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Short Communication

Nitro-Carba test, a novel and simple chromogenic phenotypic method for rapid screening of carbapenemase-producing Enterobacteriaceae

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ABSTRACT

Objectives: In this study, a rapid and simple chromogenic method for screening of carbapenemase-producing Enterobacteriaceae (CPE), namely the Nitro-Carba test (NCT), was developed.
Methods: The NCT was validated using a total of 31 carbapenemase-producing isolates [9 *Klebsiella pneumoniae* carbapenemase (KPC), 11 metallo-β-lactamase (MBL) and 11 OXA-48] and 56 non-carbapenemase-producing isolates. The assay relies on the hydrolysis of nitrocefin by carbapenemases in the presence of carbapenem antibiotics. Carbapenemases were extracted with lysis buffer prior to addition to wells with and without imipenem (IPM), meropenem (MEM) and ertapenem (ETP). Following addition of nitrocefin, a change in colour from yellow to red, indicating carbapenemase production, was observed within 20 min. The susceptibility profiles of each bacterial isolate were also investigated.
Results: The NCT detected all 31 CPE within a timeframe of only 10 s to 12 min. All carbapenemase-producers hydrolysed nitrocefin in all wells. No colour change in wells with carbapenems was observed in non-carbapenemase-producers. The sensitivity for all three carbapenems was 100%, whilst the specificity of IPM, MEM and ETP was 64.3%, 91.1% and 100%, respectively. IPM, MEM and ETP had minimum inhibitory concentrations (MICs) against all carbapenemase-producing strains ranging from 0.5 μg/mL to ≥256 μg/mL, 0.25 μg/mL to ≥256 μg/mL and 1 μg/mL to ≥256 μg/mL, respectively. OXA-48-producing isolates showed lower MICs compared with MBL- and KPC-producing isolates.
Conclusion: This assay is a promising method for detecting CPE rapidly. The NCT is a simple and reliable method capable of detecting CPE even in carbapenem-susceptible strains.

1. Introduction

Resistance to carbapenems, last-resort β-lactam antibiotics for treating infections caused antimicrobial-resistant Enterobacteriaceae, has been increasingly documented worldwide as a consequence of their increased use for the treatment of extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase-producing bacteria [1–3]. Carbapenem resistance in Enterobacteriaceae is mediated by the production of carbapenemase enzymes [4,5]. Overproduction of AmpC β-lactamases combined with porin loss/modification have also been reported in Enterobacteriaceae [6].

A number of carbapenemase-encoding genes have been identified recently, however the most prevalent carbapenemases in Enterobacteriaceae in the clinical setting and in community-acquired infections are Ambler class A *Klebsiella pneumoniae* carbapenemases (KPCs), class B metallo-β-lactamases (MBLs) [e.g. New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and imipenemase (IMP)] and class D oxacillinase with carbapenemase activity (e.g. OXA-48 and its variants) [7].

To treat these carbapenem-resistant infections effectively, early detection of carbapenemase production is necessary. Therefore, in the present study a rapid and simple method for screening of carbapenemase-producing Enterobacteriaceae (CPE) was developed, namely the Nitro-Carba test (NCT). The method developed in this study is able to detect carbapenemase production within 20 min. Nitrocefin is a chromogenic substrate that is commonly utilised to detect the production of β-lactamase enzymes in bacteria. It has been previously found that meropenem prevents the hydrolysis of nitrocefin by AmpC β-lactamase (CMY-2) [8]. In addition, ertapenem prevented nitrocefin hydrolysis by ESBLs, AmpC β-lactamases and co-produced ESBLs + AmpC β-lactamases [9].

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2. Materials and methods

2.1. Bacterial isolates

The performance of the NCT for rapid screening of carbapenemase production in Enterobacteriaceae was evaluated against isolates characterised in our previous studies [8,10], including 31 carbapenemase-producing isolates (9 KPCs, 11 MBLs and 11 OXA-48 like) and 56 molecularly confirmed non-carbapenemase-producers (15 ESBL, 32 AmpC and 9 ESBL + AmpC) as well as β-lactamase-negative *Escherichia coli* ATCC 25922. The characteristics of the organisms used are presented in Table 1.

2.2. Nitro-Carba test (NCT)

The NCT is based on the hydrolysis of nitrocefin by carbapenemases in the presence of carbapenem antibiotics. The assay was prepared and performed as follows. For each bacterial isolate, one full inoculation loop (10 μL) of colonies grown overnight at 37 °C on LB agar was selected. To extract the enzymes, the sample was re-suspended in 500 μL of lysis buffer containing 0.04% CTAB (Sigma-Aldrich, UK), 0.1 mM $ZnSO_4$ (pH 7.5) and 1% Triton™X-100 and was vortexed vigorously for 2 min. Then, 100 μL of extracted enzyme was added to wells containing either 50 μL of distilled water (control), 800 μg/mL imipenem (IPM), 200 μg/mL meropenem (MEM) or 80 μg/mL ertapenem (ETP). Following incubation at room temperature for 5 min, 50 μL of 1 g/L nitrocefin in rehydrating fluid (Oxoid™; Thermo Scientific) was added to each well to given final concentrations of IPM, MEM and ETP of 200, 50 and 20 μg/mL, respectively. Upon addition of nitrocefin, a colour change from yellow to red both in wells containing carbapenem and wells containing water was considered indicative of carbapenemase production. The absence of any colour change (no hydrolysis of nitrocefin) in wells containing carbapenem or wells containing water indicated no carbapenemase production or no β-lactamase production, respectively. All results were interpreted within 20 min of incubation at room temperature. The interpreters were unaware of the phenotypes and molecular characteristics of the test organisms.

2.3. Minimum inhibitory concentration (MIC) determination

The MICs of carbapenem antibiotics including IPM, MEM and ETP (Sigma-Aldrich) were determined using the standard broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. The susceptibility profiles of

Table 1
Organism characteristics and minimum inhibitory concentrations (MICs) of carbapenemase-producing- and non-carbapenemase-producing isolates used in this study.

β-Lactamase group		Species (no. of strains)	MIC range (μg/mL)			Time to detection of positive result for Nitro-Carba test [minute (second)]			
			IPM	MEM	ETP	Control (H_2O)	IPM (200 μg/mL)	MEM (50 μg/mL)	ETP (20 μg/mL)
Carbapenemase-producers (*n* = 31)									
Class A (*n* = 9)	KPC	*Klebsiella pneumoniae* (6)	16 to ≥256	8 to ≥256	32 to ≥256	0(6) – 6(30)	0(10) – 7(20)	0(10) – 7(29)	0(10) – 9(45)
		Klebsiella oxytoca (1)	4	8	16	2(1)	2(10)	5(47)	5(47)
		Escherichia coli (2)	16–32	8–64	16–128	1(10) – 1(51)	1(51) – 12(4)	1(51) – 12(4)	1(51) – 12(4)
Class B (*n* = 11)	IMP	*Klebsiella ozaenae* (1)	4	16	8	0(20)	0(35)	0(35)	0(35)
	NDM	*Enterobacter cloacae* (2)	64 to ≥256	128 to ≥256	128 to ≥256	0(20) – 3(10)	0(32)	0(47)	0(47)
		K. pneumoniae (3)	16–128	32 to ≥256	32 to ≥256	0(25) – 1(23)	1(40) – 8(0)	4(30) – 12(0)	4(32) – 12(0)
		Escherichia coli (1)	≥256	≥256	≥256	0(10)	4(11)	5(4)	5(4)
	VIM[a]	*K. pneumoniae* (4)	8–64	8 to ≥256	8–128	0(15) – 1(20)	0(17) – 1(53)	0(17) – 1(53)	0(17) – 2(4)
Class D (*n* = 11)	OXA-48	*E. cloacae* (2)	2–4	2	8–16	0(8) – 0(30)	2(1) – 5(17)	5(6) – 6(14)	5(10) – 6(14)
		K. pneumoniae (5)	1 to ≥256	1–64	4 to ≥256	0(5) – 0(51)	0(16) – 5(40)	2(0) – 11(32)	1(32) – 11(30)
		E. coli (4)	0.5–8	0.25–8	1–32	0(8) – 1(2)	1(42) – 7(11)	3(1) – 8(28)	2(36) – 8(28)
Non-carbapenemase-producers (*n* = 56)									
Class A (*n* = 15)	ESBL	*E. coli* (7)	≤0.25–0.5	≤0.25	≤0.25	0(15) – 9(30)	16(30) – >20	>20	>20
		K. pneumoniae (7)	≤0.25–1	≤0.25	≤0.25	0(32) – 8(10)	16(30) – >20	>20	>20
		E. cloacae (1)	≤0.25	≤0.25	≤0.25	0(40)	0(55)	>20	>20
Class C (*n* = 32)	AmpC	*K. pneumoniae* (4)	0.5–1	≤0.25	≤0.25–1	0(7) – 2	2(23) – >20	>20	>20
		E. coli (10)	≤0.25–2	≤0.25–0.5	≤0.25–1	0(5) – 2(2)	0(12) – >20	5(20) – >20	>20
		Citrobacter freundii (2)	0.5–1	≤0.25	≤0.25–1	0(14)	4(0) – 7(0)	>20	>20
		Enterobacter aerogenes (7)	0.5–4	≤0.25	≤0.25–0.5	0(15) – 4(26)	4(30) – >20	>20	>20
		E. cloacae (7)	≤0.25–2	≤0.25	≤0.25–1	0(5) – 4(17)	4(0) – >20	10(0) – >20	>20
		Morganella morganii (2)	1–2	≤0.25	≤0.25	0(10) – 0(12)	2(39) – 9(0)	8(40) – 10(0)	>20
Class A + class C (*n* = 9)	ESBL + AmpC	*E. aerogenes* (5)	≤0.25–1	≤0.25	≤0.25	1(10) – 14(0)	10(0) – >20	>20	>20
		E. cloacae (1)	1	≤0.25	≤0.25	1(20)	>20	>20	>20
		C. freundii (2)	1	≤0.25	≤0.25	2(10) – 2(36)	>20	>20	>20
		E. coli (1)	0.5	≤0.25	≤0.25	1(30)	>20	>20	>20
Negative control		*E. coli* ATCC 25922	0.5	≤0.25	≤0.25	>20	>20	>20	>20

IPM, imipenem; MEM, meropenem; ETP, ertapenem; KPC, *Klebsiella pneumoniae* carbapenemase; IMP, imipenemase; NDM, New Delhi metallo-β-lactamase; VIM, Verona integron-encoded metallo-β-lactamase; ESBL, extended-spectrum β-lactamase.
[a] One VIM–1 + SHV-12 and one VIM–1 + SHV-102 co-producer.

carbapenems against Enterobacteriaceae isolates were interpreted according to CLSI antimicrobial susceptibility breakpoint values [12] as follows: IPM and MEM, susceptible, ≤1 μg/mL and resistant, >2 μg/mL; and ETP, susceptible, ≤0.5 μg/mL and resistant, >1 μg/mL.

3. Results and discussion

Human infections caused by CPE are considerably life-threatening due to limited treatment options. Rapid detection of the presence of carbapenemases in Enterobacteriaceae could be useful for antibiotic selection and infection control. Although several methods have been established, it still presents a challenge to researchers to develop new rapid and simple innovative methods, with high performance, that are reliable and cost effective [13]. Hence, a promising approach using the NCT for rapid screening of CPE is described here.

The NCT assay was rapidly able to detect all 31 carbapenemase-producers within a quick timeframe of between 10 s and 12 min. Representative results are illustrated in Fig. 1. All carbapenemase-producing isolates hydrolysed nitrocefin in the antibiotic-free control wells and in wells containing carbapenem antibiotics. The sensitivity of the NCT for all three antibiotics was 100%. No colour changes were observed in the wells with antibiotics within 20 min for non-carbapenemase-producing isolates. ETP was found to prevent hydrolysis of nitrocefin by AmpCs and ESBLs, whilst IPM and MEM cannot prevent hydrolysis of nitrocefin by some AmpC- and ESBL-producing strains. The specificities of IPM, MEM and ETP were 64.3% (36/56), 91.1% (51/56) and 100% (56/56). These findings indicated that ETP exhibits the best activity in the prevention of nitrocefin hydrolysis by ESBLs, AmpC β-lactamases or co-expressed ESBL + AmpC β-lactamases. In agreement with a previous study, disks containing ETP supplemented with nitrocefin were able to detect all CPE isolates within 30 min, with no false-positive or -negative results [8].

Interestingly, the concentrations of antibiotics used in the NCT are much lower compared with the Blue-Carba test [14] and the Carba NP test [15], one of the most popular methods that has been used by many pathology laboratories. The NCT developed in the current study also provides faster results in detecting carbapenemase production in Enterobacteriaceae. However, a larger sample size of CPE with a variety of resistance mechanisms should be further investigated to validate the robustness of the NCT. Subjectivity in interpretation of results remains a shortcoming of all chromogenic methods [16].

The MIC results for IPM, MEM and ETP against carbapenemase- and non-carbapenemase-producing isolates employed in this study are summarised in Table 1. IPM, MEM and ETP had MICs ranging from 0.5 to ≥256 g/mL, 0.25 to ≥256 μg/mL and 1 to ≥256 μg/mL, respectively, against all carbapenemase-producing strains. OXA-48-producing isolates showed lower MICs compared with MBL- and KPC-producers. Three OXA-48-like producers were susceptible to IPM and four to MEM. OXA-48-like carbapenemase enzymes often weakly hydrolyse carbapenems resulting in an MIC that may not be high enough to be designated resistant or intermediately resistant [17]. This is consistent with the results from the current study which demonstrate that some OXA-48-producing strains are susceptible or intermediately resistant to carbapenems.

The majority of non-carbapenemase-producers remained susceptible to carbapenem antibiotics, with the exception of an AmpC-producer (ACT-1-producing *Enterobacter aerogenes*), which was found to be resistant to IPM with a MIC of 4 μg/mL. This could be due to overexpression of some ACT types that can degrade carbapenems at low rates. However, nitrocefin can discriminate a

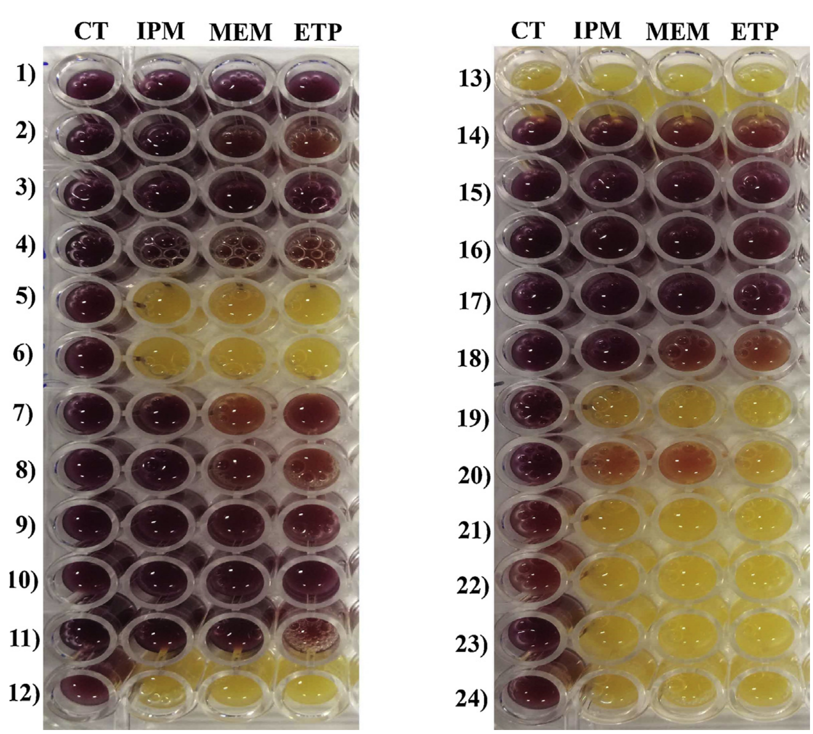

Fig. 1. Representative results from the Nitro-Carba test. CT, control (water); IPM, imipenem (200 μg/mL); MEM, meropenem (50 μg/mL); ETP, ertapenem (20 μg/mL). Row 1, VIM–1 + SHV-12-producing *Klebsiella pneumoniae* 4034; row 2, OXA-48-producing *K. pneumoniae* 4026; row 3, VIM–1 + SHV-102-producing *K. pneumoniae* 4033; row 4, OXA-48-producing *K. pneumoniae* 4012; row 5, DHA-1-producing *Escherichia coli* 2003; row 6, CTX-M–9 + ACT-18-producing *E. coli* 3003; row 7, OXA-48-producing *Enterobacter cloacae* 4003; row 8, OXA-48-producing *E. coli* 4010; row 9, NDM-1-producing *E. cloacae* 4004; row 10, KPC-3-producing *E. coli* 4006; row 11, KPC-2-producing *Klebsiella oxytoca* 4032; row 12, CTX-M–3 + ACT-1-producing *Citrobacter freundii* 3005; row 13, non-β-lactamase-producing *E. coli* ATCC 25922; row 14, NDM-1-producing *E. coli* 4011; row 15, KPC-3-producing *K. pneumoniae* 4014; row 16, KPC-producing *K. pneumoniae* 4018; row 17, OXA-48-producing *E. coli* 4007; row 18, OXA-48-producing *K. pneumoniae* 4021; row 19, DHA-1-producing *K. pneumoniae* 2001; row 20, DHA-1-producing *E. coli* 2002; row 21, CTX-M–15 + SHV-27-producing *K. pneumoniae* 1010; row 22, SHV–12 + CTX-M–9 + ACT-32-producing *E. aerogenes* 3002; row 23, ACT-32-producing *E. cloacae* 2009; and row 24, CTX-M-15-producing *E. coli* NCTC 13353.

non-carbapenemase-producer of IPM-resistant *E. aerogenes* with AmpC β-lactamase production [18]. Taken together, the findings from this study suggest that the NCT can effectively detect the presence of carbapenemases in carbapenem-susceptible isolates, in particular OXA-48-producers. The NCT can also discriminate carbapenemase-producing isolates from hyperproduction of ESBLs or AmpC β-lactamases.

4. Conclusion

A rapid and reliable method that can detect the presence of carbapenemase production in Enterobacteriaceae is important for controlling the spread of antimicrobial-resistant pathogens, screening patients and rational use of antibiotics. The NCT assay proposed here is a very simple, reliable and cost-effective method allowing rapid visual observation of results within 20 min. ETP showed the best sensitivity and specificity and thus should be selected for future investigation in a clinical setting, whilst other carbapenems can be excluded. This assay is a promising alternative method that can be applicable in any laboratory for screening CPE.

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Competing interests

None declared.

Ethical approval

Not required.

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