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Emergence of Gram-Negative Bacilli with Concomitant *bla*$_{NDM-1}$- and *bla*$_{OXA-48}$-Like Genes in Egypt

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Abstract: Multidrug-resistant Gram-negative organisms have emerged as a major threat to hospitalized patients, and are associated with serious morbidity and mortality. This study aimed to characterize carbapenem resistance genes among Gram-negative bacilli isolated from clinical samples from patients in the intensive care unit of Cairo University Hospital. A total of 211 samples were collected from patients showing clinical evidence of infection. Bacteria were isolated and identified by conventional microbiological methods. *Acinetobacter baumannii* isolates were furtherly characterized by polymerase chain reaction (PCR), using primers specific for *bla*$_{OXA-51}$-like genes. The Kirby Bauer disc diffusion method was used to determine susceptibility patterns of isolates, and carbapenem resistance was further examined by a modified Hodge test. Positive isolates were tested for the presence of *bla*$_{KPC}$, *bla*$_{OXA-48}$, and *bla*$_{NDM}$-like genes by PCR. NDM gene types were determined by direct sequencing. From the 211 samples, 229 Gram-negative bacilli were isolated. Fifty isolates (21.2%) were resistant to carbapenem. PCR analysis showed that none of the 50 isolates carried *bla*$_{KPC}$-like genes, while 24 (48%) isolates carried *bla*$_{OXA-48}$-like genes, 8 (16%) carried *bla*$_{NDM-1}$, and five isolates (10%) carried both *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$-like genes. These results indicate that continuous surveillance of these multidrug-resistant pathogens is urgently required. And that is very important is to activate the antimicrobial stewardship programs of which the most important is restriction of the big gun antibiotics like carbapenems, colistin, tigecyclin and vancomycin and restricting their prescription to privileged specialties.

Keywords: Carbapenem Resistance, Gram-negative Bacilli, *bla*$_{OXA-48}$, *bla*$_{NDM-1}$

1. Introduction

"Infections caused by Gram-negative bacilli (GNB) are of particular concern, especially in intensive care units (ICUs). These organisms are highly efficient at acquiring or up-regulating genes that code for mechanisms of antibiotic resistance, especially in the presence of antibiotic selection pressure. Furthermore, they contain a variety of resistance pathways, and often contain multiple mechanisms targeting the same antibiotic [1]". "Gram-negative bacteria resistant to three or more antimicrobial classes, known as Multidrug-resistant (MDR) Gram-negative organisms, have emerged as a major threat to hospitalized patients and have been associated with mortality rates ranging from 30–70% [2], [3]". "A range of Gram-negative organisms, including both non-fermenters (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) and fermenters (Enterobacteriaecae), are responsible for hospital-acquired infections. MDR

organisms, including *A. baumannii*, *P. aeruginosa*, and extended-spectrum β-lactamase-producing or carbapenemase-producing Enterobacteriaceae, are increasingly being reported worldwide [1]".

"Among the Enterobacteriaceae, the most clinically significant carbapenemases belong to the Ambler molecular class A (*Klebsiella pneumoniae* carbapenemase, KPC), class B (Verona-Integron Mediated, VIM; instance plasmid-mediated IMP; and New Delhi metallo-β-lactamases, NDM), and class D expanded-spectrum oxacillinase (OXA-23 and OXA-48) types [4]"."Recently, NDM-type enzymes have been identified among Enterobacteriaceae in Egypt. Like KPCs, these enzymes are frequently found on mobile genetic elements and have the potential to become widespread [5]".

"Patients in the ICU usually have serious co-morbid conditions or are compromised by invasive procedures, such as mechanical ventilation, surgery, and frequent use of vascular or urinary catheters. Infections in these patients are usually life threatening and may be caused by MDR organisms [6]". "Carbapenem antibiotics have traditionally been considered the last line of defense in treating infections caused by these bacteria but nowadays we have colistin (polymexin E) is last line of defense against MDR gram negative bacteria. [7]" "Recently Qureshi *et al.* reported Colistin-resistant *A. baumannii* almost exclusively among patients who had received colistin methansulfonate for treatment of carbapenem-resistant, colistin-susceptible *A. baumannii* infection which is very alarming data for the last backbone of treatment of MDR gram negative bacteria [8]". "Thus, the alarming spread of carbapenemases poses serious risk to these vulnerable patients, as only a few suboptimal therapeutic options remain available for treating such infections [9]". "Infections caused by carbapenem-resistant organisms have been associated with high mortality rates [5]"."Early detection and strict application of infection control measures have been reliable in reducing the likelihood of transmission in health care settings [10]". Therefore, the objective of this study was to characterize carbapenem resistance genes among GNB isolated from clinical samples from patients in one of the ICUs at Cairo University Hospital.

2. Materials and Methods

2.1. Study Design and Sample Collection

A prospective cross-sectional study was conducted over a period of 6 months (June to December 2013). Clinical samples were obtained from patients showing clinical evidence of infection at one surgical ICU of Cairo University Hospital. The research protocol was approved by the Research Ethics Committee of the Faculty of Medicine, Cairo University, Egypt.

2.2. Bacterial Isolates

A total of 211 samples were collected; 47 blood, 46 urine, 52 respiratory (sputum and bronchoalveolar lavage), 59 wound swabs, and seven fluid aspirates (pericardial, pleural, and ascitic). "Bacteria were isolated and identified by conventional microbiological methods [11]". "All suspected *A. baumannii* isolates were confirmed by PCR specifically designed to detect bla_{OXA-51}-like genes, as described by Karmostaji *et al.* [12]".

2.3. Antimicrobial Susceptibility Testing

"The antimicrobial susceptibility profiles of the isolates were determined using a modified Kirby Bauer disc diffusion method on Muller Hinton agar (Oxoid Ltd., Basingstoke, UK) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [13]". The following antibiotic discs were used (Oxoid Ltd.): ampicillin (10 μg), one of the following β-lactam/β-lactamase inhibitors (amoxicillin/clavulanic acid, 30 μg; piperacillin/tazobactam, 110 μg), one of the first generation cephalosporins (cephalothin, 30 μg; cephalexin, 30 μg; cefazolin, 30 μg), one of the second generation cephalosporins (cefaclor, 30 μg; cefoxitin, 30 μg; cefixime, 5 μg), one of the third generation cephalosporins (cefoperazone, 75 μg; ceftazidime, 30 μg; cefotaxime, 30 μg; ceftriaxone, 30 μg), cefepime (30 μg), carbapenems (imipenem, 10 μg; meropenem, 10 μg), aminoglycosides (amikacin, 30 μg; gentamicin, 120 μg), one of the fluoroquinolones (ciprofloxacin, 5 μg; levofloxacin, 5 μg; ofloxacin, 5 μg), sulfamethoxazole-trimethoprim (25 μg), tigecycline (15 μg), polymyxin B (300 units), and colistin (10 μg).

2.4. Screening of Carbapenem-Resistant Isolates

"Isolates were screened for carbapenem resistance using an imipenem and meropenem disk diffusion method. Isolates were categorized as sensitive, intermediate, or resistant according to CLSI guidelines (CLSI, 2012) [13]". Isolates were tested for carbapenemase production by modified Hodge test (MHT) as described in the CLSI guidelines. MHT-positive *K. pneumoniae* ATCC1705 and MHT-negative *K. pneumoniae* ATCC1706 were used as control strains for the assay.

2.5. Genetic Characterization of Carbapenem-Resistant Isolates

All clinical isolates found to be resistant to imipenem or meropenem and/or positive by modified Hodge test were examined for the presence of bla_{KPC}, bla_{OXA-48}, and bla_{NDM}-like genes by PCR. The PCR conditions and methodology were as described previously (14-16) (Table 1).

2.6. DNA Sequencing and Analysis

Any identified bla_{NDM} genes were sequenced to determine the NDM variant of the enzyme using the primers listed in Table 1. PCR products were purified using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified PCR products were directly sequenced on both strands using a 310 Capillary Array Sequencer and Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA, USA).

NDM sequences were analyzed using the BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA, USA), and results were compared to a reference sequence (GenBank accession no. LC032101).

2.7. Statistical Analysis

All statistical analyses were performed using SPSS version 15 for Microsoft Windows (SPSS Inc., Chicago, IL, USA). Data were statistically described in terms of frequencies (number of cases) and percentages.

3. Results

A total of 229 GNB were isolated from the 211 samples, including 75 P. aeruginosa, 62 K. pneumoniae, 43 A. baumannii, 28 Escherichia coli, 13 Proteus mirabilis, three Proteus vulgaris, three Enterobacter spp., one Morganella morganii, and one Citrobacter spp. Of these, 50 isolates (21.8%) showed resistance to both imipenem and meropenem. Among the resistant isolates, only 29 isolates were positive by modified Hodge test. These carbapenem-resistant isolates included 23 A. baumannii, 13 K. pneumoniae, 13 P. aeruginosa, and 1 E. coli (Table 2). In total, 53.5% of the A. baumannii isolates showed resistance to carbapenems, versus 21% and 17.3% of K. pneumoniae and P. aeruginosa isolates, respectively.

When studying carbapenem-resistant isolates in relation to the site of infection, we found that respiratory infections yielded the highest number of carbapenem-resistant GNB (24, 48%), followed by wound infections (13, 26%) (Table 3). All 50 carbapenem-resistant isolates were MDR, with 100% resistance to ampicillin, β lactam/β-lactamase inhibitors, first, second, third, and fourth generation cephalosporins, and carbapenems. In addition, 49 (98%) isolates were resistant to trimethoprim-sulfamethoxazole, 41 (82%) were resistant to aminoglycosides, and 39 (78%) were resistant to fluoroquinolones. However, all isolates were sensitive to tigacycline, polymyxin B, and colistin. No specific patterns were detected in relation to the absence or presence of resistance genes.

PCR analysis revealed that none of the isolates carried bla_{KPC}-like genes, while 24 of the 50 resistant isolates (48%) carried bla_{OXA-48}-like genes, eight (16%) isolates carried bla_{NDM}, and five (10%) carried both bla_{NDM} and bla_{OXA-48}-like genes (Table 4). Sequencing of the bla_{NDM} genes revealed that all isolates contained the bla_{NDM-1} variant.

Table 1. *Primers used for the detection of carbapenem resistance genes.*

Gene	Primer pair and sequence (5′–3′)	Amplicon size
bla_{OXA-51}-like	OXA-51 Forward (5'-TAATGCTTTGATCGGCCTTG-3′), OXA-51 Reverse (5'-TGGATTGCACTTCATCTTG-3′)-	353 bp
bla_{OXA-48}-like	OXA-48A (5'-TTGGTGGCATCGATTATCGG-3'), OXA-48B (5'-GAGCACTTCTTTTGTGATGGC-3')	743 bp
bla_{KPC}-like	KPC Forward (5'-ATGTCACTGTATCGCCGTCT-3′), KPC Reverse (5'-TTTTCAGAGCCTTACTGCCC-3′)	892 bp
bla_{NDM}-like	NDM Forward (5′-GGTTTGGCGATCTGGTTTTC-3′), NDM Reverse (5′-CGGAATGGCTCATCACGATC-3′)	621 bp

Table 2. *Number and percentage of carbapenem-resistant isolates among each species.*

Isolated Gram-negative bacilli (no.)	Number of isolates	Number of carbapenem-resistant isolates	Percentage of carbapenem-resistant isolates
Pseudomonas aeruginosa	75	13	17.3%
Klebsiella pneumoniae	62	13	21%
Acinetobacter baumannii	43	23	53.5%
Escherichia coli	28	1	3.57%
Proteus mirabilis	13	0	0%
Proteus vulgaris	3	0	0%
Enterobacter spp	3	0	0%
Morganella morganii	1	0	0%
Citrobacter spp	1	0	0%
Total	229	50	21.8%

Table 3. *Distribution of carbapenem-resistant isolates according to site of infection.*

	Blood No. (%)	Urine No. (%)	Respiratory samples No. (%)	Wound swabs No. (%)	Total No. (%)
Acinetobacter baumannii	3 (6%)	1 (2%)	13 (26%)	6 (12%)	23 (46%)
Klebsiella pneumoniae	2 (4%)	2 (4%)	5 (10%)	4 (8%)	13 (26%)
Pseudomonas aeruginosa	2 (4%)	3 (6%)	6 (12%)	2 (4%)	13 (26%)
Escherichia coli	0 (0%)	0 (0%)	0 (0%)	1 (2%)	1 (2%)
Total	7 (14%)	6 (12%)	24 (48%)	13 (26%)	50 (100%)

Table 4. *Distribution of carbapenem resistance genes among tested isolates (no.=50).*

	bla_{KPC} No. (%)	bla_{OXA-48} No. (%)	bla_{NDM-1} No. (%)	bla_{OXA-48} + bla_{NDM-1} No. (%)
Acinetobacter baumannii	0 (0%)	13 (26%)	3 (6%)	1 (2%)
Klebsiella pneumoniae	0 (0%)	8 (16%)	4 (8%)	4 (8%)
Pseudomonas aeruginosa	0 (0%)	2 (4%)	1 (2%)	0 (0%)
Escherichia coli	0 (0%)	1 (2%)	0 (0%)	0 (0%)
Total	0 (0%)	24 (48%)	8 (16%)	5 (10%)

4. Discussion

"In recent decades, the emergence of extended-spectrum β-lactamase-producing bacteria has led to increased use of carbapenems in clinical practice, which in turn has led to the emergence of isolates containing carbapenemases [4]". We screened GNB responsible for different clinical infections in patients admitted to one of the ICUs at Cairo University Hospital, and investigated carbapenemase production by these isolates. We identified 50 isolates with carbapenem resistance patterns among 229 isolated GNB (21.8%). Although *P. aeruginosa* and *K. pneumoniae* were the most commonly isolated species (75 and 62 respectively), *A. baumannii* isolates showed the highest incidence of carbapenem resistance (46%)." In agreement with these results, Fouad *et al.* identified 547 nosocomial infections in three ICUs from three different hospitals in Egypt between January 2011 and September 2012 and found that the majority of infections were caused by *Klebsiella* spp., whereas *A. baumannii* showed the highest levels of imipenem resistance (74%) [17]". "Our study also revealed that respiratory infections yielded the highest number of carbapenem-resistant organisms (48%). These results are in partial agreement with Maltezou *et al.*, who investigated infections caused by carbapenem-resistant GNB in hospitalized children. They isolated 71 carbapenem-resistant pathogens causing infections in 65 children, of which 25 cases were diagnosed with pneumonia (35.2%) [18]".

Although KPC-producers are now being identified at an alarming rate across the USA, France, Israel, Greece, Colombia, and China, and outbreaks of KPC-producing bacteria have been reported in many European countries, South America, and India (19-21), we did not detect any bla_{KPC}-like genes among the 50 isolates that were positive by phenotypic methods for carbapenemases production. "This is in agreement with findings reported by Shibl *et al.* [4]", who tested 60 *K. pneumoniae* isolates from Saudi Arabia (the majority of which were from patients in an ICU) and did not identify any bla_{KPC}-like genes. Together, these results indicate that bla_{KPC}-like genes are not the major source of carbapenemases in the Middle East.

"The carbapenem-hydrolyzing class D β-lactamase OXA-48 was first described in a *K. pneumoniae* strain isolated from Turkey in 2004, and is now endemic in Egypt (4). OXA-48 carbapenemases are also endemic in countries around the Mediterranean, and are rapidly spreading into other countries in Europe [22], [23]". "Although many reports have described patients becoming infected with strains carrying bla_{OXA-48}-like genes during travel to Egypt [24], [25]", few surveillance studies have focused on this gene as a cause of carbapenem resistance in Egypt. "In a recent 6-month surveillance study of carbapenem-resistant GNB isolated from a cancer hospital in Egypt, only three isolates harbored this gene [26]". Our study identified a higher number of isolates (24 isolates) (Table 3) than previously reported, which may indicate the rapid spread of carbapenem resistance in Egypt through the dissemination of bla_{OXA-48}-like genes. "Although bla_{OXA-48} is rarely detected in *Acinetobacter* spp., Goncalves *et al.* detected this gene in *A. baumannii* isolated from fecal flora of nursing home residents in northern Portugal [27]". "Importantly, bla_{OXA-48} is associated with transposons Tn1999 and Tn1999.2, which enable rapid transmission among GNB [28]".

bla_{NDM-1} was initially identified in *K. pneumoniae* and *E. coli* recovered from a Swedish patient who was previously hospitalized in India, and has rapidly disseminated to other Enterobacteriaceae in several countries [29]". "NDM-producers are of particular concern as they also harbor multiple plasmid and chromosome-encoded resistance genes, resulting in a MDR phenotype [4]". "Recently, cases of NDM-producing *A. baumannii* have been described in Egypt, China, and Israel [20], [29], [30]". "Similar to bla_{OXA-48}, the way in which bla_{NDM-1} has spread between GNB in Egypt is not yet clear, but some of the identified cases in Europe had a history of travelling to Egypt [30-32]". In this study, eight isolates were shown to harbor bla_{NDM-1} (Table 3), five of which also contained a bla_{OXA-48}-like gene (four *K. pneumoniae* and one *A. baumannii*). "Although this pattern of combined resistance has been reported previously in Lebanon and Tunisia [3], [23]", the rates identified in the current study are alarmingly high: five isolates versus one *K. pneumoniae* isolate each in Lebanon and Tunisia. However, there is little published data discussing the effect of the coexistence of several carbapenemases in GNB, an issue that requires close and continuous monitoring in infected patients.

"The emergence of such resistant strains represents a significant threat, not only to our country, but globally, especially as the dissemination of resistance genes is hastened by high rates of immigration and tourism [4]". This study has documented the emergence of NDM-1- and OXA-48-positive GNB in Egypt. NDM and OXA-48 type carbapenemases are increasingly reported in our region, with the Middle East and North Africa now regarded as secondary reservoirs for these carbapenemases. Several alarming reports have described the introduction of OXA-48- and NDM-expressing GNB to some European countries by patients previously hospitalized in Egypt. Healthcare workers, especially in ICUs, need to be aware of the emergence of these MDR isolates, as they are a significant health concern. Enhanced surveillance and detection of these MDR pathogens is urgently required so that patients can be identified quickly and appropriate infection control measures can be instituted to stop further dissemination. Further studies are also needed to clarify the epidemiological features of carbapenemase-producing isolates in Egypt. Also what is very important is to activate the antimicrobial stewardship programs of which the most important is restriction of the big gun antibiotics like carbapenems, colistin, tigecyclin and vancomycin and restricting their prescription to privileged specialties like infectious disease, intensevists, and pulmologists which can authorize other specialties in patients their clinical situation necessitates these big gun antibiotics.

5. Conclusion

This study indicates that continuous surveillance of these multidrug-resistant pathogens is urgently required. And that is very important is to activate the antimicrobial stewardship programs of which the most important is restriction of the big gun antibiotics like carbapenems, colistin, tigecyclin and vancomycin and restricting their prescription to privileged specialties.

List of Abbreviations

Polymerase chain reaction (PCR);
Gram-negative bacilli (GNB);
Intensive care units (ICUs);
Multidrug-resistant (MDR);
Klebsiella pneumoniae carbapenemase (KPC);
Verona-Integron Mediated (VIM);
Instance plasmid-mediated (IMP);
New Delhi metallo-β-lactamases (NDM);
Expanded-spectrum oxacillinase (OXA);
Clinical and Laboratory Standards Institute (CLSI);
Modified Hodge test (MHT).

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