ORIGINAL ARTICLE

NEW DELHI METALLO-BETA-LACTAMASE PRODUCING CARBAPENEM-RESISTANT GRAM-NEGATIVE BACILLI: MICROBIOLOGICAL AND GENOTYPIC ANALYSES AT A TERTIARY CARE HOSPITAL IN PAKISTAN

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Background: Metallo-beta-lactamases (MBL) catalyze the hydrolysis of beta-lactam antibiotics including carbapenems. A novel MBL subtype, New Delhi MBL (NDM), poses a serious public health problem. The aims of this study were to determine the frequency of NDM producers among the Carbapenem-resistant gram-negative bacilli (GNB) in hospitalized patients and carrying out the molecular analysis of the NDM genes as reliable data on this is not available in Pakistan. **Methods:** We carried out a cross-sectional study on prospectively collected clinical samples from 113 patients hospitalised at Shaikh Zayed Hospital Lahore, Pakistan. All the samples that were carbapenem-resistant on routine sensitivity testing were selected for this study. Various microbiological and genotypic analyses of the samples were performed. **Results:** The mean age of the patients was 47.8±20.8 years. About a quarter (25.7%) of the samples was from the urology ward and 43% were urine samples. Around two-third of the samples (n=74, 65.5%) tested positive for *Non-Enterobacteriaceae* GNB. *Pseudomonas spp* was the most common isolate among the *Non-Enterobacteriaceae* and *E-coli* amongst the *Enterobacteriaceae.* NDM gene was detected in 22 patients (19.5%). We did not find any association of the NDM gene with the demographic and clinical characteristics. **Conclusion:** NDM-positive GNB are present in our hospitalized patients, which is worrisome as these bacteria can disseminate globally and lead to an extensive and uncontrollable spread of pandemic clones for which efficient antibiotic therapy is currently not available. Systemic surveillance network and infection control strategies should be established to curtail dissemination of NDM-producing GNB in Pakistan.

Keywords: Gram-Negative Bacteria; Extended Spectrum Beta Lactamase; New Delhi metallo-beta-lactamase; Carbapenem-Resistant Enterobacteriaceae; Health Care Associated Infections; Pakistan

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INTRODUCTION

Gram-negative bacilli (GNB) cause a wide variety of infections like pneumonia, bloodstream infections, wound or surgical site infections, and meningitis in healthcare settings.[1] Recent data indicates that GNB are responsible for more than 30% of hospital-acquired infections.[2] GNB are resistant to most available antibiotics and have developed built-in abilities to acquire new ways of resistance.[1] GNB can be broadly grouped into *Enterobacteriaceae* and *Non-Enterobacteriaceae*. *Enterobacteriaceae* are a large family of GNB that normally live in the human gastrointestinal tract, and are a common cause of both community-acquired and hospital acquired infections (HAI).[3] This family includes more than 70 genera[4] and 139 species[2]. The commonest ones are *Escherichia coli, Klebsiella, Enterobacter, Citrobacter, Serratia, Salmonella, Shigella, Proteus* and *Yersinia.*[2] *Non-Enterobacteriaceae* include *Vibrio, Campylobacter, Pseudomonas* and *Acinetobacter.*[1,2]

Beta-lactam antibiotics (for example, carbapenems) act by inhibiting the cell wall synthesis and are often the mainstay of treatment in combination with other agents in patients with grave HAIs.[5,6] Unfortunately, the emergence of resistance against carbapenems has posed serious threat in the management of multi-drug resistant (MDR) bacteria world over.[6] The enzymes - beta-lactamases - that neutralize the effects of beta-lactams can be broadly grouped into two main types.[7] Serine beta-lactamases: which share a serine residue in the active site and include Class A, C, and D beta-lactamases[5,7–9] and Metallo-beta-lactamases (MBL), which use one or two zinc ions in their active sites to catalyze the hydrolysis of all classes of beta-lactam antibiotics. This group involves Class B enzymes.[5,7] Classes A, B, and D are of vital clinical importance amongst the nosocomial pathogens.[5,8,9]

Carbapenem-resistant *Enterobacteriaceae* (CRE) have been globally reported as a consequence of acquisition of carbapenemase genes[9] and have been acknowledged in health-care

settings as a cause of hard-to-treat infections associated with high mortality[4]. In hospitals, CRE infections most frequently occur amongst patients receiving treatment for other conditions or who are on long-term antibiotic therapy.[5] One report cites that CRE can contribute to death in around 40–50% of infected patients.[5] In 2009, a novel MBL subtype was recognized in a *K. pneumonia* isolate from a Swedish patient originally treated in New Delhi, India.[10] The enzyme was named NDM-1 (New Delhi MBL-1) after New Delhi, the capital city of India and was first described by Yong *et al*.[11] Since its first description, NDM carbapenemase has been reported from 40 countries worldwide, encircling all continents except South America and Antarctica[12] and poses a serious public health problem.[6,7,11,13–15]

Recent reports have shown that the plasmid-mediated bla$_{NDM-1}$ gene encoding MBL, NDM-1, is spreading globally, primarily in the members of the *Enterobacteriaceae*[16] with India and Pakistan being the main reservoirs.[10] Since Carbapenem resistance is plasmid mediated and most isolates carry the NDM genes on plasmids[10,17], molecular analysis for the determination of bla$_{NDM-1}$ gene proves to be of vital importance in identification of NDM gene carrying CRE.[16] Combination therapy will likely be required to combat the ongoing evolution of these perilous enzymes in MDR GNB.[18]

In Pakistan, the magnitude of NDM producing CRE and the data on molecular analysis are not available. We do not have reliable data on the types of NDM genes present in GNB infecting our hospitalized patients. One study is available that shows prevalence and fecal carriage of NDM in stool samples but it did not consider the types of NDM genes.[19] Another study indicates the prevalence of ESBLs and MBLs including NDM-1 at two hospitals in Pakistan[20] but they did not carry out gene sequencing and phylogenetic analysis to find out prevalent NDM genes. Hence, the aim of this study were to determine the frequency of NDM producers among the Carbapenem resistant GNB in patients and carrying out the molecular analysis of these NDM genes. This will help us establish the molecular epidemiology of NDM producing GNB in Pakistan. It will also enable us to better understand the natural history of the disease, identify possible risk factors and develop more effective and targeted medical treatment.

MATERIAL AND METHODS

This cross-sectional study was carried out between January and November 2013 on 113 consequently selected clinical samples collected from the patients hospitalised at the Federal Post Graduate Medical Institute, Shaikh Zayed Hospital Lahore, Pakistan. All the main hospital wards were included for samples collection, especially Medicine, General Surgery, Urology, Nephrology, Paediatrics, ICU, CCU, Orthopaedics and Liver Transplant Unit. All clinical samples including urine, blood, pus, body fluids, CSF, ear discharge, throat swab, sputum, tracheal aspirate and bone that were carbapenem-resistant on routine sensitivity testing on Mulleur Hinton agar, i.e., resistant to Meropenem 10 µgm disc after 24 hours incubation at 35–37 °C, were included in this study. The zone sizes were read according to CLSI recommended guidelines. The selected isolates were sub-cultured on CLED agar, labelled and incubated for 18-24 hours at 35–37 °C. Only one sample per patient was considered for analysis.

The microbiological analyses of the samples were carried out at the Federal Post Graduate Medical Institute, Shaikh Zayed Hospital Lahore, Pakistan as per the standard laboratory protocols and included gram staining, cytochrome oxidase, routine biochemical testing using Triple Sugar Iron (TSI), citrate utilization and motility tests. After identification, the organisms were stored on Nutrient agar slants at 4 °C till further processing. The genotypic analysis for the detection of NDM genes was performed by the Polymerase Chain Reaction (PCR) using NDM primers as follows:[25]

Forward primer: NDM-1_a_fw (5′-CAATATTATGCACCCGGTCG-3′)

Reverse primer: NDM-1_a_rev (5′-CCTTGCTGTCCTTGATCAGG-3′)

This is followed by Gene sequencing and Phylogenetic analysis. It was carried out at the Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan.

This study was approved by the Institutional Review Board (IRB) at the Federal Post Graduate Medical Institute, Shaikh Zayed Hospital, Lahore, Pakistan. The collected data have been kept confidential. All sensitive information was removed prior to data compilation and analysis. The cost of the microbiological and genetic testing was not paid by the patients.

Complete data comprising of age, gender, date of receiving the sample in the laboratory, hospital ward, type of sample, isolate and Carbapenem-resistance was recorded on a *pro forma* and compiled using Microsoft Excel 2007 after the removal of confidential patient identifying information. Data was exported to SPSS version 20 (IBM, NY, USA) for analysis. Descriptive statistics are presented in the form of frequencies and percentages for categorical data and mean and SD for continuous data. NDM producing GNB and gene types at the molecular level were described as frequencies and percentages. Chi-square test was used at 5% level of significance.

RESULTS

A total of 113 patients were included in the study. Table-1 provides the demographic characteristics of the study participants. There was male preponderance in the study with a male to a female ratio of 1.9:1. All age groups were represented in the study with around 19.5% of samples from the 41–50 years group followed by 51-60 years (16.8%). The mean age of patients was 47.8±20.8 years. The minimum age of the study participants was one-day with the maximum being 88 years. Table-1 also illustrates the clinical characteristics of the study participants. The samples originated from a diverse range of clinical departments. Twenty-nine (25.7%) of the samples were from the Urology ward and a large majority of the samples that were tested in the study were the urine samples 48 (42.5%) followed by the pus samples.

On microbiological analysis, 74 (65.5%) samples belonged to the *Non-Enterobacteriaceae* group. Figure-1 gives the proportion of microorganisms isolated from the clinical samples by the GNB groups. *Pseudomonas spp* was the most common isolate among the *Non-Enterobacteriaceae* and *E-coli* amongst the *Enterobacteriaceae.*

NDM gene of 475–500 base pairs (bp) was observed in 22 (19.5%) samples. Figure-2 illustrates the results of the PCR analysis.[10] NDM positive isolates were then subjected to gene sequencing which yielded the nucleotide sequences which were analysed by various bioinformatics' software namely *ENSEMBL, BLAST, MEGA-6, BioEdit* to generate a phylogenetic tree as shown in Figure-3. Two out of 10 isolates, i.e., numbers 6 and 8 could not be inferred due to a technical error. The remaining 8 sequences showed strong structural similarity with NDM-1 gene representing the prevalence of NDM-1 in our hospitalized patients. The 10 sequenced isolates were analysed using *"Automated ClustalW alignment tool"* generating diagrammatic view of multiple sequences. Multiple sequence alignment of 8 sequenced isolates displayed varying nucleotide arrangement, as shown in Figure-4.

Association of NDM gene with various characteristics is shown in Table-2. Around one-third of NDM-positive isolates were from the patients in the 71–80 years age group. Female preponderance was seen in the positive cases with a female to a male ratio of approximately 2:1. A relatively higher proportion of the NDM-positive isolates were recovered from the patients who were admitted in the nephrology ward during the study period. Urine samples had a higher proportion of positive NDM gene. The other categories had small number of samples. The differences among the categories were, however, not statistically significant (p >0.05).

Table-3 gives the association of NDM gene status with the identified bacterial groups. There were more positive isolates in the *Enterobacteriaceae* (25.6%), but this difference was not statistically significant (p=0.32). Table-3 also shows the association of NDM gene with the isolated microorganisms. Amongst the *Enterobacteriaceae*, *Citrobacter* and *Enterobacter spp* were dominant pathogens with an NDM frequency of 40% each. Amongst the *Non-Enterobacteriaceae*, *Acinetobacter spp* was found to be most common (28.1%). This finding was statistically significant (p=0.02).

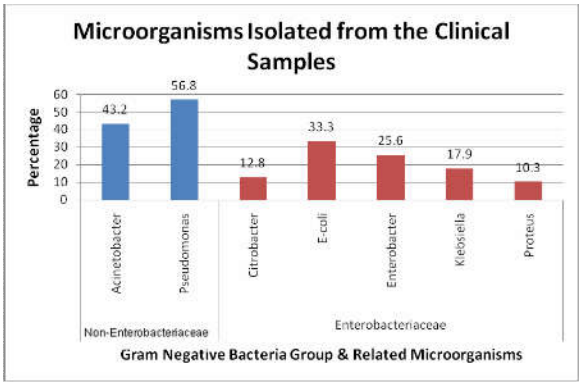

Figure-1: Microorganisms Isolated from the Clinical Samples

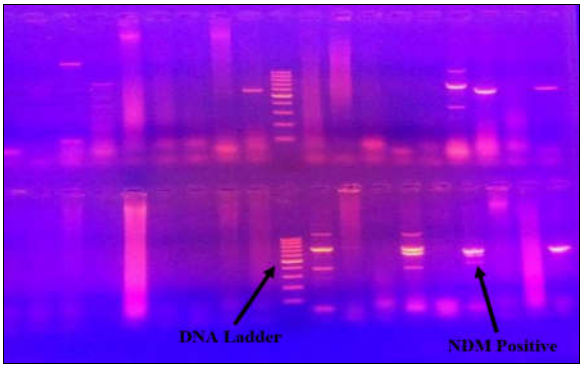

Figure-2: PCR Results for NDM Gene

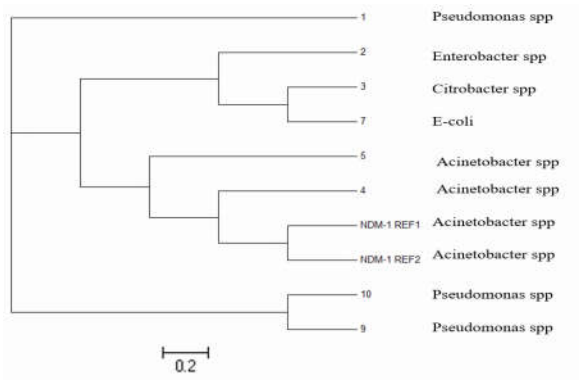

Figure-3: Phylogenetic tree of ten sequenced isolates

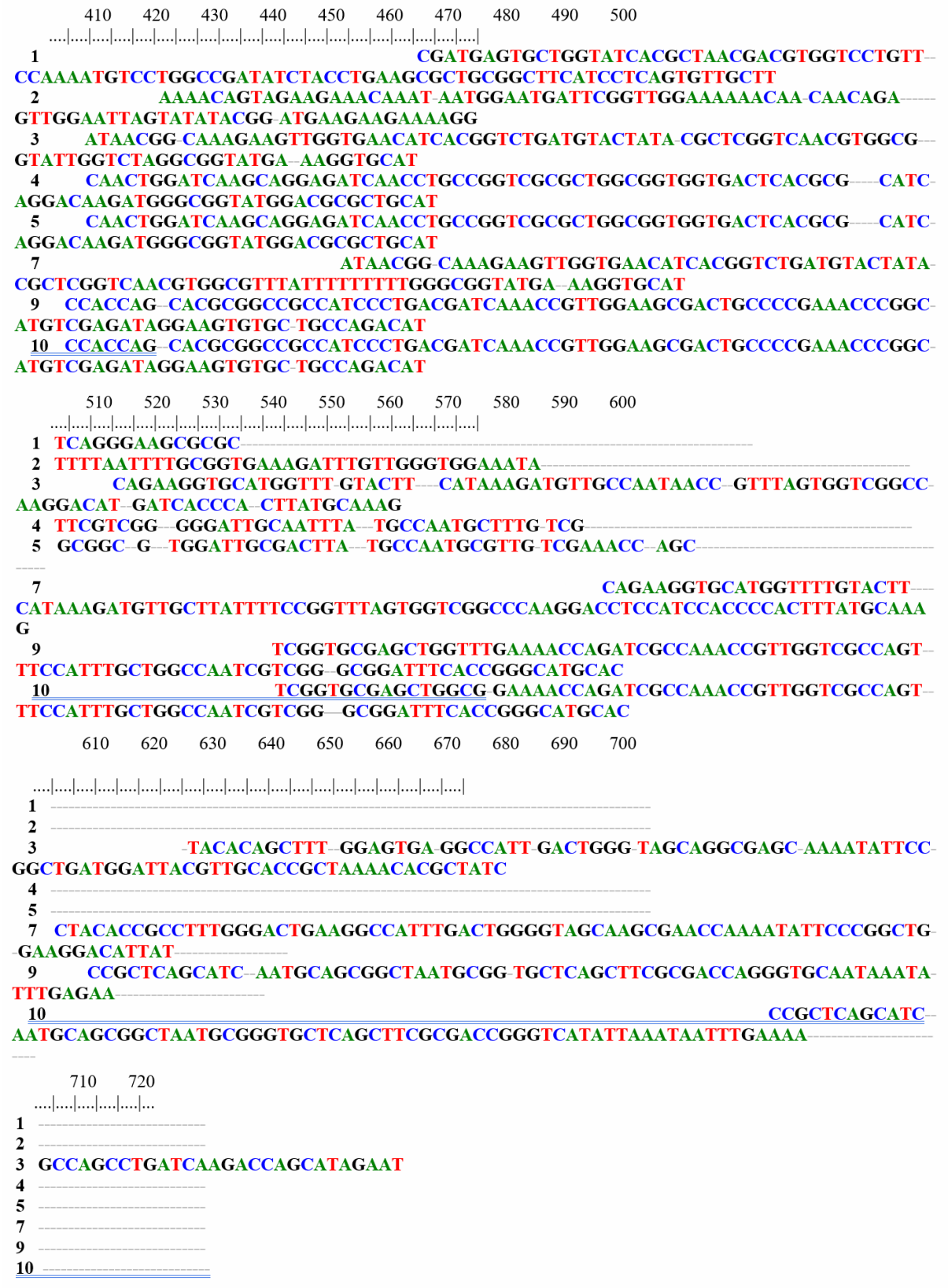

Figure-4: Multiple Sequence alignment

Table-1: Demographic and Clinical Characteristics of the Study Participants (n=113)

Characteristics		n	%
Age (years)	<10	7	6.2
	11–20	4	3.5
	21–30	12	10.6
	31–40	17	15.0
	41–50	22	19.5
	51–60	19	16.8
	61–70	16	14.2
	71–80	10	8.8
	>80	6	5.3
Gender	Male	74	65.5
	Female	39	34.5
Hospital Wards of the Participants	Alnahiyan Unit	9	8.0
	CCU	2	1.8
	Cardiothoracic Surgery	1	0.9
	ENT	1	0.9
	ICU	23	20.4
	Medical	8	7.1
	Nephrology	25	22.1
	Neurology	2	1.8
	Orthopaedics	1	0.9
	Paediatrics	5	4.4
	Surgical	7	6.2
	Urology	29	25.7
Type of Clinical Samples Used for Laboratory Analyses	Blood	9	8.0
	Bone	2	1.8
	Chest Tube	1	0.9
	CSF	1	0.9
	Central Venous Line Tip	2	1.8
	Endotracheal Tube Tip	2	1.8
	Fluid	9	8.0
	Pus	24	21.2
	Sputum	12	10.6
	Tracheal Aspirate	3	2.7
	Urine	48	42.5

Table-2: Association of NDM Gene Status with Participants Characteristics

Study Variables		Total Number within the Category	NDM Gene Status				Significance (*p*-value)
			Positive		Negative		
			n	%	n	%	
Age Groups (Years)	<10	7	2	28.6	5	71.4	0.52
	11–20	4	1	25	3	75	
	21–30	12	0	0	12	100	
	31–40	17	4	23.5	13	76.5	
	41–50	22	5	22.7	17	77.3	
	51–60	19	4	21.1	15	78.9	
	61–70	16	2	12.5	14	87.5	
	71–80	10	3	30	7	70	
	>80	6	1	16.7	5	83.3	
Gender	Male	74	11	14.9	63	85.1	0.09
	Female	39	11	28.2	28	71.8	
Hospital Wards of the Participant	Alnahiyan	9	1	11.1	8	88.9	0.56
	CCU	2	1	50	1	50	
	Cardiotho-racic	1	0	0	1	100	
	ENT	1	0	0	1	100	
	ICU	23	4	17.4	19	82.6	
	Medical	8	2	25	6	75	
	Nephrology	25	9	36	16	64	
	Neurology	2	0	0	2	100	
	Orthopaedics	1	0	0	1	100	
	Paediatrics	5	1	20	4	80	
	Surgical	7	1	14.3	6	85.7	
	Urology	29	3	10.3	26	89.7	
Type of Clinical Samples	Blood	9	1	11.1	8	88.9	0.23
	Bone	2	0	0	2	100	
	Chest Tube	1	0	0	1	100	
	CSF	1	0	0	1	100	
	CVP Tip	2	2	100	0	0	
	ETT Tip	2	1	50	1	50	
	Fluid	9	1	11.1	8	88.9	
	Pus	24	3	12.5	21	87.5	
	Sputum	12	3	25	9	75	
	Tracheal Aspirate	3	0	0	3	100	
	Urine	48	11	22.9	37	77.1	

Table-3: Association of NDM Gene Status with Bacterial Groups

Bacterial Groups	Total Number within the Category	NDM Gene Status				Significance (*p*-value)
		Positive		Negative		
		n	%	n	%	
Overall						
Non-Enterobacteriaceae	74	12	16.2	62	83.8	0.32
Enterobacteriaceae	39	10	25.6	29	74.4	
Between Main Groups						
Non-Enterobacteriaceae						
Acinetobacter	32	9	28.1	23	71.9	0.02*
Pseudomonas	42	3	7.1	39	92.9	
Enterobacteriaceae						
Citrobacter	5	2	40	3	60	0.34
E-coli	13	3	23.1	10	76.9	
Enterobacter	10	4	40	6	60	
Klebsiella	7	0	0	7	100	
Proteus	4	1	25	3	75	

*$p<0.05$

DISCUSSION

Carbapenem-resistant *Enterobacteriaceae* have recently emerged as a major public health problem.[17] The most frequent mechanism of Carbapenem resistance is the production of carbapenemases, comprising of enzymes of classes A, D and B (MBLs), with the corresponding genes often located on plasmids.[21] An escalating number of case reports and surveillance studies unveil a strong association between NDM-positive *Enterobacteriaceae* and prior hospitalization and/or travel to the Indian subcontinent.[19] According to an alert issued in the UK in 2009, an increasing number of CRE strains that were identified in the UK hospitals were mainly from the patients who were initially hospitalized in India and Pakistan and had positive NDM gene.[22] Kumarasamy *et al* documented that amongst a convenience sample of *Enterobacteriaceae* acquired from patients in India, between 31% and 55% of CRE isolates were NDM-producers. Most of these positive isolates were from the patients who had community-acquired infections.[23] Another study has documented the identification and transmission of bacteria containing the NDM in 180 cases, with 37 cases identified in the UK and 143 cases at multiple sites in Pakistan and India, suggestive of an extensive dissemination.[21]

The resistance conferred by NDM is highly alarming as carbapenems are considered to be antibiotics of last resort against MDR bacteria, particularly in ICUs and high-risk wards.[24] Though Carbapenem resistance in *Pseudomonas* and *Acinetobacter* spp is well recognized, resistance among *Enterobacteriaceae* is intensifying and has mounted from zero per cent in 2006 to 8% in 2009 in ICU blood cultures in India.[24] Due to lack of epidemiological data within Pakistan, the exact prevalence of NDM positivity is unknown. Only few studies are available out of which one indicates NDM prevalence in fecal specimens but did not include rest of clinical samples. The second study shows NDM 1 prevalence at two hospitals in Pakistan but did not include gene sequencing for determining the type of NDM genes prevalent in our setup.[20] This study was conducted to ascertain the frequency of NDM producers among Carbapenem-resistant GNB isolated from the clinical samples of hospitalized patients and gene sequencing was done to find out the types of NDM genes prevalent so far in Pakistan.

The frequency of NDM producing GNB was 19.5% in our hospitalized patients. Ninety-one isolates were non-NDM producers. A diverse range of age groups, clinical samples and hospital wards were included in the study. *Non-Enterobacteriaceae* in this study comprised 66% of clinical isolates. Amongst the *Non-Enterobacteriaceae*, *Pseudomonas* was present in 57% of cases, whereas *Acinetobacter* constituted 43% of the non-fermenters. Out of the *Enterobacteriaceae*, E-coli was the major pathogen isolated, constituting about 23% of all organisms in that group. The most vulnerable group harbouring NDM gene consisted of patients between 71–80 years who had 30% positivity for NDM. This could be due to decreased immunity and higher vulnerability of this age group to multiple diseases. Although relatively less number of female patients were included in the study, probably due to low numbers being admitted/visiting during the period of sampling, still the females were found to be at a higher risk, accounting for 28.2% NDM positive isolates, with a female to a male ratio of approximately 2:1. However, this finding was not statistically significant. Given the higher proportion of NDM gene positive samples from the females (28.2% vs. 14.9% in males) and that most of the positive samples were urine sample, we tested whether this finding could be due to a higher prevalence of urinary tract infections (UTIs) in

females. However, we did not find any statistically significant association between the female gender, urine samples, and the NDM gene positivity. The central venous line tip was associated with NDM positivity in all cases.

This study has a few limitations. It was performed in a single institution, and, therefore, may not represent the status of NDM in other parts of the country. The study involved clinical isolates from hospitalized patients, therefore, represents data of indoor patients only. So, it is difficult to assess the frequency of the NDM in the community-based cases. Nevertheless, this study provides important information on the frequency of NDM positive GNB in hospitalized patients and the associations of gene status with different types of clinical samples, age and gender groups of patients. The work can be used as a guideline for further larger studies.

Ongoing disease surveillance and close monitoring of the microbiological and antibiotic resistance patterns in the hospitals in Pakistan is the need of the hour. This will help to modify locally used antibiotics guidelines and infection control measures based on locally relevant evidence. There is also a need for the application of more restrictive infection prevention and control (IP&C) measures especially hand hygiene and environmental cleaning whilst dealing with patients who are likely to develop HAIs. In addition, regular education and training of hospital staffs in IP&C is an important step to deal with the situation.

CONCLUSION

NDM -1 positive GNB are present in our hospitalized patients. Though the frequency is not as alarming as in other regions of South East Asia, yet systemic surveillance network should be established for monitoring these resistant bacteria. The spread of NDM gene in *Enterobacteriaceae* is worrisome since these MDR bacteria could disseminate globally and lead to an extensive and uncontrollable spread of pandemic clones for which efficient antibiotic therapy is currently not available. Appropriate infection control strategies should be emphasized to ensure timely arrest of the dissemination of NDM producing GNB in Pakistan.

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Conflict of interests: We declare that we have no conflict of interests.

AUTHORS' CONTRIBUTIONS

All authors contributed to the design and execution of this work. Faryal Yunus played a major role in study planning, literature review, microbiological and genetic analyses, data compilation and manuscript writing. Faisel Yunus undertook literature review, data analysis and manuscript drafting. Mateen Izhar helped in laboratory analyses and manuscript writing. All authors had an opportunity to contribute to the interpretation of the results and have approved the final manuscript.

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