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

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Highlights:

- The present study developed Nitro-Carba Test (NCT), a rapid and simple chromogenic method for screening of Carbapenemase-producing Enterobacteriaceae (CPE).
- The NCT detected all 31 CPEs within a timeframe of only 10 seconds to 12 min.
- The sensitivity for all three carbapenems was 100%, while specificity of IPM, MEM and ETP were 64.29%, 91.07% and 100%, respectively.
- IPM, MEM and ETP against all carbapenemase-producing strains had MIC values ranging from 0.5 to ≥256 μg/mL, 0.25 to ≥256 μg/mL and 1 to ≥256 μg/mL, respectively.
- OXA-48-producing isolates showed lower MIC values compared with producers of MBL and KPC.

Abstract

Objectives: The present study developed Nitro-Carba Test (NCT), a rapid and simple chromogenic method for screening of Carbapenemase-producing Enterobacteriaceae (CPE).

Methods: The NCT was validated with a total of 31 carbapenemase-producing isolates (9 KPCs, 11 MBLs and 11 OXA-48s) and with 56 non-carbapenemase-producing strains. The assay relies on the hydrolysis of nitrocefin in the presence of carbapenems. The carbapenemases were extracted with lysis buffer prior to addition to wells with and without imipenem (IPM), meropenem (MEM) and ertapenem (ETP). Following the 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 strain were also investigated.

Results: A NCT detected all 31 CPEs within a timeframe of only 10 seconds to 12 min. All carbapenemase producers hydrolyzed nitrocefin in all wells. No colour change in the wells with carbapenems was observed in non-carbapenemase producers. The sensitivity for all three carbapenems was 100%, while specificity of IPM, MEM and ETP were 64.29%, 91.07% and 100%, respectively. IPM, MEM and ETP against all carbapenemase-producing strains had MIC values ranging from 0.5 to ≥256 µg/mL, 0.25 to ≥256 µg/mL and 1 to ≥256 µg/mL, respectively. OXA-48-producing isolates showed lower MIC values compared with producers of MBL and KPC.

Conclusion: This assay is a promising method detecting CPE rapidly. The NCT is a simple and reliable method, capable of detecting CPE in even carbapenem-susceptible strains.

Keywords: Nitro-Carba test, phenotypic chromogenic detection, carbapenemase-producing Enterobacteriaceae.

1. Introduction

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

There are a number of carbapenemase-encoded genes which have been identified recently, however, the most prevalent carbapenemases in Enterobacteriaceae in the clinical setting and community-acquired infections are Ambler class A *Klebsiella pneumoniae* carbapenenases (KPCs), class B metallo-β-lactamases (MBLs; e.g. New Delhi metallo-β-lactamases (NDM), Verona integron-encoded metallo-β-lactamases (VIM) and Imipenemase (IMP)), and class D oxacillinase with carbapenemase activity (e.g. OXA-48 and its variants)[7]. The emergence of these genotypes is different depending on geographical area. For instance, the first KPC-producing isolate was discovered in the United States and then disseminated to Israel, Greece, Italy, China and other countries worldwide[8]. The NDM and OXA-48-like carbapenemases were originally identified in the Indian subcontinent and also Turkey, while Greece and Japan are endemic areas of VIM and IMP, respectively [1, 9].

Treatment of carbapenemase-producing bacterial infections is very challenging due to paucity of optimal choice of effective antibiotics. Colistin (also known as polymyxin E), fosfomycin, and tigecycline are antibiotics of choice that have been often used for treating these

recalcitrant infections[10]. To treat these infections effectively, early detection of carbapenemase production is necessary. The present study, therefore, developed a rapid and simple method called Nitro-Carba test (NCT) for screening of carbapenemase-producing Enterobacteriaceae (CPE). The method developed in our study is able to detect carbapenemase production within 20 min. Nitrocefin is a chromogenic substrate that is commonly utilized to detect the production of β-lactamase enzymes in bacteria. It has been previously found that meropenem prevented the hydrolysis of nitrocefin by AmpC β-lactamase (CMY-2) [11]. In addition Ertapenem prevented nitrocefin hydrolysis by ESBL, AmpC and co-production of ESBL and AmpC β-lactamases [12].

2. Material and Methods

2.1 Bacterial Isolates

The performance of a NCT for rapid screening of carbapenemase production in Enterobacteriaceae was evaluated against 31 carbapenamase-producing isolates (9 KPCs, 11 MBLs and 11 OXA-48 like) and 56 molecular confirmed non-carbapenemases (15 ESBLs, 32 AmpCs and 9 ESBLs+AmpCs), all confirmed by molecular methods. A reference strain *Escherichia coli* ATCC 25922 was used as a negative β-lactamase control. These bacterial isolates have previously been described in our preceding studies[11, 13]. The characteristics of organisms used are presented in Table 1.

2.2 ***Nitro-Carba test (NCT)***

The NCT is based upon 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 tested colonies, grown overnight at 37 °C on LB agar was selected. To extract the enzymes, the sample was resuspended in 500 µL of lysis buffer containing 0.04% CTAB (Sigma-Aldrish, UK), 0.1 mM $ZnSO_4$ (pH 7.5) and 1% Triton^{TM}X-100 and vortexed vigorously for 2 min. 100 µL of extracted enzymes was added to wells containing either 50 µl of distilled water (control), 800 µg/mL of imipenem (IPM), 200 µg/mL meropenem (MEM) or 80 µg/mL of ertapenem (ETP). Following incubation at room temperature for 5 min, 50 µL of 1 g/L nitrocefin in rehydrating fluid (Oxoid^{TM}, Thermo Scientific) was added into each well (the final concentrations of IPM, MEM and ETP were 200 µg/mL, 50 µg/mL and 20 µg/mL, respectively). Upon addition of nitrocefin, a colour change from yellow to red in both 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 not aware of the phenotypes and molecular characteristics of organisms.

2.3 Minimum inhibitory concentration (MIC) determination

The MICs of carbapenem antibiotics including IPM, MEM and ETP (Sigma-Aldrich, UK) were determined using a standard broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline [14]. An overnight culture was harvested, washed and adjusted to reach 0.5 standard McFarland equivalent. Bacteria (approximately 5 x 105 CFU/mL) were treated with a two-folded dilution series of carbapenems in Mueller-Hinton (MH) medium. Wells in absence of antibiotics and bacteria were used as controls. Following incubation at 37 °C overnight, the lowest concentration showing no visible growth was denoted as a MIC value. The experiments were carried out in three independent replicates. The susceptibility profiles of carbapenems against Enterobacteriaceae isolates were interpreted in according to CLSI antimicrobial susceptibility breakpoint values[15]: IPM and MEM; susceptible ≤ 1 and resistant > 2 µg/mL and for ETP; susceptible ≤ 0.5 and, resistant > 1 µg/mL.

3. Results and Discussion

Human infections caused by CPEs are considerably life-threatening due to limited treatment options. Rapid detection of the presence of carbapenemases in Enterobacteriaceae could be useful for antibiotic administrations 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 [16]. Hence, a promising approach using the NCT for rapid screening of CPE is described here in the present study.

The NCT assay was rapidly able to detect all 31 carbapenemase producers within a quick timeframe of between 10 seconds and 12 min. The representative results are illustrated in Figure 1. All carbapenemase-producing-isolates hydrolysed nitrocefin in the antibiotic free control wells and wells containing carbapenem antibiotics. The sensitivities of the NCT for all three antibiotics were 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, while IPM and MEM cannot prevent hydrolysis of nitrocefin by some AmpC and ESBL-producing strains. The specificities of IPM, MEM and ETP were 64.29% (36 out of 56), 91.07% (51 out of 56) and 100% (56 out of 56). These findings indicated that ETP exhibits the best activity in prevention of nitrocefin hydrolysis by ESBL, AmpC or co-expression of ESBL and AmpC β-lactamases. In agreement with a previous study, discs containing ETP supplemented with nitrocefin were able to detect all CPE isolates within 30 min, with no false-positive and negative results [11].

In comparison with Carba NP, one of the most popular methods that has been used by many pathology laboratories, the concentration of antibiotics used in NCT is much lower than

those indicated in the Carba NP [17] and also Blue-Carba test manuals [18]. 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 NCT. Subjectivity in interpretation of results remains the shortcoming of all chromogenic methods [19].

The MIC results of IPM, MEM and ETP against carbapenemase- and non-carbapenemase-producing isolates employed in this study are summarized in Table 1. IPM, MEM and ETP against all carbapenemase-producing strains had MIC values ranging from 0.5 to ≥256, 0.25 to ≥256 and 1 to ≥256 µg/mL, respectively. OXA-48-producing isolates showed lower MIC values compared with producers of MBL and KPC. Three OXA-48 carbapenemase producers found to be susceptible to IPM, while four OXA-48 carbapenemase producers were sensitive to MEM. OXA-48 like carbapenemase enzymes often weakly hydrolyse carbapenems resulting in the MIC value which may not be high enough to be designated resistant or intermediately resistant [20]. This is consistent with the results from the current study which demonstrates that some OXA-48-producing strains are sensitive and intermediately resistant to carbapenems.

The majority of non-carbapenemase producers were still susceptible to carbapenem antibiotics, with the exception of an AmpC producer (ACT-1-producing *E. aerogenes*) which was found to be resistant to IPM at 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 non-carbapenemase producer of IPM-resistant *E. aerogenes* with AmpC β-lactamase production [21]. Taken together, the findings from this study suggest that the NCT

can effectively detect the presence of carbapenemase in carbapenem-susceptible isolates, in particular OXA-48 producers. The NCT can also discriminate carbapenemase-producing isolates from hyperproduction of ESBL 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 antibiotic resistant pathogens, screening patients and rational use of chemotherapy. The NCT assay we propose here is a very simple, reliable and cost-effective method, allowing rapid visual observation of results within 20 min. ETP shows the best sensitivity and specificity, and so 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.

Declarations

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Competing Interests: There are no Conflicts of Interest.

Ethical Approval: Not required

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Figure legend

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

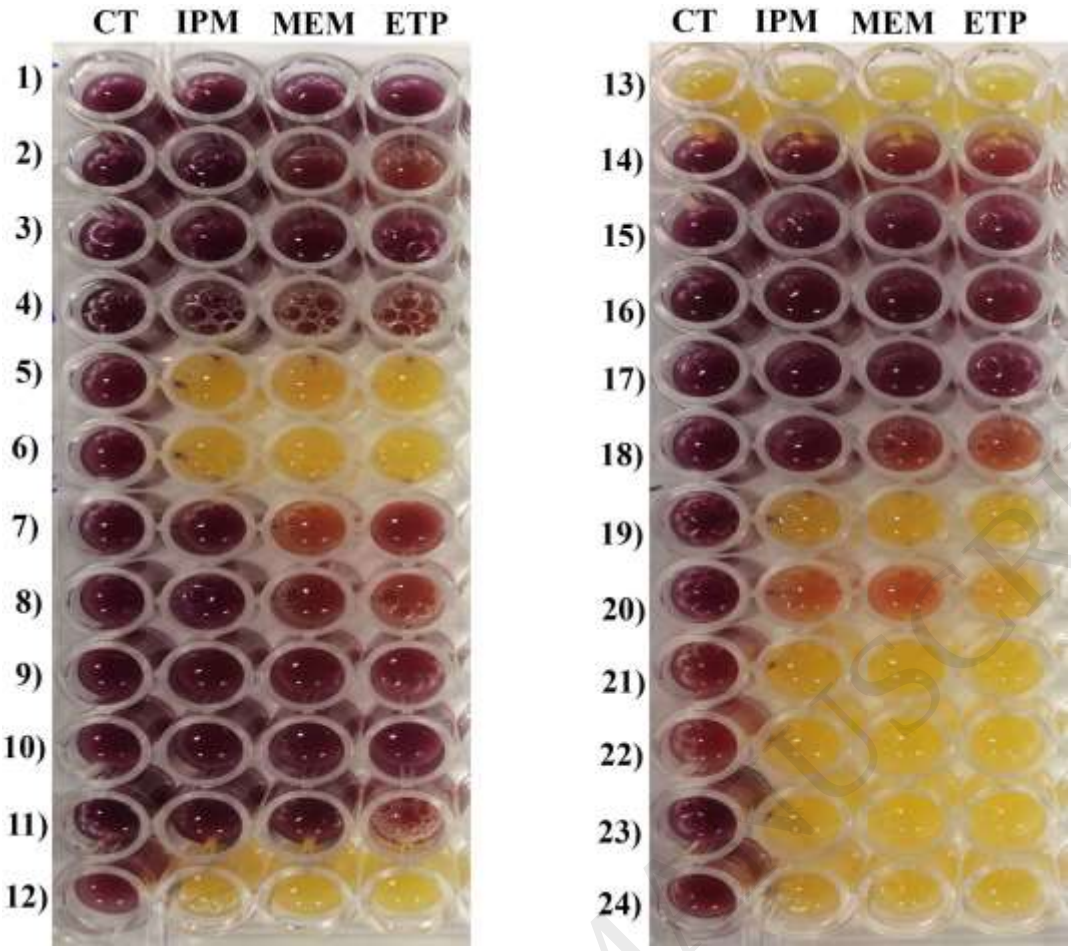

Table 1. Organism characteristics and minimum inhibitory concentrations (MICs) of carbapenemase-producing-and non-carbapenemase-producing isolates used in this study.

Group of β-lactamases		**Species (no. of strains)**	**MIC(s) (μg/mL)**				**Nitro-Carba test Time to detection of positive result [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	*K. pneumoniae* (6)	16 - ≥ 256	8 - ≥256	32 - ≥256		0(6) - 6(30)	0(10) - 7(20)	0(10) – 7(29)	0(10) - 9(45)
		K. oxytoca (1)	4	8	16		2(1)	2(10)	5(47)	5(47)
		E. 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	*K. ozaenae* (1)	4	16	8		0(20)	0(35)	0(35)	0(35)
	NDM	*E. cloacae* (2)	64 - ≥256	128 - ≥256	128 - ≥256		0(20) - 3(10)	0(32)	0(47)	0(47)
		K. pneumoniae (3)	16 - 128	32 - ≥256	32 - ≥256		0(25) - 1(23)	1(40) - 8(0)	4(30) – 12(0)	4(32) - 12(0)
		E. coli (1)	≥256	≥256	≥256		0(10)	4(11)	5(4)	5(4)
	VIM*	*K. pneumoniae* (4)	8 – 64	8 - ≥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 - ≥256	1 - 64	4 - ≥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
		C. freundii (2)	0.5 – 1	≤0.25	≤0.25 – 1		0(14)	4(0) - 7(0)	>20	>20
		E. 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
		M. 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

*One VIM-1+SHV-12 and one VIM-1+SHV-102 co-producer