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ORIGINAL ARTICLE

Characterization of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* from a healthcare region in Hong Kong

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Abstract Carbapenem-resistant Enterobacteriaceae represents a major public health issue. This study investigated the clonality and resistance mechanisms of 92 carbapenem-resistant *E. coli* ($n = 21$) and *K. pneumoniae* ($n = 71$) isolates collected consecutively from clinical specimens and patients at high risk of carriage between 2010 and 2012 in a healthcare region in Hong Kong. Combined disk tests (CDTs) and the Carba NP test were used for phenotypic detection of carbapenemases. PCR assays were used to detect carbapenemase genes. All isolates were intermediate or resistant to at least one carbapenem. Nine (9.8 %) isolates were genotypic carbapenemase producers and included six *K. pneumoniae* (one ST1306/$bla_{\text{IMP-4}}$, one ST889/$bla_{\text{IMP-4}}$, two ST11/$bla_{\text{KPC-2}}$, one ST258/$bla_{\text{KPC-2}}$, one ST483/$bla_{\text{NDM-1}}$) and three *E. coli* (one ST131/$bla_{\text{IMP-4}}$, two ST744/ $bla_{\text{NDM-1}}$) isolates. All nine isolates carrying carbapenemase genes could be detected by the CDTs and the Carba NP test. PCR identified $bla_{\text{CTX-M}}$ and bla_{AmpC} alone or in combination in 77.8 % (7/9) and 96.4 % (80/83) of the carbapenemase-producers and non-producers, respectively. Porin loss was detected in 22.2 % (2/9) and 59.0 % (49/83) of the carbapenemase-producers and non-producers, respectively. Overall, the *E. coli* clones were diverse (14 different STs), but 36.6 % (26/71) of the *K. pneumoniae* isolates belonged to ST11. In conclusion, the prevalence of carbapenemases among carbapenem-nonsusceptible *E. coli* and *K. pneumoniae* remained low in Hong Kong. Porin loss combined with AmpC and/or CTX-M type ESBL was the major mechanism of carbapenem resistance in the study population.

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Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) is a major public health challenge because therapeutic alternatives are severely limited and serious infections are associated with poor outcome [1]. Carbapenem resistance can be mediated by the production of carbapenemases (i.e. carbapenemase-producing Enterobacteriaceae, CPE) or by the combination of porin loss and production of AmpC or extended-spectrum β-lactamases (ESBLs) [2]. Of particular concern is the emergence of acquired carbapenemases among *E. coli* and *K. pneumoniae* because the two species are common causes of many human infections. Recent epidemiological studies have highlighted the potential of acquired carbapenemase producers to cause hospital outbreaks and to become endemic in healthcare settings [2, 3]. As a consequence, detecting the production of carbapenemases and distinguishing between carbapenemases and other mechanisms of carbapenem resistance has become an important task for diagnostic laboratories [4].

Globally, the major types of carbapenemases include KPC, NDM, IMP, VIM and OXA. Both epidemic clones and mobile genetic elements including plasmids, transposons and integrons are involved in their dissemination [1, 5]. The IMP and NDM type carbapenemases are most frequently reported in the Asia-Pacific region [5, 6]. Unlike KPC type carbapenemases, IMP and NDM producers may not exhibit high level resistance to the carbapenems, especially imipenem and meropenem [1, 5, 7]. False negative screening and

phenotypic tests for IMP and NDM producing isolates have been described [4, 8]. In Hong Kong, the prevalence of carbapenem resistance among clinical isolates of *E. coli* and *K. pneumoniae* remains low [9]. Most reports concern sporadic isolates introduced by patients from high-prevalence areas [5, 7, 10]. The aim of this study was to investigate the clonality and resistance mechanisms of carbapenem-resistant *E. coli* and *K. pneumoniae*. The ability of two widely used phenotypic tests to distinguish between carbapenemase producers and non-producers were described.

Materials and methods

Bacterial strains, identification and antimicrobial susceptibility testing

Seventy-one carbapenem-nonsusceptible *Klebsiella pneumoniae* and 21 *E. coli* isolates were included in the study. The isolates were recovered consecutively between September 2010 and December 2012 from inpatients treated in a healthcare region in Hong Kong comprising of a University-affiliated hospital with 1600 beds, three extended-care hospitals with a total of 1600 beds, and one paediatric hospital with 160 beds. The collection included 13 clinical isolates (from blood, wound, urine and other body fluids) and 79 stool isolates. In the hospital, carbapenem resistance among *K. pneumoniae* and *E. coli* clinical isolates were rare (<0.1 %) [9, 11]. As part of the healthcare region's infection control policy, a combination of active surveillance cultures and "added tests" as described previously [9] were done to identify high risk patients carrying multiple drug-resistant organisms and to opportunistically identify faecal carriage of CRE among stool specimens sent for bacterial culture respectively. Admission screening was implemented for all inpatients with a history of hospitalization or surgical operation outside Hong Kong in the past 12 months [12]. Faecal samples or rectal swabs were plated on MacConkey plates supplemented with 1 μg/ml meropenem [12]. Colonies that grew on the plates were identified to species level by the VITEK GNI system (bioMerieux Vitek Inc., Hazelwood, MO, USA). Susceptibility testing of the isolates was performed by the disk diffusion method [11]. On each day of testing, standard strains (ATCC 25922 and 35218) were included as quality controls. Organisms identified as members of the *Enterobacteriaceae* were investigated further if they were found to be nonsusceptible to one or more of ertapenem (<22 mm, >0.5 μg/ml), imipenem (<23 mm, >1 μg/ml) and meropenem (<23 mm, >1 μg/ml) [11]. The 92 isolates were recovered from 88 patients, of which four patients had both *K. pneumoniae* and *E. coli*. All available isolates during the study period were included.

Phenotypic detection of carbapenemases

Two tests were used for phenotypic detection of carbapenemases. The combined disk test (CDT) was performed by using a previously described procedure [13]. Ethylene diamine tetra-acetic acid (EDTA) and 3-aminophenyl boronic acid (APBA) were obtained from Sigma. Two sets each of ertapenem (ETP, 10 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg) (all from Becton Dickinson) were placed onto Mueller-Hinton (MH) agar (EDTA-CDT) and MH agar containing 250 μg/ml cloxacillin agar (APBA-CDT) inoculated with the test isolate [13]. Immediately after the disks were placed onto the agar, 10 μl of a 29.2 mg/ml EDTA or a 30 mg/ml APBA solution was added to one of the two carbapenem disks in each set. The agar plates were incubated at 37 °C overnight. An increase of ≥5 mm in zone diameter around disks containing the β-lactamase inhibitor (APBA or EDTA), as compared with the carbapenem disk alone, was considered to be a positive result. EDTA-CDT positive and APBA-CDT positive results were used to indicate the possible presence of metallo-β-lactamase (MBL) and class A carbapenemase, respectively [13]. The chromogenic carbapenemase detection assay based on hydrolysis of imipenem, Carba NP test, was carried out and interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2015, M100-S15) [11]. All strains were grown at 37 °C for 24 hours in Columbia agar (Becton Dickinson) supplemented with 5 % horse blood. Commercial protein extract reagent (B-PER-II, Thermo Scientific Pierce, IL, USA) was used for cell lysis. *K. pneumoniae* ATCC BAA-1705 and BAA-1706 were included as controls in each testing.

Molecular studies

PCR assays were used to detect carbapenemase genes (class B families: bla_{IMP}, bla_{NDM}, bla_{SPM}, bla_{SIM}, bla_{GIM}, bla_{VIM}; class A families: bla_{KPC}, bla_{GES}, bla_{SME}, bla_{IMI}, bla_{SFO}, bla_{IBC}, bla_{PER}, bla_{SPC}; and class D family bla_{OXA-48}), acquired bla_{AmpC} genes (subgroups bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{BEC}, bla_{FOX}), and bla_{CTX-M} genes (subgroups $bla_{CTX-M1G}$, $bla_{CTX-M2G}$, $bla_{CTX-M8G}$, $bla_{CTX-M9G}$, $bla_{CTX-M25G}$) [5, 10, 14]. The alleles of bla_{IMP}, bla_{NDM} and bla_{KPC} were determined by PCR and sequencing of the full length of genes [5, 7, 10]. PCR assays were used to assign the *E. coli* isolates to phylogroups [15]. Multilocus sequence typing (MLST) of *K. pneumoniae* and *E. coli* isolates was carried out using the Pasteur Institute and the Warwick scheme, respectively [16, 17], and results were analysed by eBURST v3 [18]. Sequence types (STs) were assigned to clonal complexes (CCs) when there were at least three isolates. STs with one or two isolates were designated as singletons. CCs were generated by using the

stringent group definition (6/7 shared alleles). STs within a CC were believed to be descended from the same founding genotype [18].

Isolation and analysis of porins

Bacterial outer membrane porins were analysed by standard techniques [19]. In brief, isolates were grown in Luria broth, sonicated and centrifuged. Porins were separated by 12 % SDS-PAGE gel containing 6 M urea and stained with 0.125 % Coomassie brilliant blue. Two reference strains, *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922 were included as controls in each run.

Results

Antimicrobial susceptibilities

Among the 92 isolates analysed, rates of nonsusceptibility to ertapenem, imipenem and meropenem were 98.9 % (91/92), 91.3 % (84/92) and 95.7 % (88/92), respectively (Online Resource, Table S1). Some discordant results to the three carbapenems (ertapenem, imipenem, meropenem) were observed: 81 (88.0 %) isolates were nonsusceptible to three carbapenems, nine (9.8 %) isolates were nonsusceptible to two carbapenems and two (2.1 %) were nonsusceptible to one carbapenem. Co-resistance to other β-lactams, aminoglycosides, ciprofloxacin and cotrimoxazole was common.

Phenotypic and PCR analyses for carbapenemases

In the CDTs, the proportions of isolates with positive phenotypic MBL (i.e. EDTA-CDT positive) and class A carbapenemase (i.e. APBA-CDT positive) results in at least one carbapenem disk were 6.5 % (6/92) and 4.3 % (4/92), respectively (Table 1). Valid results were obtained for all the isolates in the Carba NP test, including nine positives and 83 negatives. PCR and sequencing showed that the nine Carba NP-positive isolates were positive for $bla_{\text{NDM-1}}$ ($n = 3$), $bla_{\text{IMP-4}}$ ($n = 3$) or $bla_{\text{KPC-2}}$ ($n = 3$) genes. No carbapenemase genes were detected in the 83 Carba NP negative isolates. Besides bla_{NDM}, bla_{IMP} and bla_{KPC}, no other carbapenemase genes were detected in the isolates. Two $bla_{\text{IMP-4}}$ isolates had false-negative results in the IPM EDTA-CDT (strain 1058) and ETP EDTA-CDT (strain 249), but positive results were obtained for the other carbapenem disks (Table 1). EDTA-CDT and APBA-CDT (IPM, MEM and ETP) gave true positive results for the remaining seven carbapenemase producers. One isolate (strain 15) had false-positive APBA-CDT (MEM and ETP) result (Table 1).

ESBL, AmpC and porin loss

At least one of the β–lactam resistance mechanisms ($bla_{\text{CTX-M}}$, bla_{AmpC}, porin loss) was detected in all except two *K. pneumoniae* isolates (Table 2). Overall, $bla_{\text{CTX-M}}$, bla_{AmpC} and porin loss were detected in 60.9 % (56/92), 73.9 % (68/92) and 55.4 % (51/92) of all isolates. $bla_{\text{CTX-M}}$ and bla_{AmpC} alone or in combination was detected in 77.7 % (7/9) and 96.4 % (80/83) of the carbapenemase producers and non-producers, respectively. Porin loss was detected in 22.2 % (2/9) and 59.0 % (49/83) of the producers and non-producers, respectively.

The $bla_{\text{CTX-M}}$ gene was more frequently detected in *E. coli* (85.7 %, 18/21) than in *K. pneumoniae* (53.5 %, 38/71) isolates (Table 2). Among *E. coli* and *K. pneumoniae*, $bla_{\text{CTX-M1G}}$ alone, $bla_{\text{CTX-M9G}}$ alone and both $bla_{\text{CTX-M1G}}$ and $bla_{\text{CTX-M9G}}$ were found in 42.9 % (9/21) and 15.5 % (11/71), 28.6 %

Table 1 Characteristics of isolates with positive result in combined disk test and Carba NP test

Species, strain identifier	CTX-M	AmpC	Porin loss	Carbapenemase gene	EDTA-CDT on MH agar				APBA-CDT on MH-CLX agar				CarbaNP
					Any	IPM	MEM	ETP	Any	IPM	MEM	ETP	
K. pneumoniae 249	+	+	−	$bla_{\text{IMP-4}}$	+	+	+	−	−	−	−	−	+
K. pneumoniae 1030	+	+	+	$bla_{\text{IMP-4}}$	+	+	+	+	−	−	−	−	+
E. coli 1058	−	−	+	$bla_{\text{IMP-4}}$	+	−	+	+	−	−	−	−	+
K. pneumoniae 380	+	+	−	$bla_{\text{NDM-1}}$	+	+	+	+	−	−	−	−	+
E. coli 396	+	−	−	$bla_{\text{NDM-1}}$	+	+	+	+	−	−	−	−	+
E. coli 397	+	−	−	$bla_{\text{NDM-1}}$	+	+	+	+	−	−	−	−	+
K. pneumoniae 24	+	−	−	$bla_{\text{KPC-2}}$	−	−	−	−	+	+	+	+	+
K. pneumoniae 79	−	−	−	$bla_{\text{KPC-2}}$	−	−	−	−	+	+	+	+	+
K. pneumoniae 292	−	+	−	$bla_{\text{KPC-2}}$	−	−	−	−	+	+	+	+	+
E. coli 15	+	−	+	None	−	−	−	−	+	−	+	+	−

[a] CDTs combined disk tests, CLX cloxacillin, EDTA ethylene diamine tetra-acetic acid, APBA 3-aminophenyl boronic acid, ETP ertapenem, IPM imipenem, MEM meropenem, + positive, − negative

Table 2 Porins and PCR analysis for β-lactamase genes

Profile[a]	*K.pneumoniae* [*n* (%)]	*E. coli* [*n* (%)]	Total [*n* (%)]	No of *K. pneumoniae* (*E. coli*) with		
				bla_{IMP-4}	bla_{NDM-1}	bla_{KPC-2}
Porin loss plus bla_{CTX-M} and bla_{AmpC}	21 (29.6)	5 (23.8)	26 (28.3)	1		
Porin loss plus bla_{AmpC}	7 (9.9)	1 (4.8)	8 (8.7)			
Porin loss plus bla_{CTX-M}	4 (5.6)	10 (47.6)	14 (15.2)			
bla_{AmpC} (± bla_{CTX-M})	33 (46.5)	1 (4.8)	34 (37.0)	1	1	1
Others[b]	6 (8.5)	4 (19.0)	10 (10.9)	(1)	(2)	2
Total	71 (100)	21 (100)	92 (100)	2 (1)	1 (2)	3

[a] For presence of bla_{CTX-M}, bla_{AmpC} gene and porin loss

[b] Including five isolates with bla_{CTX-M} (two *E. coli* and three *K. pneumoniae*), three isolates with porin loss (two *E. coli*, one *K. pneumoniae*) and two isolates with neither bla_{CTX-M}, bla_{AmpC} nor porin loss.

(6/21) and 14.1 % (10/71), and 14.3 % (3/21) and 23.9 % (17/71), respectively.

bla_{AmpC} genes were detected in 33.3 % (7/21) of *E. coli* and 85.9 % (61/71) of *K. pneumoniae* isolates (Table 2). The acquired bla_{AmpC} genes in *E. coli* included five (23.8 %) bla_{CIT} and two (9.5 %) bla_{DHA}. In *K. pneumoniae*, bla_{DHA} alone, bla_{CIT} alone and both bla_{DHA} and bla_{CIT} were detected in 57 (80.3 %), three (4.2 %) and one (1.4 %) isolates, respectively. Among *E. coli* and *K. pneumoniae*, porin loss was detected in 85.7 % (18/21) and 46.5 % (33/71) of isolates, respectively (Table 2). No bla_{MOX}, bla_{ACC}, bla_{BEC} and bla_{FOX} genes were detected.

Among *E. coli* isolates, 16 (76.2 %) displayed both OmpC and OmpF loss and two (9.5 %) displayed OmpC loss (Table 2). Among *K. pneumoniae* isolates, 24 (33.8 %) displayed OmpK36 loss, one (1.4 %) displayed OmpK35 loss, and eight (11.3 %) displayed both OmpK35 and OmpK36 loss.

Clonal structure

MLST analyses revealed 14 and 29 different STs among the *E. coli* and *K. pneumoniae* isolates, respectively (Fig. 1). The *E. coli* isolates could be grouped into three clonal complexes (CCs), including CC10/phylogroups A or B1 (52.4 %, 11/21), CC131/phylogroup B2 (14.3 %, 3/21) and CC405/phylogroup D (14.3 %, 3/21) and four singletons. In contrast, the majority (77.5 %, 55/71) of the *K. pneumoniae* isolates could be grouped to CC37, among which ST11 (36.6 %, 26/71) was the predominant ST. No association was found between the types of bla_{CTX-M}, bla_{AmpC} and porin loss, and the clones of *E. coli* and *K. pneumoniae*. The bla_{DHA} gene was found in 25 different STs of *K. pneumoniae*. The STs and carbapenemase gene combinations of the nine CPE isolates were as follows: *K. pneumoniae* (one ST1306/bla_{IMP-4}, one ST889/bla_{IMP-4}, two ST11/bla_{KPC-2}, one ST258/bla_{KPC-2}, one ST483/bla_{NDM-1}); *E. coli* (one ST131/bla_{IMP-4}, two ST744/bla_{NDM-1}).

Discussion

This study investigated the antimicrobial susceptibilities and molecular characteristics of 13 clinical and 79 stool carbapenem-nonsusceptible *E. coli* and *K. pneumoniae* isolates. Nine carbapenemase producers including one from a clinical specimen and eight from screening specimens were identified, giving a carbapenemase prevalence of 9.8 % (9/92) among the carbapenem nonsusceptible isolates. In Hong Kong, carbapenem nonsusceptibility among clinical *E. coli* and *K. pneumoniae* isolates remains rare and limited to sporadic occurrence [20, 21]. All the carbapenemase-producing strains in this study were identified in patients who had a history of hospitalization in mainland China or the United States. The importance of medical tourism as a significant risk factor for acquisition of CPE has been reported in many previous studies [2, 5]. In areas where the prevalence of CPE remains low, active surveillance culture of all patients with a history of travel should continue to be emphasized [20].

MLST revealed that our strains carrying bla_{IMP-4} or bla_{NDM-1} were multi-clonal, as these resistance determinants mainly spread among bacterial populations by mobile genetic elements [1]. On the other hand, all three *K. pneumoniae* isolates carrying bla_{KPC-2} were members of the widespread ST11 or ST258 clones [22]. Besides carbapenemases, carriage of ESBL and/or AmpC in combination with porin loss or drug efflux has been reported to contribute to carbapenem resistance in *Enterobacteriaceae* [2, 23]. In the present study, CTX-M type ESBL, AmpC and porin loss were highly prevalent among the carbapenem-nonsusceptible isolates. Among the strains negative for all carbapenemases genes, the mechanism of carbapenem resistance in about half of them could be attributed to presence of CTX-M and/or AmpC in combination with porin loss. As revealed by the MLST analysis, ST11 is also an important clonal group among *K. pneumoniae* isolates not producing carbapenemases [8].

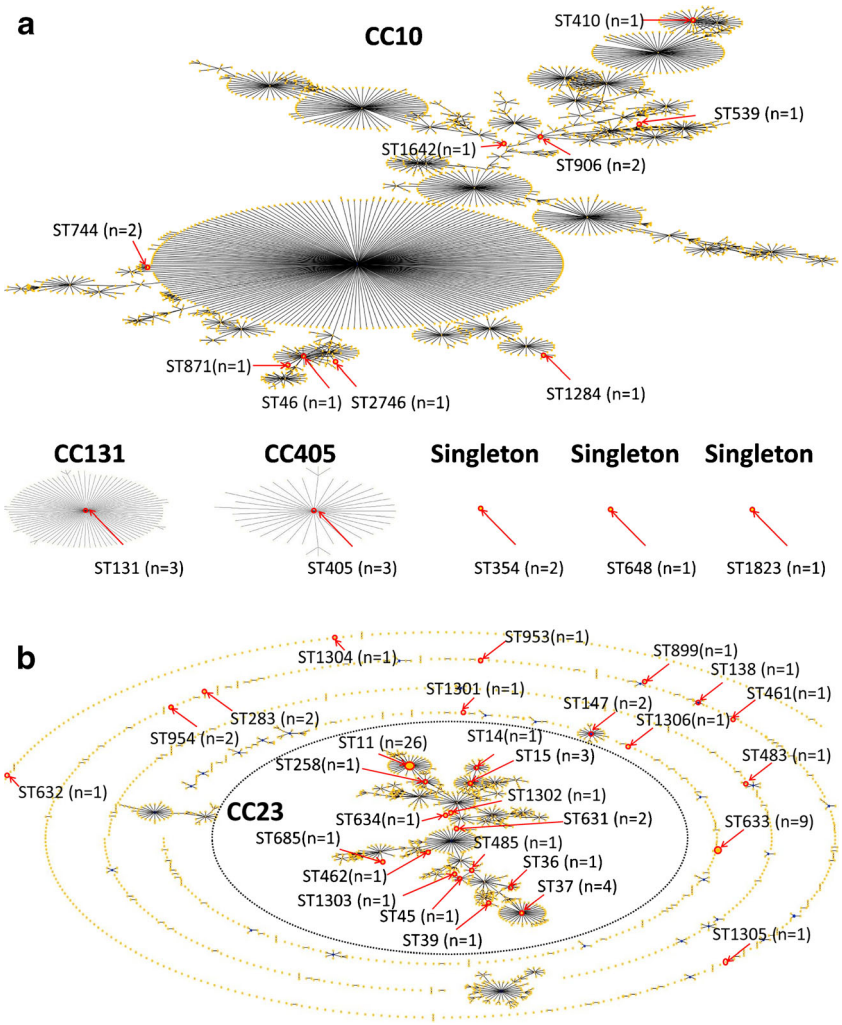

Fig. 1 Clonal structure of 21 *E. coli* and 71 *K. pneumoniae* isolates non-susceptible to carbapenems. The population snapshots were generated by eBURST v3. The STs detected in this study were labelled with arrows pointing to the circle. When more than one isolate in the indicated ST was found, the number of isolates was indicated inside the bracket. (**a**) all strains from the *E. coli* MLST database at the University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli, last accessed on 5 Nov 2015) belonging to CC10, CC131 and CC405 are depicted, together with the singleton strains found in this study. (**b**) All strains from the *K. pneumoniae* MLST database at Pasteur institute (http://bigsdb.web.pasteur.fr/, last accessed on 5 Nov 2015) belonging to CC23, together with the singleton strains detected here or related, are shown

This study used two previously validated methods for phenotypic detection of carbapenemases [13, 24, 25]. Some variations in the ability of ertapenem, imipenem and meropenem as substrates for potentiation by APBA and EDTA in CDTs were observed. Inclusion of more than one substrate in the CDT was required for phenotypic detection of all the carbapenemase producers in the collection. In contrast, the Carba NP test based upon hydrolysis of imipenem was able to detect all the carbapenemase producers and no false-positive results were observed. In diagnostic laboratories, workflow for the detection of CPE is evolving as new approaches and improvement in tests for their detection continue to be reported [13, 26]. The short turnaround time (2 hours) of Carba NP test is an important advantage. Given the greater epidemiological and clinical importance of CPE, this can allow the infection control team to make better use of isolation facilities for reducing the risk of nosocomial transmission [9]. However, false negative or equivocal Carba NP test results may occur for mucoid colonies because of difficulty in protein extraction and for enzyme types with low carbapenemase activity, especially the OXA group of enzymes [13, 27, 28]. Carba NP test may also fail to detect less common carbapenemases (SME, GES, IMI) [28–30]. Currently, the CLSI (2015, M100-S25) only recommends the Carba NP test for epidemiological or infection control purposes [11]. Its routine use in diagnostic laboratories is not recommended.

This study is limited by the small number of carbapenemase producers. We restricted the investigation of carbapenem resistance mechanisms to the most common types [1, 2]. Besides those that were investigated, drug efflux, other porins (PhoE, lamB) and other ESBLs (e.g. SHV, TEM) have also been described to contribute to carbapenem resistance [31, 32].

In conclusion, the prevalence of KPC, IMP and NDM types of carbapenemases among carbapenem-nonsusceptible *E. coli* and *K. pneumoniae* remained low in Hong Kong. Porin loss combined with AmpC and/or CTX-M type ESBL was the major mechanism of carbapenem resistance in the study population.

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Compliance with ethical standards

Transparency declaration Nothing to declare

Conflict of interest statement The authors declares that they have no conflict of interest.

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