Characterization of bla_{NDM} Gene in Multidrug-Resistant Pathogens

Ayesha Zaman[1], Hafiz Muhammad Sultan[1]*, Noor Ul Ain Malik[1], Raazia Waseem[2], Jawaria Farooq[3]

[1]Department of Biosciences, COMSATS University Islamabad, Islamabad 45550, Pakistan
[2]Department of Biotechnology, Lahore College for Women University, Lahore 44444, Pakistan
[3]Department of Zoology, Government Sadiq College Women University Bahawalpur, Bahawalpur 63100, Pakistan

Corresponding author: Hafiz Muhammad Sultan, sultanjameel909@gmail.com

Abstract: Antibiotics have been a cornerstone of treatment approaches as they protect against bacteria; however, they are often used without checks and balances. Over time, these antibiotics have become less effective due to bacterial resistance mechanisms. Through natural selection and random mutation, bacterial pathogens have developed resistance against several classes of antibiotics. One such resistance is beta-lactam resistance, caused by gene variants including the NDM gene. To date, comprehensive studies on this gene and its variants are limited in Pakistan. In our present study, multiple antibiotics were used to determine the susceptibility of bacterial samples, including *Acinetobacter*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*. 82% of the samples were bla-NDM gene positive, as determined through molecular characterization using multiplex PCR. Further studies should be conducted on a larger sample scale to obtain more impactful results regarding antibiotic resistance in various bacteria.

Keywords: bla_{NDM}; Gene; Multidrug-resistant pathogens; PCR

1. Introduction

Human mortalities formerly related to bacterial infections before the antibiotic era have tremendously improved since the discovery of penicillin. Penicillin has proven very successful in coping with bacterial infections. However, penicillin resistance has become a significant clinical problem [1]. After the introduction of antibiotics for clinical purposes, the initial optimism that every bacterial infection could be treated successfully with these antibiotics was dispelled due to the emergence of antibiotic resistance in bacterial pathogens [2].

A universal hazard to community well-being is the surge in antibiotic resistance, which often results in antibiotic failure. It is estimated that antibacterial resistance may rise by the year 2050, causing 10 million deaths every year [3]. One of the chief sources of the loss of antibiotic efficacy is the excessive use of antibiotics. Moreover, it is said that about 30 percent of the oral antibiotics prescribed are usually unnecessary [2]. As a result, several strains of pathogenic microorganisms have shown resistance to antibiotics, particularly chemotherapeutic agents [4].

A large number of infections result from frequent pathogens, namely *Enterobacteriaceae*, which are Gram-negative bacteria (GNB). *Enterobacteriaceae* have achieved carbapenem resistance through various molecular processes, mostly by producing carbapenemases [5]. New Delhi metallo-beta-lactamase 1 (NDM-1) was first found in a Swedish patient who was infected with *Klebsiella pneumoniae* and *Escherichia coli*. Antibiotics like tigecycline and colistin are used against the majority of *Enterobacteriaceae* with bla_{NDM-1} and other resistant genes. According to research conducted in Pakistan, in which stool samples were collected from patients at different military hospitals, the occurrence of NDM-1-positive *Enterobacteriaceae* was estimated to be about 18.5% on average. Of these 18.5%, 13.8% were collected from outpatients and 27.1% were from inpatients [6].

The number of bacteria that exhibit resistance against multiple drugs has been growing worldwide and are referred to as multidrug-resistant (MDR) organisms. The fast spread of MDR may depend on multiple factors like restricted laboratory diagnosis, unrestrained or inappropriate use of antibiotics, and the failure of infection-control policies [7]. A metallo-beta-lactamase (*MBL*) gene, which is *NDM-1*, has developed a mechanism to resist carbapenems. These NDM-1-producing strains are becoming a major public concern because of their stability and resistance to all presently available antibiotics [8]. Resistance against carbapenem drugs by carbapenemases has developed due to the substantial use of carbapenems, which are ineffective against these infections. Carbapenemases, especially metallo-beta-lactamases, are of major significance for further studies because of the rise of new variants of IMP and MBLs, such as NDM in the subcontinent. The majority of clinically significant pathogens carry mobile plasmids containing important *MBL* gene clusters including bla_{NDM}, bla_{VIM}, and bla_{IMP}. *Enterobacteriaceae* carbapenem-resistant epidemics are caused by oxacillinases belonging to class D, which have serine β-lactamases [9].

A major cause behind the dissemination of the *NDM-1* gene in India, Pakistan, and the UK is considered to be the availability of easily accessible and convenient transportation. In Pakistan, the exact order of resistance against metallo-β-lactamases has not been extensively investigated [10]. Imipenem was not very common in Pakistan until the end of 2000. *Pseudomonas aeruginosa* isolates collected from Lahore and Karachi were found to exhibit carbapenem resistance. It has been documented that more than 75% of isolates of Acinetobacter baumannii and P. aeruginosa taken from a hospital in Rawalpindi are MBL producers [11]. Bacteria that produce NDM are ineffective against all kinds of drugs and antibiotics.

The bla_{NDM-1} gene has been identified through multiple plasmids in NDM-1 synthesizing bacteria, posing a severe threat to humanity. The increasing worldwide prevalence of carbapenemase (NDM) producing bacterial species is a global threat. The presence of resistant genes in bacterial species like *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. poses a serious threat to healthcare worldwide [12].

In Pakistan, a study reported that the bla_{NDM} gene was present in 23.6% of the 356 clinical samples isolated from the PIMS hospital and 56 samples from Mayo Hospital, Lahore [6]. The bla_{NDM} gene was found in 1.69% of carbapenem-resistant *Acinetobacter baumannii* isolated from different hospitals in Pakistan [13]. The main aims of the current cohort are to optimize bla_{NDM} PCR amplification and sequencing of the bla_{NDM} gene from multidrug-resistant human pathogenic bacteria and to investigate genetic diversity associated with the bla_{NDM} gene. The evaluation of phylogenetic analysis of the bla_{NDM} gene to understand its evolution in our population was also part of our study.

2. Materials and methodology

2.1. Sample collection

The samples for this study were collected from the Microbiology Lab at PIMS, Islamabad, Pakistan. The demographic data of patients, type of infection, and other clinical manifestations were collected after obtaining

patient consent. The samples were inoculated on blood and MacConkey agar plates and incubated aerobically at 35–37°C for 18–24 hours. All gram-negative isolates with reduced susceptibility to imipenem and/or meropenem were included in the study and genetically characterized.

2.2. Phenotypic characterization of bacterial cultures and colonial morphology

For Gram staining, smears were made from a single discrete colony from MacConkey and CLED agar, and slides were stained with Gram stain using Preston and Morrell's modification. Slides were covered with crystal violet and kept for 30 seconds, then washed thoroughly with plain water. Slides were then covered with mordant, lugol's iodine, for 30 seconds, followed by washing with plain water. The iodine was removed with iodine-acetone for 30 seconds, and the slides were washed again. The motility of cultures was observed in a wet mount by the hanging drop method from young cultures of gram-negative isolates.

2.3. Biochemical identification using API 20E

API 20E, a standardized identification system for Enterobacteriaceae and other gram-negative organisms, was used for the biochemical profiling of bacterial cultures. The inoculum was prepared from discrete colonies on MacConkey agar with visible turbidity equivalent to 0.5 McFarland standards. The bacterial suspension was used within 30 minutes. A 0.5% turbidity standard was prepared by adding 0.5 mL of barium chloride-H_2O_2 solution to 99.5 mL of sulfuric acid with constant stirring. Before inoculation of bacterial cultures on API strips, distilled water was added to substrates charged with the prepared bacterial suspension. After 18–24 hours of incubation at 35–37°C, reagents were added and results were noted at 15 minutes. Positive results are shown in **Figure 1**.

Figure 1. API 20E

2.4. Antibiotic susceptibility testing

The bacterial cultures were tested for their susceptibility to different antibiotics. Dried lyophilized powder of Mueller-Hinton agar was weighed and added to measured distilled water in a clean, dry conical flask, then rotated gently to ensure mixing. The flask was removed from the autoclave after the pressure was released completely. Sterile Petri dishes laid on a plain table were filled to a height of 4 mm. The medium was left undisturbed until it set. The suspension was made as turbid equivalent to a 0.5% McFarland standard. The inoculum was spread evenly on the entire surface of the MHA plate in three directions. Antibiotic disks were aseptically placed at a reasonable distance from each other, equidistant on the prepared lawn. Petri plates were incubated at $35 \pm 2°C$ for 16 to 18 hours in ambient air.

2.5. Genetic characterization of bacterial isolates

All the gram-negative bacterial cultures that showed reduced susceptibility to imipenem and/or meropenem

were genetically characterized by an optimized multiplex PCR assay based on the amplification of 16S rRNA and blaNDM genes. A modified CTAB DNA isolation method optimized in the BMBB Lab was used. About 1.5 mL of bacterial culture was taken in a microfuge tube and centrifuged for 30 seconds to pellet the cells. The supernatant was discarded, and this step was repeated until the desired amount of pellet was obtained. 70 µL of 10% SDS was added to the tube and mixed gently, then incubated at 70°C for 20–30 minutes. 10–15 µL of proteinase K was added and incubated at 65°C for 2–3 hours or at room temperature overnight. 100 µL of 5 M NaCl and 100 µL of CTAB were added and vortexed, followed by incubation for 10–20 minutes at 65°C. The sample was centrifuged for 10 minutes at $11,000 \times g$, and the aqueous layer was transferred to another tube. This step can be repeated using chloroform for better results. For the purification of isolated DNA, guanidine isothiocyanate was added in a 1:2 ratio along with 10 µL of silica bead suspension. After mixing, it was incubated for 5–10 minutes at 55°C, then centrifuged for 50 seconds at 13,000 rpm. After centrifugation, the supernatant was discarded. The washing step was repeated 3 times. The pellet was air-dried for 1 hour. 70–100 µl of T.E. + RNase solution was added and the pellet was resuspended by vortexing. DNA was then stored at -20°C for further use.

2.6. Screening of bacterial cultures with 16S rRNA and bla_{NDM} multiplex PCR assay

A multiplex PCR was designed for the amplification of both bla_{NDM} and 16S rRNA genes to determine the prevalence of NDM in bacterial cultures. The 16S rRNA was used as an internal positive control for DNA isolation and PCR amplification. The 16S rRNA gene was amplified using universal 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') primer sets. The annealing temperature for 16S rRNA was 53°C. The bla_{NDM} gene was amplified using specific bla_{NDM} F (5'-AGCCGCTGCATTGATGCTGAGC-3') and bla_{NDM} R (5'-TGCGGGCCGTATGAGTGATTGC-3') primer sets. The typical PCR reaction contained about 50 ng of DNA template, Taq buffer, 200 µM of each dNTP, 1.5 units of Taq DNA polymerase, and 25 pM of each primer. The annealing temperature was 53°C. A 1% agarose gel electrophoresis was used to evaluate the PCR outputs.

2.7. Sequencing, phylogenetic, and diversity analysis of 16S rRNA and bla_{NDM} genes from selected bacterial isolates

The PCR products of the 16S rRNA and bla_{NDM} genes from selected isolates were sequenced separately by Macrogen Inc. (South Korea) Commercial DNA Sequencing Facility. The consensus sequence of forward and reverse strands for respective genes was assembled using DNA Dragon version 1.5.0. The consensus sequence was used to determine the genetic diversity of the blaNDM gene using MatGAT for nucleotide diversity range analysis, while Pair-wise Average Nucleotide Diversity per 100 sites π (Pi) and Watterson's estimator θw (Theta-w) for Population Mutation Rates per 100 sites were determined using DnaSP version 4.5. The sequence of the 16S rRNA gene was used to identify the bacterial isolates using 16S-based ID available at EzTaxon, while the bla_{NDM} gene sequence was used for phylogenetic analysis using Molecular Evolutionary Genetics Analysis software to understand the phylogenetic relations of this gene in the Pakistani population.

3. Results

3.1. Collected Sample Type and Location

The sample cohort was created by collecting 15% blood samples, 22% pus samples, 25% tracheal secretions, and 38% urine (with and without catheter) samples from patients. Different patient groups were assessed, including 32.5% from MICU + SICU, 17.5% from wards, and 50% from OPD.

3.2. Demographical characterization of cohort

In our present cohort, the ratio of males to females was equal, with 50% male and 50% female patients. The study also investigated the frequency of patients in four age groups. Patients under 20 years old comprised 17.5% of the cohort. Patients aged 20 to 40 years made up 45% of the cohort. Patients aged 41 to 60 years constituted 30% of the cohort, and those over 60 years old were 7.5% of the cohort.

A total of four types of isolates were collected: 10% *Acinetobacter*, 15% *E. coli*, 52% *K. pneumoniae*, and 23% *P. aeruginosa*, as shown in **Figure 2**.
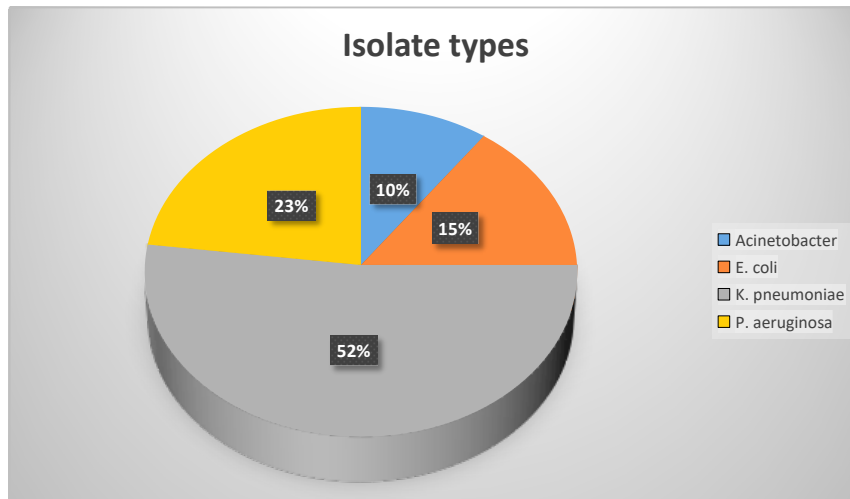

Figure 2. Percentage of each type of isolate

3.3. Susceptibility profile and molecular characterization of *bla*$_{NDM}$ gene

Isolates were selected based on reduced susceptibility to ETP, IPM, and MEM. Among aminoglycosides, AK (80%) was more potent than TOB and CN. The third-generation fluoroquinolone, LEV, was 37.5% susceptible. Chloramphenicol had relatively better coverage than SXT. Isolates were exclusively resistant to ATM, CXM, CFM, CRO, CAZ, FEP, AML, and NA. TGC was 87.1% susceptible to carbapenem-resistant isolates. CT and PB were exclusively effective against these isolates. F was 83.3% susceptible to isolates compared to FOT, both of which were only applied to isolates from urine. These isolates also showed markedly reduced susceptibility to SCF (50%) and TZP (35%), as shown in **Figure 3**. The molecular characterization of the *bla*$_{NDM}$ gene is given in **Figure 4**.
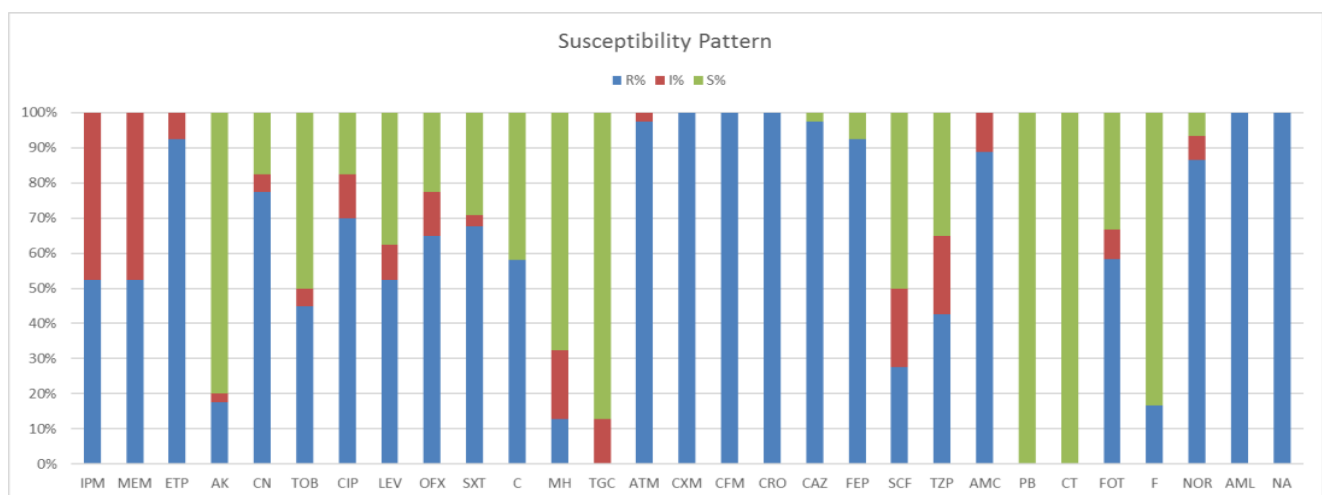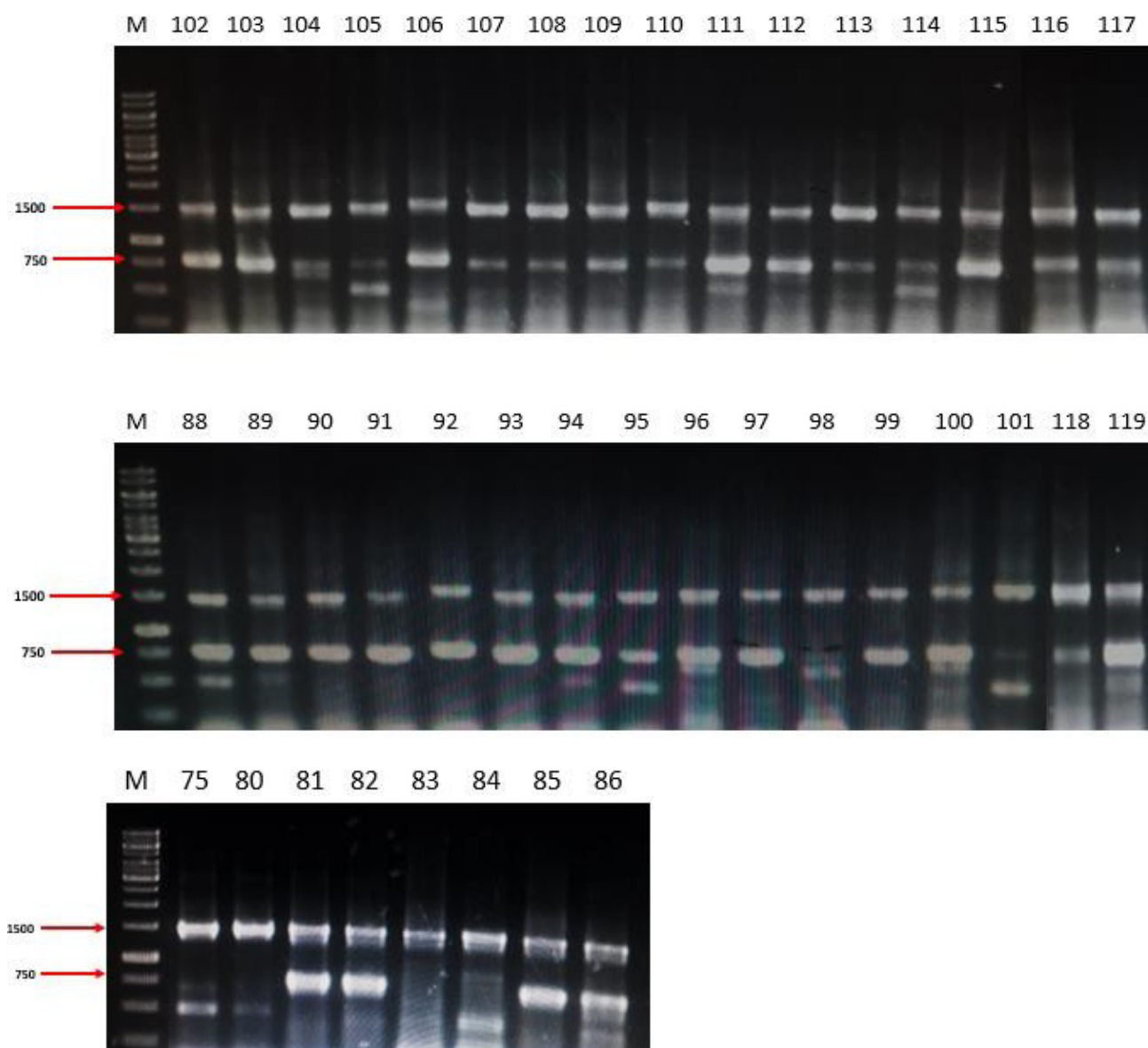

Figure 3. Pattern of susceptibility

Figure 4. PCR amplification for bla_{NDM} and 16s rRNA in multiplex PCR using bla_{NDM} and 16s rRNA primers

4. Discussion and conclusion

In the present study cohort, the minimum age of the patients was 13 years, while the maximum age was 72 years. A total of 32.5% of samples were received from intensive care units, 17.5% from wards, and interestingly, 50% from OPD, indicating that these carbapenem-resistant isolates have reservoirs in the community. Within hospitals, MICU/SICUs are the primary source of carbapenem-resistant isolates. A majority of CRE strains showed that 27.07% were obtained from the ICU unit, 9.94% from the post-anesthesia care unit (PACU), and 5.52% from the surgical ICU. These results are quite similar to our study population, except that we did not follow the PACU [15].

In our study, from the total of 40 samples, 38% were isolated from urine, 15% from blood, 22% from pus, and 25% from tracheal secretions. Urine samples were common in our study, which aligns with a previous study done in Pakistan [15]. The urinary tract was the most common site of infections, followed by bacteremia

for CRE infections. It was reported that MBL producers were most commonly isolated from nasobronchial lavage, followed by pus and urine. In the present cohort, among CR-GNR, 52% were *K. pneumoniae*, 15% were *E. coli*, 23% were *P. aeruginosa*, and 10% were *Acinetobacter*. The prevalence of organisms can vary from institution to institution depending on the use of infection control practices and antibiotics. This finding is similar to a study conducted in Karachi, Pakistan, where 63% were *K. pneumoniae*, followed by *E. coli* at 32%, *Enterobacter* species, *Citrobacter* species at 2% each, and *Serratia* species at 1%. A similar study conducted in Kuwait showed CRE *K. pneumoniae* at 66.6%, *E. coli* at 19%, and *E. cloacae* at 4.7%. In contrast to our findings, a study reported that resistant lactose fermenter *Enterobacteriaceae* isolated from clinical samples were 75% *E. coli*, 15% *K. pneumoniae*, and 5% *Enterobacter aerogenes*.

None of the CRE isolates were susceptible to ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefalothin, cefuroxime, cefoxitin, cefixime, cefotaxime, ceftazidime, ceftriaxone, cefepime, and tobramycin. All the isolates were non-susceptible to ertapenem, meropenem, and imipenem. Tigecycline was found to be the most active agent in vitro, which endorsed our findings. Contrary to our data, one study reported from Pakistan found that 38% of MBL producers were sensitive to the cefoperazone-sulbactam combination and aminoglycosides. Fourteen percent of MBL-producers were sensitive to the ampicillin-clavulanic acid combination. Thirteen percent of the MBL producers were sensitive to the piperacillin-tazobactam combination, and only 11% showed sensitivity to the tetracycline group.

In our study, the bla_{NDM} gene was present in 82% of samples. A study conducted in Pakistan [6] collected clinical samples from two hospitals in Lahore and Islamabad. On PCR amplification, 23.6% of isolates harbored the bla_{NDM-1} gene, 25.1% of isolates had the bla_{VIM} gene, and 1.5% of isolates displayed the bla_{IMP} gene. The bla_{NDM-1} gene is located on remarkably plastic plasmids–genetic structures that can move with ease from one bacterium to another. It is now very clear that bla_{NDM-1} plasmids are highly promiscuous and can easily spread among various species, transmitting resistance genes. Resistance is increasing day by day in our country. Surveillance for antimicrobial resistance among bacterial pathogens in hospitals, particularly in ICU settings with a preexisting higher resistance burden, is mandatory for establishing and/or modifying guidelines for empirical treatment of severe infections in ICU patients caused by these antimicrobial-resistant pathogens. All carbapenem-resistant isolates in their study were bla_{NDM}-positive by PCR [16], which is similar to our findings.

Our study concluded that antibiotic and multidrug resistance are major issues in the field of medicine and health. Excessive use of drugs and genetic mutation have led to the danger of antibiotic resistance. Antibiotics should not be used indiscriminately to avoid resistance in various bacterial strains. We found that a major portion of our patient samples had mutated the bla_{NDM} gene in multiple bacterial strains such as *Acinetobacter*, *K. pneumoniae*, *P. aeruginosa*, and *E. coli*. These results were obtained through molecular characterization using multiplex PCR.

Author contribution

Conceptualization: Ayesha Zaman, Hafiz Muhammad Sultan
Investigation: Ayesha Zaman, Hafiz Muhammad Sultan, Noor Ul Ain Malik, Raazia Waseem, Jawaria Farooq
Formal analysis: Ayesha Zaman
Writing – original draft: Ayesha Zaman
Writing – review & editing: Ayesha Zaman, Hafiz Muhammad Sultan, Noor Ul Ain Malik, Raazie Waseem, Jawaria Farooq

Disclosure statement

The authors declare no conflict of interest.

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