Vol. 15(6), pp. 286-294, June, 2021
DOI: 10.5897/AJMR2021.9497
Article Number: 53DF9E666937
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Molecular characterization of carbapenem resistant Gram-negative rods in Neonatal Intensive Care Unit of Mansoura University Children's Hospital

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Received 7 February, 2021; Accepted 13 May, 2021

Carbapenems are group of extended-spectrum β-lactam antimicrobials frequently used for treating multidrug-resistant Gram-negative bacilli (GNB) infections. This study aimed at detecting and characterizing carbapenem resistance (CR) genes among GNB isolated from patients treated in neonatal intensive care unit (NICU) of Mansoura University Children's Hospital (MUCH), Egypt. It is a prospective study conducted from 2015 to 2016. A total of 158 GNB isolates were examined for CR both phenotypically and genotypically. Among 158 Gram negative isolates, there were 58 (36.7%) CR strains. Extended-spectrum β-lactamase (ESBL) production was confirmed in all 58 (100%) isolates. Carbapenemase production was detected in 52 (89.5%) strains while metallo beta-lactamase (MBL) production was found in 33 (56.9%) strains. Molecular characterization of CR strains revealed that 57 (98.3%) isolates were positive for carbapenemase encoding genes. KPC gene was the most frequent detected gene (34/58). VIM, IPM, OXA and NDM genes were also detected in 15, 13, 9 and 1 isolate, respectively. Only one isolate was negative for all encoding resistance genes despite positive for ESBL phenotype. Infection with CR strains has been increasing in clinical settings which limit the use of carbapenems.

Key words: Gram-negative bacilli, carbapenem resistance, carbapenemase, metallo beta-lactamase, multiplex polymerase chain reaction (PCR), carbapenemase encoding genes, neonatal intensive care unit.

INTRODUCTION

Treating neonatal infections is challenging due to spread of multidrug-resistant bacteria (Mzimela et al., 2021). Carbapenems are final antimicrobial therapy for life threatening microbes, nevertheless, the appearance of carbapenemases in Gram negative bacteria has put the clinicians in front of restricted treatment choices

(Choudhury et al., 2018). Carbapenem resistance (CR) may be due to either carbapenemase production or other mechanisms, such as alteration of outer membrane permeability together with extended-spectrum β-lactamase (ESBL) production, over expression of *AmpC* type β-lactamases or activation of efflux pumps. The production of carbapenemases is generally a more potent mechanism of CR compared with the other mechanisms (Nordmann et al., 2012; Woodford et al., 2004).

Ambler classification categorizes the β-lactamases into four classes (A to D). Most of carbapenemase-producing bacteria related to A class (*bla* $_{KPC}$), B class (*bla* $_{VIM, IMP,}$ and $_{NDM}$), and D class (*bla* $_{OXA-48}$) Albiger et al., 2015). The *Pseudomonas* and *Acinetobacter* species releasing these enzymes have a wide geographical distribution and have been associated with hospital outbreaks (Woodford et al., 2004). Dissemination of carbapenemases is rapid and widespread in healthcare settings (Elbadawi et al., 2021).

Several methods for detecting carbapenemase production have been used. These methods include phenotypic and molecular methods (Al-Zahrani, 2018). This study aimed at molecular characterization of beta-lactamases associated with CR Gram negative neonatal infections.

METHODOLOGY

Study design

This prospective study was conducted over 2 years during 2015 and 2016 in the NICU of MUCH, which is a level III unit with 25 incubators in 5 equal-sized rooms, that admits approximately 450 neonates per/year; there are no single rooms for isolation.

Sample collection and processing

A total of 158 GNB were detected from clinical samples (blood, urine, wound, tracheal aspirate, abscess, etc.) of 350 admitted neonates. Collected specimens were sent to Microbiology Diagnostics and Infection Control Unit laboratory in less than 2 h. If delay in transportation is expected, the specimens (except blood culture bottles) were kept at 4°C in the refrigerator. Clinical samples were processed using the standard laboratory techniques. Microscopic examination of Gram-stained films of the different samples was carried out to find any bacterial cells. Followed by culture on Nutrient agar, Blood agar, MacConkey agar and CLED agar (for urine samples) plates (Oxoid, UK) using the streak plate technique. Plates were kept in incubator at 37°C for 24 h.

Bacterial identification and susceptibility testing

Identification of the bacterial isolates was performed according to standard procedures, in reference to Mahon et al. (2007/2008). GNB were identified according to colonial appearance, microscopic evaluation and biochemical reactions including Oxidase test using Oxidase strips (Oxoid, UK), Kligler iron agar (KIA) test, Lysine iron agar (LIA) test, Motility, Ornithine production, Indole (MIO) test and Citrate utilization test (Oxoid, UK).

Antimicrobial sensitivity testing was performed by the disc diffusion test using Mueller Hinton (MH) agar (BBL, Becton Dickinson, Cockeysville, MA, USA) and CLSI 2017 M100-S27 breakpoint values were used (CLSI, 2017). Sensitivity testing was performed for the following 10 antibiotics agents: Amoxycillin clavulanate (AMC: 30 µg); Cefuroxime (CXM: 30 µg); Piperacillin-tazobactam (TPZ: 110 µg); Cefoxitin (FOX: 30 µg); Cefipime (FEP: 30 µg); Ceftriaxone (CRO: 30 µg); Ceftazidime (CAZ: 30 µg); Cefotaxime (CTX: 30 µg); Imipenem (IPM: 10 µg), and Meropenem (MEM: 10 µg). Bacterial isolates were diagnosed as CR if they were resistant (diameter ≤ 19 mm) to at least one of the used carbapenems (IPM and MEM).

Phenotypic detection of carbapenemase activity and ESBL production

The production of ESBL, carbapenemase and MBLs were tested using cephalosporin/clavulanic acid (BD Diagnostics, Franklin Lakes, NJ, USA) combination disc, The Modified Hodge test (MHT) and synergy combined disc test (CDT), respectively.

Phenotypic ESBL production was detected with the combination disc diffusion test with clavulanic acid. The inhibition zone surrounding the cephalosporin (Cefotaxime, Ceftazidime and Cefepime) discs combined with clavulanic acid is compared to the zone around the disc with the cephalosporin alone. The reaction was positive if the inhibition zone was 5 mm larger with clavulanic acid than without (Al Naiemi et al., 2012).

The Modified Hodge test

a 1:10 dilution of the *Escherichia coli* ATCC 25922 (NAMRU-3 Institute, Naval Medical Research Unit Three, Cairo, Egypt) was made by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of saline. This dilution was streaked on the surface of MH agar plates using a swab, and allowed to dry for 3 to 5 min. Thereafter, 10 µg imipenem disc was placed in the center of the test plate. The test organism was streaked in a straight line from the disk to the edge of the plate. Plates were Kept at 35°C for 24 h. Plates were examined for a clover leaf like indentation of the test isolate and the reference strain of *E. coli* within the zone of inhibition of the imipenem disc (Tamma and Simner, 2018).

Detection of MBL (class B)

CDT was performed according to Joji et al. (2019). Two 10 µg imipenem discs and two 30 µg ceftazidime discs (Becton Dickinson) were put on a plate inoculated with the test bacteria. 10 µL of sterilized 0.5 M EDTA solution (dissolve 186.1 g disodium EDTA in 1000 ml distilled water at pH 8.0) was supplemented to one disc of each antibiotic. After that, inhibition zones of the imipenem and imipenem + EDTA and ceftazidime and ceftazidime + EDTA discs were compared after 18 to 24 h of incubation at 35°C. A zone diameter difference between the imipenem and imipenem + EDTA discs or the ceftazidime and ceftazidime + EDTA discs ≥7 mm was

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Table 1. Oligonucleotide primers.

Gene	Primer sequence (5´→3´)	Amplicon size (bp)
bla $_{KPC}$	Forward: CGTCTAGTTCTGCTGTCTTG Reverse: CTTGTCATCCTTGTTAGGCG	798
bla $_{VIM}$	Forward: GATGGTGTTTGGTCGCATA Reverse: CGAATGCGCAGCACCAG	390
Bla $_{IMP}$	Forward: GGAATAGAGTGGCTTAAYTCT Reverse: CGGTTTAAYAAAACAACCACC	232
bla $_{OXA-48}$	Forward: GCGTGGTTAAGGATGAACAC Reverse: CATCAAGTTCAACCCAACCG	438
bla $_{NDM-1}$	Forward: GGTTTGGCGATCTGGTTTTC Reverse: CGGAATGGCTCATCACGATC	621

defined as a positive result for MBL production.

Molecular characterization of carbapenemase encoding genes

DNA template

Genomic DNA was extracted using Gene JET DNA Purification Kit (Thermo Scientific, For 50 preps, Lot. 00189391, The European Union (EU) Lithuania) following the manufacturer instructions.

Multiplex PCR method

The primers used in this study were based on primers published by Poirel et al. (2011) and are listed in Table 1. The amplification of DNA and thermal cycling conditions were done as described by Karuniawati et al. (2013). One multiplex PCR reaction was done detecting *bla*$_{IMP}$ and *bla*$_{VIM-2}$ genes. Second multiplex PCR reaction was done detecting *bla* $_{KPC}$ and *bla* $_{OXA-48}$ genes. Third reaction was done for detecting *bla*$_{NDM-1}$ gene. DNA amplification was performed through a 50 µl reaction mix having 10 mM Tris-HCL, 50 mM KCL, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 mM of each of the deoxynucleoside triphosphate, 1U of DNA polymerase, 5 µl of template DNA, and 0.4 mM of each primer.

The thermal cycler program was adjusted by using PTC-100TM Programmal Thermal Controller, Peltier-Effect Cycling, MJ. The amplification conditions include: initial denaturation at 94°C for 5 min; 30 cycles of a final elongation at 72°C for 5 min. For multiplex PCR of the carbapenemase genes, the annealing temperature was 55°C for amplification of *bla* $_{VIM}$, *bla* $_{IMP}$ and *bla* $_{KPC}$ genes, 45°C for *bla* $_{NDM}$ gene and 57°C for amplification of *bla* $_{OXA-48}$ genes (Karuniawati et al. 2013). The amplicons were analyzed by electrophoresis in a 1.5% agarose gel.

Statistical analysis

Data were statistically analyzed using Microsoft Excel 2010 and Statistical Package of Social Science (SPSS) software version 22 (SPSS Inc., Chicago, IL, USA) and categorical variables were presented as counts and percentages.

RESULTS

Distribution of the isolated CR Gram-negative species

During the study period, 58 CR Gram-negative species were identified from 158 GNB. The most common species were *Klebsiella pneumoniae (*37.9%), followed by *E. coli* (24.1%). Additionally, *Pseudomonas aeruginosa* accounted for 8.6%, *Proteus mirabilis* (6.9%), *Serratia marcescens* and *Enterobacter cloacae* (5.2% each), *Enterobacter aerogenes* and *Citrobacter freundii* (3.4% each), *Proteus vulgaris*, *Klebsiella oxytoca* and *Acinetobacter baumannii* (1.7% each). Most of the 58 strains were isolated from blood (63.8%), and from tracheal aspirates (20.7%). The remaining strains were isolated from umbilical venous catheter (5.2%), long line (3.4%), urine (3.4%), and others (3.4%) (Table 2).

Antibiotic resistance pattern among CR Gram-negative isolates

The overall resistance to carbapenems was high (75.9% for imipenem and 57% for meropenem). High rate of CR was detected among *K. pneumoniae* (86.4% for imipenem and 63.6% for meropenem) and *E. coli* strains (78.6% for imipenem and 71.4% for meropenem). The best susceptibility for these highly resistant strains was detected with pipracillin/tazobactem combination (*K. pneumoniae* resistance was 18.2% and *E. coli* resistance was 14.3%). Amoxicillin/Clavulinate had some activity against few strains (only one *E. coli*, one *Enterobacter cloacae* and one *Enterobacter aerogenes* strains) (Table 3).

Table 2. Occurrences of CR Gram-negative species according to clinical samples.

Species	Sample						Total [No. (%)]
	Blood	Tracheal aspirate	UVC	Long line	Urine	Others	
Klebsiella pneumoniae	16	3	1	1	1	0	22 (37.9)
Escherichia coli	9	3	0	0	1	1	14 (24.1)
Pseudomonas aeruginosa	4	1	0	0	0	0	5 (8.6)
Proteus mirabilis	2	1	1	0	0	0	4 (6.9)
Serratia marcescens	2	1	0	0	0	0	3 (5.2)
Enterobacter cloacae	2	1	0	0	0	0	3 (5.2)
Enterobacter aerogenes	1	0	1	0	0	0	2 (3.4)
Citrobacter freundii	0	1	0	0	0	1	2 (3.4)
Proteus vulgaris	0	1	0	0	0	0	1 (1.7)
Klebsiella oxytoca	1	0	0	0	0	0	1 (1.7)
Acinetobacter baumannii	0	0	0	1	0	0	1 (1.7)
Total No. (%)	37 (63.8)	12 (20.7)	3 (5.2)	2 (3.4)	2 (3.4)	2 (3.4)	58 (100)

UVC: Umbilical venous catheter.

Table 3. Antibiotic resistance rates of isolated CR Gram-negative rods.

Species	% Antibiotic resistance rate									
	AMC	CXM	TZP	FOX	FEP	CRO	CAZ	CTX	IPM	MEM
Klebsiella pneumoniae (n=22)	1 00	90.9	18.2	95.4	77.3	90.9	100	100	86.4	63.6
Escherichia coli (n=14)	92.8	92.8	14.3	85.7	71.4	92.8	92.8	92.8	78.6	71.4
Pseudomonas aeruginosa (n=5)	100	60	20	60	40	80	60	80	60	40
Proteus mirabilis (n=4)	100	75	25	50	50	75	75	75	75	50
Serratia marcescens (n=3)	100	100	0	66.7	33.3	100	66.7	100	66.7	33.3
Enterobacter cloacae (n=3)	66.7	66.7	0	66.7	33.3	66.7	66.7	66.7	66.7	33.3
Enterobacter aerogenes (n=2)	50	100	50	50	50	50	50	100	50	50
Citrobacter freundii (n=2)	100	100	50	100	50	50	100	100	50	50
Proteus vulgaris (n=1)	100	100	100	100	0	100	100	100	0	100
Klebsiella oxytoca (n=1)	100	100	0	100	0	100	100	100	100	0
Acinetobacter baumannii (n=1)	100	100	0	100	0	100	100	100	100	0
Total (n=58)	94.8	87.9	18.9	82.7	60.3	86.2	87.9	93.1	75.9	57.0

AMC: Amoxicillin/clavulanic acid, CXM: cefuroxime, TZP: piperacillin/tazobactam, FOX: cefoxitin, FEP: cefepime, CRO: ceftriaxone, CAZ: ceftazidime, CTX: cefotaxime, IPM: imipenem, and MEM: meropenem.

Phenotypic characterization of carbapenem resistance

ESBL production was confirmed in all 58 (100%) isolates by the cephalosporin/clavulanic acid combination disc. Detection of carbapenemase production by MHT (Figure 1) was found in 52 (89.6%) isolates while MBL production was detected in 33 (56.9%) isolates by synergy CDT (Figure 2 and Table 4).

Genotypic characterization of carbapenem resistance

The molecular characterization of the 58 isolates showed that 57 (98.3%) were positive for carbapenemase encoding genes. *bla* $_{KPC}$ was the most prevalent gene detected in 34 isolates (58.6%), next to it was the *bla* $_{VIM}$ gene which was present in 15 (25.8%), *bla* $_{IMP}$ in 13 (22.4%), *bla* $_{OXA-48}$ in 9 (15.5%) and *bla* $_{NDM-1}$ in one isolate (1.7%). Five isolates were positive for both *bla* $_{OXA}$ *and bla* $_{KPC}$ and 4 isolates were tested positive for both *bla* $_{IMP}$ and *bla VIM*. One strain was negative for all the tested genes despite positive ESBL phenotype (Table 4).

DISCUSSION

The emergence and global spread of acquired CR isolates are designated a “global sentinel event” (Woodford et al., 2004). The overall rate of CR Gram

Figure 1. Modified Hodge test.

negative strains in our NICU during the study period was high (36.7%). This finding agrees with another Egyptian study conducted by Makharita et al. (2020) who detected 36% isolated Enterobacteriacea were carbapenemase producers. Much lower CR isolation rates have been reported in Turkey 2.82% (Baran and Aksu, 2016) and the United States 1.4 to 4.2% (Pollett et al., 2014). Moreover, one Chinese study reported 0.9% CR isolates (28/3286) (Liao et al., 2014). The reason for higher rate of CR detected in our study could be related to the extensive use of carbapenems to treat life threatening infections in neonatal patients and absence of antibiotic stewardship program. In this study all CR strains were retrieved from the NICU in which many CR infection and colonization factors, including impaired immune status, prolonged hospital stay, and frequent use of antibiotics, were present. For this reason, proper hand hygiene and infection control measures must be emphasized. Monitoring of CR must be encouraged to reduce CR infection rate.

K. pneumoniae (37.9%) was the most common CR species isolated followed by *E. coli* (24.1%). This is in

Figure 2. Combined disc synergy test.

agreement with other CR studies in Egypt, United States, Europe and China (Metwally and Elnagar, 2019; Centers for Disease Control and Prevention, 2013; Akova et al., 2012; Yang et al., 2018) and is consistent with the CDC's recommendations that *K. pneumoniae*, *E. coli*, and *Enterobacter* spp. are the key health care-associated pathogens to focus on in the control of US CR epidemic (Centers for Disease Control and Prevention, 2014). That is why attention should be raised when any of these bacteria is isolated from clinical specimens.

In the present study, the overall resistance to carbapenems was high (75.9% for imipenem and 57% for meropenem). Higher rate of resistance was detected by Pollett et al. (2014); Mohamed et al. (2018), who found low rate of susceptibility to meropenem (4.3%, 3%) and to imipenem (1.7%, 3%). On the other hand, lower rate of resistance was detected by Okoche et al. (2015) who found that only 18.4% of study isolates were resistant to meropenem. The varied range in susceptibility/resistance rate of carbapenems among GNB in different studies may be due to different antibiotic usage patterns in different geographic regions.

It is difficult to compare the present results with those of others taking into consideration variations in study

Table 4. Phenotypic and genotypic characterization of CR among 58 Gram-negative clinical isolates.

Carbapenem resistance characterization	Number of isolates (N=58)
Phenotypic characterization	
Cephalosporin/Clavulanic combination disc test:	
ESBL positive	58
ESBL negative	0
ESBL (%)	100
Modified Hodge test:	
MHT-positive	52
MHT-negative	6
Carbapenemase (%)	89.60
Synergy combined disc test:	
CDT-positive	33
CDT-negative	25
MBLs (%)	56.90
Genotypic characterization	
bla $_{KPC}$	34
bla $_{VIM}$	15
bla $_{IMP}$	13
bla $_{OXA-48}$	9
bla $_{NDM-1}$	1
bla $_{OXA-48}$+ *bla* $_{KPC}$	5
bla $_{IMP}$ + *bla* $_{VIM}$	4
PCR-negative	1
Carbapenemase encoding genes (%)	98.27

ESBL: Extended spectrum beta-lactamase, MHT: modified Hodge test, MBL: metallo beta-lactamases, CDT: combined disc test.

populations either pediatrics or adults, different carbapenem breakpoints, and different definitions of CR (Vading et al., 2011).

In the present study, all CR strains were ESBL producers and carbapenemase production was detected in 52 isolates (89.6%), while MBL production was detected in 33 (56.9%) isolates. Similarly, Fattouh et al. (2015) identified 88.14% of CR isolates as carbapenemase producers but lower MBLs activity was detected in 33.9% of the isolates by CDT. ESBL production was confirmed by Netikul and Kiratisin (2015) in 76.2% of the CR isolates and only 14.4% were positive for MHT. In accordance, Makharita et al. (2020) detected 33.2% MBLs positive isolates while only 33.6% of the CR isolates were positive MHT. On the contrary to the present results but also in Mansoura, carbapenamase activity was detected in 61.9% of CR *K. pneumoniae* isolated from ICUs of Mansoura University hospitals by MHT method (Moemen and Masallat, 2017).

Molecular characterization of the isolates revealed that 57 (98.3%) were positive for carbapenemase encoding genes. In accordance with the present results, Makharita et al. (2020) found 98.6% (74/75) of CR strains were carrying *KPC* gene, moreover 97.3% (73/75) were carrying *GES* gene. Also, similar to the present results, Yang et al. (2018) found that, among all CR strains *bla* $_{KPC}$ carrying rate was 60.3% and *bla* $_{IMP}$ were detected in 4.5% of total CR strains. Not so far from the present results. Pollett et al. (2014) stated that, a carbapenemase-encoding gene was found in 81.7% (94/115) of CR with *bla* $_{KPC}$ the most prevalent (78.3%).

These results were different with Elbadawi et al. (2021) who detected that the most prevalent gene was NDM-1 gene. On the other hand, Baran and Aksu (2016). reported that the *OXA-48* gene was the most frequent gene as it was detected in 86/181 (47.5%) strains, *NDM-1* gene in 6 (3.3%) strains, and *VIM* gene in 1 (0.6%) strain. *IMP* and *KPC* genes were not identified. Both *OXA*-48 and *NDM*-1 were produced by 3 strains and both *OXA-48* and *VIM* were produced by one strain.

It is cleared that carbapenemase genes tend to be frequent in certain countries. It was found that *bla* $_{KPC}$

genes are dominant in USA, Greece and Egypt, while *bla* $_{NDM}$ genes are commonly detected in isolates recovered from India, Pakistan and Far East (Elbadawi et al., 2021).

A study in our locality conducted by Moemen and Masallat (2017) detected that 92.9% of the CR isolates were positive for one or more carbapenemase genes. Five of the 39 carbapenemase gene carrying isolates harbored two or more genes. The most prevalent gene was *bla* $_{KPC}$ 47.8% followed by *bla* $_{VIM1}$ 21.7%.

One strain that was negative for all the tested genes was detected despite positive ESBL phenotype. Similarly, Moemen and Masallat (2017) found that, none of the tested carbapenemase genes tested (bla $_{KPC}$, bla $_{NDM}$, bla $_{VIM-1}$, bla $_{IMP}$, and bla $_{OXA-48}$-like) were detected in three isolates. Carbapenem resistance of these isolates is mostly due to a combination of ESBLs and changes in outer membrane proteins (ESBL/Omp) (Endimiani et al., 2010).

This study had several limitations. First, the study did not check tested isolates for sensitivity to last-line antimicrobials, such as colistin, tigecycline, and fosfomycin. Secondly, only isolates that showed imipenem or meropenem in the resistant were tested. Unlikely, carbapenemases have been found in carbapenem-sensitive Enterobacteriaceae, specially *bla* $_{OXA-48}$ (Nordmann et al., 2012). Finally, clinically significant isolates were mainly examined and this undervalues the colonized patients, which may forcefully propagate CR (Viau et al., 2012). For this reason, given that CR are increasing in this region, augmentation of local infection control measures beyond core elements to active surveillance may be useful, as stressed by the CDC (Centers for Disease Control and Prevention, 2014).

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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