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RESEARCH ARTICLE

DISSEMINATION OF NEW DELHI METALLO-B-LACTAMASE (*BLA*$_{NDM}$) GENE IN *PSEUDOMONAS AERUGINOSA* ISOLATES FROM BURN CENTER IN NAJAF, IRAQ

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ABSTRACT

Carbapenems are the most potent β-lactams and widely used as the mainstay and empirical for treatment of MDR *P. aeruginosa*. Carbapenems resistant *P. aeruginosa* has now emerged and is disseminating worldwide.Identified *bla*$_{NDM}$in the isolates of *P. aeruginosa*, that are recovered from Burn Center in Najaf.Cross- sectional study involved 123samples were collected from Burn Center in Najaf. All samples culture on blood and MacConkey agar, and then isolation and identification by conventional methods and confirmed by VITEK-2 compact to diagnosis *P. aeruginosa*. The antimicrobial susceptibility test for 13 antibiotics were tested by using MIC method.35*P. aeruginosa*isolates from 123 samples taken from Burn Center (28.5%), 22/35 (63%) isolates carbapenem resistant *P. aeruginosa* (CRPA), there were 8/22 (36.4%) of samples positive for *bla*$_{NDM}$ gene. The findings of this study underscore the high prevalence of NDM-type MBL among carbapenemase producing isolates.

INTRODUCTION

Pseudomonas aeruginosa is one of the major opportunistic and common hospital-acquired (nosocomial) pathogen that causes many severe and often fatal infections [(1)]. In case of these diseases, antibiotics can become a difficult mission, because *P. aeruginosa* is intrinsic resistant to various antibiotics [(2)]. Besides that, *P. aeruginosa* possesses several mechanisms of antimicrobial resistance during discovery of mutations in chromosomal genes or by lateral development of immune determinants [(3)]. *P. aeruginosa* easily acquires resistance devices to numerous classes of antibiotics even through the treatment course. Different mechanisms of resistance oftenexist simultaneously, thus conferring combined resistance [(4)]. Carbapenem resistance can result from decreased permeability of the outer membrane, up-regulation efflux pumps, overproduction of a chromosomal AmpC-type cephalosporinases or the construction of carbapenemases [(5)]. *P. aeruginosa* pathogens are problematic to therapy because of inherent resistant to several antibiotics (MDR) and high risk of tolerance through treatment [(6)]. It is cause hospital-acquired infections and community acquired, infections with this bacteriumhigh morbidity and mortality have been linked with other bacterial infections [(7)]. WHO has newly recorded resistant to carbapenem of *P.aeruginosa* as 1 of 3 species of bacteria for which there's also a significant need to develop novel antimicrobial agents to combat pathogens [(8)]. Carbapenems are deliberated of most consistent agents for therapy of bacterial infections of bacteria, and the appearance and extent resistance to these antimicrobial agents institute, the primary problem for public health [(9,10)]. Carbapenems, including meropenem and imipenem are the most potent antibiotics used to the therapy of infections, started with multidrug resistant Gram-negative bacilli [(11)]. Carbapenem resistant *P. aeruginosa* and *Enterobacteriaceae*, are main causes of health related infections and have been listed as urgent threats to public health [(12)]. Class B enzymes are predominantly in the β-lactamase class, which has the capability to hydrolyze carbapenems which is exposed to destruction by (EDTA), chelators of Zn^{2+} and another divalent reactions. The hydrolysis mechanism relies on the association of β-lactam agents with zinc ions in the active site of enzyme. The most frequent MBLs contain the NDM, VIM, IMP, GIM, SIM, and SPM enzymes, that are situated within variation of integrons structures, when these integrons are attached to transposons or plasmid, communication between bacteria is eagerly facilitated [(11, 13)]. The New Delhi MBL (NDM-1) originated from India and Pakistan, the first detected from *K. pneumonia* was isolated in 2009, from patient Swedish of Indian origin, who had developed medical treatment in 2007, New Delhi- India [(14)]. The most discovered recently metallo-β-lactamase is NDM-1, which was spread quickly and has been published worldwide.

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MATERIAL AND METHODS

Bacterial isolates: Cross-sectional prevalence investigation was performedduring the surveillance period between three months November, December 2018 and January 2019, a total of 123 samples were obtained from patient in Burn Center, Najaf.Previous to the star of this study, ethics approval were achieved from Kufa University/ College of Medicine, Ministry of Health, in addition to, the research ethics committees. The samples were collected from patients have infections from burn exudate. Infections were considered as either, hospital acquired or community related consistent with standard epidemiological definitions established by Kumarasamy (2010)[15]. For each patients, medical records were included name, gender, age, hospitalization, address, and antibiotic receiving. The samples were labeled and transport to the Kufa University, College of Medicine, Microbiology Laboratory for processing as describe by Collee *et al. (*1996)[16].Theburn exudate were collected by sterile swab from infected site, swab was moisted with normal saline before collected samples, avoid drying.The samples were transport to the laboratory , and culture on MacConkey and blood agar dishes and incubate aerobically at 37 C to 24 hours,and culture on MacConkey and blood agar dishes and incubate aerobically at 37 C to 24 hours,when count of bacteria 10^5 colony forming unit (CFU)/ml. After 24 hours incubation, the culture media were examined for the presence of *P.aeruginosa* growth. All isolates were initially identified by conventional methods based on Holt *et al.* (1994) and MacFaddin (2000)[17,18], and species were identification, then confirmed with VITEK-2 automated system using the GN 222 card.*P.aeruginosa* isolated in nutrient broth containing 15% glycerol in the tube and stored in deep freeze. This study followed to identify the occurrence of carbapenem resistant *P.aeruginosa* isolates carrying carbapenemase genes in clinical samples attained from Burn Center in Najaf.

Antibiotic susceptibility testing: All identified *P. aeruginosa* isolates were exposed to antimicrobial susceptibility test against 13 different antibiotics of 8 classes. Selected based on CLSI (2019)[19]. *E. coli* ATCC 25922 strain that are used quality control of the antibiotics tested. Three to five isolated colonies were transferred into test tube enclosing two ml for nutrient broth, incubate for two hrs. at 37°C, and then, density of broth was attuned with McFarland 0.5 by normal saline addition. The Muller-Hinton agar was streaking by sterile loop or disposable loop for different directions of plate and leaved to five minutes to dry, and distributed antibiotic disks on the surface of plate , then incubated for 18-24 hours at 37 °C. According to CLSI (2019), measured of inhibition zone around colony by the ruler or special measuring device. Any isolates of bacteria that were resistance against at least 1 antibiotic in 3 or more classes called Multi Drug Resistance (MDR), isolates were resistant to at least one antibiotic in all but one or two classes of antibiotics called Extensive Drug Resistance (XDR), the isolates exhibited resistance to all classes of antimicrobial agents called Pan Drug Resistance (PDR) [20].The multiple antibiotic resistant types of the isolates are summarized in Table (1). Consequently, resistance to ≥ 3 anti-pseudomonal categories was seen in 3 isolates (13.6%), which characterized as MDR *P. aeruginosa* and were categorized in three of these isolates, 2 (9.1%), and 1 (4.5%) were resistant to 5 and 4 antibiotic categories employed, respectively. Importantly, resistance to ≥ 6 anti-pseudomonal categories were seen in 19 isolates (86.4%), which characterized as XDR *P. aeruginosa*, of which 14 (63.6%) were seen resistant to 6 antibiotic categories, and 5 (22.7%) were resistant to 7 antibiotic categories. Pan-drug resistance for carbapenem resistant *P. aeruginosa* was not yet identified in this study.Colistin retained in vitroactivity against 90.9% of MDR and XDR isolates. All isolates exhibited resistance to imipenem and meropenem were further tested for minimum inhibition concentration (MIC) by VITEK-2 automated system, using GN AST 222 card and results were interpreted based on CLSI (2019) break points.

DNA extraction and PCR confirmation chromosome DNA was extracted from each *P. aeruginosa* isolated by DNA extraction kit Favorgen (Taiwan) according to manufactures instruction. The bacteria were confirmed using polymerase chain reaction (PCR) method for NDM of the *P. aeruginosa*. PCR was carried with 2 µl template DNA, 2 µl of each primer (F: GGTTTGGCGATCTGGTTTTC) and (R: CGGAATGGCTCATCACGATC) and 5 µl of Taq DNA polymerase and 5 µl of free DNA water in a total volume of 20 µl . The DNA was amplified using the following protocol: initial denaturation 95 °C for 120 sec. followed by 30 cycles of denaturation 95 °C for 0.5 min., annealing 63.4 °C for 0.5 min. and extension of 72°C for 0.5 min. with a single finalextension of 72 °C for 5 minutes.

Agarose gel preparation: The Preparation of Agarose gel according to Bartlett and Stirling (1998)[21] as the following:
10 ml for TBE buffer diluting by 90 ml of DW, added 100 ml in the flask, and then 1.5gm Agarose into 100 ml of diluting buffer, mix well and heating until the mixture become clear. Allowed to cooling at 40 °C , and then added 5µl of red safe stain. Followed, Agarose was empty carefully in gel tray of electrophoresis, left until cooled and become firm. Then, 5 µl product of PCR were loweded into wells of gel and followed by add DNA lowed marker in the first well, fixed the gel tray in electrophoresis and fuelled by TBE buffer, performed at 65 volt for 2 hours. Finally, identified the result by the documentation of the gel system.

Statistical analysiswasperformed using SPSS-21 software (SPSS verison-21) for finding signification relationship among of bacteria, virulence gene and pattern resistance of *P. aeruginosa* isolated from clinical samples

RESULTS

A total of 123 samples were recovered from Burn Center in this study, 35/123 (28.5%)of *P. aeruginosa* isolates and 22/35 (63%) isolates carbapenem resistant *P. aeruginosa* (CRPA) Table (2)**.** The 22 *P. aeruginosa* isolates , which exhibited resistance toward meropenem and/or imipenem, were further confirmed by minimal inhibitory concentration (MIC) by VITEK-2 compact system using GN AST 222 card. Breakpoints of antibiotics resistance were determined according to CLSI (2019) recommendations. The MIC values of antibiotics for all of the isolates are provided in Table (3).

Molecular screening of carbapenemase producers: All 22 carbapenem resistant *P. aeruginosa* samples were divided by conventional PCR for potential gene determinants encoding carbapenemases using a specific primers for Ambler class B MBL (bla_{NDM}).

Table 1. Multiple anti-pseudomonal resistance of carbapenem resistant *P. aeruginosa* (n=22)

Resistant type No. (%)	No. of antibiotics resistance Classes (n= 8)	Number of isolates	Isolate symbol
MDR 3 (13.6)	5	2	Pa4, Pa10
	4	1	Pa12
XDR 19 (86.4)	6	14	Pa1, Pa2, Pa3, Pa5, Pa6, Pa7, Pa8, Pa9,Pa11, Pa13, Pa14, Pa16, Pa21, Pa22
	7	5	Pa15, Pa17, Pa18, Pa19, Pa20
PDR 0 (0)		0	0

MDR, multidrug resistance; XDR, extensive drug resistance; PDR, pan-drug resistance

Table (2): Occurrence of *P. aeruginosa* isolates in burn centerpatients

Sample source	No. of samples	No. (%) of *P. aeruginosa* isolates	No. (%) of *P. aeruginosa* (CRPA) isolates
Burn center	123	35 (28.5)	22 (63)

Table (3): Minimum inhibitory concentration (MIC, µg/ml) of *P. aeruginosa* carbapenem resistant isolates from burn center (n=22) according to the CLSI (2019)

Isolate Symbol	Ticarcillin	Ticarcillin-clavulanic acid	Piperacillin	Ceftazidime	Cefepime	Aztreonam	Imipenem	Meropenem	Amikacin	Gentamicin	Tobramycin	Ciprofloxacin	Colistin
Pa1	≥128	≥128	≥128	16	≥32	≤8	8≥	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa2	≥128	≥128	≤16	16	≥32	≤8	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa3	≥128	≥128	≥128	16	≥32	≤8	8≥	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa4	≥128	≥128	≥128	≥32	≥32	≤8	8≥	8≥	≤16	8	≤4	2≥	≤ 2
Pa5	≥128	≥128	≥128	≥32	≥32	≤8	8≥	8≥	≤16	16≥	≤4	2≥	≤ 2
Pa6	≥128	≥128	≥128	≤8	≥32	≤8	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa7	≥128	≥128	≥128	≥32	≥32	≤8	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa8	≥128	≥128	≥128	≤8	≥32	≥32	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa9	≥128	≥128	≥128	≥32	≥32	≤8	8≥	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa10	≥128	≤16	≥128	≥32	≥32	≤8	8≥	8≥	≤16	16≥	16≥	≤ 0.5	≤ 2
Pa11	≥128	≥128	≥128	16	≥32	≤8	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa12	≥128	≥128	≥128	≤8	≤8	≤8	8≥	8≥	≤16	≤4	≤4	≤ 0.5	≤ 2
Pa13	≥128	≥128	≥128	16	≥32	≤8	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa14	≥128	≥128	≥128	16	≥32	≥32	4	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa15	≥128	≥128	≥128	≥32	≥32	≤8	8≥	8≥	64≥	16≥	16≥	2≥	≥ 4
Pa16	≥128	≥128	≤16	≤8	≥32	≥32	8≥	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa17	≥128	≥128	≥128	≥32	≥32	≥32	8≥	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa18	≥128	≥128	≥128	≥32	≥32	≥32	8≥	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa19	≥128	≤16	≥128	≥32	≥32	≥32	8≥	8≥	64≥	16≥	16≥	1	≤ 2
Pa20	≤16	≤16	≥128	≥32	≥32	≥32	≥8	8≥	64≥	16≥	16≥	2≥	≥ 4
Pa21	≥128	≥128	≤16	16	≥32	≤8	≥8	8≥	64≥	16≥	16≥	2≥	≤ 2
Pa22	≥128	≥128	≤16	16	≥32	≤8	≥8	≥8	64≥	16≥	16≥	2≥	≤ 2
Total resistance (%)	21 (95.4)	19 (86.4)	18 (81.8)	10 (45.5)	21 (95.5)	7 (31.8)	15 (68.2)	22 (100)	18(81.8)	20 (91)	19 (86.4)	19 (86.4)	2 (9.1)

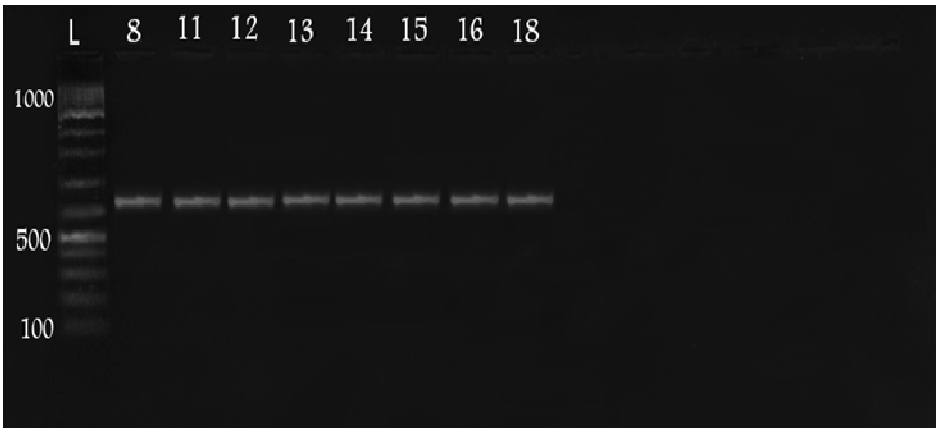

Figure (1): Agarose gel with red save stained of mono-plex PCR amplified product from extractDNA of*P. aeruginosa* samples with bla_{NDM} gene primer.The electrophoresis performed at 65 volt for 120 minutes. Lane (L) is molecular size of DNA marker (1000-bp ladder). Lanes8, 11, 12, 13, 14, 15, 16, 18 showed positive results by bla_{NDM} gene (623 bp)

Importantly, the PCR products obtained in this work revealed that *bla*$_{NDM}$ MBL gene, that was prevalent (8, 36.4%) among carbapenem resistant isolates (Figure 1).

DISCUSSION

To date, twelve transferable MBL typesthat hydrolyze carbapenems have been identified in Gram-negative bacilli; though, the most confusing MBLs are certainly, the NDM-type MBLs [22,23].The emergences of NDM producing nosocomial pathogens worldwide, has raised a major public health threat and represent a big challenge for physicians to treat infected patients [24]. The NDM was observed for the first time in *K. pneumoniae* samples in 2008 from aSwedish case of Indian descent [14].Moreover, presenceof NDM-type MBLs in *P. aeruginosa*was identified in 2011 for the first time from patients in Serbia [25]. Later then, NDM producing *P. aeruginosa* samples have been recovered throughout diverse locations [26, 27, 22]. In 2011, Iraqi Ministry of Health attentionhas focused on *K. pneumoniae* regarding the worldwide dissemination of these isolates containing the NDM-type MBLs (laboratories-dep@yahoo.com). However, the presence of NDM in Iraq was first documented in 2014 in two *P. aeruginosa* isolates recovered from hospitalized patients in Najaf [28]. In addition, Al-Hasnawi (2020)[29] reported the first detection of *bla*$_{NDM}$ in 4 (18.2%) of carbapenem resistant *K. pneumoniae* isolates in Najaf hospitals. Whereas, in a recent study on 97 *P. aeruginosa* isolates by AlKhudhairy and Al-Shammari (2020)[30], the *bla*$_{NDM}$ gene was not detected in Najaf.However, the complete picture of the true dissemination of NDM-type MBLs producing *P. aeruginosa* in Najaf hospitals is yet unknown. One of the significant finding in the current study is the presence of *bla* $_{NDM}$ gene in isolates tested. Surprisingly, the results indicated that 8 (36.4%) of the 22 resistant to carbapenem of *P. aeruginosa* were positive for NDM (Figure1), suggesting possible the incidence of endemic pathogens with *bla*$_{NDM}$ gene harboring *P. aeruginosa* samples through the study period,which can be a serious concernand is worthy of further epidemiological studies. The *bla*$_{NDM}$ gene *P. aeruginosa* carrying isolates are mainly hazardous since most plasmids detected in these isolates are transferable, suggesting a widespread horizontal transmission among pathogens and probable high dissemination of unrecognized asymptomatic carriers in hospitals [31].

Current study also confirmed that NDM producing *P. aeruginosa* is one of the challenges in Najaf hospitals, which has been became even more urgent since the current detection of colistin resistant in one isolate (Pa15) carried *bla*$_{NDM}$ gene recovered from patient admitted in Burn Center. The spread of this isolate in Najaf hospitals has end all hopes to control of *P. aeruginosa* infections. In association with present study, Al-Hasnawi (2020) found for the first time co-existence of colistin resistant *mcr-1* and *bla*$_{NDM}$ genes in two isolates of MBLs producing *K. pneumoniae* in Al-Sader Medical City. Present study, moreover revealed that detection high rate of *bla*$_{NDM}$ suggested not only for the high degree of genetic transmission among pathogenic bacteria in Najaf hospitals, but also possibilities to Human variables for example hygiene and travel. The *bla*$_{NDM}$ has been linked with international travel, mainly to the India, where it is the main reservoir of NDM-1 producers [32,33, 34].Remarkably, high frequency of *bla*$_{NDM}$ MBL was identified in current study despite the fact that many Iraqi peoples, including the inhabitants of Najaf province, travel to India for medical care (medical tourism), which may help in the acquisition of this gene. In addition, contact with healthcare in endemic countries for NDM producers such as India, has been linked to cases presenting in European countries [35].In national comparable with present study,a recent survey conducted on different hospitals and specialized health centers in Al-Diwaniyah province revealed that 37.5% of 24 resistant to carbapenem of *P. aeruginosa* isolates were NDM-type MBLs producers [36]. Further, Al-Taie (2019)[37] reported a high prevalence (78%) of NDM in 27 resistant to carbapenem resistant of *P. aeruginosa* samples from Baghdad hospitals. The results of current investigation are interesting in that this is the second work to show that the dissemination of carbapenems resistant *P. aeruginosa* isolates carrying *bla*$_{NDM}$ gene in Najaf hospitals. Present result line with several reports that found widespread incidence of NDM-1 in Indian and also has been reported from geographically various areas of the world such as UK, the USA, Japan, India, Singapore, Australia the Middle East and several countries in Europe [38,39]. This dissemination of NDM is just one example of how antimicrobial resistance will spread rapidly globally.

Conflict of Interest: The authors declare no conflict of interest.

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