JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2016. Vol. **10**(4), p. 2585-2591

Comparison of Existing Phenotypic and Genotypic Tests for the Detection of NDM and GES Carbapenemase-Producing Enterobacteriaceae

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http://dx.doi.org/10.22207/JPAM.10.4.14

(Received: 28 June 2016; accepted: 11 August 2016)

The efficient detection and distinction of carbapenem-resistant and carbapenemase-producing Enterobacteriaceae continues to pose a major challenge to clinical microbiology laboratories, particularly in resource-constrained countries. Disc diffusion (DD), micro-broth dilution (BMD), Vitek II, Carba NP test, modified Hodge's test (MHT) and real-time PCR were evaluated on known carbapenem-resistant and carbapenemase-producing clinical Enterobacteriaceae isolates in terms of their sensitivity and specificity using whole genome sequencing (WGS) as the gold standard. DD with meropenem (MRP), real-time PCR, DD with imipenem (IMP), BMD, Carba NP test, and BMD with IMP had sensitivities of 100%, 97.96%, 97.96%, 97.96%, 95.92%, and 95.92% respectively. Real-time PCR and Carba NP test had the highest specificities (100%) and shortest turnaround times (< 3 hours). DD or BMD using meropenem, followed by Carba NP test and PCR were the best protocols for detecting and confirming CPEs clinically. We recommend the Carba NP test and/or DD specifically for resource-constrained laboratories for detection and control of carbapenemase-producing Enterobacteriaceae.

Keywords: Carbapenemase; Carba NP test; modified Hodge's test; Vitek II; Carbapenem resistance; whole genome sequencing.

The limited therapeutic options available for infections caused by carbapenem-resistant Enterobacteriaceae (CRE) and carbapenemase-producing Enterobacteriaceae (CPE), alongside the relatively higher mortality rates associated with the infections they cause are challenges affecting clinical medicine and antibiotic chemotherapy[1,2]. A major clinical conundrum facing clinical microbiologists is the detection and discrimination of CPE from CRE using antimicrobial sensitivity testing (AST) results as some CPE have been shown to be susceptible to carbapenems and not all CRE are CPE. Consequently, screening and detection methods that are rapid, reliable and reproducible are critical to the early identification and containment of CRE and CPE, particularly in resource-constrained clinical settings, to pre-empt their escalation to epidemic proportions[3].

Whereas molecular tests using conventional PCR or multiplex/real time PCR followed by sequencing and whole genome sequencing (WGS) are able to detect all known carbapenemases present in an isolate, the cost and skills required for these tests are beyond the affordability of many clinical microbiology laboratories. The same can be said for the UV spectrophotometric method[3]. Subsequently, simpler tests such as disc diffusion (DD), micro-broth dilution (BMD), Vitek II, the modified Hodge's test (MHT), meropenem-EDTA synergy test, and

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the Carba NP tests are suggested for resource-constrained health-care settings. Imipenem (IMI) and meropenem (MRP) are the commonly used carbapenems for detecting CREs and CPEs, albeit MRP and in some cases ertapenem have been reported to be more sensitive[3]. This study evaluated the specificity and sensitivity of phenotypic and genotypic methods in detecting CPE from CRE using WGS as the gold standard on clinical Enterobacteriaceae.

MATERIALS AND METHODS

Ethical clearance

The study was approved by the Biomedical Research and Ethical Committee of the University of KwaZulu Natal, South Africa (reference number BE040/14).

Bacterial isolates

Forty-eight clinical Enterobacteriaceae isolates from various sources, viz., rectal swabs, blood culture, catheter tips, urine, vaginal swabs, sputum, abdominal swab, pus swabs, peritoneal fluids, and tracheal fluids from patients of both sexes between the ages of nine months and 82 years that had been well-characterised by WGS were used (unpublished data).

E. coli ATCC 25922 and *K. pneumoniae* ATCC BAA 1706, which are carbapenem sensitive and non-CPE respectively, were used as negative controls. Standard well-characterised CRE isolates (purchased from Service de Bacteriologie-Virologie INSERM U914, Emerging Resistance to Antibiotics Hopital de Bicetre) producing class A, B and D carbapenemases viz., *E. coli* - LIL-2- KPC-2, *S. marcescens* BM -18- IMP-1, *E. cloacae* -CHE- GES-5, *P. rettgeri* IR-38 -NDM-1, *K. Pneumoniae* -DIH-VIM-19, and *E. coli*-BOU-OXA-48 were used as positive controls.

Carbapenemase screening tests (DD, MBD, and Vitek II with IMP and MRP)

DD, BMD, and Vitek II were used to determine the susceptibilities of the isolates to IMP and MRP using EUCAST 2016 guidelines and breakpoints (v 6.0)[4]. All isolates that were non-susceptible to any of the two carbapenems (Table SI) were interpreted as a CRE and a potential CPE.

MHT, Carba NP and real-time PCR

A DD assay using MRP was set-up as previously described[5]. The MHT was carried out and results interpreted as described by the CLSI[6]. The CNP was carried out and interpreted using the modifications described by Vasoo *et al.* (2013) (Table SI)[7]. Real-time PCR was used to screen for the presence of known carbapenemases (KPC, GES, OXA-48, NDM, SPM, VIM, IMP, SIM and GIM) using already described conditions[8,9].

WGS

The isolates had been already characterised by WGS in a previous study (Table SI) (unpublished). The WGS results were used as the gold standard to evaluate all the other phenotypic and real-time PCR tests to determine their sensitivities and specificities.

Statistical analysis

Methods described by Parikh *et al.* (2008) were used to determine the sensitivity and specificity (Table I) of all the phenotypic and real-time PCR tests using WGS as the gold standard to calculate the true positives, true negatives, false positives, and false negatives obtained from each test with a confidence interval (CI) of 95% (Tables I and II)[10].

RESULTS AND DISCUSSION

Tables I-III summarises the results of the phenotypic and real time PCR tests.

Analysis

As shown in Table I, DD with MRP test had the highest sensitivity, but a poorer specificity. Although the sensitivity of the real-time PCR, DD with IMP, and BMD with MRP were equal, real-time PCR had the best specificity (100%), which was equalled only by that of the Carba NP test (Table I). In spite of the higher sensitivities of the DD and BMD, their lower specificities were of concern. However, DD with IMP had high sensitivity (97.96%) and relatively high specificity (71.43%). With the exception of Vitek II, MRP provided a higher sensitivity than IMP in all tests (DD, Vitek II and BMD), albeit with a lower specificity; in keeping with studies that advocate for the use of MRP in DD [3]. We therefore suggest that MRP alone could be used in BMD for CPE detection as it had better sensitivity and specificity than BMD with IMP (Table I); MRP's specificity was lower than IMP in DD.

The lower sensitivities and specificities of Vitek II and the MHT make them unsuitable as

Table 1. Sensitivity, specificity, NPV, and PPV of micro-broth dilution (BMD), disc diffusion (DD), Vitek II, modified Hodge's test (MHT), Carba NP test, and PCR for carbapenemase-producing Enterobacteriaceae

Detection test		Carbapenemase producers and positive controls (n=49)	Non-carbapenemase producers and negative controls (n=7)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predictive value (PPV) (%) (95% CI)	Negative predictive value (NPV) (%) (95% CI)
Micro-broth dilution[1]	MRP[2]	48	4	97.96 (89.15-99.95)	57.14 (18.41-90.10)	94.12 (83.76-98.77)	80.00 (28.36-99.49)
	IMP[3]	47	2	95.92 (86.02-99.50)	28.57 (3.67-70.96)	90.38 (78.97-96.80)	50.00 (6.76-93.24)
Disc diffusion	MRP	49	3	100 (92.75-100.00)	42.86 (9.90-81.59)	92.45 (81.79-97.91)	100 (29.24-100.00)
	IMP	48	5	97.96 (89.15-99.95)	71.43 (29.04-96.33)	96.00 (86.29-99.51)	83.33 (35.88-99.58)
Vitek II	MRP	39	2	75.59 (65.66-89.76)	28.57 (3.67-70.96)	88.64 (75.44-96.21)	16.67 (2.09-48.41)
	IMP	44	3	89.80 (77.77-96.60)	42.86 (9.90-81.59)	91.67 (80.02-97.68)	37.50 (8.52-75.51)
Modified Hodge's Test		40	5	81.63 (67.98-91.24)	71.43 (29.04-96.33)	95.24 (83.84-99.42)	35.71 (12.76-64.86)
Carba NP Test		47	7	95.92 (86.02-99.50)	100.00 (59.04-100.00)	100.00 (92.45-100.00)	77.78 (39.99-97.19)
PCR		48	7	97.96 (89.15-99.95)	100.00 (59.04-100.00)	100.00 (92.60-100.00)	87.50 (47.53-99.68)

[1] This was used as the gold standard to assess the efficiency of the other tests in predicting an NDM-1 phenotype
[2] Meropenem: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)
[3] Imipenem: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)

CPE screening or detection tests (Tables I and II), as has been suggested in other studies[3] Interpretation of MHT results is subjective. Although BMD is seen as the gold standard for susceptibility tests, its sensitivity and specificity for CPEs were relatively low. The lower sensitivity of DD with IMP has been cited as a reason for the use of ertapenem in screening for CPEs[3]. The major setback in these culture-based tests is the one-day incubation period required to obtain results and their inability to differentiate between carbapenem resistance mediated by carbapenemases, overproduction of ESBLs, and/or AmpCs coupled with lower membrane permeability. As discussed elsewhere[3], several *in vitro* carbapenem-susceptible isolates were resistant *in vivo* whilst isolates resistant *in vitro* were actually non-carbapenemase producers. Subsequently, the initiation of therapy based on just MICs and breakpoints results has the potential to result in therapeutic failure and the exacerbation of resistance. Further reducing the breakpoint averages as suggested and/or augmenting with carbapenemase-detection tests will be an appropriate step towards carbapenem stewardship[3].

Most of the false-negative results (Tables II and SI) that resulted in low specificities were due to the GES-5 carbapenemases as they have lower hydrolysis rates (Table SI). This was the reason for the lower Carba NP test sensitivity rate of 95.92%, a phenomenon also observed by the test's developers[3,11], whilst a false-negative detection of OXA-232 was the reason behind the real-time PCR's sensitivity rate of 97.96% (Tables I, II and SI). The substantial presence of GES genes in South Africa[1] requires that the Carba NP test is followed up with PCR, if accessible and affordable, to enable the effective detection of GES genes that would otherwise not be detected. The CNP had a higher sensitivity than that reported in Belgium by Yusuf *et al*. (2013)[3,12] and a lower sensitivity than that reported by Vasoo and peers at the Mayo clinic, USA[3,7]. Compared to the culture-based sensitivity/screening and detection tests, the Carba NP test and real-time PCR had shorter turn-around times as well as relatively higher sensitivities and perfect specificities (100%). These make them ideal for detecting CPEs for a faster infection control intervention and for carbapenem (antibiotic) stewardship. Multiplex real time PCR has been used to screen for CPEs from rectal, peri-anal and throat swabs and stool samples with faster turnaround time and high detection efficiency; but the skill and costs associated with these tests are not available to microbiology labs in many under-resourced countries[3].

Table 2. True positive, true negative, false positive and false negative values of micro-broth dilution (BMD), disc diffusion, Vitek II, modified Hodge's test (MHT), Carba NP test, and PCR for carbapenemase-producing Enterobacteriaceae

Detection test		True positive	True negative	False positive	False negative
BMD[1]	MRP[2]	48	4	3	1
	IMP[3]	47	2	5	2
Disc diffusion	MRP	49	3	4	0
	IMP	48	5	2	1
Vitek II	MRP	39	2	5	10
	IMP	44	3	4	5
MHT[5]		40	5	2	9
Carba NP		47	7	0	2
PCR		48	7	0	1

[1] Micro-broth dilution: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)
[2] Meropenem: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)
[3] Imipenem: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)
[5] Modified Hodge's Test

Table 3. Results of disc diffusion (DD), micro-broth dilution (BMD), Vitek II, modified Hodge's test (MHT), Carba NP test (CNP), real-time PCR, and whole genome sequencing (WGS) on the Enterobacteriaceae isolates

Isolate	Disc diffusion (DD)		MD[1] MIC (mg/L)		Vitek II (mg/L)		MHT [2]	CNP [3]	Real-time	WGS [4] PCR
	MRP [5]	IMP [6]	MRP	IMP	MRP	IMP				
K. pneumoniae										
C(UNN_S3)	R[7]	R	256	>64	≥16	≥16	+	+[8]	bla_{NDM-1}	bla_{NDM-1}
D(UNN_S4)	R	R	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
I(UNN_S9)	R	R	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
J(UNN_S10)	R	R	128	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
3_S2	R	R	128	>64	≥16	≥16	+	-[9]	-	$bla_{OXA-232}$
12_S5	R	R	64	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
13_S6	I[10]	S[11]	128	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
15_S8	R	R	16	>64	≥16	8	-	+	+	bla_{GES-5}
18_S10	R	R	128	>64	4	≥16	+	+	+	bla_{GES-5}
20_S11	R	R	128	>64	4	8	+	+	bla_{NDM-1}	bla_{NDM-1}
21_S12	R	R	512	>64	≥16	8	+	+	bla_{NDM-1}	bla_{NDM-1}
29_S13	R	R	512	>64	4	8	+	+	bla_{NDM-1}	bla_{NDM-1}
30_S14	R	R	256	>64	≥16	≥16	-	+	+	bla_{GES-5}
32_S15	R	I	128	>64	≥16	8	+	+	bla_{NDM-1}	bla_{NDM-1}
34_S16	R	R	512	>64	4	≥16	[12]	-	+	bla_{GES-5}
35_S17	R	R	512	>64	4	≥16	-	+	+	bla_{GES-5}
36_S18	R	R	128	>64	4	≥16	-	-	+	bla_{GES-5}
38_S19	R	R	16	>64	≥16	≥16	?	+	+	bla_{GES-5}
47_S22	R	S	128	>64	≥16	2	?	-	-	-
52_S26	R	R	512	>64	4	≥16	-	+	+	bla_{GES-5}
53_S27	R	R	256	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
Serratia marcescens										
B (UNN38 _S2)	R	R	>512	>64	≥16	≥16	-	+	bla_{NDM-1}	bla_{NDM-1}
E (UNN41_S5)	R	R	128	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
G (UNN43_S7)	R	R	16	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
K (UNN47_S11)	R	R	128	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
L (UNN_S12)	R	R	64	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
7_S3	R	R	64	0.5	≥16	≥16	-	+	bla_{NDM-1}	bla_{NDM-1}
45_S21	R	S	0.5	>64	≥16	≥16	-	-	-	-
56_S29	R	R	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
59_S30	R	I	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
67_S33	R	R	32	>64	4	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
68_S34	R	R	64	32	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
71_S36	R	R	>512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
Enterobacter cloacae										
A (UNN37_S1)	R	R	1	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
F (UNN42_S6)	R	R	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
H (UNN44_S8)	R	R	>512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
1_S1	S	S	2	>64	≥16	≥16	+	-	-	-
16_S9	R	I	256	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
43_S20	R	R	>512	>64	4	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
49_S24	R	R	512	0.5	16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
51_S25	R	R	>512	>32	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
55_S28	R	R	512	>64	4	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
63_S31	R	R	512	32	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
65_S32	R	R	512	>64	≥16	≥16	+	-	-	-
Escherichia coli										
10_S4	R	R	>512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-5}

Citrobacter freundii										
14_S7	R	R	64	32	≥16	≥16	+	-	-	-
48_S23	R	R	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}
Klebsiella michiganensis										
69_S35	R	I	512	>64	≥16	≥16	+	+	bla_{NDM-1}	bla_{NDM-1}

[1] Microbroth dilution: EUCAST breakpoints (2016) were used for the interpretation
[2] Modified Hodge's Test
[3] Carba NP Test
[4] Whole genome sequencing results
[5] Meropenem: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)
[6] Imipenem: EUCAST breakpoints (2016) were used for the interpretation (Resistant > 8mg/L or < 16mm zone diameter)
[7] Resistant
[8] Positive
[9] Negative
[10] Intermediate resistant. This was counted as resistant in the analysis
[11] Susceptible[12] Indeterminate i.e. the results are elusive such that an absolute decision (positive or negative) cannot be made. This was counted as negative in the analysis.

CONCLUSION

WGS is the most ideal tool for detecting CPEs clinically, albeit undescribed carbapenemases cannot be identified. Real-time PCR can be used directly as a screening and detection tool to identify NDM and GES CPEs without the need for a culturing step, thus saving time and efforts. The Carba NP test is recommended for resource-constrained clinical settings due to its simplicity, shorter turn-around time and lower cost while DD and BMD with MRP can serve as an initial NDM and GES CPE screening and detection tests in the absence of the Carba NP test.

ACKNOWLEDGEMENTS

We are especially grateful to Bettina Aasnæs for significant technical assistance with the WGS and to Bjørg Christina Haldorsen for help in the real-time PCR of the isolates; both at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway. We acknowledge the contribution of the Norwegian Sequencing Centre, University of Oslo, and Center for Bioinformatics at UiT –The Arctic University of Norway for generating the sequence data.

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