confirmation tests should be performed to avoid delayed reporting of carbapenemase-producers to the clinic. Recommended phenotypic confirmation tests are the modified Hodge test as well as carbapenemase inhibition tests with boronic acid for Ambler class A carbapenemases and with ethylene diamine tetra-acetic acid (EDTA) or dipicolinic acid for metallo-carbapenemases.

1. Introduction

The rapid emergence and dissemination of Enterobacteriaceae that are resistant to carbapenems such as imipenem and meropenem poses a considerable threat to clinical patient care and public health. Carbapenemase-producing strains are characterised by their resistance to virtually all β -lactam antibiotics, including the cephalosporins and carbapenems, as well as to fluoroquinolones, aminoglycosides and co-trimoxazole. Invasive infections with these strains are associated with high rates of morbidity and mortality [1,2].

The carbapenemases fall into three classes according to their amino acid sequence: Ambler class A (serine carbapenemases); class B (metallo-carbapenemases); and class D (OXA carbapenemases). Within these classes, further divisions are made (see Table 1) and new variants are frequently encountered [3]. The rapid emergence and spread of carbapenemase-producing strains is mainly caused by epidemics of bacteria bearing plasmid-mediated KPC (class A), VIM-1 and NDM (class B) and OXA-48 (class D) enzymes. Carbapenem minimum inhibitory concentrations (MICs) observed in carbapenemase-producing microorganisms can exhibit considerable variation depending on the type and expression of carbapenemase enzyme, the bacterial species and the presence of other resistance mechanisms such as cephalosporinases [extended-spectrum β -lactamase (ESBL) and AmpC], reduced permeability and/or efflux pumps [4–6]. Increased carbapenem MICs in Enterobacteriaceae may also result from high expression of AmpC or CTX-M ESBLs in combination with porin alterations [5,7].

2. Purpose of this guideline

This guideline aims to standardise and improve carbapenemase detection in Enterobacteriaceae for routine diagnostics in clinical microbiology laboratories. Although this guideline reflects, as much as possible, the current knowledge on carbapenemases, regular updating will remain necessary owing to ongoing technological developments and emergence of new carbapenemase variants.

Adequate detection of carbapenemase-producing microorganisms in the routine diagnostic laboratory is essential for patient care because (i) it is vital for the correct choice of antibiotic therapy and (ii) appropriate hospital hygiene precautions are indicated for patients harbouring carbapenemase-producing microorganisms as these strains are associated with multiresistance and epidemics that result in reduced patient safety and increased costs.

3. Strategy for carbapenemase detection

The detection strategy includes a screening step followed by a phenotypic and genotypic confirmation step (see Fig. 1).

Screening is based on detection of reduced susceptibility to carbapenems by carbapenemase-producing isolates compared with isolates of the wild-type population (Table 2). For each class of carbapenemases, and for each species and isolate, the MIC may vary from MICs of the wild-type population to >256 mg/L, dependent on the presence of other resistance mechanisms.

Setting of the screening breakpoints should therefore be guided by the following principles:

1. the breakpoint MIC should be higher than the highest MIC of the wild-type population (<http://www.eucast.org>), as the specificity of the screening test may otherwise become too low; and
2. the MIC breakpoint should be lower than the lowest carbapenem MICs described in the literature for strains shown to have a carbapenemase gene.

3.1. Meropenem

Based on these criteria, the MIC screening breakpoint for meropenem could be set at ≥ 0.5 mg/L for all Enterobacteriaceae (Table 2), enabling detection of the vast majority of carbapenemase-producers. Only sporadic carbapenemase-producers with meropenem MICs < 0.5 mg/L will not be detected using this breakpoint [4].

The zone diameter screening breakpoint for meropenem has been set at ≤ 23 mm. The meropenem zone diameter breakpoint is slightly less sensitive than the MIC screening breakpoint of 0.5 mg/L (84% vs. 100%, respectively) [5], but it was shown to detect all VIM- and KPC-producing isolates [9]. Although a meropenem zone diameter screening breakpoint of ≤ 27 mm was reported to have a sensitivity of 100% for carbapenemase production [5], this would result in an unacceptably high level of false-positive isolates based on the published meropenem zone diameter distributions (<http://www.eucast.org>).

3.2. Imipenem

For imipenem it is not possible to set a breakpoint for all Enterobacteriaceae as some species (*Proteus* spp., *Serratia* spp., *Providencia* spp. and *Morganella morganii*) have a high imipenem MIC owing to mechanisms other than carbapenemase production (see Table 2 for epidemiologic cut-offs for imipenem MICs for these species). For pragmatic reasons, and based on the available wild-type MIC distributions, we have chosen to make a distinction in this guideline between species for which the MIC for imipenem can be used as a breakpoint and species for which it cannot. For *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp. and *Citrobacter* spp. the imipenem breakpoint is ≥ 2.0 mg/L or a zone diameter ≤ 21 mm. Although the imipenem MIC screening breakpoint of ≥ 2.0 mg/L was shown to have a sensitivity of 79% [5], the breakpoint was not set lower because the imipenem MIC distribution of the wild-type population is up to 1 mg/L (Table 2) (<http://www.eucast.org>). It has been shown that the sensitivity of the imipenem zone diameter screening breakpoint ≤ 21 mm was 100% [5,9].

3.3. Ertapenem

Ertapenem is not advised as an indicator carbapenem in this guideline since it has lower specificity than imipenem and meropenem. Ertapenem is less specific because isolates with AmpC/ESBL and decreased permeability have higher MICs for ertapenem than for imipenem or meropenem [7]. However, an ertapenem screening breakpoint of 0.5 mg/L could be used (Table 2).

Phenotypic confirmation of carbapenemase production is based on detection of a diffusible carbapenemase and in vitro inhibition of carbapenemase activity upon

addition of an inhibitor. The genotypic confirmation consists of polymerase chain reaction (PCR) detection and sequencing of carbapenemase genes.

3.4. Screening methods for carbapenemases

Carbapenemase screening should be a standard component of the susceptibility testing on all Enterobacteriaceae isolated in routine diagnostics. This can take place by assessing the carbapenem MICs or by an alert from the expert system (see Fig. 1).

When using automatic systems for susceptibility testing (e.g. Phoenix, Vitek or MicroScan), panels containing meropenem are preferred. The preferred lowest concentration in the panels should be 0.25 mg/L for meropenem and 1 mg/L for imipenem. If ertapenem is used then the preferred lowest concentration should be 0.25 mg/L.

The laboratory should be aware that strains with an MIC above the carbapenemase screening breakpoint but below the clinical breakpoint might nevertheless have a carbapenemase gene (see Table 2). Strains with an MIC for meropenem of 0.5, 1 or 2 mg/L or an MIC for imipenem of 2 mg/L are sensitive according European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints but should still be tested for the presence of a carbapenemase gene (Table 1).

Use of a correct inoculum is important both for broth microdilution methodology and the automatic systems since a moderate decrease in the inoculum may lead to inaccurate susceptibility results. It was shown in a study with 29 KPC-positive

isolates that if the standard inoculum was decreased from 10^5 to 10^4 colony-forming units/mL, the imipenem MIC was on average 1.6 doubling dilutions lower [1].

To exclude technical errors and to limit the number of strains to be confirmed for carbapenemase production, a carbapenem MIC above the screening breakpoint measured by an automatic system can be confirmed by Etest (bioMérieux, Marcy l'Etoile, France) with meropenem or imipenem (Fig. 1) on Muller–Hinton agar (Iso-Sensitest agar may underestimate carbapenem MICs of metallo-carbapenemase-producers owing to low zinc concentrations [10]). Determining the MIC of carbapenemase-positive strains by Etest can be complicated because mutant colonies with higher MICs than the dominant population may be found in the inhibition ellipse. These colonies should be included when interpreting the Etest in accordance with the manufacturer's instructions.

3.5. Phenotypic confirmation of carbapenemase production

On the first isolate from a patient with a positive carbapenemase screen test, a PCR-based test should be performed to confirm the presence of carbapenemase genes (Fig. 1). However, if genotypic confirmation is not immediately available, phenotypic confirmation tests can be performed in order to avoid delayed reporting of potential carbapenemase-producers to the clinic. Phenotypic confirmation may be performed using one or two methods, the modified Hodge test and the carbapenemase inhibition tests [5,11,12]. The modified Hodge test is used for detection of diffusible carbapenemases, and the inhibition tests are used to distinguish between the different classes of carbapenemases (Tables 3 and 4). Test characteristics and

validation reports of the inhibition tests for the routine setting, only some of which are commercially available, are limited [11].

3.5.1. Modified Hodge test

The modified Hodge test (Fig. 2) should be performed according to the guidelines of the Clinical and Laboratory Standards Institute [13]. The modified Hodge test has a high sensitivity (95–100%) [5,11]. Disadvantages of this test are possible interpretation difficulties and the fact that different classes of carbapenemases cannot be distinguished. Specificity may be low because CTX-M ESBL- or AmpC-producing isolates with reduced or absent porin expression may give false-positive results [5,12]. However, for detection of class A carbapenemases the specificity of the modified Hodge test can be increased by modifying the test as described by Pasteran et al. [12] (see Fig. 2).

3.5.2. Inhibition tests of carbapenemase activity (synergy tests)

The confirmation step is based on in vitro inhibition of carbapenemase activity by addition of an inhibitor specific for a class of carbapenemases (resulting in a reduction in the MIC for the carbapenem). This phenomenon is called synergy between the carbapenem and the inhibitor.

For detection of class A carbapenemases, the inhibitor used is boronic acid (3-aminophenylboronic acid). For detection of class B metallo-carbapenemases, ethylene diamine tetra-acetic acid (EDTA) or dipicolinic acid can be used as an inhibitor.

It is recommended that these tests be carried out using either combination disks or an Etest strip that contains both a carbapenem and an inhibitor. Table 3 shows the details of the combination tests as recommended by a group of experts from EUCAST and the ESCMID Study Group for Antibiotic Resistance Surveillance (ESGARS) as well as how these should be interpreted [11]. Double-disk synergy tests (disk approximation methods) are not recommended since the sensitivity depends on the optimal distance between the disks, which cannot be predicted.

3.6. Quality control

The following strains are recommended for quality control: *Klebsiella pneumoniae* ATCC BAA-1705 (KPC-positive); *E. coli* ATCC 25922 (carbapenemase-negative); and *K. pneumoniae* ATCC BAA 1706 (resistant to carbapenems by mechanisms other than carbapenemase, modified Hodge test-negative).

3.7. Genotypic confirmation

Genotypic confirmation comprises PCR detection and sequencing of carbapenemase genes. The high diversity of genes with ever-increasing numbers of new variants implies that isolates with a negative genotypic result in the local laboratory setting should be sent to a reference laboratory for further genotypic confirmation.

In The Netherlands, the reference centre is a collaboration between RIVM (Centre for Infectious Disease Control) and the Medical Microbiology Laboratory of University Medical Centre Utrecht. The carbapenemase genes named in Table 1 can currently

be detected by PCR and sequencing at the University Medical Center Utrecht using in-house PCRs as well as recently published PCRs [14].

3.8. Report to the clinic

Strains confirmed to be carbapenemase-positive should be reported to the clinic as susceptible or resistant according to the clinical breakpoints of EUCAST (<http://www.eucast.org>). Strains shown to have a carbapenemase gene and an MIC for meropenem of 0.5, 1 or 2 mg/L or an MIC for imipenem of 2 mg/L are therefore reported as susceptible. The clinical significance of the presence of a carbapenemase gene in an isolate with a carbapenemase MIC below the clinical EUCAST breakpoint is currently unknown. The published data on this issue are controversial since a few case reports described carbapenem therapy failure in patients infected with KPC-positive *K. pneumoniae* with carbapenem MICs in the susceptible range [15,16]. In contrast, two studies from Greece in patients with bloodstream infections observed no difference in mortality between VIM-negative *K. pneumoniae* infections and infections with VIM-positive *K. pneumoniae* having an imipenem MIC \leq 4 mg/L. Mortality during carbapenem treatment was increased in those patients with VIM-positive *K. pneumoniae* infections and an imipenem MIC $>$ 4 mg/L [17,18]. A comment should therefore be added to the results to indicate that the susceptibility of the microorganism to carbapenems is not clear and that treatment of any infection should be determined in consultation with a microbiologist-physician or an infectious diseases consultant.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Detection scheme for carbapenemases in Enterobacteriaceae. ^a The zone diameter screening breakpoint for meropenem has been set at ≤ 23 mm with a disk content of 10 μg . For *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. the zone diameter screening breakpoint for imipenem has been set at ≤ 21 mm with a disk content of 10 μg [5]. MIC, minimum inhibitory concentration; APB, 3-aminophenylboronic acid; DPA, dipicolinic acid; EDTA, ethylene diamine tetra-acetic acid.

Fig. 2. Protocol for the modified Hodge test. (1) A 0.5 McFarland suspension of *Escherichia coli* ATCC 25922 should be diluted 1:10 in physiological saline solution (NaCl 0.9%). (2) Inoculate a Muller–Hinton agar plate with this dilution as for a standard Etest or disk diffusion test. (3) Place a 10 μg meropenem, imipenem and ertapenem disk on the plate. (4) Use an inoculation loop or swab to pick up three to five colonies from an overnight culture of an isolate suspected of carbapenemase production and streak from the edge of the carbapenem disk. The streak should be 20–25 mm long. (5) Incubate overnight at 35 ± 2 °C in an aerobic atmosphere. (6) Following incubation, examine the intersection of the edge of the inhibition zone and the streak of the test isolate (see figure). The test result is non-determinable if the growth of *E. coli* ATCC 25922 is inhibited by the test isolate (inhibition zone parallel to the streak of the test isolate). Further modification of modified Hodge test ('double-modified Hodge test'): to avoid false-positive results of the modified Hodge test in isolates producing AmpC and/or extended-spectrum β -lactamase (ESBL) with decreased permeability, the test may be additionally performed with disks containing a carbapenem plus oxacillin to inhibit AmpC β -lactamases and with disks containing

a carbapenem plus 3-aminophenylboronic acid to inhibit AmpC and class A carbapenemases [12].

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Table 1

Carbapenemase genes

Ambler Class A	9 families (KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC, IBC)
Ambler Class B	6 families (VIM, GIM, SIM, NDM, IMP, SPM)
Ambler Class D	2 families (OXA, PSE)

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Table 2

Carbapenem screening breakpoints, clinical breakpoints and epidemiological cut-off values (mg/L)

Species	Meropenem		Imipenem		Ertapenem	
	<i>Escherichia coli</i> , <i>Klebsiella</i> spp., <i>Enterobacter</i> spp., <i>Salmonella</i> spp.	<i>Citrobacter</i> spp., <i>Serratia</i> spp., <i>Proteus mirabilis</i> , <i>Morganella morganii</i> , <i>Providencia</i> spp.	<i>E. coli</i>	<i>Klebsiella</i> spp., <i>Enterobacter</i> spp., <i>Salmonella</i> spp., <i>Citrobacter</i> spp.	<i>Serratia</i> spp., <i>P. mirabilis</i> , <i>M. morganii</i> , <i>Providencia</i> spp.	<i>E. coli</i> , <i>Klebsiella</i> spp., <i>Enterobacter</i> spp., <i>Salmonella</i> spp., <i>Serratia</i> spp., <i>P. mirabilis</i> , <i>M. morganii</i> , <i>Providencia</i> spp.
Carbapenemase screening breakpoint	≥0.5 ^a	≥0.5 ^a	≥2 ^b	≥2 ^b	N/A	≥0.5 ^c
Epidemiological cut-off wild-type	S ≤0.125	S ≤0.25	S ≤0.5	S ≤1	S ≤4 (<i>Serratia</i> S ≤2)	S ≤0.064
EUCAST breakpoint	S ≤2	S ≤2	S ≤2	S ≤2	S ≤2	S ≤0.5
CLSI breakpoint	S ≤1	S ≤1	S ≤1	S ≤1	S ≤1	S ≤1

N/A, not applicable; S< susceptible; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute.

- ^a The zone diameter screening breakpoint for meropenem has been set at ≤ 23 mm with a disk content of 10 μg .
- ^b For *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. the zone diameter screening breakpoint for imipenem has been set at ≤ 21 mm with a disk content of 10 μg .
- ^c No zone diameter screening breakpoint has been set for ertapenem owing to the low specificity, although a breakpoint of ≤ 27 mm (disk content 10 μg) was reported to have a sensitivity of 97% and a specificity of 90.5% for KPC-producing *K. pneumoniae* and *E. coli* [8].

Table 3

Phenotypic carbapenemase confirmation tests for Class A and B carbapenemases

Carbapenemase	Method	Antibiotic	Concentration	Inoculum	Medium	Carbapenemase positive when:	References
Class A	Combination diffusion tablet (Rosco)	Meropenem + boronic acid	Meropenem 10 µg	0.5 McF	MHA	Rosco: an increase of ≥5 mm of the zone surrounding the combination of meropenem + boronic acid relative to the zone with meropenem only	http://www.rosco.dk

	Combination diffusion disk (in-house)	Meropenem + boronic acid	Meropenem 10 µg, boronic acid 600 µg	0.5 McF	MHA	In-house disks: an increase of ≥4 mm of the zone surrounding the combination of meropenem + boronic acid relative to the zone with meropenem only	[4]
Class B	Combination diffusion tablet (Rosco)	Imipenem + EDTA ^a	Imipenem 10 µg, EDTA 750 µg	0.5 McF	MHA	Rosco: an increase of ≥7 mm of the zone surrounding the combination of imipenem + EDTA relative to the zone with imipenem only	http://www.rosco.dk

Combination diffusion disk (in-house)	Imipenem + EDTA ^a	Imipenem 10 µg, EDTA 0.5 M	0.5 McF	MHA	In-house disks: an increase of ≥5 mm of the zone surrounding the combination of imipenem + EDTA relative to the zone with imipenem only	[4]
Combination diffusion tablet (Rosco)	Meropenem + DPA	Imipenem 10 µg	0.5 McF	MHA	Rosco: an increase of ≥5 mm of the zone surrounding the combination of imipenem + DPA relative to the zone with imipenem only	http://www.rosco.dk

MBL Etest ^b	Imipenem + EDTA ^a	N/A	0.5 McF	MHA	MIC ratio ≥ 8 or phantom effect or deformation of ellipse	http://www.abbiodisk.com
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McF, McFarland; MHA, Mueller–Hinton agar; EDTA, ethylene diamine tetra-acetic acid; DPA, dipicolinic acid; MBL, metallo- β -lactamase; N/A, not applicable; MIC, minimum inhibitory concentration.

^a The brand of Muller–Hinton agar may influence the test characteristics of the metallo-carbapenemase inhibition tests with EDTA [10].

^b To avoid false-negative results in MBL-producing isolates with imipenem MICs ≤ 4 mg/L, and according to the instructions of the manufacturer, the result of the MBL Etest should be interpreted as non-determinable if the Etest result is imipenem < 4 mg/L and imipenem + EDTA < 1 mg/L.

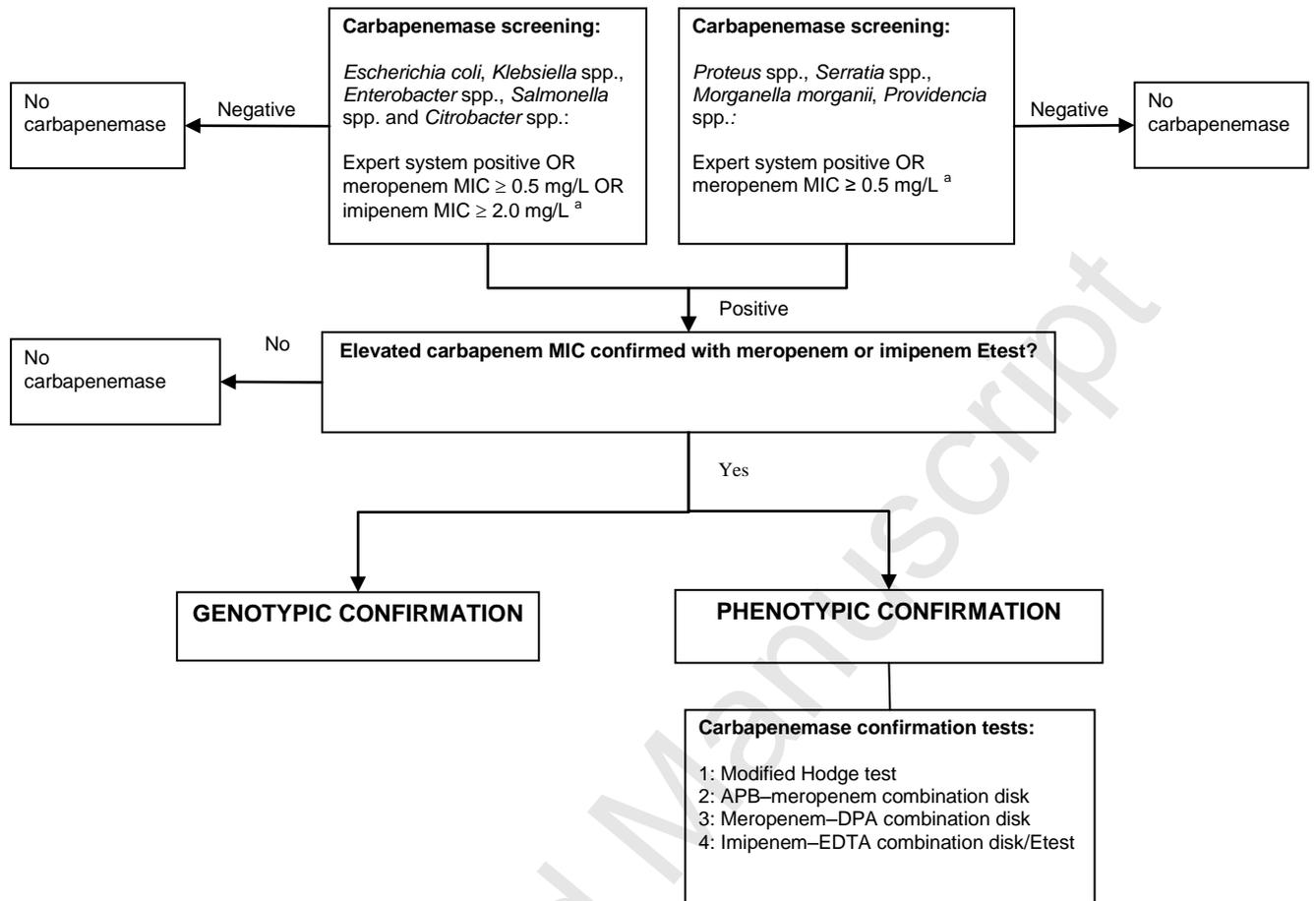
Table 4

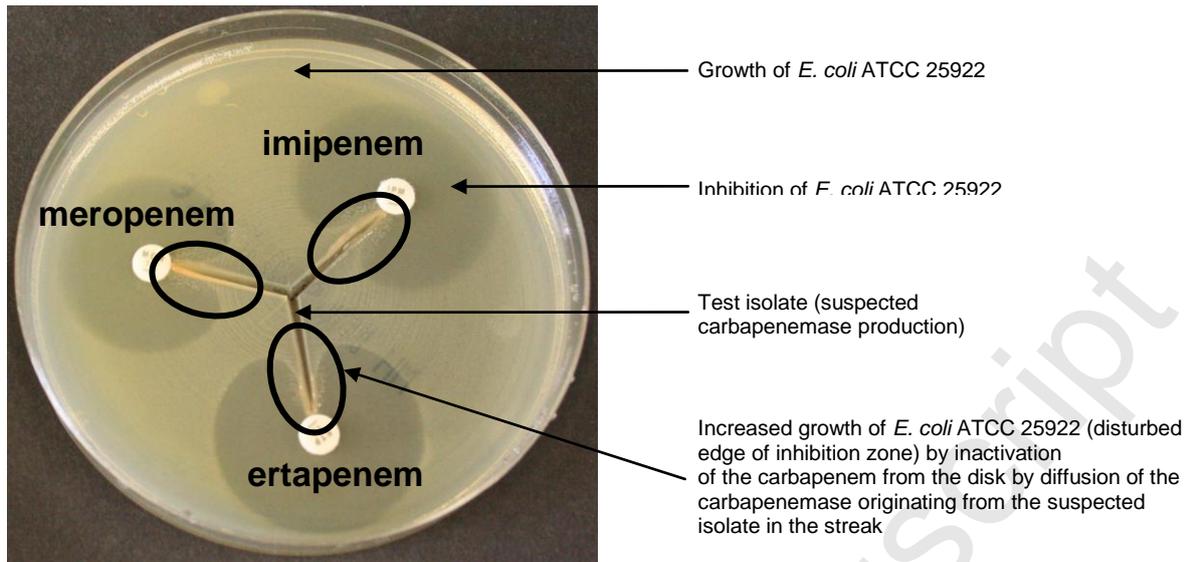
Interpretation chart of phenotypic carbapenemase confirmation tests

Confirmation test	Carbapenemase			AmpC with reduced permeability	ESBL with reduced permeability
	Class	Class	Class		
	A	B	D		
Modified Hodge test	+	+	+	+/-	+/-
Meropenem \pm boronic acid	+	-	-	+/-	-
Meropenem \pm Cloxacillin ^a	-	-	-	+/-	-
Imipenem \pm EDTA	-	+	-	-	-
Meropenem \pm DPA	-	+	-	-	-

ESBL, extended-spectrum β -lactamase; EDTA, ethylene diamine tetra-acetic acid;
DPA, dipicolinic acid.

^a Disk content 10 μ g (meropenem) plus 750 μ g (cloxacillin) (Rosco).





Example of positive modified Hodge test (photo Neil Woodford, Health Protection Agency, UK)

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