Formation of a carbapenemase resistance detection algorithm for use in the routine laboratory

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ABSTRACT

The increasing prevalence of carbapenem non-susceptible Gram negative organisms demands prompt and accurate identification of resistance mechanisms to limit their transmission. The aim of this study is to evaluate the rapid CARB screen (Rosco Diagnostica, Denmark), the 'KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit' (Rosco Diagnostica), Check-MDR Carba and Check-Direct CPE kits (Check-Points, The Netherlands). The purpose of this study is the formation of a carbapenemase resistance detection algorithm that can be used in the routine laboratory. Results of the rapid CARB screen kit were improved when isolates were tested from Muller Hinton agar with a meropenem 10 μg disc instead of blood agar. The rapid CARB screen (performed in 2–3 h) demonstrated overall 98.7% sensitivity and 87.7% specificity (n=133). The KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit (which requires overnight incubation) demonstrated a high number of false-positive results giving 38.6% specificity and 100% sensitivity (n=44). The Check-MDR Carba (performed in 5 h), detecting carbapenemase presence, provides a cabapenemase-positive or -negative result demonstrated 96.7% specificity and 98.6% sensitivity (n=132). The Check-Direct CPE (performed in 3 h), which identifies KPC, NDM/VIM or OXA-48 type carbapenemases, demonstrated 96.5% specificity and 97.1% sensitivity (n=97). The Check-Direct CPE, however, failed to detect dual carbapenemase genes present in two out of four isolates. The principal conclusion is the recommendation of the rapid CARB screen and Check-MDR Carba for incorporation into a carbapenemase detection algorithm which, when used in combination, will yield results with 97.3% sensitivity and 99.6% specificity.

KEY WORDS: Carbapenemase.
Enterobacteriaceae.
Pseudomonas.
Acinetobacter.

Introduction

The prevalence of carbapenem-resistant organisms (CROs) is increasing worldwide, mainly due to carbapenemase production. Carbapenemase-producing organisms (CPOs) have intrinsic or acquired carbapenemases. Intrinsic carbapenem resistance may occur due to non-transferable carbapenemases, impermeability or porin loss.[1–3] Carbapenemases are a form of β-lactamase that confer resistance or reduced susceptibility to all or nearly all members of the β-lactam antibiotics. There are very few treatment options for CRO infections and they are associated with significant morbidity and mortality.[4–6] Acquired carbapenemases are carried on mobile genetic elements allowing them to be transmissible between organisms.[2,7]

Carbapenemases are classified by the Ambler system into class A, B and D, based on their amino acid homology. Serine carbapenemases include class A, which may be inhibited by clavulanic acid, tazobactam, sulbactam, avibactam and boronic acid, and class D, which may demonstrate high-level resistance to temocillin. Class B (metallo-β-lactamases [MBLs]) require zinc and are inhibited by zinc chelators such as ethylenediaminetetraacetic acid (EDTA) and dipicolonic acid (DPA).[4,5,7–11] In Ireland the most prevalent carbapenemase genes include bla_{KPC} (*Klebsiella pneumoniae* carbapenemase), bla_{NDM} (New Delhi metallo-β-lactamase), bla_{VIM} (Verona Integron Encoded metallo-β-lactamase), bla_{IMP} (Imipenem metallo-β-lactamase) and bla_{OXA-48} (oxacillin-hydrolysing β-lactamase).[2,12–17]

One of the primary methods of carbapenemase detection is when an isolate demonstrates reduced susceptibility to carbapenems. There are currently disagreements in what is considered carbapenem-resistant within the microbiology community. Carbapenems testing as susceptible have been associated with regrowth and therapeutic failure.[18,19] European Committee on Antimicrobial Susceptibility Testing (EUCAST) has implemented lower breakpoints as of December 2013 for detection of carbapenem resistance in Enterobacteriaceae to <27 mm for meropenem (10 μg) and ertapenem (10 μg) in countries where OXA-48 is endemic, such as Ireland,[20] while CLSI reports resistance at <18 mm for meropenem (10 μg)[21] and the British Society for Antimicrobial Chemotherapy (BSAC) reports resistance at ≤15 mm for ertapenem (10 μg) and ≤19 mm meropenem (10 μg).[1] The HPSC has issued separate breakpoints for therapeutic decision-making, reporting resistance to meropenem (10 μg) at ≤23 mm, ertapenem (10 μg) at ≤24 mm and imipenem (10 μg) at ≤22 mm.[22] The lack of consensus between these regulatory bodies in carbapenemase detection highlights the need for more work in this area.

There are few strategies available for the rapid, reliable detection of all of these carbapenemases. Phenotypic methods available for carbapenemase detection include the modified Hodge test (MHT) and Etest MBL strips,

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automated expert analysis (AES) and combination disc tests. The MHT and Etest MBL strips have been reported to demonstrate false-positive results and low sensitivity.[8,18,23] Automated expert analysis systems vary in ability to detect carbapenemase profiles.[2] Combination disc tests are optimised for Enterobacteriaceae and demonstrate low sensitivity for *Pseudomonas aeruginosa* MBL detection.[24]

Accurate detection of carbapenemase-producing *Pseudomonas* species and *Acinetobacter* species is particularly important due to the rise of carbapenem resistance in these organisms and the spread to Enterobacteriaceae.[8] Metallo-β-lactamase production in *P. aeruginosa* is increasing in prevelance worldwide.[24] The main types of carbapenemase found in *Acinetobacter* spp are due to OXA-type carbapenemase, with several MBL carbapenemases being detected to a lesser extent.[8]

Pseudomonas spp. and *Acinetobacter* spp. may also harbour *Klebsiella pneumoniae* carbapenemase enzymes.[25] *Acinetobacter baumannii* possessing KPC genes have been found in Central America; however, they have not yet been discovered in Europe.[1] Currently, an inadequate level of carbapenemase screening is being performed in non-Enterobacteriacae. This may be in part due to the resources involved in investigation and the lack of phenotypic tests that are easily interpreted and suitable for use in a routine diagnostic laboratory.[26] Current phenotypic detection methods in use do not provide comprehensive carbapenemase detection, and therefore the aim of this study is to examine other methods available. The four methods evaluated here include:

- The KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit (Rosco Diagnostica, Denmark), a disc potentiation inhibitor-based test for carbapenemase detection in non-fermenters. This kit detects KPC, AmpC-β-lactamase (AmpC) and MBL producers in *P. aeruginosa* and *Acinetobacter* species.[20] MacConkey agar is used instead of Muller Hinton agar; as larger synergistic zones for MBL detection is hypothesised to be due to the enhanced release of MBL by oxgall in the MacConkey agar.[8]
- The Carbapenemase Nordmann-Poirel test (CARBA-NP) is a new carbapenemase detection method based on the hydrolysis of the β-lactam ring in imipenem;, an increase in pH results in a colour change from red to yellow with phenol red solution. This method should detect all known and emerging carbapenemases.[10] The rapid CARB screen is a commercially available kit developed by Rosco Diagnostica, based on the CARBA-NP method, and may be performed in three hours. Several studies have assessed the CARBA NP test. Tijet *et al*.[27] reported 100% specificity and 80% sensitivity for the CARBA NP, with false negatives associated with mucoid colonies and isolates with weak carbapenemase activity. Dortet *et al*.[29] found use of the CARBA NP test in *Pseudomonas* species demonstrated 100% specificity and 94.4% sensitivity; however, several guiana extended-spectrum (GES)-type carbapenemases were not detected.
- The Check-MDR Carba (Check-Points, Wageningen, The Netherlands) is a molecular based assay for carbapenemase-positive or -negative detection using a real-time polymerase chain reaction (PCR) assay. The Check-MDR Carba detects all known variants of bla_{KPC}, bla_{NDM} and bla_{OXA-48}. This kits detects all bla_{VIM} variants except bla_{VIM-7} and the nine most prominent bla_{IMP} variants in Enterobacteriaceae. Cuzon *et al*.[30] demonstrated 100% sensitivity and specificity using this assay.

Table 1. List of organisms.

	Organism	No. of Isolates
OXA-48 positive	*Klebsiella pneumoniae*	21
	Escherichia coli	3
OXA-51 positive	*Acinetobacter baumannii*	1
OXA-51 and -58 positive	*A. baumannii*	4
KPC and OXA-48 positive	*K. pneumoniae*	2
VIM positive	*Enterobacter cloacae*	2
	Citrobacter species	3
	Escherichia coli	2
	Klebsiella species	3
	Pseudomonas putida	1
NDM positive	*C. freundii*	2
	E. coli	6
	K. pneumoniae	2
NDM and OXA positive	*K. pneumoniae*	2
KPC positive	*K. pneumoniae*	17
IMP positive	*P. aeruginosa*	2
	K. oxytoca	1
CPO negative	Enterobacteriaceae	22
	P. aeruginosa	35

- The Check-Direct CPE (Check-Points) is a multiplex real-time PCR assay that detects specific carbapenemase genes. The Check-Direct CPE assay detects all known variants of bla_{KPC}, bla_{NDM}, and bla_{OXA-48} genes. All bla_{VIM} variants are detected except bla_{VIM-7} and $bla_{OXA-162,\ -181,\ -204}$ and rarer OXA-48 type carbapenemase genes found in Enterobacteriaceae.[31–33] The kit does not detect bla_{IMP} gene or differentiate between bla_{NDM} and bla_{VIM} genes. The Check-Direct CPE has demonstrated 100% sensitivity and specificity in a study by Nijhuis *et al*.[34]

There are no current molecular methods for carbapenemase detection in routine use at Our Lady's Children's Hospital Crumlin (OLCHC), with isolates being sent to the National Reference Laboratory for investigation. Overall, the introduction of a rapid carbapenemase detection algorithm would decrease sample turnaround time from weeks to days

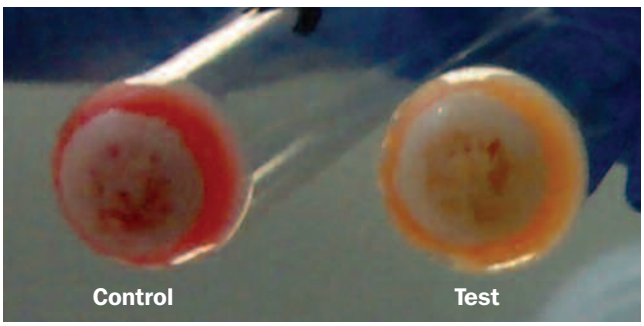

Fig. 1. Positive rapid CARB screen. Two Diatabs are present with each test, one negative control Diatab (should remain red throughout the test) and one test Diatab. If the test is positive the test Diatab will turn yellow, as indicated. If the test isolate is negative for a carbapenemase it will remain red.

Table 2. Interpretation of the KPC/MBL by *P. aeruginosa/Acinetobacter* Confirm kit.

Result	Organism		Meropenem + phenylboronic acid	Meropenem + cloxacillin (high)	Dipicolinic acid
Not KPC/MBL	*P. aeruginosa*	Meropenem 10 μg	–	≥5 mm	
KPC	*P. aeruginosa*	Meropenem 10 μg	≥4 mm	<3 mm	
		Meropenem (+) Cloxacillin (high)	≥4 mm		
MBL	*P. aeruginosa*	Imipenem 10 μg	–	–	Synergism
	Acinetobacter		–	–	Synergism

Interpretation criteria for the KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm kit with zone sizes and effect of synergism being compared to determine if a β-lactamase is present.

at OLCHC. This would facilitate prompt infection control measures and improved patient management. The main objectives of this study is to develop a carbapenemase resistance detection algorithm using phenotypic and genotypic detection that demonstrates greater than 95% sensitivity and 95% specificity for combined testing with some or all of these four methods.

Materials and methods

Ethical approval

This study does not involve human subjects, their tissue or their data, and therefore did not require ethical approval.

Storage conditions

All Isolates were stored at –80°C on Microbank beads (Pro-Lab Diagnostics). Isolates taken from beads, cultured onto blood agar (Oxoid, Basingstoke, England; CM0271) and incubated at 35°C for 18 h for use with each of the kits.

Isolate collection

The carbapenemase detection methods were performed using 67 characterised carbapenemase- producing Enterobacteriaceae (23 bla_{OXA-48}, 16 bla_{KPC}, 10 bla_{VIM}, 10 bla_{NDM}, two bla_{IMP}, two bla_{NDM} and bla_{OXA-48}, two bla_{KPC} and bla_{OXA-48}, and two bla_{MBL}), two *Pseudomonas* species (bla_{IMP} and bla_{VIM}), five *Acinetobacter* spp. (bla_{OXA}). BAA1705 *Klebsiella pneumoniae* (bla_{KPC}), *P. aeruginosa* ATCC 10145 (bla_{IMP}), *Escherichia coli* ATCC 25922, 21 non-CPOs (11 AmpC, eight ESBL and two AmpC and ESBL producers) and 35 carbapenem non-susceptible *P. aeruginosa* (Table 1).

Carbapenemase-producing organism were collected from clinical specimens from OLCHC, another Dublin hospital, and the National Reference Laboratory in Galway. *P. aeruginosa* isolates were recovered from carbapenemase screening media between October 2013 and August 2014. Carbapenemase screening media contained 250 mg/L flucloxacillin, 0.25 mg/L ertapenem and 70 mg/mL zinc sulphate, based on a modified version of SUPERCARBA medium.[10] Isolates were excluded if they were susceptible to meropenem/imipenem (10 μg) by disc diffusion.

The KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit (Rosco Diagnostica) was performed using two MBL-producing *P. aeruginosa* and five OXA-producing *Acinetobacter*, *P. aeruginosa* ATCC 10145 (positive control), *E. coli* ATCC 25922 (negative control) and 35 *P. aeruginosa* isolates with reduced carbapenem susceptibility.

The rapid CARB screen kit (Rosco Diagnostica) was performed with the 75 characterised CPOs, 21 non-CPOs, *K. pneumoniae* BAA1705 (positive control), *E. coli* ATCC 25922 (negative control) and 35 *P. aeruginosa* isolates with reduced carbapenem susceptibility (Table 1).

The Check-MDR Carba (Check-Points) was performed with the 75 characterised CPOs, *K. pneumoniae* BAA1705, 21 non-CPOs and 35 *P. aeruginosa* isolates with reduced carbapenem susceptibility (Table 1).

The Check-Direct CPE (Check-Points) kit was performed with the 75 characterised CPOs, *K. pneumoniae* BAA1705, and 21 non-CPOs (Table 1).

Identification and antibiograms

Identification was performed on all isolates by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Antibiograms of the CPOs, ESBL and AmpC-producing isolates were obtained by analysis with the Vitek 2XL (bioMérieux, Durham). Meropenem, imipenem and ceftazidime sensitivities were performed on the 35 carbapenem non-susceptible *P. aeruginosa* by disc susceptibility testing. Breakpoints were interpreted according to EUCAST (www.eucast.org/clinical_breakpoints). All isolates were labelled with a unique identifier based on the resistance mechanism possessed. Evaluation of the carbapenemase detection methods was performed by comparison to the gold standard (PCR).

KPC/MBL in P. aeruginosa/Acinetobacter *Confirm Kit*

The KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit was performed by lawning a 0.5 McFarland suspension onto two MacConkey agar plates (Oxoid; CM0007). The Diatabs were placed at required distances, depending on carbapenem susceptibility, as per the manufacturer's instructions, and incubated at 35°C overnight. KPC detection was determined by measuring the inhibition zones between meropenem (10 μg), meropenem+phenylboronic acid and meropenem+cloxacillin high. MBL detection was through synergism between DPA and imipenem (10 μg) and/or meropenem (10 μg) (Table 2). Synergism being regarded as the increase in the inhibition zone caused by the presence of an inhibitor.[8]

Rapid CARB screen

Isolates were tested with the method recommended by the manufacturer using isolates cultured on blood agar (Rosco Diagnostica). Several loops of the isolate were added to lysis

buffer (B-PER II; Bacterial Protein Extraction Reagent, Thermo Scientific) and emulsified to obtain a concentration of a 4 McFarland standard suspension. This was vortex-mixed and after incubation an aliquot of the bacterial suspension was added to two fresh test tubes with saline. To one tube the control Diatab was added and to the other the test Diatab. A change of colour from red to yellow indicated a positive reaction (i.e., that the test strain produces a carbapenemase). If the negative control Diatab changed from red to yellow the test was recorded as invalid. Modifications were made to the method for the rapid CARB screen (Rosco Diagnostica) to increase the performance as follows:

- A heavier inoculum was used.
- The addition of 100 µL zinc sulphate heptahydrate (7 mg; Merck, Darmstadt, Germany) to 100 mL phosphate buffered serology saline (Inverclyde Biologicals, Lanarkshire) was used instead of saline.
- A 0.5 MacFarland of isolate lawned onto Muller Hinton agar (Oxoid) with a meropenem (10 µg) disc was used; the inoculum used for the bacterial suspension being taken from around the meropenem disc.

Seventy-five characterised CPOs, 21 non-CPOs, *K. pneumoniae* BAA1705 (positive control) and *E. coli* ATCC 25922 (negative control) were tested after culturing from blood agar and Muller Hinton agar. As initial results from isolates grown on Muller Hinton demonstrated the highest sensitivity and specificity, the 35 *P. aeruginosa* isolates with reduced carbapenem susceptibility were also tested after being cultured on Muller Hinton agar (Table 1).

Molecular methods

KPC-, NDM/VIM- and IMP-positive controls for the Check-Direct CPE were combined in equal volumes. The Check-MDR Carba and Check-Direct CPE were performed using the same bacterial crude lysate prepared as follows: isolates were inoculated on blood agar plates and incubated at 35˚C overnight. A 0.5–1.0 McFarland bacterial suspension was prepared for each isolate using molecular-grade water

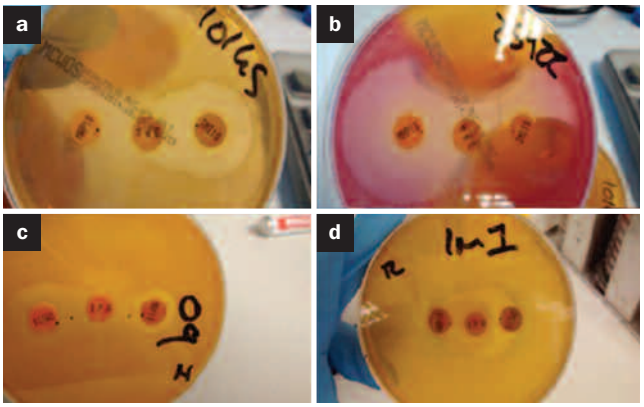

Fig. 2. Optical comparison of synergism with KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit. **a)** Positive synergy using *Pseudomonas aeruginosa* ATCC 10145. **b)** No synergy using *Escherichia coli* ATCC 25955. **c)** OXA carbapenemase. **d)** Carbapenemase-producing organism with imipenem metallo-β-lactamase.

(Sigma-Aldrich, Wicklow, Ireland). For each bacterial suspension 10 µL Check-MDR Carba internal control and 5 µL Check-Direct CPE internal control was added. Samples were vortex-mixed briefly and then heated at 98˚C for 10 min. The samples were vortex-mixed for 30 sec after heating and centrifuged for 2 min at 10,000 xg. Supernatants were aliquoted, stored at 4˚C on ice for one day and at –20˚C for a week. The PCR assays were performed on both assays as per the manufacturer's instructions (Check-Points) using the parameters specified in Table 3 on the 7500 FAST real-time PCR system (Applied Biosystems, Warrington, UK). Molecular method results were considered valid based on external control, positive control and internal control results meeting set criteria defined by the manufacturer.

Statistical analysis

The methods were examined statistically using test efficiency and 95% confidence intervals (CI).

Test efficiency was calculated as true positives + true

Table 3. a) ABI 7500 real-time cycling parameters. **b)** Real-time PCR set-up.

a	Check-Direct CPE			Check-MDR Carba*		
Step	Temperature	Time	Cycles	Temperature	Time	Cycles
1	50˚C	2 min	1	50˚C	2 min	1
2	95˚C	10 min	1	95˚C	10 min	1
3	95˚C	15 sec	45	95˚C	15 sec	40
	60˚C	60 sec	45	60˚C	60 sec	40

b	Target	ABI 7500 Detector	Target	ABI 7500 Detector
	KPC	FAM		FAM
	NDM, VIM	VIC	Carbapenemase	
	OXA-48	Texas Red (TXR)		
	Internal control	Cy5	Internal Control	Cy5

a) Protocol for the Check-MDR Carba and Check-Direct CPE, highlighting the cycling parameter.
b) Details of the detectors used for each target.

*The Check-MDR Carba real-time PCR is preceded by a two-hour ligation step whereby the samples are heated at 95˚C for 3 min, then 65˚C for 120 min, 98˚C for 2 min, and held at 4˚C.

Table 4. Results of KPC/MBL Confirm ID kit in *P. aeruginosa/Acinetobacter*.

No. of isolates	Species	Result KPC/MBL Rosco	PCR result	Rapid CARB Screen result	Dipicolinic acid sensitivities (mm)	Ceftazidime sensitivities
1	*P. aeruginosa* ATCC 10145	MBL	MBL	Positive	9	S
1	*E. coli* ATCC 25922	Negative	Negative	Negative	9	S
1	*A. baumannii*	MBL	OXA	Negative	9	R
3	*A. baumannii*	MBL	OXA	Positive	15	R
1	*A. baumannii*	Negative	OXA	Positive	16	R
1	*P. aeruginosa*	MBL	MBL	Positive	9	R
1	*P. putida*	MBL	MBL	Positive	30	R
15	*P. aeruginosa*	MBL	Negative	Negative	9	R
1	*P. aeruginosa*	MBL	Negative	Negative	9	S
3	*P. aeruginosa*	MBL	Negative	Negative	15	S
1	*P. aeruginosa*	MBL	Negative	Negative	16	S
1	*P. aeruginosa*	MBL	Negative	Negative	17	S
1	*P. aeruginosa*	KPC and MBL	Negative	Negative	9	S
1	*P. aeruginosa*	KPC and MBL	Negative	Negative	9	R
2	*P. aeruginosa*	Negative	Negative	Negative	9	S
7	*P. aeruginosa*	Negative	Negative	Negative	9	R
1	*P. aeruginosa*	Negative	Negative	Negative	20	R
1	*P. aeruginosa*	Negative	Negative	Negative	19	S
1	*P. aeruginosa*	Negative	Negative	Negative	18	S

negatives/ total disease + total non-disease x 100. CI were calculated according to Robert Newcombe's method adapted from that of Wilson.[35,36]

95% confidence intervals = $p \pm 1.96 \times \sqrt{p(1-p)/n}$. Combined sensitivity and specificity was calculated according to Kanchanaraksa's method. Net sensitivity = sensitivity 1 x sensitivity 2. Net specificity = Spec1 + Spec2 – (Spec1 x Spec2).

Results

KPC/MBL in* P. aeruginosa/Acinetobacter *Confirm Kit

To evaluate the KPC/MBL in *P. aeruginosa/Acinetobacter* Confirm Kit 44 samples were examined using three MBL-positive *Pseudomonas* spp. and 41 MBL- and KPC-negative isolates, with results compared to PCR. All MBL isolates tested positive with this kit. A high number of false-positive results were observed, with 27/41 KPC/MBL-negative isolates, resulting in a calculated assay specificity of 38.6% (95%CI: 0.25–0.55) and 100% sensitivity (95% CI: 0.31–1).

Four out of the five *Acinetobacter* species containing OXA carbapenemases demonstrated synergy when tested with imipenem/meropenem and DPA, which was unexpected (Figure 2c). All isolates were retested for DPA susceptibility alone. From the four false-positive OXA carbapenemases, one was fully DPA resistant. Seventy percent of isolates were found to be fully DPA resistant (31/44). The susceptibility to DPA did not account for the false-positive synergistic effect. The synergy observed was due to the combined effect of the carbapenem and DPA, implying that an MBL was present, although this was not the case. The nine isolates demonstrating >9 mm diameter with DPA, combined with MBL synergy, had larger syngerstic zone with DPA and carbapenem combined (Table 4).

The kit manufacturer states that ceftazidime susceptible isolates may cause false MBL positives. In this study, six out of the 27 false positives were susceptible to ceftazidime. The controls worked as expected. The positive control strain *P. aeruginosa* ATCC 10145 was ceftazidime susceptible (Table 4).

Rapid CARB screen kit

The rapid CARB screen was modified as initial testing yielded weak-positive or negative results for carbapenemase-producing isolates. Initially, eight out of 10 NDM-producing isolates gave weak positive or negative reactions when cultured from blood agar. The use of a higher inoculum produced stronger reactions; however, six out of 10 NDM-producing isolates still produced weak positive reactions, with one OXA and one IMP carbapenemase remaining undetected when cultured from blood agar.

Addition of zinc to saline was performed in an attempt to enhance MBL detection; however, 11 uninterpretable results were obtained from the 78 isolates tested, whereby the negative control Diatab turned positive. No results were observed whereby the negative control Diatab turned positive while the test Diatab remained negative.

When Muller Hinton agar was used with a meropenem (10 μg) disc, the rapid CARB screen detected 70/76 CPOs. An additional five CPOs produced uninterpretable results; these isolates contain one of each of KPC, OXA-48, VIM and NDM and OXA genes and an ESBL isolate. Growth of isolates on Muller Hinton resulted in the highest sensitivity and specificity for the rapid CARB screen kit when compared to growth from blood agar and blood agar with the addition of zinc to saline (Table 5).

Table 5. Comparison of rapid CARB screen kit methods.

	Specificity	95% CI	Sensitivity	95% CI
Blood agar	68.2%	0.45–0.85	97.4%	0.90–1
Muller Hinton	68.2%	0.45–0.85	98.7%	0.92–1
Blood agar and zinc	50%	0.29–0.71	96.1%	0.88–0.99

Effects of three different methods used with the rapid CARB screen. The 98 isolates were tested three ways using the rapid CARB screen. Isolates were grown on blood agar, Muller Hinton agar with a meropenem (10 μg) disk and blood agar using zinc supplementation. In order to determine sensitivity and specificity, the uninterpretable, weak positive and positive isolates were grouped together as these would all be followed up. Values determined by comparison to PCR.

The *P. aeruginosa* isolates with reduced carbapenem susceptibility were cultured on Muller Hinton agar with a meropenem (10 μg) disc and tested by the rapid CARB screen. For the 133 isolates the calculated rapid CARB screen kit sensitivity was 98.7% (95% CI: 0.92–1) and 87.7% specificity (95%CI: 0.76–0.95) when grown on Muller Hinton agar (Table 6). Three ESBL- and two AmpC-producing isolates produced false positive results.

All weak positives/negatives were improved on culturing from Muller Hinton agar with a higher inoculum, with the exception of one OXA-51 from an *Acinetobacter baumannii*, which remained undetected (Table 7).

Check-MDR Carba

The Check-MDR Carba demonstrated 96.7% specificity (95% CI: 0.88–0.99) and 98.6% sensitivity (95% CI: 0.91–1) from the 132 isolates (76 CPOs and 56 non-CPOs) tested, with a calculated test efficiency of 97.7%. As OXA-51 and OXA-58 carbapenemase variants are not detected with this assay, 71 CPOs were detected. From this cohort, one IMP-producing standard strain, *P. aeruginosa* ATCC 10145, was not detected. This strain was not detected on repeat (Table 8). Of the 61 target negative samples, two samples were initially detected with positive cycle threshold (Ct) values but did not demonstrate true amplification plots; both samples were identified as PCR-negative on repeat. The assay required approximately 5 h to perform, including extraction, ligation and PCR.

Check-Direct CPE

Ninety-seven isolates (76 CPOs and 21 non-CPOs) were tested with the Check-Direct CPE, resulting in 96.5% specificity (95% CI: 0.80–1) and 97.1% sensitivity (95% CI: 0.89–1) with a calculated test efficiency of 96.9%.

As IMP, OXA-51 and OXA-58 carbapenemase variants are not detected with this assay, 68 CPOs were detected. Of the 29 target negatives, one sample was determined as positive (Ct: 39.64). No repeat was performed on this sample. Two of the bla_{NDM} were not detected initially, but were detected on repeat. Two of the 68 known carbapenemase-positive samples gave aberrant results with dual enzymes (Table 9). The first sample was a known KPC and OXA-48 producer, but only OXA-48 was detected. The second sample was also a known KPC and OXA-48 producer with only KPC being detected. Statistics were based on repeat PCR analysis. The Check-Direct CPE method required around 3 h to perform both the bacterial extraction and PCR.

Discussion

Carbapenemase spread poses a worldwide health threat in terms of the limited options for treating CPO infections. Routine detection methods have various levels of effectiveness due to the complex nature of class A, B and D carbapenemases, and variable phenotypic expression in levels of carbapenemase production.

Phenotypic carbapenemase testing in the laboratory is essential, providing a cost-effective way of detecting known and new carbapenemases. Results of the KPC/MBL *P. aeruginosa/Acinetobacter* Confirm Kit synergism were subjective in interpretation (Fig. 2). The high number of MBL false positives would incur considerable costs, with more isolates being followed up as possible carbapenemase producers. Acinetobacter species containing OXA carbapenemases demonstrated synergism when tested with a carbapenem in combination with DPA, which should not occur as these isolates have been confirmed to possess only class D carbapenemases by PCR.

The kit insert advises that ceftazidime-sensitive isolates may cause false positives (Rosco Diagnostica). Excluding ceftazidime-susceptible isolates, there still remained 21/44 isolates producing false-positive MBL synergism. The reason for this is unclear. Investigation of DPA susceptibility did not correlate with the false-positive results. DPA susceptibility did, however, make synergism harder to interpret as the increased DPA zone sizes were close to the zone sizes of imipenem/meropenem. DPA has previously been reported to have 'no inhibition of bacterial growth' by Shin *et al.*;[38] however, this study identified that 29.5% (13/44) of isolates

Table 6. Performance of Rapid CARB screen kit using Muller Hinton agar.

	Test positive	Test negative	Uninterpretable
Carbapenemase producers	93.4% (71)	1.3% (1)	5.3% (4)
Non-carbapenemase producers	10.5% (6)	87.7% (50)	1.8% (1)

The breakdown of positive, uninterpretable isolates and negative results for the rapid CARB screen using Muller Hinton agar. Uninterpretable isolates being those where the negative control Diatab was found to be positive.

Table 7. Rapid CARB screen carbapenemase detection using Muller Hinton.

Gene	No. of isolates tested	Sensitivity (%)	Specificity (%)
bla_{OXA-48}	24	100	100
bla_{OXA-51} and bla_{OXA-58}	4	100	100
bla_{OXA-51}	1	0	0
bla_{KPC} and bla_{OXA-48}	2	100	100
bla_{VIM}	10	100	100
bla_{NDM}	10	100	100
bla_{NDM} and bla_{OXA-48}	2	100	100
bla_{KPC}	17	100	100
bla_{IMP}	3	100	100
$bla_{VIM/NDM}$	3	100	100

Details the ability of the rapid CARB screen to detect each of the carbapenemase genes tested. In order to determine sensitivity and specificity the uninterpretable and weak positive, isolates were grouped as positive as these would all be investigated further. Values determined by comparison to PCR.

Table 8. Check-MDR Carba assay sensitivity and specificity for all genes detected.

Gene	No. of isolates tested	Sensitivity (%)	Specificity (%)
bla_{OXA-48}	24	100	100
bla_{KPC} and bla_{OXA-48}	2	100	100
bla_{VIM}	10	100	100
bla_{NDM}	10	100	100
bla_{NDM} and bla_{OXA-48}	2	100	100
bla_{KPC}	17	100	100
$bla_{VIM/NDM}$	3	100	100
bla_{IMP}	3	67	100

Details of the ability of the Check-MDR Carba to detect the carbapenemase genes for which it possesses targets. These targets are detected as either positive or negative results. Values are determined by comparison to independent, external PCR. One bla_{IMP} gene was not detected with the Check-MDR Carba.

demonstrated some level of inhibition due to DPA. A recent study by Hansen *et al*.[39] also found a low specificity of 67% using the KPC/MBL *P. aeruginosa/Acinetobacter* Confirm Kit performed with Muller Hinton. While Hansen *et al*.[39] used a different medium, in comparison to this study the results demonstrate a high false-positive rate with MBL-negative *P. aeruginosa*.

This study suffers from the low numbers of KPC/MBL-positive *P. aeruginosa/Acinetobacter* isolates examined. In future work, it would be beneficial to analyse more KPC/MBL-positive *P. aeruginosa/Acinetobacter* isolates to increase statistical power and examine the potential of this kit for KPC detection in *P. aeruginosa/Acinetobacter* species. *P. aeruginosa* and *Acinetobacter* spp. isolates collected on in-house carbapenemase detection media, which contains low-level ertapenem (0.25 mg/L) tested sensitive to meropenem and imipenem. The effect of decreased sample numbers can be seen in terms of power, with the large confidence interval of 0.31–1 for sensitivity. The confidence interval for specificity was found to be low (0.25–0.55). The *P. aeruginosa/Acinetobacter* Confirm Kit has demonstrated inadequate specificity for recommendation in this carbapenemase detection algorithm.

The rapid CARB screen method provides a comprehensive detection method where all class A, B and D carbapenemases that hydrolyse the imipenem β-lactam ring should be detected. This method provided a rapid result in less than 3 h, at a low cost of less than €2 per test. A drawback of this kit is the significant percentage of isolates that gave uninterpretable results, as the control tablet became positive. Similar findings were reported by Dortet *et al*.[40] where almost 30% of strains gave uninterpretable results with the Carba NP test using MacConkey agar.

The use of different media highlighted in Table 5 shows that Muller Hinton (Oxoid) performed optimally, as it demonstrated the highest sensitivity and specificity. Only 50% of bla_{NDM} producers were detected with Gram-negative selective media in a study by Dortet *et al*.[40] This is comparable to this project where culture on blood agar produced suboptimal detection of NDM producers, with bacterial inoculum being increased to produce the desired effect. In addition, the authors found that addition of zinc to Muller Hinton agar improved detection rates using bioMerieux Muller Hinton agar and Bio-Rad Muller Hinton.[40] However, the addition of zinc to saline in this study increased the number of uninterpretable results. The difference in detection may be due to the difference in zinc content of various media to enhance MBL activity. The test may also be affected by several external components (e.g., pH), as most carbapenemases demonstrate optimal activity at pH 6.8.[41] No GES-type carbapenemases were used in this study. In future work it would be beneficial to examine if these carbapenemases would be detected with the rapid CARB screen.

The false positives with the rapid CARB screen are hypothesised to be due to the production of a weak but constant carbapenem degradation process where much enzyme (AmpC/ESBL) is being produced. As noted by Woodford *et al*.,[42] imipenem has demonstrated relative susceptibility to slow hydrolysis by AmpC enzymes or

Table 9. Check-Direct CPE assay sensitivity and specificity for all genes detected.

Gene	No. of isolates tested	Sensitivity (%)	Specificity (%)
bla_{OXA-48}	24	100	100
bla_{KPC} and bla_{OXA-48}	2	0*	0*
bla_{VIM}	10	100	100
bla_{NDM}	10	100	100
bla_{NDM} and bla_{OXA-48}	2	100	100
bla_{KPC}	17	100	100
$bla_{VIM/NDM}$	3	100	100

*Single genes detected only

Details of the ability of the Check-Direct CPE to detect the carbapenemase genes for which it possesses targets. Values are determined by comparison to independent external PCR.

ESBLs. The three false-positive ESBL isolates in this study demonstrated cefoxitin resistance. This suggests an additional mechanism of resistance such as AmpC upregulation or impermeability.[42–45]

The bla_{OXA-51} in *Acinetobacter baumannii*, which was not detected by the rapid CARB screen kit, may benefit from the development of a modified version of the CARBA NP. This modified method is called the CarbAcineto NP, described by Dortet *et al*.[46] and is designed specifically for carbapenemase detection among *Acinetobacter* spp. The CarbAcineto NP test provides a cost-effective improvement in phenotypic carbapenemase detection in *Acinetobacter* spp. OXA-51 carbapenemases may be found intrinsically; however, over-expression and association with mobile genetic elements pose an infection control risk.[47]

The rapid CARB screen was found to be technically demanding and required careful handing to minimise bubbles created with the buffer. A recent paper by Yusuf *et al*. evaluated the rapid CARB screen (Rosco Diagnostica) and found it had sensitivities of 73.3% and 66.7% for Enterobacteriaceae and *P. aeruginosa*, respectively, while demonstrating 100% specificity.[48] These results are in contrast to those found in this study, with just 88% specificity and 99% sensitivity for both Enterobacteriaceae and non-Enterobacteriaceae. A study by Huang *et al*. likewise found 76% specificity and 98% sensitivity, with a high proportion of uninterpretable results. The report found that unlike the commercial rapid CARB screen (Rosco Diagnostica), using the Carba NP test demonstrated 97% sensitivity and 100% specificity.[49] High sensitivity is considered more important than high specificity in phenotypic screening methods as query carbapenemase isolates will be examined further with molecular methods. The rapid CARB screen (Rosco Diagnostica), has been shown to be a useful method for detection of carbapenemase producers. This screen needs to be used in combination with other methods for carbapenemase detection. Ultimately, the use of phenotypic tests is dependent on the level of β-lactamase expression, which can vary between enzymes and isolates.

Molecular testing confirms the presence/absence of carbapenemase genes and provides a means for epidemiological studies. Epidemiology allows development of effective strategies for monitoring CPOs. Analysis of the Check-MDR Carba demonstrated 96.7% specificity and 98.6% sensitivity, at approximately €92 to perform one test (including the negative extraction control and positive control). If batching samples, the cost could be reduced significantly (e.g., three tests with controls would cost approximately €125). As the Check-MDR Carba includes a two- hour ligation step, it takes 5 h from bacterial extraction to results. The Check-MDR Carba provides a wide range of carbapenemase targets but only defines carbapenemase-positive/negative results, and does not detect the type of carbapenemase present. The lack of detection of the one bla_{IMP} strain with this kit in this study was unexpected as this was a standard *P. aeruginosa* ATCC 10145 strain. A drawback of this kit is that it has been designed for use on isolates only and is not validated for use directly from stool specimens.

Analysis of the Check-Direct CPE demonstrated high sensitivity of 97.1% and specificity of 96.5%. This assay cost approximately €98 to perform for one test, including the negative extraction control and positive control, and took around 3 h. If batching samples, the cost could be reduced, with three tests and controls costing approximately €135. The assay identifies specific KPC, NDM/VIM or OXA-48 carbapenemase genes. One of the main drawbacks of the Check-Direct CPE is that IMP carbapenemase genes are not detected and VIM/NDM carbapenemases are not differentiated. This assay, however, is designed for use with isolates or rectal/perianal swabs, unlike the Check-MDR Carba. This study examined the application of the Check-Direct CPE on isolates only. Future studies would need to examine a range of faecal samples for carbapenemase detection. This would improve turnaround time considerably without the need to isolate the organism first.

Performing multiplex PCR can be deleterious to PCR reaction chemistry. This was clearly demonstrated in this study whereby several of the internal controls with the Check-Direct CPE were detected at higher Ct values or not at all when a carbapenemase was present. This study identified specific shortcomings in the Check-Direct CPE reactions chemistry in cases where two carbapenemase targets were present. Suboptimal reaction chemistry has resulted in the failure of the kit to detect dual enzyme activity. Of the four isolates with dual enzymes, it was of concern that the assay only detected one of the carbapenemases from the two KPC and OXA-48 producers. In terms of epidemiological linkage, failure to detect dual enzymes could provide incomplete data. The use of molecular methods has demonstrated more definitive and less-subjective results in comparison to the phenotypic methods above. Both Check-MDR Carba and the Check-Direct CPE incur high costs, even when isolates are batched, which may not be cost-effective for routine diagnostic laboratories. The Check-Direct CPE, however, will not be used in the algorithm due to its lack of ability to detect dual carbapenemase genes clearly, high cost and lack of IMP carbapenemase detection.

This study recommends the use of the rapid CARB screen, to be followed up by the Check-MDR Carba (which demonstrated >95% sensitivity and specificity) to be incorporated into a carbapenemase resistance detection algorithm (Fig. 3). The carbapenemase resistance detection algorithm demonstrates greater than 95% sensitivity and 95% specificity using these two methods and is accepted as a whole. Therefore, any false positives obtained with the rapid CARB screen can be excluded with the Check-MDR Carba. The sensitivities and specificities combined for the rapid CARB screen and Check-MDR Carba produce 97.3% net sensitivity and 99.6% net specificity. The introduction of the proposed algorithm would lead to an improved turnaround time of four days from isolation to carbapenemase detection. However, isolates testing positive for carbapenemase genes need characterisation of specific genes to be confirmed by the National Reference Laboratory.

Future promising developments include the CT103XL Check-MDR Microarray (Check-Points) which detects multiple β-lactamases in a single isolate. Emerging and rarer carbapenemases (e.g., GES, German imipenemase [GIM] and Sao Paulo metallo-β-lactamase [SPM]) can be identified along with several carbapenemases found in *A. baumannii*.[50] These methods are, however, very costly to perform and may not be feasible for a routine diagnostic laboratory.

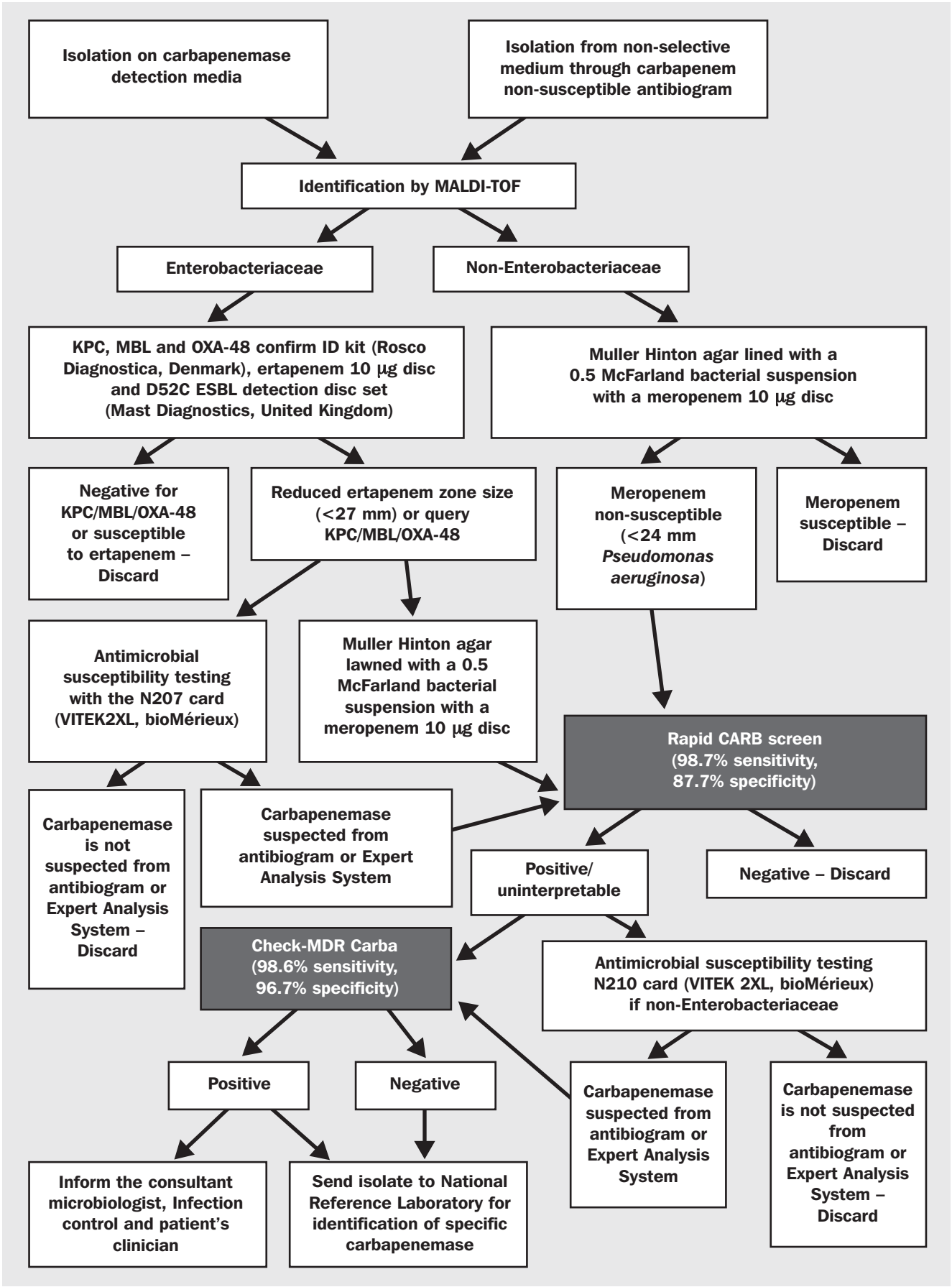

Fig. 3. The workflow from isolation of a possible carbapenemase to detection. The rapid CARB screen and the Check-MDR Carba are incorporated in this proposed method of detection.

Conclusions

In conclusion, the KPC/MBL in P. aeruginosa/Acinetobacter Confirm Kit provided subjective results and low specificity. The Check-Direct CPE provided incomplete results for dual carbapenemases and is not designed for IMP carbapenemase detection. Therefore, neither of these assays is recommended for carbapenemase detection. Check-MDR Carba demonstrated greater than 95% sensitivity and 95% specificity, but should be used in conjunction with other phenotypic detection methods such as the rapid CARB screen and the KPC, MBL and OXA-48 confirm ID kit (Rosco Diagnostica). The inclusion of these methods in a carbapenemase detection algorithm provides a system to decrease patient length of stay in isolation, reduces the number of days using broad-spectrum antibiotics, and prevents the spread of carbapenemase genes. The development of a combination of phenotypic and molecular methods is required for rapid, sensitive and specific carbapenemase detection. The algorithm generated through this study involves the incorporation of the rapid CARB screen and the Check-MDR Carba, which provides a comprehensive basis for carbapenemase detection. Limitation of carbapenemase spread requires further development of multiplex PCR assays and microarrays to provide affordable detection of the main carbapenemase genes in Ireland. □

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