Original article

Colonization of intestinal microbiota with carbapenemase-producing Enterobacteriaceae in paediatric intensive care units in Cairo, Egypt

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ABSTRACT

Background and study aims: Colonized patients with carbapenamase producing Enterobacteriaceae (CPE) are vulnerable to invasive infections from their endogenous flora. We aimed to assess faecal colonization with (CPE) among children admitted to Cairo University paediatric intensive care units (ICUs). The phenotypic and genotypic characterizations of carbapenemase-producing Enterobacteriaceae were also studied.

Patients and methods: A total of 413 Enterobacteriaceae isolates have been isolated from cultured rectal swabs of 100 children. All swabs were inoculated on ChromID™ CARBA agar to screen for carbapenem resistant Enterobacteriaceae (CRE). Disk diffusion method, Modified Hodge test (MHT) and further genotypic detection of carbapenemases genes (bla_{OXA-48}, bla_{KPC} and bla_{NDM-1}, bla_{VIM} and bla_{IMP}) by multiplex PCR were done.

Results: Out of 413 Enterobacteriaceae isolates; 100 isolates were defined as CRE. Bla_{OXA-48} was detected in (33%); Escherichia coli (n = 11), Klebsiella oxytoca (n = 3) and Klebsiella pneumoniae (n = 19), while (27%) carried bla_{NDM-1} Escherichia coli (n = 7), and Klebsiella pneumoniae (n = 20).

Conclusion: Prevalence of carbapenem resistant Enterobacteriaceae was 24%, various genes of carbapenemases were detected in 80% of carbapenem resistant Enterobacteriaceae with dominance of bla_{OXA-48}. Understanding the colonization status of our patients with strict infection control measures can reduce the risk of horizontal gene transfer of carbapenemases.

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Introduction

Carbapenem resistant Enterobacteriaceae (CRE) are considered harmless but may cause serious infections in debilitated patients due to severe illness, old age, invasive procedures, or indwelling catheters [1,2]. Admission to intensive care units (ICUs) and sharing a room with known CRE carriers considered a major risk factor for acquiring carbapenem resistant Enterobacteriaceae [3,4]. Patients colonized with carbapenem resistant Enterobacteriaceae may seem healthy and have no symptoms but still require specific infection control precautions and cohorting measures when admitted to healthcare settings to prevent contamination of the environment and spread to vulnerable patients. Meanwhile decisions regarding surveillance of CRE depends on the local epidemiology and accessible financial resources [5].

Resistance to carbapenems in Enterobacteriaceae may be due to different mechanisms the most common mechanism is production of carbapenamases enzymes in this case organisms will be called carbapenemase-producing Enterobacteriaceae (CPE). Carbapenamases enzymes are typically located on plasmids that facilitate transmission within and between bacterial species [2–4].

Carbapenemases of universal importance include Klebsiella pneumoniae carbapenemase (KPC), New Delhi metallo-β-lactamase-type 1 (NDM-1), Verona integron encoded metallo-β-lactamase (VIM), imipenemase metallo-β-lactamase (IMP), and oxacillinase-48 (OXA-48) [6]. Non-carbapenemase carbapenem resistance is mediated by a combination of mechanisms, typically by production of an extended spectrum β-lactamase (ESBL or AmpC) plus decreased permeability of the bacterial cell wall (e.g., porin mutations) or overproduction of efflux pumps [6]. Unfortunately, the antibiotic agents for treating CRE infections are extremely limited and are often associated with adverse reactions.

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We aimed in our study to assess faecal colonization by carbapenemase-producing *Enterobacteriaceae* among children admitted to Cairo University paediatric intensive care units over a period of 6 months. The phenotypic and genotypic characterizations of carbapenemase-producing *Enterobacteriaceae* were also studied with sequencing of the most prevalent carbapenemase gene.

Patients and methods

Over the period of 6 months a total of 413 *Enterobacteriaceae* isolates have been isolated from 100 cultured rectal swabs taken from 100 children admitted to Cairo University paediatric intensive care units (ICUs) from September 2015 till February 2016.

Timing of rectal swabbing was done according to both the CDC and European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommendations. Periodic screening (e.g., weekly), for hospitalized patients in high-risk units such as ICUs and initial screening for patients referred from other hospitals or from different hospital wards [1,2]. Patients data were extracted from their medical records per a prepared questionnaire including; demographics (age, gender, ward class), specific co-morbid conditions (cardiovascular, renal, pulmonary, etc.), exposure (≥ 1 day(s)) to antimicrobials (imipenem, meropenem, ertapenem, doripenem, ciprofloxacin, vancomycin, cephalosporins, piperacillin-tazobacatam, metronidazole).

All rectal swabs (APTACA® Amies Charcoal transport medium Italy) were transmitted to microbiology unit of Cairo university hospital to be cultured on blood agar, MacConkey agar (Oxoid Co. England) and ChromID CARBA agar (Biomerieux, France) for screening of CRE per manufacture recommendations. All cultured plates were incubated overnight at 35 ± 2 °C, ambient air. All isolates were subjected to:

1. Identification by Vitek2 compact automated system (Biomerieux, France).
2. Antimicrobial susceptibility testing by disc diffusion method (Modified Kirby Bauer technique) using Muller Hinton agar (Oxoid Co. England), aerobic incubation at 35 °C for 16–18 h. Antimicrobial discs, imipenem (10 mcg), meropenem (10 mcg), gentamicin (10 mcg), ciprofloxacin (5 mcg), amikacin (30 mcg), cotrimoxazole (25 mcg), cefepime (30 mcg), cefotaxime (30 mcg), cefotaxime + clavulanic acid(30/10 mcg), aztreonam (30 mcg), ceftazidime (30 mcg), ceftazidime + clavulanic acid (30/10 mcg), amoxicillin-clavulanic acid (20/10 mcg), and cefoxitin (30 mcg) were obtained from Oxoid Co. (Oxoid Limited, Basingstoke, Hampshire, England). Phenotypic screening and confirmation of extended spectrum β lactamases (ESBL) production was done per CLSI (2015) using the combined disc tests [7].

One hundred carbapenem resistant *Enterobacteriaceae* clinical isolates grown on ChromID CARBA agar (Biomerieux, France) were further tested for

1. The Modified Hodge test as described in CLSI (2015) [7].
2. Minimum inhibitory concentration (MIC) of colistin and tigecycline by broth microdilution per CLSI recommendations. Resistant and susceptible to tigecycline were defined as MIC 2 mg/L and ≤ 1 mg/L respectively (European committee on antimicrobial susceptibility testing; EUCAST 2011).
3. Genotypic characterization of carbapenemase-producing *Enterobacteriaceae* was done in the molecular laboratory of the microbiology and botany department, faculty of science, Cairo university by multiplex PCR for bla_{VIM}, bla_{IMP}, bla_{NDM-1}, bla_{KPC},

and bla_{OXA-48} genes in a single reaction with primers and methodology described by Poirel et al. (2011) [8] as shown in Table 1. Total DNA was isolated by boiling lysis method. Briefly, a single bacterial colony was picked, suspended in 100 µl of PCR-grade water and boiled for 10 min. Following centrifugation at 16,000 rpm for 5 min, the supernatant was used as a template. PCR analysis was performed in a total volume of 50 µl containing 2 µl total DNA extract, $1 \times$ PCR buffer {20 mMTris-HCl [pH 8.3], 20 mMKCl and 5 mM $(NH_4)_2SO_4$}, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 10 mM of each primer, and 2 U of Maxima™ Hot Start *Taq* DNA Polymerase (Thermo Scientific, USA). Amplification was carried out as follows: 10 min at 95 °C and 36 cycles of amplification consisting of 30 s at 95 °C, 40 s at 52 °C, and 50 s at 72 °C, with 10 min at 72 °C for the final extension. Amplicons were analysed by electrophoresis in a 2% agarose gel at 100 V for 1 h in $1\times$ Tris-acetate-EDTA (TAE) buffer containing 0.5 µg/ml ethidium bromide. The Gene Ruler 100 bp DNA ladder was used as a DNA size marker (Thermo Scientific, USA).

4. Sequencing reactions were done for OXA48 gene because it was the most prevalent carbapenamase in our study. Sequencing was done at Macrogen Company (South Korea) using ABI PRISM BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the forward primer (5'-TTGGTGGCATCGATTATCGG-3') and the reverse one (5'-GAGCACTTCTTTTGTGATGGC-3'). The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730x1 sequencer (Applied Biosystem, USA). Nucleotide sequence similarities were determined using other known sequences found in the GenBank database using BLAST program of National Center for Biotechnology Information (NCBI) databases. The sequences were submitted to GenBank sequence database of NCBI for analysis using the online program http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS 21.0 (SPSS, Chicago, IL, USA). Data was presented by percentages for categorical variables. Mean – SD for quantitative data. Tests of significance were used to assess difference between two study groups; *t*-test was used for assessing significant difference between two study groups in quantitative variables. The chi square test was used to test for statistical significance of categorical variables and was set at a p value less than 0.05.

Table 1
Set of primers used in this study.

Primer	Sequence (5'–3')	Gene	Product size (bp)
IMP-F IMP-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	bla_{IMP}	232
VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	bla_{VIM}	390
OXA-F OXA-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	bla_{OXA-48}	438
NDM-F NDM-R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	bla_{NDM}	621
KPC-Fm KPC-Rm	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	bla_{KPC}	798

Results

Over the period of 6 months our study population were 100 children aged from 0.03 month to 144 months (mean 11.39), median 1.85 ± 25 SD. Co -morbidities associated with carbapenem resistant *Enterobacteriaceae* faecal carriage were shown in Fig. 1.

Our phenotypic screening revealed 100 carbapenem resistant *Enterobacteriaceae* (CRE) clinical isolates. All CRE carriers were empirically treated with most frequently used antibiotics as follows; (vancomycin 37%, aminoglycosides 35%, cephalosporins 34%, carbapenems 29%, quinolones 11%, and aminoglycosides 8%). Previous antibiotic intake was highly associated (p value <0.001) with carriage of carbapenems resistant *Enterobacteriaceae.*

Prolonged hospital stay was highly associated with CRE faecal carriage (p value <0.001). We noticed that maximum incidence of CRE carriage lies within hospital stay 15–21 days (88%) in patients referred from other hospitals and the lowest within 4–7 days of hospital stay. The studied CRE isolates were identified as follows; *Escherichia coli* (n = 25), *Enterobacter cloacae* (n = 4), *Klebsiella oxytoca* (n = 1) and *Klebsiella pneumoniae* (n = 70). By modified Hodge test 41% of CRE isolates were positive. While sensitivity of MHT in detection of bla_{VIM}, bla_{OXA-48} and bla_{NDM-1} was 88.9%, 75% and 57.4% respectively. The prevalence of CRE was 24%, while 80% of carbapenem resistant *Enterobacteriaceae* were also carbapenamase producers with dominance of bla_{OXA-48} gene as shown in Table 2 and Fig. 2.

Sequencing of *bla* $_{OXA-48}$ gene in *Klebsiella pneumoniae* revealed 99% similarity to OXA-48 (Accession number AHY82488.1).
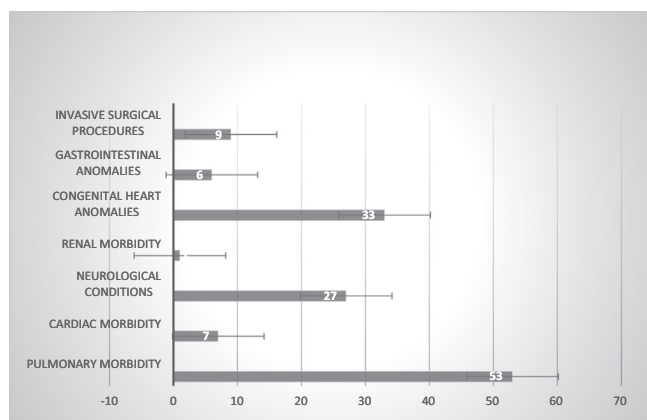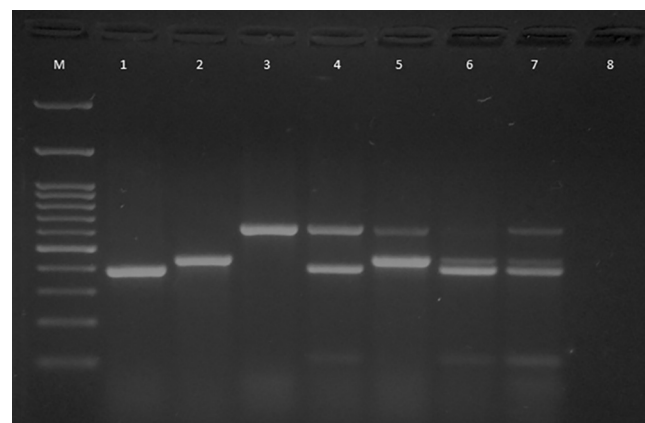
Fig. 2. Multiplex PCR for carbapenemase genes. M: 100 bp DNA Ladder; Lane 1: VIM; Lane2: OXA-48; Lane3: NDM-1; Lane4: VIM + NDM-1; Lane5: OXA-48 + NDM-1; Lane6: VIM + OXA-48; Lane7: VIM + OXA-48 + NDM-1; Lane8: Negative Control.

Discussion

The load of antibiotic resistance is a public health distress and may contribute to increased mortality, prolonged hospital stays and increased expenses of healthcare, especially in developing countries like Egypt where data on antibiotic resistance are very limited [9].

Scarce studies have described paediatric faecal carriage by carbapenem producing *Enterobacteriaceae* in non-outbreak conditions. From our study the high prevalence of CRE 24% could be explained by several factors; 1 – over-the-counter use of antibiotics, unfortunately Egypt is among the three countries in the world where marketing sales of carbapenems have significantly increased per The Lancet Infectious Diseases Commission report [10]. In our study, all carbapenem resistant *Enterobacteriaceae* faecal carrier patients were empirically treated with most frequently used antibiotics at the time of sampling. 2 – The availability of substandard quality carbapenems and sub therapeutic doses used in addition to absence of regulations controlling the antibiotic use. 3 – Inadequate hygiene in hot humid climate that favours bacterial growth. 4 – Insufficient attention to antimicrobial stewardship. 5 – Limited resources, deficient infection-control practices, inability to imply isolation precautions or regular screening of high-risk individuals.

Fig. 1. Associated comorbidities with CRE faecal carriage.

Table 2
Genetic characterization of carbapenemases in CRE clinical isolates.

			Bacteria				Total
			E. coli	*Enterobacter coloacae*	*Klebsiella oxytoca*	*Klebsiella pneumoniae*	
Carbapenemase genes	bla_{NDM-1}	n	7	0	0	20	27
		%	28%	0%	0%	28.6%	27%
	bla_{NDM-1}, bla_{OXA-48}	n	1	0	0	4	5
		%	4%	0%	0%	5.7%	5%
	bla_{NDM-1}, bla_{OXA-48} *and* bla_{VIM}	n	0	0	0	1	1
		%	0%	0%	0%	1.4%	1%
	bla_{OXA-48}	n	11	3	0	19	33
		%	44%	75%	0%	27.1%	33%
	bla_{OXA-48}, bla_{VIM}	n	1	0	0	5	6
		%	4%	0%	0%	7.1%	6%
	bla_{VIM}	n	0	0	0	8	8
		%	0%	0%	0%	11.4%	8%
	Negative PCR	n	5	1	1	13	20
		%	20%	25%	100%	18.6%	20%
Total		n	25	4	1	70	100

In the current study production of carbapenamases enzymes was the main mechanism of resistance to carbapenems as 80% of CRE were carbapenamases producers as detected by multiplex PCR with dominance of bla_{OXA-48}. The phenotypic detection of bla_{OXA-48} producers is particularly problematic due to low carbapenem MICs and/or susceptibility to cephalosporins in the absence of a coproduced ESBL or AmpC enzyme [11]. Yet, it is one of the carbapenemases progressively reported worldwide however, most reports are from Europe, North America and Asia. This enzyme was described in various *Enterobacteriaceae*, including *Klebsiella pneumoniae*, *E. coli*, *Enterobacter cloacae* isolates. Similarly, bla_{OXA-48} producing *Enterobacteriaceae* has been found in different hospitals in Morocco. The Middle East and North Africa are considered as reservoirs of bla_{Oxa-48} producers [12–16]. Whereas bla_{NDM-1} was the second common carbapenamase in our study as it was found totally in (27%) of the studied isolates mainly in *Klebsiella pneumoniae*. Now the Middle East consider as an additional reservoir of bla_{NDM-1} producers while Central and South America represented the last zone without description of blaNDM-1 [17,18]. While bla_{VIM} gene was totally found in (8%) of all isolates. bla_{VIM} producers have been reported in Egypt and other Mediterranean countries such as France [19–21]. Unexpectedly bla_{IMP} and bla_{KPC} genes were not detected in our isolates in concordance with Shibl et al. (2013) study in Riyadh in Saudi Arabia [16]. Other mechanisms of carbapenem resistance other than production of carbapenamases are expected in the remaining 20 PCR negative isolates such as combined production of an extended spectrum β-lactamase and porin mutations or production of other carbapenamases [6]. Expectedly colistin and tigecycline susceptibility was preserved in all isolates in concordance with other Egyptian studies done in surgical ICUs 75% of our isolates were XDR while 25% were MRD [20,22].

In conclusion, the high prevalence of carbapenem resistant *Enterobacteriaceae* faecal carriage with dominance of bla_{OXA-48} emphasizes the importance role of the intestinal tract as a main reservoir of CRE and increases the risk of patient to patient transmission. Our results highlight the necessity for active surveillance program in hospitals not only during outbreaks but also as a routine measure for early detection of carbapenem resistant *Enterobacteriaceae* carriers. As well as applying effective strategies for antimicrobial therapy and infection control measures to decrease the abuse and misuse of antimicrobial agents against resistant strains and to prevent their spread especially in long-term care facilities.

Conflicts of interest

All authors report no conflicts of interest relevant to this article.

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