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Detection of carbapenemases in *Enterobacteriaceae*: a challenge for diagnostic microbiological laboratories

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Abstract

Carbapenemase-producing bacteria have now spread all over the world. Infections caused by those bacteria are difficult to treat. Therefore, there is an urgent need for accurate and fast detection of carbapenemases in diagnostic laboratories. In this review, we summarize screening methods for suspected isolates, direct assays for confirmation of carbapenemase activity (e.g. the Carba NP test and matrix-assisted laser desorption ionization time-of-flight mass spectrometry carbapenem hydrolysis assay), inhibitor-based methods for carbapenemase classification, and molecular-genetic techniques for precise identification of carbapenemase genes. We also propose a workflow for carbapenemase identification in diagnostic laboratories.

Keywords: β-Lactamase, Carba NP, carbapenemase, detection, *Enterobacteriaceae*, MALDI-TOF MS
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Introduction

Progress in human medicine, especially in surgery, transplantology, neonatology, haemato-oncology, and intensive medicine, has been followed by prolonged hospitalization, and excessive and long-term antibiotic treatment of patients enfeebled by immunodeficiencies and invasive procedures. In the environment of intensive-care units (ICUs), incubators, and haemato-oncology cleanrooms, nosocomial infections have become a serious problem, with decisive effects on mortality rates and treatment outcomes [1]. In an international study on the prevalence and outcomes of infections in ICUs with >1200 participants, 51% of patients were considered to be infected, and the mortality rate of the infected patients was at least twice as high as that of the uninfected patients [2]. Members of the *Enterobacteriaceae* are among the major causative agents of nosocomial infections [3].

At the beginning of a new millennium, multiresistant enterobacteria producing extended-spectrum β-lactamases (ESBLs) and acquired AmpC-type cephalosporinases have already spread worldwide, mainly as nosocomial pathogens, but also in the community [4]. It is difficult to control all of the factors responsible for the spread of carbapenemase-producing bacteria. However, the high consumption of carbapenem antibiotics connected with this epidemic situation has been one of the main reasons for their rapid spread. Additionally, other factors may include co-selection by other antibiotics (e.g. cephalosporins), spread of those bacteria in community, uncontrolled spread in the hospital environment, owing to undiagnosed carriers, and other, still unknown, factors [5,6].

Carbapenemase-producing *Enterobacteriaceae* (CPE) have already been detected all over the globe, with a marked endemicity according to enzyme type. In Europe, the most critical situations have been reported in Greece, with 60.5% carbapenem-resistant *Klebsiella pneumoniae* (a total of 1460 isolates), and in Italy, with 28.8% carbapenem-resistant *K. pneumoniae* (a total of 841 isolates), recovered from blood within the EARS-Net project in 2012 (EARS Report 2012). The prevalence of CPE in the community is not well known, and it is most probably diverse, as for ESBL-producing *Escherichia coli*

(3–5% in France; >80% in India) [5]. Besides medical settings, carbapenemase-producing bacteria have been detected in environmental samples, especially from sewage water, as well as in veterinary and wildlife samples [7–10].

Enterobacteriaceae are known as some of the most common pathogens causing both community-acquired and hospital-acquired infections, including infections of the urinary and gastrointestinal tracts, pneumonia, peritonitis, meningitis, sepsis, and medical device-associated infections. The infections caused by CPE are associated with significant mortality. The mortality rates revealed in small clinical studies ranged from 22% to 72%. [11,12]. The reasons for this are most probably multifactorial, including underlying diseases, delays in the initiation of effective therapy, and the lack of effective antimicrobials [13].

The production of acquired carbapenemases makes the choice of antibiotic treatment of infections caused by Gram-negative bacteria very limited. The horizontal transmission of carbapenamase genes mediated by mobile genetic elements carrying additional resistance elements, which confer resistance to various groups of antibiotics, results in multidrug resistance, including bacteria resistant to all available antibiotics [14]. *In vitro* susceptibility to colistin, tigecycline and aminoglycosides is mostly preserved, but the impact of these antibiotics *in vivo* is still uncertain and the mortality rates remain high, despite treatment according to the results of susceptibility testing [15]. Some studies have suggested that combination therapy could be associated with a better outcome than monotherapy [15,16]. However, there is still not an equal substitute for carbapenem antibiotics in the treatment of severe infections caused by multiresistant Gram-negative bacteria.

As a part of the human intestinal flora, *Enterobacteriaceae* are easily spread (hand carriage, and contaminated food and water) and difficult to eliminate, especially in countries with low levels of hygiene. An alarming example has been the successful worldwide spread of *E. coli* ST131 producing CTX-M-15 [4,17]. High rates of, mainly, community-onset extraintestinal infections caused by this successful clone have been detected in Europe, North America, Japan, and Korea. Even higher rates are estimated from limited data in Asia, the Middle East, and Africa. The same clone has been reported in companion animals, non-companion animals, and foods [17]. The epidemic situation regarding CPE will probably develop in the same way as for ESBL producers. The only hope is an efficient infection control strategy applied in as many countries as possible.

Data from Israel have shown that nosocomial infections caused by carbapenem-resistant *Enterobacteriaceae* can be controlled by strict epidemiological intervention. Acquisition of CPE declined in acute care from a monthly high of 55.5 cases per 100 000 patient-days to an annual low of 4.8 cases per 100 000 patient-days [18]. However, for such a successful intervention, there is an urgent need for diagnostic laboratories to introduce rapid and sensitive methodologies for the detection of carbapenemase producers.

Screening for carbapenemase producers

Susceptibility of CPE and indicator antibiotics

Proper selection of suspicious isolates with reduced susceptibility to indicator antibiotics (e.g. carbapenems) is crucial for the identification of carbapenemase-producing isolates. These isolates should be further examined for confirmation of carbapenemase production. However, some carbapenemase-producing bacteria might show lower MICs for carbapenems than the clinical breakpoints proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). MIC distributions are available from http://mic.eucast.org/ (accessed 28 December 2013) and the CLSI [19]. Therefore, previous studies suggested that enterobacterial isolates showing even a small reduction in susceptibility to carbapenems should be further examined for carbapenemase production [20].

Recently, the EUCAST subcommittee for detection of resistance mechanisms and specific resistance of clinical and/or epidemiological importance published a recommendation for detection of CPE (EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_guidelines_detection_of_resistance_mechanisms_121222.pdf (accessed 28 December 2013)). Screening cut-off values were proposed for meropenem, imipenem, and ertapenem (Table 1). Meropenem was additionally proposed as the indicator antibiotic with the best balance of sensitivity and specificity, but, for bacteria producing OXA-48-type β-lactamases, temocillin was proposed as an indicator antibiotic with high sensitivity, as those isolates showed very high MICs for this antibiotic (≥256 mg/L) [21]. However, the specificity of this feature remains to be more extensively evaluated [22].

Huang *et al.* [23] recently published a precise analysis of 1354 enterobacterial isolates, including 435 confirmed carbapenemase producers. Most of the analysed CPE possessed class D OXA-48-type β-lactamases. On the basis of their data, it can be hypothesized that the current cut-off values for carbapenems proposed by the EUCAST were not able to identify a significant number of OXA-48 producers. In geographical areas with a high prevalence of CPE, including

TABLE 1. Clinical breakpoints of selected carbapenems proposed for *Enterobacteriaceae*, and European Committee on Antimicrobial Susceptibility Testing (EUCAST) proposals for screening cut-offs for the detection of carbapenemases

	Clinical breakpoints (susceptible category)				Screening cut-off	
	MIC (mg/L)		Disk diffusion inhibition zone diameter (mm)			
	EUCAST	CLSI	EUCAST	CLSI	MIC (mg/L)	Disk diffusion inhibition zone diameter (mm)
Ertapenem	≤0.5	≤0.25	≥25	≥23	>0.125	<25
Imipenem	≤2	≤1	≥22	≥23	>1	<23
Meropenem	≤2	≤1	≥22	≥23	>0.125	<25

OXA-48 producers, the use of temocillin disks (30 μg) and piperacillin–tazobactam disks (100/10 μg) can significantly increase the sensitivity of screening procedures. New cut-offs were proposed for those antibiotics (temocillin, <12 mm; piperacillin–tazobactam, <16 mm) [23]. The use of the combination of modified zone diameter cut-offs can exclude the presence of any carbapenemases that could have been missed with other selection criteria, and avoid unnecessary additional testing for confirmation of CPE [23].

Recently, Hartl *et al.* [24] proposed the determination of MICs for temocillin (cut-off of ≥128 mg/L) combined with the meropenem double-disk synergy test (DDST) as an algorithm that could be introduced into diagnostic laboratories for the identification of carbapenemase production and differentiation of Ambler classes in CPE.

Another screening carbapenem antibiotic, faropenem, was proposed by Day *et al.* [25]. They tested 166 enterobacterial strains producing various carbapenemases, and 82 non-carbapenemase producers. In most carbapenemase-producing strains, no inhibition zones (i.e. zone diameter of 6 mm) around the faropenem disk (10 μg) were observed. For OXA-48 producers, inhibition zone diameters ranged from 6 mm to 12 mm. For all but one OXA-48 producer with an inhibition zone of >6 mm, large numbers of colonies growing up to the disk were observed. MICs of faropenem were ≥64 mg/L for all VIM-producing, IMP-producing and KPC-producing strains, and 99% of NDM-1-producing strains. Faropenem MICs of OXA-48-producing strains ranged from 2 mg/L to 32 mg/L. Similar results were obtained in five of 82 non-carbapenemase-producing *Enterobacteriaceae* with the disk diffusion test. Four non-carbapenemase-producing strains had MICs of >32 mg/L. The authors of this study hypothesized that the faropenem disk diffusion test can be used as a sensitive (99%) and specific (94%) screen for carbapenemase-producing bacteria. Similar results (sensitivity of 98% and specificity of 87%) were confirmed in the collection of isolates referred to a UK reference laboratory. However, further validation of this test should be performed.

Finding specific and sensitive indicator antibiotics that are similar to cefoxitin for screening of methicillin-resistant *Staphylococcus aureus* is a challenge for the diagnosis of carbapenemase-producing bacteria. Because of the great diversity of carbapenemases, with different biochemical properties, solution of this problem will be difficult.

Screening cultivation media

The recommended samples for the screening of CPE are faecal or rectal swabs, as gastrointestinal carriers represent the most important potential source of cross-transmission in the healthcare setting. All patients at risk (e.g. patients repatriated from a foreign country or from a healthcare setting with a high prevalence of CPE, patients in ICUs, transplant patients, and immunocompromised patients) should be screened. The patients should be kept in strict isolation until a negative result is announced (at least 24–48 h) [5].

An ideal screening medium should be designed to detect all classes of carbapenemase with a high sensitivity and sufficient specificity to obtain a reliable answer to the question of whether a patient is or is not colonized by CPE as soon as possible. Attention should be given to the price and the screening frequency in each laboratory, as well as to the increasing number of tested samples. Faster results could be achieved with an agar-based chromogenic medium for direct plating, without any necessary enrichment in liquid medium. However, no appropriate medium is available at this moment.

Several commercial media have been tested on molecularly well-characterized carbapenemase-producing strains and on clinical samples. Adler *et al.* [26] have compared three agar-based screening media for the direct detection of CPE from rectal swabs: CHROMagar-KPC (Hy-Labs, Rehovot, Israel), MacConkey agar supplemented with imipenem at 1 mg/L, and MacConkey plates with imipenem, meropenem and ertapenem disks. The highest sensitivity (85%) and specificity (94.3%) have been observed for MacConkey agar supplemented with imipenem. However, only KPC producers were detected in this survey.

In a comparative study, Nordmann *et al.* suggested that a culture medium designed for screening of ESBL producers (ChromID ESBL; bioMérieux, La-Balme-Les-Grotte, France) could achieve higher sensitivity than a medium designed to

select carbapenemase producers (CHROMagar KPC; CHROMagar, Paris, France) [27,28]. However, low specificity caused by co-detection of ESBL producers necessitates further diagnostic tests before reporting of the definitive results.

A prototype chromogenic screening medium, ChromID CARBA (bioMérieux), designed for the detection of CPE, was tested against a group of 200 rectal swabs (133 presumptive CPE; 92 confirmed by phenotypic and genotypic tests) [29]. Direct plating onto ChromID CARBA medium was performed in parallel with four other screening methods: (i) overnight selective enrichment in 5 mL of tryptic soy broth with a 10-μg ertapenem disk, followed by plating onto MacConkey agar; (ii) short, selective enrichment in 9 mL of brain–heart infusion broth with a 10-μg ertapenem disk, followed by plating onto ChromID ESBL medium; (iii) direct plating onto ChromID ESBL; and (iv) direct plating onto MacConkey agar supplemented with meropenem (1 mg/L). Direct plating on ChromID CARBA showed higher sensitivity (92.4%) and specificity (96.9%) than the rest of the methods.

None of the media mentioned above is suitable for detecting producers of OXA-48-type carbapenemases, because these strains are usually susceptible to cephalosporins, and often show low-level resistance to carbapenems. An overnight culture in broth, supplemented with ertapenem, followed by cultivation on Drigalski agar medium, with ertapenem and imipenem E-tests, was suggested as the preferable methodology for the selection of OXA-48 producers by Ruppe *et al.* [30,31].

Singh *et al.* compared the efficiency of direct plating on two different selective media (CHROMagar ESBL and vancomycin, amphotericin B, ceftazidime and clindamycin plates) with a real-time PCR assay for bla_{KPC} genes after a cultivation step in an enrichment broth for the selection of KPC producers from rectal specimens. PCR demonstrated higher sensitivity (97.0%) than culture with selective screening media (77.3%), with a significantly shorter turn-around time (24 h for the PCR-based method vs. 64–72 h for culture) [32]. These results indicate that PCR-based screening of CPE is the most effective procedure. This conclusion, however, does not take into account the high cost of PCR assays and the numerous samples examined, which force microbiologists to develop a screening medium for direct plating with comparable sensitivity. The specificity of CPE detection with current screening media, usually mentioned in some publications, depends greatly on the collection of isolates used for the validation, and cannot be extrapolated to regions with different epidemiological situations.

Recently, a novel screening medium was developed by Nordmann *et al.* [33]. This medium showed very high sensitivity (96.5%) in also detecting OXA-48-producing strains [34]. This medium is based on Drigalski agar supplemented with ertapenem, zinc sulphate, and cloxacillin. SUPERCARBA medium has superior sensitivity to other, commercially available, media (CHROMagar KPC and Brilliance CRE) [34].

Phenotypic tests

Numerous studies have described and evaluated the performance of simple phenotypic tests for the specific detection of carbapenemase-producing strains, mainly of *K. pneumoniae* and *E. coli*.

Modified Hodge test

In the last two decades, detection of β-lactamase production with a modification of the disk diffusion test has been of interest for many research groups. Three-dimensional tests for the detection of extended-spectrum and AmpC β-lactamases were described in 1990 and 2000 [35–37]. All of the described methods were based on the application of a heavy bacterial inoculum or a crude extract of tested isolates into a slit or a cup cut in the agar, at different distances from the antibiotic disks. The deformation of the inhibition zone in the vicinity of the inoculated slit or cup cut was indicative of the presence of ESBLs or AmpC-type β-lactamases. A similar technique was developed in 1976 by Masuda *et al.* [38], and was later modified by Marchiaro *et al.* and Pasteran *et al.* [39,40]. Disks soaked with the tested culture or cell-free extract were placed near the indicator antibiotic disks onto agar plates, which had been previously inoculated with an *E. coli* susceptible strain. Carbapenemase production was suggested by the growth of the indicator *E. coli* cells around the disks loaded with the tested isolates or crude extracts. However, all of the above methods are labour-intensive, and they have not been widely used in diagnostic laboratories.

One of the most extensively used methods is based on techniques first described by Ørstavik and Ødegaard in 1971 [41], and later by Hodge *et al.* in 1978 [42]. The latter method was used for the detection of penicillinase production in *S. aureus*, *Neisseria gonorrhoeae*, and other microorganisms. The plate was inoculated with a susceptible strain of *S. aureus*, a penicillin disk (10 U) was placed in the centre of the plate, and tested isolates were streaked outward from the disk. β-Lactamase production was verified as a distortion of the inhibition zone.

In 2001, Lee *et al.* [43] published a modification of the Hodge test. The modified Hodge test was used for the detection of carbapenemase production in *Pseudomonas* species and *Acinetobacter* species [43]. In the modified Hodge test, penicillin-susceptible *S. aureus* was replaced with *E. coli*

ATCC 25922, and a 10-U penicillin disk with a 10-μg imipenem disk. Two years later, the same authors published an evaluation of their test [44]. In the latter study, Lee *et al.* showed that the sensitivity of the modified Hodge test for the differentiation of metallo-β-lactamase (MBL) producers could be improved by the addition of zinc sulphate to an imipenem disk or to Mueller–Hinton agar.

Although the modified Hodge test is simple and cheap, a high frequency of false-positive results has been observed, especially in carbapenem-resistant *Enterobacteriaceae* that produce ESBLs (e.g. CTX-M type) or AmpC β-lactamases with porin deficiency in their cell walls [45,46]. Pasteran *et al.* [40,47] proposed further modifications of the modified Hodge test to exclude false-positive results in some AmpC and ESBL producers. Also, the modified Hodge test failed to detect carbapenemase production in some isolates. Performance of the modified Hodge test with MacConkey agar and ertapenem disks gave better sensitivity for MBL producers, owing to enhanced release of β-lactamases from cells [48]. However, Girlich *et al.* [49] observed very low sensitivity for the latter modification, especially in NDM-1-producing bacteria. False-negative results were minimized after the addition of zinc sulphate to Mueller–Hinton agar; nevertheless, the test still remained negative for some NDM producers (the sensitivity increased from 77.4% to 94.0%).

Two recent studies reported different sensitivities and specificities for the modified Hodge test [50,51]. In the first study, Cury *et al.* [50] found an excellent negative predictive value (100%). Unfortunately, the study was limited to KPC-producing *Enterobacteriacea* only. The authors also found a high frequency of inconclusive results. In contrast, Doyle *et al.* [51] described only 58% sensitivity and 93% specificity.

A disadvantage is that the assay is time-consuming, as it requires 24–48 h for the first results after isolation of a suspected carbapenamase-producing strain, and cannot distinguish the carbapenemase type.

Despite the problems with the interpretation of the modified Hodge test and the relatively high rates of false-positive and false-negative results with some isolates, the CLSI proposed this test for confirmation of putative carbapenemase producers [52]. On the basis of the results reviewed above, however, the modified Hodge test should not be used for final confirmation of carbapenemase production.

Detection of MBLs based on inhibition by chelating agents

Phenotypic detection of MBL producers is based on the specific inhibition of MBLs by chelating agents. Several tests relying on the synergy between MBL inhibitors, most commonly EDTA [43,53], and an oxyimino-cephalosporin or a carbapenem, have been developed. Additionally, various tests utilizing other chelating agents, such as dipicolinic acid (DPA) [54], 1,10-phenanthroline [55], and thiol compounds (2-mercaptopropionic acid and sodium mercaptoacetic acid) [44,56], or the use of a combination of chelators (e.g. EDTA plus 2-mercaptopropionic acid) [57], have been proposed. These compounds inactivate MBLs that act against β-lactams, by depriving them of hydrolytically essential Zn^{+} divalent cations. The DDST and the combined disk test (CDT) are the most commonly used formats of MBL detection assays. The DDST uses a β-lactam disk placed closed to a disk with a given amount of an MBL inhibitor (most commonly EDTA). The formation of a synergy pattern is indicative of MBL production. Alternatively, in the CDT variant, the β-lactam disk is potentiated with an inhibitor, and the diameter of its inhibition zone is then compared with that of the β-lactam disk alone. An increase in the inhibition zone diameter above a predefined cut-off value indicates MBL activity. Both formats of MBL detection test show high sensitivity even with isolates with low carbapenem resistance levels [20]. However, in the case of the DDST, the interpretation of this test is subjective and cannot be quantified.

Investigators have proposed various techniques for EDTA-based detection tests, mainly in the DDST format. In the first study, presented by Arakawa *et al.* [56], a ceftazidime (30 μg) disk was placed near a filter disk with a centre-to-centre distance of 10–25 mm. To the filter disk, 5 μL of a 0.1 M EDTA solution was added. EDTA expanded the growth-inhibitory zone between the two disks, but its appearance and reproducibility were relatively poor, even when a 0.5 M EDTA solution was added to the filter disk. In a study presented 1 year later, the use of an imipenem (10 μg) disk and an EDTA (*c.* 1.5 mg) disk at a distance of 10 mm (edge to edge) was proposed [43]. A 1.5-mg EDTA disk was prepared by adding 10 μL of a 0.5 M EDTA solution (pH 8.0) to a blank filter paper disk. Lee *et al.* found that the sensitivity and specificity for the EDTA-based DDST test were both 100% as compared with the imipenem hydrolysis assay. In a later study, Lee *et al.* showed that addition of sodium mercaptoacetic acid (*c.* 2 mg) to an EDTA disk improved detection and elimination of a non-specific inhibition zone for both *Pseudomonas aeruginosa* and acinetobacters [44]. In that study, however, disks containing a smaller concentration of EDTA (750 μg) were used, because they alone did not produce the undesirably large inhibition zones. A CDT format of an EDTA-based detection test was presented by Yong *et al.* in 2002 [53]. In the latter test, the inhibition zones of imipenem (10 μg) and imipenem–EDTA (0.750 μg) disks were compared. This method, using a ≥ 7-mm inhibition zone with the imipenem–EDTA disks, differentiated all MBL-producing pseudomonads, and the sensitivity and specificity for acinetobacters were 95.7% and

91.0%, respectively. Recently, a group of experts from the EUCAST and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) published a recommendation for the detection of class A and B enzymes in *Enterobacteriaceae*. In the procedures recommended, the production of class B enzymes is suspected when a difference of ≥5 mm in the zone diameter is observed between meropenem (10 μg) and meropenem plus EDTA (0.25 M) [58].

On the same principle, the E-test MBL strip (AB bioMérieux, Paris, France) is one of the methods advocated for the detection of MBL-producing isolates [20]. This method, which uses imipenem and imipenem plus EDTA, is efficient in detecting MBL producers showing high carbapenem resistance levels, but may fail to detect MBL-producing isolates with low-level resistance to imipenem. Recently, novel E-test strips, containing meropenem and meropenem plus EDTA, have been credited with high sensitivity and specificity for the initial characterization of MBL-producing *Enterobacteriaceae* [58]. The sensitivity of MBL detection methods was significantly increased if the growth media were supplemented with zinc [49,59].

In general, MBL detection methods based on β-lactam–chelator combinations perform well for *K. pneumoniae* and *E. coli*, but they have not been systematically tested for other enterobacterial species. Also, it must be pointed out that the specificity of MBL detection methods might be impaired by the effects of chelating agents, acting non-specifically and affecting other processes [60–62]. Therefore, the results should be cautiously interpreted, and confirmed with a reference methodology (carbapenem hydrolysis-based assays—see below).

Detection of KPCs based on inhibition by boronic acids

Phenotypic detection of KPC production is based on the inhibitory effects of boronic acid and its derivatives, phenylboronic acid (PBA) and 3-aminophenylboronic acid (APBA). Boronate derivatives that structurally resemble β-lactams have long been used in probing the function of β-lactamases, especially class C enzymes. In 2008, Pasteran *et al.* [63] observed that boronates preferentially inhibit KPC-type β-lactamases. This report was soon followed by studies proposing the use of PBA combined with a carbapenem for the identification of KPC producers [64–66]. These studies differed in the performance of boronate-based tests, and the observed sensitivity and specificity. In the study performed by Pasteran *et al.* [64], the DDST approach was found to work well. However, so far, the CDT format has been used more and has evaluated as being better. Of several indicator β-lactams tested, either imipenem [64] or meropenem [65,66] showed better sensitivity and specificity for the identification of isolates producing KPCs or any other members of the class A carbapenemases. Also, different cut-off values of zone diameter differences between carbapenem disks and carbapenem disks supplemented with PBA were used. Pasteran *et al.* [64] proposed an increase of ≥4 mm in the zone diameters of imipenem disks combined with PBA over those for imipenem alone. In the study performed by Doi *et al.* [65], a potentiation of ≥5 mm was observed for all KPC-producing isolates when APBA was added to meropenem disks. The use of a ≥7 mm cut-off for meropenem disks, with and without 400 μg of PBA, was proved to be excellent for identifying KPC-producing *K. pneumoniae* strains and differentiating them from plasmidic AmpC-producing *K. pneumoniae* and *E. coli* [66].

Apart from the disk diffusion approaches, a method in which MICs of carbapenems were determined in the absence and in the presence of 0.3 mg/mL APBA has been developed [64]. A ≥3-fold reduction in the MICs of imipenem has been proposed as the cut-off for differentiating class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria with maximum sensitivity and specificity (both being 100%).

As with the MBL detection tests, experience with boronate-based detection of KPC producers is limited, mainly, to *K. pneumoniae*. In general, boronate-based tests show high sensitivity in the detection of KPC producers. However, specificity problems may arise with isolates showing reduced susceptibility to carbapenems, owing to high-level expression of AmpC-type β-lactamases (cephalosporinases) and porin defficiency [64]. This could be explained by the observed significant inhibition of AmpC enzymes by boronic acids [67]. The problem can be partly overcome by the simultaneous use of cloxacillin, which preferentially inhibits cephalosporinases [68].

Differentiation of MBLs and/or KPCs based on combined disk methods

Recently, the emergence of *K. pneumoniae* strains co-producing KPCs and VIM β-lactamases in Greek hospitals was documented [69–71]. Hospital infections caused by *K. pneumoniae* co-producing KPCs and MBLs have also been described in other regions, such as Germany, Italy, China, and Colombia [72–76]. The spread of double carbapenemase producers has compromised the performance of the chelator-based and boronate-based tests described above. Previous studies have shown that double carbapenemase producers may appear to be negative for one or even both carbapenemases [69,70,74].

In 2010, Tsakris *et al.* [77] described an assay for the phenotypic differentiation of MBLs and class A carbapenemases in enterobacterial isolates. The described assay used four disks: meropenem, meropenem plus PBA, meropenem plus EDTA, and meropenem plus PBA and EDTA. The method

failed to detect one of the double carbapenemase producers tested, and it misclassified two ESBL and AmpC producers as KPC producers.

Recently, Miriagou *et al.* presented two modifications of the KPC/MBL kit (ROSCO Diagnostica, Taastrup, Denmark) (Giakkoupi *et al.*, 22nd ECCMID, 2012, Abstract P1721). The KPC/MBL kit contains four tablets: meropenem (10 μg), meropenem plus APBA (600 μg), meropenem plus DPA (1000 μg), and meropenem plus cloxacillin (75 μg) (www.rosco.dk) [68,79]. In the first modification (Method A), an additional tablet containing meropenem plus APBA and EDTA was included. The second modification (Method B) differed from the first one only in that APBA was replaced with PBA (400 μg). The performance of both modifications was compared with that of the original KPC/MBL kit and the phenotypic assay (Method C) described previously by Tsakris *et al.* [77]. All four methods showed excellent specificity, and performed well against single carbapenemase producers. However, significant problems were encountered with the subset of double carbapenemase producers. The lowest sensitivity was observed with the KPC/MBL kit, which missed 32 double carbapenemase producers. Methods A and C also showed low sensitivity. The sensitivity was significantly improved for Method B, which failed to detect only one double carbapenemase producer. Miriagou *et al.* [78] suggested that the key changes explaining the observed high diagnostic value of Method B were the inclusion of additional combined tablets containing meropenem plus two inhibitors (DPA for MBLs, and a boronic acid derivative for KPCs) and the replacement of APBA with PBA. The algorithm used to interpret the results of Method B, with some modifications, is shown in Fig. 1. The main limitation of that study is that all four methods were tested against a single enterobacterial species, *K. pneumoniae*.

None of the detection tests reviewed above is suitable for the identification of isolates producing acquired OXA-type carbapenem-hydrolysing class D β-lactamases. The enzymatic properties of OXA-type carbapenemases, which are not inhibited by clavulanic acid, tazobactam, sulbactam, or any zinc chelator, prevented the development of specific phenotypic tests for their detection. High-level resistance to both temocillin (MICs of >64 mg/L) and piperacillin–tazobactam in *Enterobacteriaceae* showing reduced susceptibility or resistance to at least one carbapenem may be predictive of production of OXA-48 [21]. Regarding the proposed modification (Method B) of the KPC/MBL kit, Miriagou *et al.* [78] suggested that isolates that are negative with both 'double' disks and the 'triple' disk must be further examined for other carbapenem resistance mechanisms, most importantly OXA-48 production. Definite confirmation of such isolates requires molecular techniques (e.g. PCR-based methods) for the precise identification of carbapenemase genes.

Molecular-genetic Techniques

Molecular techniques remain the reference standard for the precise identification of carbapenemase genes [5]. Most of

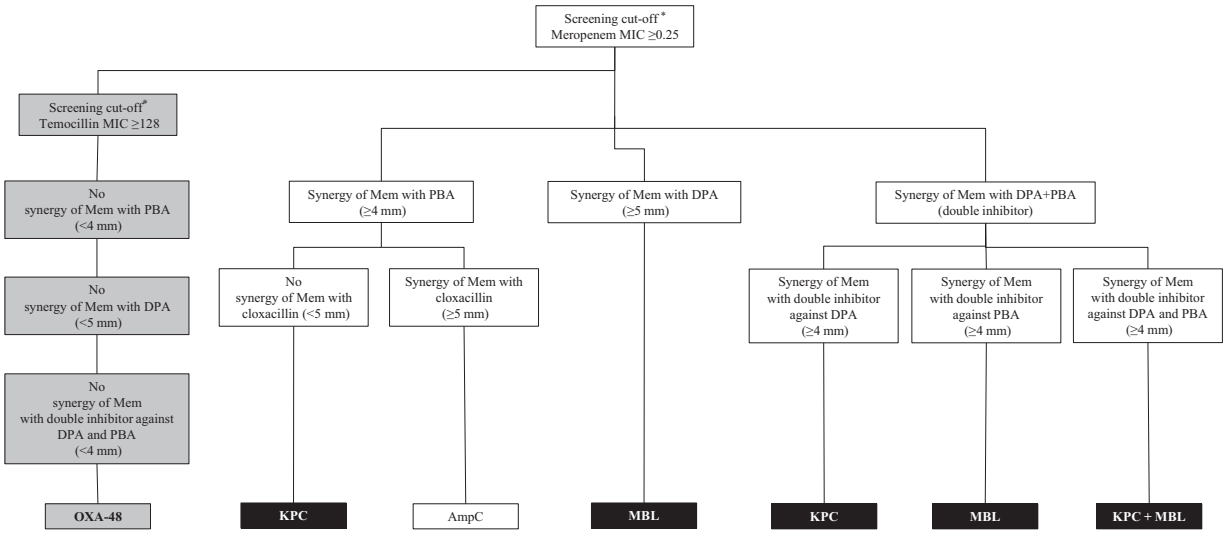

FIG. 1. Algorithm proposed by Miriagou *et al.* [78] for interpretation of results obtained with the KPB/MBL confirmation-based Method B. The algorithm has been modified for the detection of OXA-48-type carbapenemases and the use of screening cut-offs proposed by the European Committee on Antimicrobial Susceptibility Testing. *Ertapenem MIC ≥0.25 mg/L, imipenem MIC ≥2 mg/L or piperacillin–tazobactam (inhibition zone of <16 mm) can also be used. DPA, dipicolinic acid; MBL, metallo-β-lactamase; PBA, phenylboronic acid.

these techniques are based on PCR technology, and may be followed by sequencing of the entire coding region if identification of the β-lactamase gene is required.

Also, hybridization techniques have been commonly used for the identification of carbapenemase genes in research laboratories and reference centres. Combination of hybridization techniques and Southern blotting is used to determine whether the carbapenemase gene resides on a plasmid or is chromosomal [80,81]. Additionally, some other laboratories have used colony blot hybridizations to efficiently screen large numbers of clinical isolates for the presence of carbapenemase genes [80,82].

Currently, many clinical laboratories routinely use 'in-house' PCR-based methods in order to deal with the problems of phenotypic detection methods and reduce the detection time. A PCR technique performed directly on colonies can give results within 4–6 h, with excellent sensitivity and specificity. Additionally, as mentioned above, PCR-based methods allow the detection of OXA-type carbapenemases. PCR-based methods are either simplex [83] or multiplex PCR assays [84–88]. Real-time assays, which further reduce the detection time, have also been utilized [89–92]. Recently, microarray technology was added to the list of molecular techniques, with the aim of achieving the rapid and reliable detection of multiple resistance determinants [93,94].

The main disadvantages of molecular techniques are their cost and the requirement for trained microbiologists. An additional issue with molecular methods is that the range of resistance genes to be detected is predefined. Thus, these methods may not detect novel carbapenemase gene types.

PCR methods

When the presence of a carbapenemase is suspected, PCR is the fastest way to determine which family of β-lactamase is present. Numerous simplex PCR assays targeting a simple carbapenemase type have been used in previous studies. Queenan and Bush provided, in their review, a selection of published primers that have been used in simplex PCR assays to detect all of the families and subgroups known at this time [83].

The increasing frequency of carbapenemase-producing bacteria underlined the necessity of having tools available to monitor the emergence and the spread of each family of carbapenemase gene types. In 2007, Ellington *et al.* [84] developed a multiplex PCR assay for the detection and differentiation of the five different families, VIM, IMP, SPM, GIM, and SIM, of acquired MBL genes in a single reaction. The assay showed excellent performance, as it detected and distinguished all of the reference strains, and found IMP and VIM alleles in all isolates tested. Later, an update of the described assay was presented by Poirel *et al.* [88]. The updated assay, which consisted of three multiplex PCR reactions, was aimed at additionally detecting the emerging carbapenemase genes encoding the class A KPC, the class B NDM-1, and the class D OXA-48, as well as the recently identified genes encoding DIM-1, BIC-1, and AIM-1.

Following those reports, a set of six multiplex PCRs and one simplex PCR for the rapid detection of the most widespread β-lactamase genes encoding the OXA-1-like broad-spectrum β-lactamases, ESBLs, plasmid-mediated AmpC β-lactamases and class A, B and D carbapenemases was created by a research group from France [86]. However, no carbapenemase genes were detected in the clinical isolates tested. In 2011, an update of the assay, described by Dallenne *et al.* [86], was aimed at detecting the majority of genes encoding clinically important β-lactamases causing third-generation cephalosporin or carbapenem resistance. In the updated assay, Voets *et al.* [87] developed seven multiplex PCRs for the detection of plasmid-mediated AmpC β-lactamases (ACC, ACT, DHA, CMY, FOX, LAT, MIR, and MOX), metallo-carbapenemases (GIM, NDM, SIM, and SPM), serine carbapenemases (IMI, SME, and NMC-A) and OXA β-lactamases (OXA groups 23, 24, 48, 1, 2, 51, 4, and 58), and they used four multiplex PCRs of Dallenne *et al.* for the detection of genes encoding the CTX-M, SHV, GES, VEB, PER, KPC, VIM and IMP β-lactamase families. All of the multiplex PCRs were evaluated for performance in a single amplification protocol.

In 2010, Endimiani *et al.* presented a PCR-based method for the detection and identification of bla_{KPC} genes among *Enterobacteriaceae* [95]. However, that protocol makes use of PCR/electrospray ionization–mass spectrometry (MS) technology, a promising genotyping system that has high multiplexing capacity and can be used for detecting different genes in a single strain. This system can also detect single-nucleotide polymorphisms, including mutations corresponding to changes in amino acids. In the described study, all bla_{KPC-2}-possessing and bla_{KPC-3}-possessing strains were correctly reported. Additionally, all tested strains were correctly defined at the species level.

Apart from the 'in-house' assays, there are also commercially available kits based on PCR and hybridization, e.g. Hyplex MBL ID (BAG Health Care, Lich, Germany) for the detection of bla_{VIM}(1–13) and bla_{IMP}(1–22), and Hyplex CarbIxa ID (BAG Health Care) for the detection of bla_{OXA} carbapenemase genes. This methodology allows the detection of carbapenemase-encoding genes from clinical samples (19th ECCMID, 2009, Abstract P1054); however, its diagnostic usefulness has to be further assessed in multicentre studies that also include settings with a low prevalence of carbapenemase producers.

Real-time PCR methods

Several multiplex real-time PCR methods, which further reduce the detection time for carbapenemase genes, were developed by research groups at the same time. Real-time PCR methods can be followed by a melting curve step, allowing the accurate differentiation of carbapenemase gene variants. In 2007, Mendes *et al.* [90] described the first multiplex real-time PCR assay for the detection of genes encoding MBL-type enzymes (IMP and VIM types, SPM-1, SIM-1, and GIM-1) identified up to that point. The MBL identification was based on the characteristic amplicon melting peak. In a study published a few months later, the specific detection of bla_{VIM} and bla_{IMP} genes in Gram-negative bacteria was achieved in <1 h with a real-time PCR assay [89]. The authors showed that melting curve analysis of the real-time PCR products clearly differentiated the genes into four groups: (i) bla_{VIM-1}-like; (ii) bla_{VIM-2}-like; (iii) bla_{IMP-1}-like; and (iv) bla_{IMP-2}-like.

In 2011, Chen *et al.* [91] developed a multiplex real-time PCR scheme that could identify bla_{KPC} gene variants. In contrast to other PCR-based methods for the detection of bla_{KPC} genes, such as two real-time PCR assays [97,98] and a method that uses PCR/electrospray ionization–MS [95], the described protocol made use of the technology of molecular beacon probes, which are able to detect the presence of single-nucleotide polymorphisms. Thus, the described real-time PCR could distinguish between different bla_{KPC} variants [91], and therefore provides information of both epidemiological and evolutionary significance. Later, Chen *et al.* [99], using the technology of molecular beacon probes, designed a multiplex real-time PCR assay capable of identifying both the epidemic *K. pneumoniae* ST258 clone and bla_{KPC} carbapenemase genes in a single reaction. That assay showed excellent sensitivity (100%) and specificity (100%), and seemed to be a useful tool for screening of *K. pneumoniae* isolates and surveillance of the epidemic ST258 clone in both community and healthcare settings. Although the initial cost of investment for the molecular beacons is significant, it is offset by the reduced requirements for template quality, reaction volume, and time [100].

Recently, Monteiro *et al.* [92] developed a single multiplex real-time PCR assay for the identification of the most common types of serine carbapenemase (KPC, GES, and OXA-48) and metallo-carbapenemase (IMP, VIM, and NDM), already described in enterobacterial isolates, using high-resolution melting curves. The entire assay, including DNA extraction, sample preparation, a multiplex PCR run, and analysis of results, was performed in 3 h. In the two studies that followed: (i) the performance of a real-time PCR for identification of bla_{VIM}-type and bla_{KPC} carbapenemases in an ultrarapid single reaction was compared with the performance of other PCR-based methods [101]; and (ii) a multiplex real-time PCR assay for the detection of OXA-48, VIM, IMP, NDM and KPC carbapenemase genes was evaluated in a multicentre study [102].

Both the sensitivity and the specificity of all of the real-time protocols presented above were 100%. These results indicate that real-time PCR assays are robust, reliable and rapid protocols for the detection of the most prevalent carbapenemase genes. Therefore, molecular methods should be used for screening for the presence of the carbapenemase genes, so that their epidemiological spread can be monitored.

Microarrays

DNA hybridization techniques in a microarray format allow the simultaneous detection of many sequences. In 2010, Ulyashova *et al.* [103] developed a microarray able to identify KPC, OXA, IMP, SPM, VIM, SIM and GIM carbapenemases. Commercially available microarrays for the detection of ESBLs and carbapenemases have been recently validated [93,104]. This method provides excellent sensitivity and specificity. Carbapenemases and other β-lactamases can be also detected directly from blood cultures. However, sensitivity can be influenced by the method used for DNA extraction.

Except for blood cultures, microararrays have not yet been validated for the detection of β-lactamases from clinical specimens. Their labour-intensive nature and relatively high cost still prevent them from becoming routine for the detection of carbapenemases in diagnostic laboratories. Despite this, microarrays may provide useful data for epidemiological typing of the isolates.

Direct Methods for Detection of Carbapenemase Activity

In contrast to molecular-genetic techniques, which are only able to detect genes that are already known, direct detection of carbapenemase activity is also able to detect novel carbapenemases. Therefore, these methods should be available as reference standard methods for the detection of carbapenemase activity in routine diagnostic laboratories.

Colorimetric assays

Some of the simplest and cheapest methods for the detection of β-lactamases are colorimetric assays that can detect hydrolysis of the amide bonds of β-lactams according to colour changes. Specific tests use a chromogenic β-lactam (e.g. nitrocefin) that changes colour after being hydrolysed [105,106]. Non-specific tests are based on non-specific interactions with hydrolysed β-lactam molecules or on the

detection of pH changes. A well-known non-specific test is the starch–iodine assay [105–107]. After hydrolysis of the indicator β-lactam, the colour of the reaction mixture or plate around colonies of β-lactamase producers changes from dark blue to colourless. For the detection of β-lactamase-producing *S. aureus*, *Haemophilus influenzae*, and other bacteria, a phenol red-based method has been developed. β-Lactamase production is indicated as a change of colour from red to yellow [106,108]. Recently, a phenol red-based method was modified for the detection of carbapenemases in *Enterobacteriaceae* and *P. aeruginosa*, known as the Carba NP test [22,109]. In this test, a bacterial culture is mixed with lysis solution. After lysis, the cell-free supernatant is mixed with a solution of phenol red, zinc sulphate, and imipenem. In carbapenemase-producing bacteria, the colour of the reaction changes from red to yellow or to light red. This method also allows the preliminary identification of carbapenemase type (class A, B, or D) based on the inhibition profile with tazobactam and EDTA [109].

In 2013, Tijet *et al.* [111] published another validation of the Carba NP test performed on a collection of 244 enterobacterial and *P. aeruginosa* isolates. They reported 100% specificity of the test. However, the sensitivity (72.5%) was lower than the 100% for *Enterobacteriaceae* and 94.4% for *P. aeruginosa* reported originally [22,109,110]. The test gave false-negative results in some mucoid isolates and especially in OXA-48-producing *Enterobacteriaceae*. The authors hypothesized that this behaviour could be attributable to low expression of carbapenemase. With a two-fold higher bacterial concentration, the sensitivity increased to 80%.

Recently, Dortet *et al.* [112] validated the Carba NP test for the detection of carbapenemase producers from blood cultures. Although there is one more 3-h step for incubation in brain–heart infusion in the procedure, this modification can reduce the time needed for carbapenemase identification from approximately 24 h to 3–5 h.

The Carba NP test is a reliable method for the detection of carbapenemases, especially of Ambler classes A and B, with superior specificity. Whether or not the test is reliable for the detection of all OXA-48-type carbapenemase producers and other bacteria with low production or inefficient carbapenemases must be determined in multicentre studies.

MS

In 2011, the first methods for the detection of carbapenemase activity by the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) were published. In those assays, fresh bacterial culture is mixed with carbapenem solution (meropenem or ertapenem) [113,114]. After incubation at 35–37°C for 2–4 h, the reaction mixture is centrifuged, and the supernatant is measured by MALDI-TOF MS. In spectra, the carbapenem molecule, its sodium salts, and degradation products in the case of carbapenemase hydrolysis, are visible. This method allows the detection of real hydrolysis, without false-positive results. Some OXA-48-type-producing strains, however, can give false-negative results. In some slime-producing strains, measurement can be complicated by interactions with polysaccharides. The mass spectra of such strains are usually difficult to interpret.

The MALDI-TOF MS carbapenem hydrolysis assay has been modified and validated by other research groups [115–118]. Interestingly, this method gives superior results for the detection of OXA-type carbapenemases (CHDL) in *Acinetobacter baumannii* [119].

Another modification of the MS method was proposed by Carvalhaes *et al.* [120]. This group used liquid chromatography (LC)–MS for the detection of carbapenem molecules and their degradation products in CHDL-producing, IMP-producing, NDM-producing, VIM-producing, GIM-producing, KPC-producing and GES-producing bacteria. Incubation of the reaction mixture for 4 h increased both the sensitivity and specificity of the method to 100%. Recently, similar results were obtained by Carricajo *et al.* [121], using ultra-performance LC–tandem MS. Unfortunately, the equipment required for LC–MS is not currently available in most diagnostic laboratories, in contrast to MALDI-TOF mass spectrometers.

MALDI-TOF MS has now been introduced into many microbiological laboratories across Europe as an identification procedure for bacteria and fungi. Although the costs of the measurement (identification of bacteria and fungi) are low, the equipment remains expensive for countries with a low economic status. Recently, this technology was approved by the US Food and Drug Administration. Therefore, the MALDI-TOF MS carbapenem hydrolysis assay may become a reliable tool for confirmation of carbapenemase activity in both routine and reference laboratories. Unfortunately, no automatic software for the analysis of acquired spectra has yet been available. Therefore, the users must have some experience with MS measurement, in contrast to MALDI-TOF MS for microorganisms. Another multicentre validation of this method is also needed.

Spectrophotometric assays

Spectrophotometric detection of carbapenem hydrolysis has been proposed as a reference method for confirmation of carbapenemase activity [122]. Although this approach is labour-intensive, it cannot be used routinely in diagnostic laboratories. For the detection of carbapenemase activity, bacterial crude extract, usually prepared by sonication, is added to buffered carbapenem solution (imipenem), and hydrolysis of the β-lactam ring is measured in UV spectra

[123–125]. Bernabeu *et al.* [123] demonstrated the ability of the imipenem hydrolysis spectrophotometric assay to detect a broad range of carbapenemases (100% sensitivity), including OXA-48-type class D enzymes Laraki et al. [125]. Only one *Enterobacter cloacae* strain overexpressing intrinsic AmpC was misidentified as a carbapenemase producer (98.5% specificity).

Workflow in Routine Diagnostic Laboratories

Because of the global spread of carbapenemase-producing bacteria, it is necessary to introduce methods for their detection into all clinical diagnostic laboratories. For this purpose, we recommend a workflow, shown in Fig. 2, for carbapenemase identification.

During an outbreak, in regions with a known epidemiological situation, PCR-based screening can be performed directly from clinical material, and the results will be available to hospital epidemiologists within a few hours. Simultaneously, the samples should be cultivated on screening cultivation media to obtain an isolate for further molecular-epidemiological typing. For other screening purposes, a sample should be first cultivated on a proper selective cultivation medium. Carbapenemase production must be confirmed by direct detection of carbapenem hydrolysis. Carbapenemase genes can be identified by PCR amplification or a microarray technique. Before molecular testing, inhibition (e.g. EDTA or boronic acid) and susceptibility (e.g. temocillin) profiles of the isolate may provide helpful information for the selection of a more targeted PCR-based technique. Apart from screening samples, detection of carbapenemase production should be performed in any enterobacterial isolate (Table 1) with a suspicious susceptibility profile (usually isolates with clinical significance).

Conclusion

New, reliable methods for the detection of carbapenemases have now been developed (e.g. the Carba NP test and the

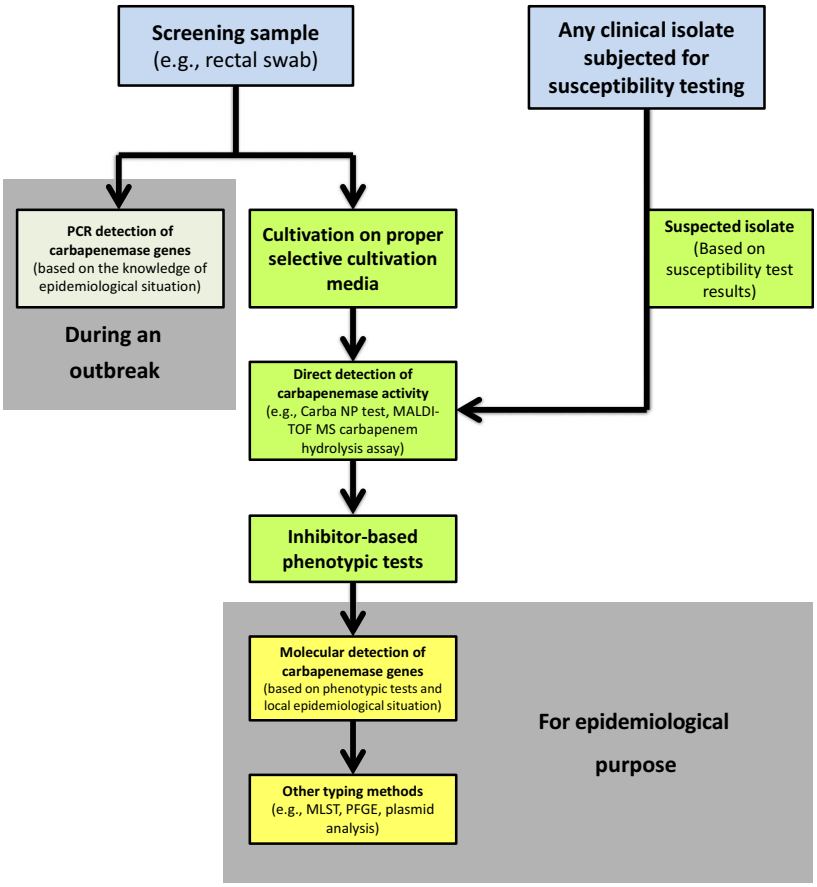

FIG. 2. Proposed workflow for the detection of carbapenemases in diagnostic laboratories. MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis.

MALDI-TOF MS hydrolysis assay). All of the methods, however, need experienced laboratory personnel. Therefore, in countries with a low prevalence of CPE, final confirmation of carbapenemase production should be performed in reference laboratories. For the improvement of CPE diagnosis, national authorities or international institutions should provide: (i) education to the diagnostic laboratory staff (e.g. interpretation of test results); and (ii) external quality controls for the evaluation of laboratories responsible for the diagnosis of CPE. Research on diagnostic methods should be further focused on interlaboratory/multicentre validation of the method, and the development of new methods for direct detection of CPE colonies on selective cultivation media.

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Transparency Declaration

All authors have no conflict of interests to be declared.

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