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REVIEW ARTICLE

Review of established and innovative detection methods for carbapenemase-producing Gram-negative bacteria

J. Osei Sekyere, U. Govinden and S. Y. Essack

Antimicrobial Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

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Correspondence
John Osei Sekyere, Antimicrobial Research Unit, School of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.
E-mail: jod14139@yahoo.com

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Summary

The minimal antibiotic options for carbapenemase-producing Gram-negative bacteria necessitate their rapid detection. A literature review of a variety of phenotypic and genotypic methods is presented. Advances in culture methods and screening media are still subject to long incubation hours. Biochemical methods have shorter turnaround times and higher sensitivities and specificities, but cannot differentiate between various types and variants. Spectrophotometric methods are cheap and efficient, but are uncommon in many clinical settings, while the MALDI-TOF MS is promising for species identification, typing and resistance gene determination. Although next generation sequencing (NGS) technologies provide a better platform to detect, type and characterize carbapenem-resistant bacteria, the different NGS platforms, the large computer memories and space needed to process and store genomic data and the nonuniformity in data analysis platforms are still a challenge. The sensitivities, specificities and turnaround times recorded in the various studies reviewed favours the use of the biochemical tests (Carba NP or Rapid Carb screen tests) for the detection of putative carbapenemase-producing isolates. MALDI-TOF MS and/or molecular methods like microarray, loop-mediated isothermal amplification and real-time multiplex PCR assays could be used for further characterization in a reference laboratory. NGS may be used for advanced epidemiological and molecular studies.

Introduction

Increasing resistance to carbapenems, the antibiotic of last resort against many multi-drug resistant bacteria, is a worldwide clinical concern. Resistance is largely attributed to the carbapenem hydrolysing enzymes, carbapenemases, which hydrolyse most β-lactams. Carbapenemases are associated with diverse mobile genetic elements, including but are not limited to, plasmids, transposons and integrons. These enzymes carry multiple resistance genes, thereby conferring resistance to several antibiotic classes, such as aminoglycosides, fluoroquinolones, tetracyclines, trimethoprim, sulphonamides and phenicols (Queenan and Bush 2007; Nordmann 2014).

Carbapenemases have been classified molecularly into two groups based on their active sites: metallo-β-lactamases (MBL or class B), and serine-based (classes A and D) carbapenemases. The differences in the active sites of carbapenemases affect carbapenem hydrolysis rates and resistance to other β-lactam antibiotics and β-lactamase inhibitors (Queenan and Bush 2007; Bush 2013). MBLs have one or two zinc atom(s) at their active sites that are necessary for their hydrolytic ability. They hydrolyse all β-lactams except monobactams and are inhibited by ethylene diamine tetra acetic acid (EDTA), 1, 10-phenanthroline and dipicolinic acid (DPA) (Rasheed *et al.* 2013; Dijk *et al.* 2014). Class A types hydrolyse all β-lactam antibiotics, including monobactams, and are inhibited by the β-lactamase inhibitors clavulanic acid, sulbactam, tazobactam and avibactam (Queenan and Bush 2007). While Class D carbapenemases are also serine types with relatively weaker hydrolytic activity towards the carbapenems and oxy-iminocephalosporins they are varyingly inhibited by β-lactamase inhibitors (Nordmann and Poirel 2013).

Carbapenem-resistant phenotypes may also be attributed to the hyper-production of extended spectrum

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β-lactamases (ESBLs) or AmpC beta-lactamases (plasmid or chromosomally mediated class C β-lactamases), coupled with outer membrane impermeability due to efflux pump up-regulation and/or porin loss or mutation (Livermore and Woodford 2006; Bush 2013). The complexity and diversity of carbapenem resistance mechanisms, particularly carbapenemase-mediated resistance, necessitates rapid and accurate detection to inform appropriate therapy.

Phenotypic detection methods

While a number of tests may be used to identify carbapenemase producers, six established phenotypic methods are increasingly being adopted by most clinical microbiology laboratories and have been evaluated in several multicentre studies. These are the Modified Hodge Test (MHT), disc-inhibitors synergy test, chromogenic and nonchromogenic screening media, biochemical tests, spectrophotometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Phenotypic detection is subdivided into screening and detection tests. Screening media are used to determine colonization by carbapenemase-producing isolates.

Screening tests

Chromogenic and nonchromogenic screening media

The use of dehydrated chromogenic media for screening began with ESBL producers, and has been extended to carbapenem-resistant Enterobacteriaceae (CREs) (Gazin *et al.* 2012). Chromogenic media incorporates chromogens, which are molecules that serve as substrates for species-specific enzymes and change colour upon degradation to identify particular species of the Enterobacteriaceae, different colony colours being used to identify the various species or genera (Gazin *et al.* 2012; Day *et al.* 2013). The ChromID ESBL (bioMerieux, La Balme Les Grottes, France) chromogenic media was originally designed to detect ESBLs, but was found to have a higher sensitivity (>95%) and a lower limit of detection (LOD) (10–80 CFU ml^{-1}) than CHROMagarKPC (10^1–2×10^5) for class A and B carbapenemases (Gazin *et al.* 2012; Nordmann *et al.* 2012a). ChromID ESBL incorporates an extended spectrum cephalosporin, cefpodoxime, which is hydrolysed by class A and B but not class D carbapenemases, thus allowing this media to detect the former two classes effectively, with Nordmann *et al.* (2012a,b) reporting a sensitivity of 87·7% for ChromID ESBL compared to 40·3% for CHROMagar KPC (CHROMagar Microbiology, Paris, France) (Table 1). It is only able to detect class D carbapenemases when the host co-expresses an ESBL due to the inability of OXA-48 type enzymes to hydrolyse cefpodoxime (Gazin *et al.* 2012; Nordmann *et al.* 2012a).

CHROMagar KPC efficiently detects VIM and KPC carbapenemases (Kruse *et al.* 2013), with poor sensitivity for OXA-48 producers (Nordmann *et al.* 2012a; Girlich *et al.* 2013b), which accounts for its overall lower sensitivity. The highest sensitivity recorded for CHROMagar KPC was 76·6% (Vasoo *et al.* 2014), with associated specificity, positive and negative predictive values of 75·7, 59·0 and 87·6% respectively (Table 1). CHROMagar KPC is noted for its sensitivity for/enhanced ability to detect highly resistant CREs with higher MICs (Nordmann *et al.* 2012a; Girlich *et al.* 2013b).

Brilliance™ CRE (BC) media is another chromogenic media incorporating a carbapenem and two chromogens (Gazin *et al.* 2012; Kotsakis *et al.* 2013). Kotsakis *et al.* (2013) reported a higher sensitivity and specificity (98·8 and 79·3% respectively) for BC in detecting carbapenemase-producing *Klebsiella pneumoniae* than for other members of the Enterobacteriaceae, the overall sensitivity and specificity being 92·6 and 85·1% respectively. In the same study, the BC detected all *Acinetobacter baumannii* strains with an LOD of 10^1 CFU ml^{-1} and 91·3% of *Pseudomonas aeruginosa* with a specificity of 100% (Table 1).

A study conducted in Pakistan with mostly NDM-1 producing Enterobacteriaceae from stool samples reported a very low sensitivity (62·5%) for BC, due to the detection of 153 false positives, which were ESBLs or AmpC producers (Day *et al.* 2013). However, undetected NDM producers, possibly inhibited by the overgrowth of other Enterobacteriaceae, were detected with appropriate colours when re-inoculated onto BC, with only two carbapenemase-producing Enterobacteriaceae (CPE) species failing to produce a coloured colony. Consequently, the specificity, sensitivity and positive predictive values of BC were reduced to 59, 34 and 16% respectively when the colours of the colonies were used as a benchmark to determine CPEs (Table 1). In this same study, the ChromIDCARBA (bioMerieux, La Balme Les Grottes, France) was 100% sensitive, with a specificity and positive predictive value of 98 and 91% respectively, with all colonies being coloured on this media.

Girlich *et al.* (2013a) reported that the ChromID CARBA (bioMerieux, La Balme Les Grottes, France) had the lowest sensitivity in detecting OXA-48 CPEs, with a sensitivity <30% relative to SUPERCARBA (see below). It showed a sensitivity of 93–100% for an LOD between 10^1 and 10^2 CFU ml^{-1}) and ChromeID OXA-48 (bioMerieux, La Balme Les Grottes, France), which showed a sensitivity of 91·2 and 96·5% respectively for an LOD of 10^2 and 10^5 CFU ml^{-1} respectively. The specificity was higher (67·5%) than only the SUPERCARBA (52·5%) and the

Table 1 Performance characteristics of established and innovative carbapenemase detection methods

Test/Method	Turnaround time	Target species (*n**)	Target carbapenemases	Sensitivity (%)	Specificity (%)	PPV† (%)	NPV‡ (%)	Reference
EDTA-IMI disc	18–24 h	*Pseudomonas* spp.(116)	MBLs§	100·0	95·7	ND¶	ND	Yong *et al.* (2002)
		Acinetobacter spp.(23)	MBLs	91·0	100·0	ND	ND	
Combined Disc Test	18–24 h	Enterobacteriaceae (189)	Classes A & B	94·8	100·0	100·0	94·8	Pournaras *et al.* (2013)
Direct Ertapenem disc test	18–24 h	Enterobacteriaceae (150)	KPC	83·0	73·8	59·1	90·5	Vasoo *et al.* (2014)
Disc-diffusion assay	18–24 h	Enterobacteriaceae (128)	Classes A, B & OXA-48	95·0–100·0	96·0–100·0	ND	ND	Dijk *et al.* (2014)
OXA-48 disc test	18–24 h	Enterobactericeae (254)	OXA-48	96·3	97·7	ND	ND	Tsakris *et al.* (2015)
AVI-DDST	18–24 h	Enterobacteriaceae (23)	OXA-48 and Class A	100·0	100·0	ND	ND	Porres-Osante *et al.* (2014)
MAST-CDS	24 h	Enterobacteriaceae (38)	Class A and B	91·0	100·0	ND	ND	Saito *et al.* (2015)
ChromID ESBL	18–24 h	Enterobacteriaceae (114)	Classes A, B, D	87·7	24·2	ND	ND	Nordmann *et al.* (2012a)
CHROMagar KPC	18–24 h	Enterobacteriaceae (114–150)	Classes A, B, D	40·3–76·6	75·7–85·5	59·0	87·6	Nordmann *et al.* (2012a); Vasoo *et al.* (2014)
Brilliance™	CRE 18–24 h	Enterobacteriaceae (175–230)	Classes A, B	59·0–98·8	34·0–100·0	16·0	ND	Kotsakis *et al.* (2013); Day *et al.* (2013)
ChromID CARBA	18–24 h	Enterobacteriaceae (117)	Classes A, B, D	100·0	98·0	91·0	ND	Girlich *et al.* (2013b)
		Enterobacteriaceae (117)	OXA-48	<30·0	67·5	ND	ND	
ChromID OXA-48	18–24 h			91·2–96·5	96·5	ND	ND	
SUPERCARBA	18–24 h			93·0–100·0	52·5	ND	ND	
		Enterobacteriaceae (114)	Classes A, B, D	95·6	82·2	ND	ND	Nordmann *et al.* (2012a)
REMEL SPECTRA	18 h	Enterobacteriaceae (150)	KPC	97·8	86·4	76·7	98·9	Vasoo *et al.* (2014)
Rapid Carb Screen Kit	5–180 min	Enterobacteriaceae *Ps. aeruginosa* (92–133)	Classes A, B, D	73·3–98·7	87·7–100·0	84·6	88·7	Yusuf *et al.* (2014); Miriagou *et al.* (2013); Boran *et al.* (2015)
Carba NP test	5–120 min	Enterobacteriaceae *Ps. aeruginosa* (108–244)	Classes A, B, D	72·5–100·0	100·0	100·0	69·2–92·2	Dortet *et al.* (2012a,b); Tijet *et al.* (2013, 2014)
CarbAcineto NP test	5–120 min	*Ac. baumannii* (151)	Classes A, B, D	94·7	100·0	ND	ND	Dortet *et al.* (2014)
Spectrophotometry	1–3 hr	Enterobacteriaceae (44)	Classes A, B, D	100·0	98·5	ND	ND	Bernabeu *et al.* (2012)
MALDI-TOF MS	75 min-12 h	Gram-negative bacilli (101–149)	Classes A, B, D	95·2–100·0	90·0–100·0	ND	ND	Kempf *et al.* (2012); Lee *et al.* (2013); Hrabák *et al.* (2012); Vogne *et al.* (2014)
Real-time multiplex PCR	60–120 min	Gram-negative bacilli (58–465)	Classes A, B, D	100·00	89·9–100·00	46·6–16·6	100·0	Monteiro *et al.* (2012); Hanemaaijer *et al.* (2014); Lowman *et al.* (2014); Milillo *et al.* (2013)
Check-MDR Carba assay	4·5–5 h	Enterobacteriaceae (132–465), Nonfermenting Gram-negative bacilli (10)	NDM, VIM, IMP, OXA-48, KPC	90·5–100·0	87·7–100·0	100·0	100·0	Hanemaaijer *et al.* (2014); Cuzon *et al.* (2013); Boran *et al.* (2015); Vogne *et al.* (2014)

(*Continued*)

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Table 1 (*Continued*)

Test/Method	Turnaround time	Target species (*n**)	Target carbapenemases	Sensitivity (%)	Specificity (%)	PPV† (%)	NPV‡ (%)	Reference
Check-Direct CPE	<180 min	Gram –negative bacilli (83–450)	KPC, VIM, NDM, OXA-48	97·1–100·0	94·0–100·0	100·0	0·0–70·0	Nijhuis *et al.* (2013); Findlay *et al.* (2015); Boran *et al.* (2015); Huang *et al.* (2015)
LAMP/eazyplex® superBug complete A	25–60 min	Gram –negative bacilli (14–450)	KPC, VIM, NDM, OXA-48	100·0	100·0 (83·0 for OXA-48 like genes)	ND	ND	Findlay *et al.* (2015); Liu *et al.* (2012); Nakano *et al.* (2015)
TaqMan PCR	<120 min	Enterobacteriaceae (59, 1308)	Classes A, B & D	100·0	100·0	ND	ND	Swayne *et al.* (2013)
NucliSENSEasyQKPC	<120 min	Enterobacteriaceae (300)	KPC only	100·0	100·0	ND	ND	Spanu *et al.* (2012)
Xpert®Carba-R kit	52 min	Gram-negative bacilli (450)	KPC, VIM, NDM, OXA-48	100·00	100·0 (83·0 for OXA-48 like genes)	ND	ND	Findlay *et al.* (2015)
Microarray (Alere technologies)	2–8 h	Gram-negative bacilli (117)	Classes A, B, D	98·2	97·4	ND	ND	Braun *et al.* (2014)
Microarray (Verigene BC-GN)	2 h	Gram-negative bacilli (104)	Classes A, B, D	96·8	100·0	ND	ND	Sullivan *et al.* (2014)
Microarray (Check-MDR CT101-103)	≤6 h	Gram-negative bacilli (57–187)	Classes A, B, D	90·5–100·0 (KPC=85·0)**	95·7–100·0	97·6–100·0	99·0–100·0	Sullivan *et al.* (2014); Woodford *et al.* (2011); Stuart *et al.* (2012); Naas *et al.* (2011a); Cuzon *et al.* (2012); Castanheira *et al.* (2013)
Xpert MDRO assay	<1 h	Gram-negative bacilli (328)	KPC, NDM, VIM	100·0	99·0–99·4	81·8–93·0	100·0	Tenover *et al.* (2013)
Next generation sequencing platforms	>8 h	All cells (103–110)	Classes A, B, D	100·0	100·0	ND	ND	Deraspe *et al.* (2014); Espedido *et al.* (2013); Perichon *et al.* (2014); Barisic *et al.* (2014)

AVI-DDST, avibactam-double disc synergy test; CPE, carbapenemase-producing Enterobacteriaceae; CRE, carbapenem-resistant Enterobacteriaceae; LAMP, Loop-mediated isothermal amplification; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MBL, metallo-β-lactamases.

*Sample size.

†Positive predictive value.

‡Negative predictive value.

§Metallo beta lactamase.

¶Not determined by author.

**Naas *et al.* found sensitivity and negative predictive value for KPC to be <100·0% with the Check-MDR CT102.

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LOD was above 10^5 CFU ml^{-1}. The ChromID OXA-48 was designed by to augment the OXA-48 deficiencies of the ChromID CARBA (Girlich *et al.* 2013a).

The SUPERCARBA is a nonchromogenic media that was designed in-house by Nordmann and colleagues in 2012 to identify all carbapenemases classes, incorporating lower ertapenem concentration with zinc sulphate and cloxacillin to inhibit AmpCs and noncarbapenemase CREs in a Drigalski agar base (Nordmann *et al.* 2012a). It had a higher sensitivity and specificity (95·6 and 82·2% respectively) than ChromID ESBL and CHROMagar KPC. It was able to detect class A, B and D carbapenemases, with sensitivities of 100, 90 and 100% respectively (Table 1) (Nordmann *et al.* 2012a). The SUPERCARBA had a relatively lower LOD (10^1–10^2 CFU ml^{-1}) for NDM, KPC and OXA-48 producers, with the LOD ranging between 10^1–10^6 CFU ml^{-1} for VIM and IMP producers. It is able to detect CREs and nonCPE CREs. In another study, the SUPERCARBA had a lower specificity (60·7%) than CHROMagar KPC due to the false positive detection of AmpCs and ESBLs hyperproducers that had poor membrane permeability (Girlich *et al.* 2013b). The SUPERCARBA is the only media able to detect all carbapenemases types (A, B, OXA-48).

Another chromogenic media, Remel Spectra™ CRE (Thermo Fisher Scientific, Basingstoke, UK), has been shown to have slightly higher sensitivity, specificity, positive and negative predictive values (97·8, 86·4, 76·7 and 98·9% respectively) when compared with CHROMagar KPC and the direct ertapenem disc method, which uses an ertapenem disc-diffusion method to determine sensitivity to ertapenem (83·0, 73·8, 59·1 and 90·5% respectively) (Table 1). As with the disc-inhibitors tests, the major disadvantage of these media is the 1 day incubation required before results are obtained. In addition, the absence of chromogen-specific enzymes in certain strains of a targeted species results in false negatives, as the expected colony colour are not observed (Vasoo *et al.* 2014).

These media are mainly limited to the Enterobacteriaceae and cannot detect nonfermenting bacilli. Their ability to detect various carbapenemases types is highly variable and, except for SUPERCARBA and the ChromID OXA-48, they are unable to detect OXA-48 producers. They are also not able to absolutely inhibit non-CPE CREs, which results in false positives. In addition, these tests assume the phenotypic expression of the resistance genes, creating the potential for false negative results in the event of their being unexpressed or minimally expressed carbapenemase genes. The use of these screening media must therefore be confirmed by genotypic tests, increasing the detection time and costs (Kruse *et al.* 2013; Hrabák *et al.* 2014).

Detection tests

The modified Hodge test

The cloverleaf technique, or MHT, is based on the inactivation of a carbapenem by carbapenemase-producing strains that enables a carbapenem-susceptible indicator strain to extend growth towards a carbapenem-containing disc, along the streak of inoculum of the tested strain (Girlich *et al.* 2012). The MHT has been fraught with false positive detections (especially among AmpC and CTX-M hyperproducers) in several studies (Wang *et al.* 2011; Clinical and Laboratory Standards Institute (CLSI), 2015) and poor detection of NDM producers (Girlich *et al.* 2012).

Disc-inhibitors synergy test

Carbapenemase class-specific inhibitors have been used to detect and differentiate between Class A and B carbapenemases. Yong *et al.*(2002) demonstrated the ability of EDTA to inhibit MBLs by chelating with the zinc ions needed at their active sites, thus inhibiting their activity (Yong *et al.* 2002). Using 750 μg EDTA with 10 μg imipenem disc, they were able to detect MBLs in 100% of *Pseudomonas* spp., tested with a specificity and sensitivity of 95·7 and 91·0% respectively being obtained for *Ac. baumannii* (Table 1). Pitout *et al.* (2005) proved meropenem to be a better alternative to imipenem, and recommended its use with EDTA tests to identify MBLs. Doi *et al.* (2008) used 3-amino phenyl boronic acid (PBA) with ertapenem to detect class A *Kl. pneumoniae* carbapenemase (KPC)-producers among *Escherichia coli* and *Kl. pneumoniae*. The mechanism behind the inhibition of class A carbapenemases by PBA is still unknown (Pitout *et al.* 2005; Doi *et al.* 2008).

Pournaras *et al.* (2013) improved these tests by using both EDTA (10 μl of 0·5 mol l^{-1}) and PBA (10 μl of 40 mg ml^{-1}) with 10 μg meropenem discs on a single plate to detect and differentiate between carbapenemase types A and B from rectal swabs, with sensitivity and specificity of 94·8 and 100% respectively in Enterobacteriaceae. In this assay, meropenem discs alone were used as a control and standard to detect changes in the inhibition zones after inoculating an additional three meropenem discs with EDTA, PBA and EDTA-PBA. A difference of ≥5 mm between the meropenem and meropenem-impregnated discs were considered positive for MBL, class A or both carbapenemases respectively (Pournaras *et al.* 2013).

In a more complicated method, Birgy *et al.* (2012) impregnated meropenem discs with EDTA, PBA, clavulanate and cloxacillin with two Mueller-Hinton agar plates to detect MBLs, class A carbapenemases, ESBL and AmpCs respectively. This was done in thirty well-charac-

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terized Enterobacteriaceae with different resistance mechanisms, viz., ESBLs, plasmid-borne AmpCs, porin mutations and all carbapenemases types. These combinations were able to detect and differentiate between carbapenemase classes, ESBL or AmpC producing carbapenem resistance phenotypes. As AmpCs are inhibited by PBA, cloxacillin was used to differentiate between AmpCs and Class A carbpanemases by comparing the zone sizes of cloxacillin-impregnated carbapenem discs to those of PBA. Class A ESBLs are, however, uninhibited by PBA or cloxacillin (Birgy *et al.* 2012).

By using DPA, PBA and temocillin with meropenem discs, Dijk *et al.* (2014) were able to detect and identify MBLs and the serine-based classes A and D carbapenemases in Enterobacteriaceae respectively through inhibition. Class D carbapenemases were detected with a sensitivity and specificity of 100% by resistance to temocillin, indicated by zone diameters of ≤10 mm together with the absence of synergy between meropenem discs and DPA, PBA or both. Class A and B carbapenemases were detected with sensitivities of 95% and 90% and specificities of 99 and 96% respectively. While Dijk *et al.* (2014) were able to phenotypically detect all OXA-48-like oxacillinases using inhibitors and meropenem discs with apparent efficiency, Oueslati *et al.* (2015) suggest that detection and screening strategies based on temocillin resistance are valid for OXA-48-like enzymes (OXA-48, OXA-162, OXA-181 and OXA-204) with significant carbapenemase activity only (Dijk *et al.* 2014; Oueslati *et al.* 2015). Porres-Osante *et al.* (2014) developed the avibactam-double disc synergy test, which used ertapenem and avibactam-impregnated discs on cloxacillin containing Mueller-Hinton agar. The cloxacillin inhibited AmpC producers, and allowed the test to identify KPC, IMI and OXA-48 producing Enterobacteriaceae, as well as porin-deficient extended spectrum β-lactamase (ESBL) producing isolates, with 100% specificity and sensitivity. However, the sample size was limited and further tests are necessary to establish its validity (Porres-Osante *et al.* 2014). Tsakris *et al.* (2015) designed a disc test to identify OXA-48 producing Enterobacteriaceae using an imipenem disc and two blank discs each impregnated with EDTA and EDTA plus PBA respectively. A distortion of the inhibition halo around the two blank discs was read as OXA-48 positive, and the test was 96·3% sensitive and 97·7% specific (Tsakris *et al.* 2015).

A commercial carbapenemase detection kit, the MAST-CDS (Mast Group, Merseyside, UK), detects class A and B CPE through inhibitor-impregnated meropenem discs. Disc A contains only meropenem with no inhibitor, B contains 1000 μ DPA to inhibit MBLs, C contains 600 μg aminophenylboronic acid to inhibit class A-type carbapenemases and D contains 750 μg cloxacillin to inhibit AmpC producers. CLSI guidelines for disc-diffusion tests are used in this test. Differences in zone sizes between A and B (≥5 mm) and A and C (≥4 mm) are read as MBL and KPC-type positive. Where zone size differences of ≥4 mm and ≥5 mm are observed in both C and D respectively, compared to A, then test isolates are porin-deficient AmpC producers. This test does not detect OXA-48 producers, and has a sensitivity of 91% and a specificity of 100% for class A and B types (Saito *et al.* 2015).

Although the disc-inhibitor synergy tests are simple, cheaper and relatively efficient in detecting and differentiating between carbapenemases, they require at least 18 h incubation to obtain results. This longer turnaround time is the main drawback of these inhibitor synergy tests with carbapenem antibiotic discs. As with all phenotypic tests, the potential exists for false negative results in the event of unexpressed or minimally expressed resistance genes.

Biochemical tests

The progressively derived Carba NP I and II and the CarbAcineto tests are three biochemical tests with relatively high sensitivities and specificities, and have faster turnaround times than culture based methods. They were developed in 2012 by Nordmann and colleagues (Dortet *et al.* 2012b, 2014) to detect carbapenemase producers in Enterobacteriaceae, *Pseudomonas* spp. and *Ac. baumannii.* These tests are all based on the hydrolysis of imipenem, with a colour change of a phenol indicator from red to yellow due to the acidic product engendered from imipenem hydrolysis. The CarbAcineto test is specifically optimized for detecting *Ac. baumannii* carbapenemase producers through the use of higher inoculum sizes and NaCl instead of a lysis buffer in the Carba NP test (Dortet *et al.* 2014). The Carba NP II further detects and differentiates classes A and B carbapenemase types by including EDTA and tazobactam to inhibit MBLs and class A carbapenemases respectively. Isolates that are carbapenemase positive, but fail to be inhibited by either class of inhibitors, are defined as class D carbapenemase producers (Dortet *et al.* 2012b).

The Carba NP test has been reported as having a sensitivity and specificity of 100% by almost all the studies conducted using Enterobacteriaceae and *Pseudomonas* spp. (Dortet *et al.* 2012a; Nordmann *et al.* 2012b; Yusuf *et al.* 2014). Although a sensitivity ranging between 91 and 100% is commonly recorded, Tijet *et al.* (2013) found a lower sensitivity of 72·5%, and a negative predictive value of 69·2% using 244 Enterobacteriaceae isolates, due to the inability of the test to identify carbapenemases in mucoid and swarming cells, and/or in those harbouring carbapenemases with low hydrolytic activity (Tijet *et al.* 2013, 2014). A new commercial version of the

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CARBA NP test (RAPIDEC® CARBA NP), with a reported specificity and sensitivity of 97·8%, is available (http://www.biomerieux-diagnostics.com/rapidec-carba-np) (Poirel and Nordmann 2015). The Carba NP test has been added to the MHT as the carbapenemase detection method officially recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015).

The Rapid CARB Screen Kit (RoscoDiagnostica A/S, Taastrup, Denmark) is another commercial chromogenic media that was reported to have sensitivity, specificity, positive and negative predictive values of 73·3, 100, 84·6 and 88·7% respectively when compared with the Carba NP test, which had values of 91·1, 100, 100 and 92·2% respectively (Yusuf *et al.* 2014). Its detection of OXA-48 producers and weakly hydrolytic carbapenemases is ineffective and should be used with caution in regions with high OXA-48 producers (Miriagou *et al.* 2013; Yusuf *et al.* 2014).

The Blue-CARBA was designed by Pires *et al.* (2013) as a modification of the Carba NP test, the aim being to contain costs and increase simplicity. This test uses whole cells without a protein extraction step, a more economical brand of imipenem, and a bromothymol indicator with a pH range of 6·0–7·6 optimal for most β-lactamases, which changes colour within 2 h, making it faster and cheaper. It detects carbapenemase production with a reported 100% sensitivity and specificity for all Gram-negative carbapenemase-producing bacteria. However, higher inocula are required for OXA-48 producers. Few studies have been done to evaluate this method, and additional analyses is required to prove the concept of this faster and cheaper method for all class D carbapenemases (Pires *et al.* 2013).

The biochemical tests hold much promise over other phenotypic methods, due to their relatively lower cost, faster turnaround time, simplicity, and minimum skill required in conducting them. They are also able to differentiate between carbapenemase producers and noncarbapenemase-producing CREs, ESBLs and/or AmpCs hyperproducers with membrane impermeability. However, their inability to differentiate between carbapenemase types, especially for isolates expressing multiple carbapenemases, and to detect isolates with low carbapenemase hydrolysis activity, makes them less effective than molecular tests. In addition, like all other phenotypic tests, these assume the phenotypic expression of resistance genes, creating the potential for false negative results in the event of unexpressed or minimally expressed carbapenemases.

Spectrophotometry

Spectrophotometry is an established reference method used by Bernabeu *et al.* (2012) to detect and differentiate carbapenemases in Gram-negative bacilli using imipenem as the substrate. Supernatant obtained from centrifuged and sonicated 24 h-culture cells is added to imipenem. Absorbance per minute of imipenem alone was compared with that of imipenem and bacterial extract. This was used to determine the various types of carbapenemases, with a sensitivity and specificity of 100 and 98·5% respectively compared to conventional PCR, while identifying specific carbapenemases and excluding noncarbapenemase producers from a specimen. (Bernabeu *et al.* 2012). This method is easy, fast and cheap, but requires skills and a spectrophotometer not found in many clinical microbiology laboratories in resource-constrained countries. This technique efficiently detects VIM, IMP and SIM producers, while NDM and carbapenem hydrolysing Ambler class D β-lactamase producers remain difficult to detect (Dortet *et al.* 2014).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry

The MALDI-TOF MS is a proteomics tool that indirectly identifies resistance genes using expressed proteins (enzymes) (Hrabák *et al.* 2012, 2014). It is becoming common in many clinical microbiology laboratories in developed countries to identify bacterial species, subspecies and their resistance genes. The charge to mass ratio of an ionized carbapenem, and its degradation products, is detected after incubating the carbapenem with extracted bacterial proteins for 1–4 h, forming spectra of the carbapenem and its degradation products. This is used to identify the carbapenemase type and variant involved with 100% specificity and sensitivity in most Gram-negative bacilli (Kempf *et al.* 2012; Lartigue, 2013; Lee *et al.* 2013).

Hrabák *et al.* (2012) reduced the incubation time required by adding sodium dodecyl sulphate (SDS) to the bacterial cells before incubation with the meropenem substrate. They used this modification to detect NDM-1, VIM-1, KPC, OXA-48 and OXA-162 in Enterobacteriaceae within 4 h with 100% sensitivity and specificity. Kempf *et al.* (2012) used an imipenem substrate with an ethanol, acetone and tri-fluoroacetic acid matrix in an Ultraflex I mass spectrometer to obtain clear imipenem, and imipenem degradation products' spectra, with no background peaks or noise to detect carbapenemases in *Ac. baumannii.* An ertapenem substrate was used to detect IMP-6, VIM-2, NDM-1, SIM-1, KPC-1, OXA-23 and OXA-51 carbapenemases with 100% specificity and sensitivity within 2 h of incubation for MBLs and KPC types, and 4 h for class D enzymes. Although not reported in this study, there is the likelihood that the detected OXA-51 enzymes were hyper-produced as they cannot be detected under normal levels. The same study

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established the appropriate volume and proportion of bacteria that should be used for incubation to obtain the correct spectra, the bacterial volumes with excellent results being ≥20 μl (Hrabák *et al.* 2012; Kempf *et al.* 2012; Lee *et al.* 2013).

Hoyos-Mallecot *et al.* (2014) used blood cultures to determine the presence of carbapenemases with a MALDI-TOF MS, and obtained a sensitivity and specificity of 100 and 90% respectively within 4·5 h. Sauget and colleagues (Sauget *et al.* 2014) described a sensitivity of 98·9% and a specificity of 97·8% compared with PCR in a MALDI-TOF MS to OXA-48 producing Enterobacteriaceae, where an imipenem substrate was used after 1 h incubation with bacterial extracts and a turnaround time of 90 min (Hoyos-Mallecot *et al.* 2014; Sauget *et al.* 2014).

MALDI-TOF MS, in combination with PCR, is suggested as being an advanced and faster method of identifying resistance genes to improve clinical antibiotic therapy (Lupo *et al.* 2013). Its main disadvantage is the initial cost of the instrument, making it unaffordable to many laboratories in resource-constrained settings (Lupo *et al.* 2013; Hrabák *et al.* 2014). It also works indirectly at the genetic level by detecting proteins that have been translated from messenger RNAs transcribed from a gene. Despite its advantages, it cannot detect unknown resistance genes.

Genotypic methods

Molecular methods

Conventional PCR, with subsequent gel electrophoresis, is giving way to real-time PCR that is able to detect and quantify resistance genes simultaneously by using fluorescent probes or dyes. The results are displayed on a screen as fluorescent intensity against a melting temperature graph that is used in a melting curve analysis. Genes have unique melting curves that are used to identify them in real time without the need for a gel electrophoresis (Wang *et al.* 2012a,b). Other real-time assays use probes with unique molecular fluorescent beacons that attach to the template DNA and ligate (probe ligation), making them very specific. This is followed by amplification by universal primers, and fluorescent detection of the unique beacons and probes using their cycle threshold, a mechanism used by many commercial microarrays such as the Check-Direct CPE (Check- points, Wageningen, the Netherlands) (Nijhuis *et al.* 2013; Hanemaaijer *et al.* 2014).

Multiplex PCR uses a set of multiple primers to detect several genes simultaneously (Doyle *et al.* 2012), and requires the use of highly specific primers that do not self-react. The cycling conditions must also be ideal for all primers, and the amplicon sizes of the target genes must be different for easy resolution and identification (Lupo *et al.* 2013). By combining real-time and multiplex PCR, it is now possible to detect a variety of resistance genes in a single reaction with a shorter turnaround time.

Real-time multiplex assays

In-house

Monteiro *et al.* (2012) were first to report the use of multiplex real-time PCR to detect all carbapenemase classes (NDM, KPC, GES, IMP, VIM and OXA-48) within 3 h (including DNA extraction) in a single reaction using a melting curve analysis. This enabled identifying all carbapenemase types and variants with 100% sensitivity and specificity in Enterobacteriaceae, *Ac. baumannii* and *Ps. aeruginosa,* an improvement on those achieved using conventional PCR. Multiplex real-time PCR has been used in initial screenings to detect NDM, VIM, IMP, GES, KPC and OXA-48 CPEs in rectal and throat swabs, as well as in peri-anal and stool samples, with a sensitivity, specificity, positive and negative predictive values of 100, 93·3, 46·6 and 100% respectively (Hanemaaijer *et al.* 2014; Lowman *et al.* 2014). Naas *et al.* (2011a,b) have optimized real-time quantitative PCR (qpcr) for detecting NDM-1carbapenemases directly from stool samples with comparable, if not superior, detection limits and a faster turnaround time of results (4 *vs* 48 h) compared with culture techniques (Naas *et al.* 2011b).

Commercial

The GenExpert® system (Cepheid, Sunnyvale, CA) recently developed a real-time PCR technique with ready to use cartridges to rapidly detect the clinically relevant bla_{KPC},bla_{IMP},bla_{KPC}, bla_{VIM}, bla_{NDM} and bla_{OXA-48} carbapenemases. The OXA-48 group may be extended spectrum β-lactamase producers, and therefore resistant to expanded-spectrum cephalosporins, while others may be of reduced susceptibility or resistant to carbapenems e.g. OXA-163 (differing from OXA-48 by a single amino substitution and a four amino acid deletion), which confers resistance to broad spectrum cephalosporins but hydrolyses carbapenems only marginally. OXA-181 differs from OXA-48 by four amino acids, but possesses very similar hydrolytic activity towards β-lactams. Detection of OXA-48-like carbapenemases requires a subsequent sequencing step as PCR alone cannot differentiate between carbapenemase and ESBL producers. Decousser *et al.* (2015) reported failures in detecting bla_{OXA-48} like genes in *Escherichia coli* with the GenExpert® system using the Xpert Carb-R®cartridge, while a positive carbapenemase

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result was obtained with the Carba NP® test. Hence, Decousser *et al.* (2015) suggest that molecular diagnostic techniques in the current phase of their development should not be considered as reference standards for detecting CRE carriers, as molecular-based techniques may detect only a limited number of known genes (Decousser *et al.* 2015).

Using the commercial Check-MDR Carba assay (Check-Points), also based on real-time multiplex PCR technology, Hanemaaijer *et al.* (2014) detected KPC, NDM, OXA-48, VIM and IMP from rectal and throat swabs after incubation and DNA extraction. The bacterial isolates were incubated overnight in a solution of vancomycin (50 mg l^{-1})-ertapenem (0·25 mg l^{-1}) and vancomycin (50 mg l^{-1})-ceftazidime (2 mg l^{-1}), after which both solutions were mixed, lysed with a chromopeptidase at 37°C for 15 min, and centrifuged to obtain a supernatant that was used for the Check-MDR Carba assay (Hanemaaijer *et al.* 2014; Findlay *et al.* 2015). Cuzon *et al.* (2013) used the Check-MDR Carba to detect the same enzymes in 183 Gram-negative rods within 5·5 h (including DNA extraction) by detecting FAM labelled fluorescence probes within an FAM channel of 21·0 and 32·0 threshold cycle (Cuzon *et al.* 2013).

The Xpert®Carba-R kit (Cepheid) is a commercial multiplex real-time-based kit that has been evaluated to detect most carbapenemases, except IMP, among Gram-negative bacilli within 52 min with 100% sensitivity and specificity to all tested carbapenemases, except some OXA-48 and OXA-181 types as recently discovered, decreasing the specificity to 71% (Table 1) (Decousser *et al.* 2015; Findlay *et al.* 2015). The kit has an Xpert®Carba-R Assay cartridge and sample reagent that is loaded into a GeneXpert GXXVI platform to run the reaction. More studies would be necessary to further establish this detection kit's reliability and reproducibility.

Wang *et al.* (2012a,b) used SYBR Green fluorescent molecules in an internally controlled real-time PCR to detect KPC from 120 stool and 128 nasal swabs samples with an LOD of 80 CFU ml^{-1}, and attained a 100% and 99% sensitivity and specificity respectively (Table 1) (Wang *et al.* 2012a,b). This assay could not differentiate between various KPC variants, although it was fast (<2 h). Subsequent studies by the same authors (Wang *et al.* 2012b) with 159 clinical isolates (*Kl. pneumoniae*, *Klebsiella oxytoca*, *E. coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Proteus mirabilis*, *Ac. baumannii* and *Ps. aeruginosa*) were, however, 100% sensitive and specific, with an LOD of 0·8 CFU ml^{-1} and a turnaround time of ≤4 h. The SYBR Green is a cheaper and more sensitive (albeit less specific) method compared to the fluorescent tagged probes (Wang *et al.* 2012a,b).

The Check-Direct CPE (Check-Points) is a new commercial multiplex real-time PCR assay designed by the same manufacturers of the Check-MDR Carba assay to simultaneously detect KPC, OXA-48, VIM and NDM from rectal swabs. However, as a surveillance tool, it is not able to differentiate between NDM and VIM, as the probes of both latter genes have same fluorescent tags (Nijhuis *et al.* 2013; Hanemaaijer *et al.* 2014). Notwithstanding, Findlay *et al.* (2015), using a BD MAX™ (Becton Dickinson, Oxford, UK) platform, were able to distinguish NDM and VIM. IMP types are not detected by this assay (Findlay *et al.* 2015). An advantage is that it has a lower turnaround time of ≤3 h, with an LOD of 200–2 × 10^4 CFU ml^{-1} and a 100% sensitivity and specificity (Table 1). Not all variants of the same class (A, B and D) and families (IMP, SME, NMC, SIM, DIM, AIM, GES etc.) of carbapenemases were included, and more evaluation is thus necessary to assess the assay's ability to detect all variants (Nijhuis *et al.* 2013).

Innovative PCR assays

A new PCR-based assay (the Xpert MDRO PCR assay), consisting of a cartridge containing reagents for DNA extraction, amplification and detection, was evaluated (Tenover *et al.* 2013) on 328 rectal, peri-rectal and stool samples. It detects KPC with sensitivity, specificity, positive and negative predictive values of 100, 99, 93 and 100% respectively. It also detects VIM with sensitivity, specificity, positive and negative predictive values of 100, 99·4, 81·8 and 100% respectively.

Swayne *et al.* (2013) used novel and improved TaqMan PCR assays to detect MBLs (IMP, SIM, GIM, VIM, NDM and SPM), serine carbapenemases and ESBLs with a mixture of primers and probes. Using the threshold cycle to identify the enzymes, the assay was 100% sensitive and specific. Milillo *et al.* (2013) used a similar method of primers and probes with 16S RNA to identify NDM and KPC directly from cultures with 100% sensitivity and specificity. Easy QKPC (bioMerieux, Marcy l'Etoile, France) is a new real-time PCR assay that works on the NUCLiSENS platform to identify KPCs (Spanu *et al.* 2012), the NUCLiSENSEasyQ analyser amplifies, and detects and automatically interprets results in real time. Spanu *et al.* (2012) showed that the Easy QPC was 100% sensitive and specific with a faster turnaround time (≤2 h).

Microarray

A microarray based assay (Alere Technologies GmbH, Loebstedter, Jena, Germany) was evaluated with DNA extracted from 117 clinical Enterobacteriaceae, *Ps. aerugi-*

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nosa and *Ac. baumannii* strains to identify the species, carbapenemases, ESBLs and narrow spectrum β-lactamases genes in a single reaction, with 98·2 and 97·4% sensitivity and specificity respectively (Braun *et al.* 2014). This commercial microarray provides a report on the species and resistance genes after analysing signals picked from hybridized probes on an Array Mate Reader (Braun *et al.* 2014). In this evaluation, an OXA-23 and OXA-48 genes were not detected, and two *K. oxytoca* and one *Kl. pneumoniae* isolates were not identified.

A new commercial microarray, the Verigene Gram-negative blood culture nucleic acid test (BC-GN) (Nanosphere, Northbrook, IL) was evaluated by Sullivan and peers (Sullivan *et al.* 2014). It was able to identify nine different Gram-negative bacterial genus/species and six resistance determinants (including KPC, NDM, VIM, IMP, OXA) directly from positive blood cultures within 2 h. The sensitivities and specificities of the species' and resistance genes' identification were 89 and 100 and 96·7 and 100% respectively (Table 1). However, detecting species in polymicrobial bacteraemia were inefficient, and more evaluations would be needed to establish the reliability of this relatively new assay. In the same study, the Check-MDR CT102 microarray was used as a parallel reference standard with perfect concordance with real-time PCR (Table 1). A more recent version of the Check-MDR CT102, the Check-MDR CT103, was evaluated on 87 Gram-negative bacilli to detect both carbapenemases and different ESBLs and AmpCs concurrently, and was found to be efficient, rapid and robust (Table 1) in detecting the commonest of these enzymes (Cuzon *et al.* 2012).

Loop-mediated isothermal amplification

To overcome inherent deficiencies in PCR, like the cost involved in purchasing consumables and the inactivation of *Taq*polymerase by inhibitors (heparin and blood serum) in crude biological samples, the loop-mediated isothermal amplification (LAMP) assay, which only needs a temperature-controlled water bath to ensure isothermal conditions, has been developed (Liu *et al.* 2012; Nakano *et al.* 2015). It works by autocycling strand displacement DNA synthesis, using *Bst*DNA polymerase and a set of four to eight primers that attaches to various portions of the DNA, thereby increasing its sensitivity and specificity (Liu *et al.* 2012; Nakano *et al.* 2015). The amplification process can be directly observed by measuring the turbidity of the reaction solution with a spectrophotometer or a fluorescent reagent (Liu *et al.* 2012; Nakano *et al.* 2015). The assay is cheap, fast (works within 1 h) and simple with higher sensitivities for DNA in clinical samples (Liu *et al.* 2012; Nakano *et al.* 2015) compared to PCR.

Unlike multiplex real-time PCR that can take up to a year to modify its primers and conditions to detect new genes, LAMP offers greater flexibility in rapidly responding to new target genes and seeks each target in an individual reaction (Findlay *et al.* 2015). This assay has been used to detect NDM-1 and all KPC variants from clinical samples at 10^0 CFU ml^{-1}, an LOD 10–100 fold greater than that of PCR (10^1 CFU ml^{-1}) (Nakano *et al.* 2015). Findlay *et al.* (2015) evaluated eazyplex® Superbug complete A kit (Amplex, Gieβen, Germany), a commercial kit that works on LAMP technology with 450 clinical Gram-negative bacterial isolates. The kit detected NDM, KPC, OXA-48 and VIM genes but not IMP and OXA-181 within 25 min with 100% sensitivity and specificity for all genes, except OXA-48-like genes for which the sensitivity was 83%, albeit with 100% specificity (Table 1) (Findlay *et al.* 2015).

Next generation sequencing

Subsequent to the capillary based sequencing techniques, which used >20 000 large bacterial artificial chromosomes clones to build libraries for sequencing whole genomes with great labour and cost (Mardis 2008), 'Next Generation Sequencing' (NGS) technologies have emerged to revolutionize biomedical research by affording a speedy and cost effective sequencing of the whole genome (Mardis 2008; Buermans and Den Dunnen 2014). By avoiding the use of bacterial clones, NGS technologies are able to sequence DNA with platform-specific adaptor libraries that are subsequently amplified isothermally prior to sequencing, or sequenced directly without an amplification step (Bertelli and Greub 2013; Buermans and Den Dunnen 2014) within hours at relatively low cost. Carbapenemase genes are detected from NGS data by annotating NGS raw reads and/or assembled contigs with online servers like RAST (Aziz *et al.* 2008), CARD (McArthur *et al.* 2013), ResFinder (Zankari *et al.* 2012) and PGAAP (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) (Singh *et al.* 2013).

NGS affords clinical microbiology the tool to sequence single-cell DNA and DNA of unculturable bacteria from all clinical samples without a culturing step, thus effectively avoiding the time required for culturing and subsequent PCR and sequencing. As a result of whole genome sequencing (WGS) through NGS, PCR-based characterization of carbapenemases, their genetic environment and mobile genetic elements (integrons, transposons and plasmids) can be achieved in a single reaction compared to the several laborious PCR, restriction enzyme digestion and gel electrophoresis steps employed previously (Barisic *et al.* 2014; Deraspe *et al.* 2014). Moreover, NGS technology provides data on the strain types, species identity and

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data on the whole resistome and virulome of the isolate, thus providing a comprehensive molecular view of the isolate under investigation. Consequently, epidemiological and carbapenem resistance mechanisms studies are now accomplished in a single reaction, and Carbapenemases that could not be identified by PCR due to their novelty or their primers not being included in a study can be easily identified and characterized by WGS (Ho *et al.* 2011; Espedido *et al.* 2013; Hammerum *et al.* 2015).

In terms of efflux and porin profiles analysis as a membrane permeability factor in carbapenem resistance, which was historically achieved by qPCR followed by serial analysis of genetic expression and/or SDS-PAGE, WGS provides a better tool. The expression levels of efflux and porins in resistant and susceptible strains can now be compared to effectively describe their roles in membrane permeability changes (Martinez *et al.* 2015). The interplay between regulatory proteins-encoded genes, operons, repressors and inducers can also be analysed epigenetically (Mardis 2008; Bertelli and Greub 2013; Buermans and Den Dunnen 2014).

WGS, unlike all the other methods described above, is able to identify all carbapenemase types, known or unknown, within 8 h, with 100% sensitivity and specificity compared with PCR. Other molecular assays have to be optimized to identify common local or regional carbapenemases, as they cannot identify all carbapenemase types (Findlay *et al.* 2015). The major drawback to WGS is the absence of a universal data analysis platform to easily compare, analyse and share information within the global scientific community. This is due in part to the differences in NGS platforms used, the plethora of data to be added to a central database, the compatibility of NGS data with older sequencing data and a standard bioinformatics software to incorporate and analyse all current WGS and older sequencing data (Bertelli and Greub 2013; Buermans and Den Dunnen 2014).

Conclusion

Carbapenemase-producing strains are increasingly associated with high mortality and morbidity worldwide due to their unprecedented association with multi- or pan-drug resistance, and to the absence of standardized and clinically effective detection methods for early identification (Kruse *et al.* 2013; Tuite *et al.* 2014). Appropriate treatment and infection control rely on efficient and timely identification of carbapenem-resistant bacteria, as well as the mechanisms responsible for carbapenem resistance. Advances in culture methods like the MHT, disc synergy tests and screening media are still bedevilled with long incubation hours. The sensitivities, specificities and turnaround times recorded in the various studies reviewed favours the use of the biochemical tests (Carba NP or Rapid Carb screen tests) for the detection of putative carbapenemase-producing isolates. MALDI-TOF MS and/or molecular methods like microarray, LAMP and real-time multiplex PCR assays could be used for further characterization in a reference laboratory. NGS may be used for advanced epidemiological and molecular studies. Advancement in technology, as evidenced by nanopore sequencing, will make molecular methods automated, faster, more efficient, user friendly and cost effective.

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Conflict of Interest

The authors have no transparency to declare.

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