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ORIGINAL ARTICLE

Detection of NDM-1-producing *Klebsiella pneumoniae* Isolated from Sohag University Hospital, Upper Egypt

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ABSTRACT

Background: *Resistance to carbapenems is a worldwide problem, NDM-1 carbapenemase is rapidly spreading in many parts of the world. Few studies in Egypt reported the enzyme.* ***Objectives:*** *to detect the NDM-1 in carbapenems resistant K. pneumoniae isolated from patients hospitalized in Sohag University hospital.* ***Methodology:*** *The study was conducted during the period between June 2017 to December 2017. K. pneumoniae was isolated from the clinical samples collected from patients with HAI in Intensive Care Units (ICUs), different wards. These samples included blood, urine, sputum/endotracheal aspirates, and pus. Samples were inoculated on primary culture media. The identification and antibiotic susceptibility testing of all suspected K. pneumoniae isolates were performed by the Vitek2 compact system. Combined disk test using EDTA was used for phenotypic testing of Metallo-β-lactamase production. PCR used to detect specific gene for bla NDM-1.* ***Results:*** *200 samples were collected. Fifty-four (54) K. pneumoniae were isolated and (28)55% were resistant to imipenem and meropenem by Vitek2 compact system. Ten (35.7%) of them were positive for production of Metallo-β-lactamase by combined disk test with EDTA and eight (28.5%) of them were positive blaNDM-1 gene by PCR.* ***Conclusion****: This study is the first report of detection of blaNDM-1in Sohag university hospital, Upper Egypt. Appling of infection control measures is a must to prevent the spread of blaNDM-1. Also, strict antibiotic policy is mandatory to decrease antimicrobial resistance in our hospital.*

INTRODUCTION

Carbapenems were considered the last line drugs against multiple drug resistant (MDR) bacteria. Resistance to carbapenems is mainly due to the production of hydrolyzing enzymes, carbapenemases.

New Delhi metallo- beta-lactamase-1(NDM-1) is one of the most recent and important carbapenemases. It belongs to class B enzymes of the Ambler classification which require the presence of zinc for their activity (so referred to as Metallo-β-lactamases, MBL)[1]. It was firstly isolated from a Swedish patient who acquired the organism in New Delhi, India[2].

NDM-1 has intrinsic ability to destroy most known beta-lactam antibiotics; penicillins, cephalosporins, and carbapenems with the exception of aztreonam[3]. NDM-1 is carried by a mobile genetic element that was easily transferred to other enterobacteriaceae and gram-negative bacteria. This genetic element often contained other drug resistance genes, including a gene encoding another broad-spectrum beta-lactamase and genes inactivating erythromycin, ciprofloxacin, rifampicin, and chloramphenicol[4].

It was firstly reported in Egypt from *K. pneumoniae* isolate in Cairo[5]. It was also detected in *Pseudomonas aeruginosa* isolates[6], *Acinetobacter baumannii* isolates[7], indicating the spread of this gene among gram negative organisms. In the last years spread of NDM-1 to the middle east[8] and the Mediterranean region has been reported.[9]

The goal of the study was to detect the presence of bla NDM-1 in carbapenem resistance *Klebsiella pneumonia* isolated from Sohag University hospital in Upper Egypt.

METHODOLOGY

Bacterial isolates

This study was carried out at the Medical Microbiology and Immunology Department, Faculty of Medicine, Sohag University during the period between June 2017 to December 2017. Samples were collected from the admitted patients with hospital acquired infections in Intensive Care Units (ICUs), different wards as urology and surgery, chest departments at Sohag University Hospital. Hospital- acquired infections were defined according to CDC/NHSN criteria for hospital- acquired infections[10]. In cases of HAP/VAP non- invasive samples as spontaneous expectoration, induced Sputum, endotracheal aspirate

was done in agreement of IDSA[11]. Respiratory samples and urine were collected aseptically in a sterile container. Sterile swabs used for wounds and sterile blood collecting tube for blood. Samples were labeled and transported immediately to the laboratory, blood samples were collected on blood culture bottles and subcultured on agar plates. Other samples were cultured directly on blood, MacConkey and EMB agar plates (Oxoid, UK) for 24h at 37^0C, mucoid lactose fermenter colonies were stained by gram stain for microscopic examination. Gram negative bacilli were subjected to oxidase testing, subculture on TSI (Oxoid, UK). Suspected *klebsiellae spp*. identification and their antimicrobial resistance pattern were done by the automated Vitek compact 2 systems using ID and AST cards (BioMérieux, France). The study was approved by the Research Ethics Committee of Sohag Faculty of Medicine.

Determination of MBLs producing isolates

K. pneumoniae resistant to one or more of carbapenems were subjected to phenotypic detection of Metallo β-lactamases (MBLs) production which was done by combined disk tests (CDTs). Klebsiella isolates (0.5 McFarland) were streaked on Muller Hinton agar plates. Two imipenem discs (10 µg) were placed apart (25mm) on the plate surface. Ten (10) µl of 0.1M (292 µg) anhydrous Ethylene diamine tetraacetic acid (EDTA) was added to one imipenem disc. The inhibition zones of imipenem and its EDTA-impregnated discs were compared after overnight incubation at 37°C. A zone size difference of >4 mm was indicative of MBLs production[12,13]. Identified colonies were preserved in 15% glycerol and tryptone soya broth at -70 °c for subsequent PCR testing.

Detection of bla NDM-1 gene by PCR

DNA extraction

Extraction was done by the boiling method; few colonies from overnight growth were diluted in 50 ul distilled water then heated to100^0 C for 10 minutes then centrifuged at 3000 rpm for 10 minutes. The clear supernatant is collected into a new tube and used.

DNA amplification:

In a sterile thermal cycler tube, 25µl PCR reaction mix containing 12.5µl PCR Master Mix (Bioscience, Germany), 8 µl PCR grade water, 1.25 µl of each primer (Invitrogen, USA) and 2 µl of the extracted DNA sample was added. The negative control was prepared by replacing the DNA template with PCR grade water. The primers used in this study were NDM-F (5`-GGTTTGGCGATCTGGTTTTC-3`), and NDM-R (5`-CGGAATGGCTCATCACGATC-3`), which amplified an internal fragment of 621 bp of the blaNDM-1[12]. The cycling conditions included the following: initial denaturation at 95°C for 5 minutes, followed by 36 cycles of 95°C for 1min (denaturation), 55°C for 30 seconds (annealing) and 72°C for 1 min (extension), then final elongation at 72°C for 10 minutes followed by a hold at 4°C. Biometra thermal cycler (Biometra, Germany) was used for amplification of DNA. The amplified DNA products &100bp ladder (molecular weight marker) was separated by agarose gel electrophoresis on 2% agarose gel stained with 0.5 µg/mL ethidium bromide using electrophoresis power supply (Biometra, Germany). The bands were visualized and photographed using Ingenius3 gel documentation system **(**Ingenius, Syngene, USA**)**.

RESULTS

A total of 200 samples were collected from ICU and different wards of Sohag University hospital. Fifty-Four *K. pneumoniae* were identified. The highest prevalence of *K. pneumoniae* was in respiratory samples14 (46.6%) followed by blood cultures10(33.3%). The prevalence of *K. pneumoniae* in different samples is shown in table 1.

The resistance rates among the 54 isolated *K. pneumoniae* shows ESBL production in more than 50% of the isolate, resistance to imipenem and meropenem was 28 (51.8%). Resistance to ampicillin-sulbactam was 49(90%), ceftriaxone was 49(90%), aztreonam was 38(70.3%) and to levofloxacin was 40 (74%). About 70% of *K. pneumoniae* isolates were multidrug-resistant (MDR) showing resistance to three or more drugs in different antimicrobial classes (table 2).

Carbapenem resistant *K. pneumoniae* isolates 28 (51.8%) show a higher rate of resistance compared to carbapenem sensitive *K. pneumoniae* isolates 27(48.2%). The Carbapenem resistant *K. pneumoniae* were resistant to 28(100%) ampicillin, cefazolin, ceftriaxone, cefepime, ceftazidime, aztreonam. Ciprofloxacin resistance was 26(92.8%). However, all of them were sensitive to tigecycline,68% were sensitive to amikacin and 57.2% were sensitive to gentamicin (table3).

Table 1: Number and percentage of K. pneumoniae in different samples

Samples type	Number of samples	Number(%) of Klebsiella
total	200	54(100%)
Pus swabs	80	15(18.7%)
Urine	70	15(21.4%)
Endotracheal aspirate/ sputum	30	14(46.6%)
Blood culture	30	10(33.3%)

Table 2: Antibiotic resistance of isolated K. pneumoniae from different samples

Antibiotic	Pus (15)	Respiratory sample (14)	Blood (10)	Urine (15)	Total (54)
ESBL	8 (53.3%)	5 (35.7%)	4(40%)	13(75%)	30 (55.5%)
Ampicillin	15(100%)	14 (100%)	10(100%)	15(100%)	54(100%)
Ampicillin-sulbactam	13(85%)	14(100%)	10(100%)	12(80%)	49(90%)
Piperacillin-tazobactam	10 (66.7%)	8(57%)	10(100%)	12(80%)	40(88%)
Cefazolin	13(85%)	14(100%)	10(100%)	12(85%)	49(90%)
Cefoxitin	11 (73.3%)	12(85.7%)	10(100%)	13(90%)	46(85.1%)
Ceftriaxone	12 (80%)	14(100%)	10(100%)	13(90%)	49(90%)
Ceftazidime	12 (80%)	14(100%)	9(90%)	14(95%)	49(90%)
Cefepime	12 (80%)	14(100%)	8(80%)	13(85%)	47(87%)
Aztreonam	11 (73.3%)	12(85.7%)	5(50%)	10 (66.7%)	38(70.3%)
Imipenem	8 (53.3%)	11(78.5%)	4(40%)	5(30%)	28(51.8%)
Ertapenem	8 (53.3%)	11(78.5%)	4(40%)	4(26.6%)	27(50%)
Meropenem	8 (53.3%)	11(78.5%)	4(40%)	5(30%)	28(51.8%)
Amikacin	1 (6.6%)	8(57%)	0 (0.0%)	1(5%)	10(18.5%)
Gentamicin	1 (6.6%)	11(78.5%)	6(60%)	1(5%)	19(53%)
Tobramycin	10 (66.7%)	11(78.5%)	6(60%)	1(5%)	28(51%)
Ciprofloxacin	5(33.3%)	11(78.5%)	9(90%)	14(95%)	39(72.2%)
Levofloxacin	6 (40%)	11(78.5%)	9(90%)	14(95%)	40 (74%)
Moxifloxacin	5(33.3%)	10 (71.4%)	8(80%)	14(95%)	37(68%)
Tigecycline	0 (0.0%)	(0.0%)	(0.0%)	(0.0%)	(0.0%)
Nitrofurantoin	5(33.3%)	8(57%)	1(10%)	(0.0%)	14(25%)
trimethoprim sulfamethoxazole	3 (20%)	13(92.8%)	1(10%)	7(65%)	24(44%)

Table 3 : Antibiotic resistance in carbapenem resistant and carbapenems sensitive K. pneumoniae isolates.

Antibiotic	Carbapenem resistant	Carbapenem sensitive	Total
Total	28(100%)	26(100%)	54(100%)
ESBL	8(28.5%)	22(84.6%)	30 (55.5%)
Ampicillin	28(100%)	26 (100%)	54(100%)
Ampicillin-sulbactam	28(100%)	21(80.7%)	49(90%)
Piperacillin-tazobactam	26 (92.8%)	14(53.8%)	40(88%)
Cefazolin	28(100%)	21(80.7%)	49(90%)
Cefoxitin	27 (96.4%)	19(67.8%)	46(85.1%)
Ceftriaxone	28(100%)	21(80.7%)	49(90%)
Ceftazidime	28(100%)	21(80.7%)	49(90%)
Cefepime	28(100%)	19(67.8%)	47(87%)
Aztreonam	28(100%)	10(38.4%)	38(70.3%)
Imipenem	28(100%)	0	28(51.8%)
Ertapenem	27 (96.4%)	0	27(50%)
Meropenem	28(100%)	0	28(51.8%)
Amikacin	9(32%)	1(3.8%)	10(18.5%)
Gentamicin	12(42.8%)	7(26.9%)	19(53%)
Tobramycin	19(67.8%)	9(34.6%)	28(51%)
Ciprofloxacin	24(85.7%)	15(57.6%)	39(72.2%)
Levofloxacin	26(92.8%)	14(53.8%)	40 (74%)
Moxifloxacin	26(92.8%)	11(42.3%)	37(68%)
Tigecycline	0 (0.0%)	0 (0.0%)	0(0.0%)
Nitrofurantoin	9 (32 %)	5(19.2%)	14(25%)
Trimethoprim/ sulfamethoxazole	16(66.6%)	8(30.7%)	24(44%)

Phenotypic testing of Metallo-β-lactamases production by combined disk tests (CDTs) was done to 28 (51.8%) organisms showing resistance to one or more carbapenems drugs (imipenem, meropenem and ertapenem), 10 *K. pneumoniae* were positive.

Molecular analysis of blaNDM-1 gene is by PCR revealed that; 8(28.5%) isolates were positive out of the 28 carbapenems resistant *K. pneumoniae*. All of the blaNDM-1 gene positive- isolates were isolated from ICU patients; two from blood and 3 from urine, and 3 from endotracheal aspirate.

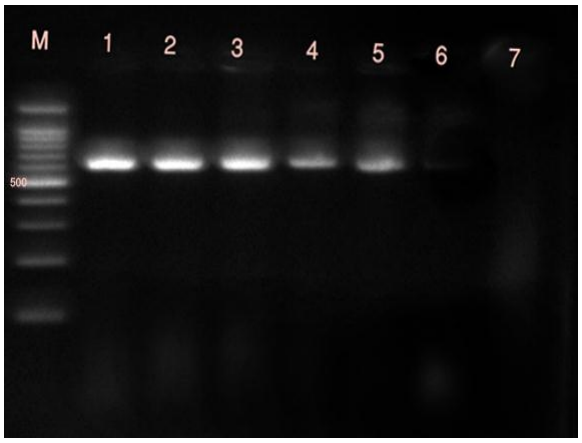

Fig. 1: Agarose gel electrophoresis of PCR-amplified products of the NDM-1gene. Lane M; DNA ladder (100 bp DNA Marker). Lanes 1-5; the NDM-1 gene (621 bp). Lane 6: negative sample. Lane 7; negative control.

DISCUSSION

K. pneumoniae is an important nosocomial pathogen causing urinary tract, respiratory tract, and bloodstream infections. In the present study, 54(22.5%) *K. pneumoniae* were isolated from 200 clinical samples collected from nosocomial infections. the percentage of isolated *K. pneumoniae* were highest in respiratory samples ;14(46.6%) followed by blood culture samples 10(33.3%) similar results were reported in 2 studies in Egypt [14,15].

Carbapenems are the most important therapeutic agents against MDR Gram-negative bacteria. The current emergence of carbapenemase-producing bacteria represents a major threat. In our study resistance to imipenem and meropenem were 28(51.8%). The high rate of carbapenems resistance is recently reported in several studies in Egyptian hospitals; in Assuit university hospital; 26(48.1%) of isolated gram negative bacilli were carbapenem- resistant [15]. and in Cairo university hospital, 50 (21.2%) of isolated gram negative were resistant to carbapenem[16]. In Tanta university hospital, the overall resistance among Enterobacteriaceae was 47 (62.7%) with a rate of resistance in *klebsiellae spp.*was 11(23%) [17]. The rate of resistance in our study is similar to *Fazeli et al* in Iran who reported that 43.7% of isolated *klebsiellae pneumonia* were resistant to carbapenems. The unrestricted use of carbapenems in the treatment of bacterial infections leaded to the selection of resistant strains and the emergence of imipenem-resistant *K. pneumonia* strains.

In our study, *K. pneumoniae* isolates were multidrug resistant in 70 % of total isolates. Higher rates were reported by a previous Egyptian study as 71.1% of *K. pneumoniae* were MDR [18]. The misuse of antibiotics and lack of infection control policy is responsible for the high rates of antimicrobial resistance detected in our study.

All the isolates were sensitive to tigecycline, making it the last treatment option. Also, many Egyptian studies confirmed the sensitivity of carbapenem- resistant strains to tigecycline[14,15,19]. However, recent resistance to tigecycline was reported in a study from Saudi Arabia[20]and another study in Kuwait[21].

This study used combined disk tests (CDTs) (IMP/IMP +EDTA) method for phenotypic detection of carbapenemase of Metallo-β-lactamases types. EDTA is a chelating agents that inactivate the Metallo β-lactamase enzymes by binding to zinc which presents in the active site of the enzyme. Comparing the results of this test to the PCR results in our study, the method was (100%) sensitive and (90%) specific. The high sensitivity and low specificity of the test was reported by many studies[15,22]. False positive results may be explained by the presence of other MBL genes as VIM (Verona-Integron Mediated) or IMP (Instance plasmid-mediated)[23]; which were not detected in this study and necessitate further investigations.

The blaNDM-1 is a recently reported class B carbapenemase identified mostly in *K. pneumoniae* and *E. coli*. It originated in India and spread rapidly worldwide in few years by international travel. the gene is endemic in the Indian subcontinent which acts as a reservoir for the gene. It was detected in small outbreaks or sporadic cases in the middle east and Mediterranean countries [9]

In this study, PCR results demonstrated that 8(28.5%) out of 28 carbapenem-resistant isolates expressed the New Delhi Metallo-beta-lactamase (blaNDM-1). The gene was detected in carbapenems resistant *klebsiellae* in a recent Egyptian studies[22,16,24,19]. However, a recent study in Tanta university hospital reported the absence of the gene in their isolates[17]. The gene was also reported in kuwait[21], Oman [25] and Saudi Arabia [26].

The New Delhi Metallo-β -lactamase is a broad spectrum carbapenemase with the ability to inactivate β -lactams except for aztreonam[27]. All carbapenem-resistant isolates in our study were also resistant to

aztreonam indicating the presence of other resistant genes. this was also reported by other study from Egypt[18]. Aztreonam is inactivated by ESBLs or KPC carbapenemases which may be associated with NDM-1 positive isolates[28].

Control of this resistant strains can be achieved through early detection of resistance genes among bacterial isolates and limiting the dispersal of these organisms which can rapidly spread among wide range of gram negative organisms by plasmids. Spread to *E. coli* which is part of the normal human gut flora can lead to carriage of the gene in GIT and spread by faeco-oral route. This can make the NDM producers endemic in community and hospitals which will make their control of difficult[1].

CONCLUSION

K. pneumoniae is the causative agent of hospital acquired infections, such as pneumonia, bloodstream infections, wound infections and UTI. Carbapenem resistant is prevalent in our setting which necessitate applying strict antibiotic policies that govern the use of antibiotics in hospitals. The rapid detection and appropriate treatment of NDM producers is mandatory. Infection control measures should be strictly applied to prevent spread of these highly resistant pathogen in hospitals and community.

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