RESEARCH ARTICLE

Dissemination of NDM-1 in pseudomonas aeruginosa and klebsiella pneumoniae isolated from pus samples in tertiary care hospitals of Quetta, Pakistan

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Abstract

Objective: To highlight the prevalence and epidemiology of New Delhi metallo-ß-lactamase-1. producers in pus samples.

Methods: The cross-sectional study was conducted from April to August 2018 at the Biotechnology Laboratory, Balochistan University of Information Technology Engineering and Management Sciences, Quetta, Hi-tech Laboratory, Centre for Advance Studies in Vaccinology and Biotechnology, University of Balochistan, Quetta and Microbiology Laboratory, Bolan Medical Complex Hospital, Quetta, Pakistan. Biochemical and molecular approaches were used for the identification of the isolates and Modified Hodge Test for the phenotypic detection of class-A carbapenemase activity. Minimum Inhibitory Concentration was performed using E-test and broth microdilution method. Molecular basis of carbapenemase activity was ascertained by the recognition of blaNDM-1 gene in the isolates.

Results: Of the 300 pus samples taken from surgical/burn units, 6(2%) blaNDM-1 harbouring isolates were found; 3(50%) each being Klebsiella pneumoniae and Pseudomonas aeruginosa. Klebsiella. pneumoniae isolates were extensively drug-resistant. The Pseudomonas aeruginosa isolates displayed resistance against 21 antibiotics of tetracyclines, quinolones, β-lactams, aminoglycosides, monobactams, sulphonamides, macrolides, cephalosporins, phosphonic acid and polypeptide groups, suggesting pan-drug resistance.

Conclusion: The resistance pattern of the bacterial isolates poses a significant clinical threat in the region.

Keywords: blaNDM-1, Carbapenemase, XDR, PDR, Quetta. (JPMA 71: 228; 2021)

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Introduction

Carbapenem group of antibiotics is one of the most potent drugs that has been used as a last resort to treat infections caused by multi-drug resistant (MDR) gram-negative bacilli.[1] According to standardised international terminology created by the European Centre for Disease Control (ECDC) and the Centre for Disease Control and Prevention (CDC), Atlanta, MDR was defined as non-susceptible to at least one agent in three or more antimicrobial categories, extensively drug-resistant (XDR)as those non-susceptible to at least one agent in all but two or fewer antimicrobial categories, meaning that bacterial isolates remain sensitive to only one or two categories, and pan-drug-resistant (PDR) as those non-susceptible to all agents in all antimicrobial categories.[2] Indiscriminate use of carbapenems without proper diagnosis has, however, resulted in the emergence of carbapenem-resistant bacteria,[3] reported from countries around the globe.[4] To deactivate the drugs, such bacteria encode diverse carbapenemases which are beta (β)-lactamases, having the ability to hydrolyse most β-lactams, including carbapenems[5]. Infections caused by bacteria resistant to carbapenems often fail to respond to conventional therapy, and there are reports that blood stream infections due to such bacteria can lead to 50% mortality.[4] A variety of carbapenemases have been reported in enterobacteriaceae which are classified into Ambler class A, B and D β-lactamases.[4,6] Class-B β-lactamases are considered to be the most efficient carbapenemases that possess highest activity, and often of the imipenemase (IMP), veronaintegron-encoded metallo-β-lactamase (VIM) and New Delhi metallo-β-lactase-1(blaNDM-1) types.[4] The blaNDM-1 is a broad-spectrum β-lactamase with the ability to hydrolyse most of the β-lactams except aztreonam.[6] It was initially discovered in 2008 in a Swedish patient of Indian origin,

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but now have been reported worldwide with most of the cases tracked to India and Pakistan.[7,8] Cases have been reported from various places, including the United Kingdom, Canada, France, China, Japan, Oman, Iraq, Bangladesh and the United States.[8,9] The emergence of carbapenem resistance, mostly associated with enterobacteriaceae, rose with the ability of rapid clonal distribution due to its presence on mobile elements such as plasmids.[7] In the subcontinental context, the isolation of bla$_{NDM-1}$ from common bacterial species, like Klebsiella(K.) pneumonia and Escherichia(E.) coli and approximately 1.5 billion population reservoirs are deeply disconcerting public health worldwide.[4] There are random reports of the isolation of bla$_{NDM-1}$, mainly of E. coli and K. pneumoniae from different parts of Pakistan.[7,10-12] However, such studies have never been reported from the province of Balochistan. The current study was planned to identify the prevalence and epidemiology of bla$_{NDM-1}$ producers in clinical samples from 2 tertiary care hospitals in Balochistan, Pakistan.

Patients and Methods

The cross-sectional study was conducted from April to August 2018 at the Biotechnology Laboratory, Balochistan University of Information Technology Engineering and Management Sciences (BUITEMS), Quetta, Hi-tech Laboratory, Centre for Advance Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, and Microbiology Laboratory, Bolan Medical Complex Hospital, Quetta, after approval from the ethics committee of Bolan Medical Complex Hospital, Quetta.

Patients regardless of gender and age were recruited from surgical wards, outdoor patient departments (OPDs), burns wards, OPD and burns intensive care unit (ICU) of Bolan Medical Complex Hospital and Sandeman Provincial Hospital, two tertiary care hospitals in Quetta, which treat patients from the whole province as well as from Afghanistan. Informed consent was taken from all recruited subjects. The sample size was calculated and interpreted in accordance with the Principles and Procedures of Statistics: A Biometrical Approach.[13] According to the data taken from the burns and surgical units of the hospitals, the number of patients per annum varied from 3000 to 3500 from 2016 to 2018. In 2018, the number was 3048 and 10% of it was taken as the sample size which was rounded off to 300 patients.

Detailed clinical history was recorded for each patient regarding wound, time and duration of pus discharge, previous antibiotic therapy, pre-existing clinical complications and the areas/climate from where they belonged to. Those included were patients taking selected antibiotic therapy after going through culture sensitivity test, and were in good healing condition. Those with irrational, combination and multiple antibiotic therapies or with diabetes history or belonging to poor hygienic environment and were in poor healing condition were excluded.

Those patients were preferred whose pus was still oozing from their wounds after several antibiotic therapies. Antibiotic therapy of the patients was discontinued for 72 hours before sample collection, because specimen contain more bacterial load before antibiotic therapy.[14]

Samples were taken aseptically in sterile 5cc syringes (Becton Dickinson, USA) and with sterilised cotton swabs, autoclaved on 121°C at 15 psi for 20 minutes. The surface area of the wound was cleaned with sterilised cotton. Efforts were made to collect fresh pus from inside the wound after applying slight pressure. Samples were labelled and transported to the microbiology laboratory immediately. All the samples were inoculated within one hour of collection.

Conventional microbiological procedures were adopted for bacterial isolation from the pus samples. Each sample was streaked simultaneously onto Blood, MacConkey and Cetrimide agar plates (Oxoid, United Kingdom) and were incubated aerobically at 37°C for 24 hours.[15] Plates were observed for bacterial growth and the isolated colonies were further triple-cloned. Isolates were identified by using the analytical profile index, (API) 20E and API 20NE system (bioMerieux, France) according to the manufacturer's directions. Bacterial genomic deoxyribonucleic acid (DNA) was extracted (Thermo Scientific Genomic Purification Kit, Lithuania), following the manufacturer's instructions. Further, 16S ribosomal DNA (rDNA) gene was amplified using universal primers, 27F-5'- AGA GTT TGA TCC TGG CTC AG -3' and RD1-5'- AAG GAG GTG ATC CAG CC -3' for the amplification of internal fragment of 1500bp with initial denaturation 95°C for 2 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 2 minutes at 72°C. The final extension was carried out at 72°C for 10 minutes.[16]

Standardised antibiotic sensitivity test was performed on Mueller-Hinton agar plates using disc diffusion Kirby Bauer technique and 0.5 McFarland turbidity standard methods. Interpretation of the results was based on the Clinical and Laboratory Standards Institute (CLSI) guidelines.[17] The minimum inhibitory concentrations (MICs) were determined by the E-test method (Oxoid UK, Liofilchem, Italy) and broth microdilution method. Results were interpreted according to CLSI[17] and Food

and Drug Administration (FDA)[18] breakpoints and recommendations.

Modified Hodge Test (MHT) was performed for the phenotypic detection of class Acarbapenemase enzyme activity of bacterial isolates, as described by CLSI.[17] American Type Culture Collection (ATCC) strains of E. coli 25922, K. pneumoniae BBA 1705 as positive control, and K. pneumoniae BBA 1706 as negative control, were used in the assay.

Plasmid DNA was extracted for selected phenotypically carbapenem-resistant isolates using Gene JET Plasmid Miniprep Kit (Thermo Fisher Scientific, Lithuania), according to the manufacturer's instructions. The bla$_{NDM-1}$ gene was amplified by polymerase chain reaction (PCR) using primers; blaNDM-1 F-5'- GGG CAG TCG CTT CCA ACG GT-3' and blaNDM-1 R-5'- GTA GTG CTC AGT GTC GGC AT -3'. The conditions for PCR were set toinitial denaturation at 95°C for 5 minutes, followed 30 cycles of 95°C for 40 seconds, 58°C for 30 seconds, 72°C for 30 seconds with final extension 72°C for 5 minutes.[6,19]

Sequencing of the PCR product of 16S ribosomal ribonucleic acid (rRNA) and bla$_{NDM-1}$ genes of the representative samples was carried out commercially through Macrogen, South Korea. Deduced sequences were aligned using basic local alignment search tool (BLAST).[20]

Results

Of the 300 samples, 100(33.3%) were from the burns wards, and 50 (17.7) each from the burns OPD, burns ICU, surgical OPD and surgical wards. Overall, 127(42.33%) samples showed no bacterial growth, 173(57.66%) exhibited bacterial growth on blood, MacConkey and Cetrimide agar plates after aerobic incubation at 37°C for 24 hours. Based on cultural, biochemical and morphological characterisation, 9 different bacterial types were tentatively identified, with: staphylococcus (S.) aureus being the most common (Figure-1).

All gram-negative isolates of different spp. subjected to antibiogram were sensitive to a range of antibiotics (Table-1) except P. aeruginosa 3(1%) and K. pneumonia 3(1%). These 6(2%) isolates were tested against 21 antibiotics. *P. aeruginosa* isolates Pa-1, Pa-152 and Pa-287exhibited a high level of resistance against β-lactam and non β-lactam groups of antibiotics and were kept in the PDR category (Figure-2). *K. pneumoniae* isolates Kp-58, Kp-117 and Kp-269 were resistant to β-lactams as well but Kp-58 and Kp-269 were sensitive to polymyxin B, tigecycline and colistin, whereas Kp-117 was sensitive to tigecycline only. The resistance pattern of these isolates indicated they were XDR (Table-2).

Out of the 6 suspected bla$_{NDM-1}$ producers, 5(83.3%) gave positive clover-leaf-like indentation, hileKp-269 was negative.

All the 6(100%) isolates were successfully amplified for the 16S rDNA gene. The retrieved sequences showed

Table-1: Sensitivity pattern of non- Multi Drug Resistant (MDR)Pseudomonas (P.) aeruginosa and Klebsiella (K.) pneumoniae isolates.

Bacterial strains Drug resistance	TOB	SXT	CIP	CN	CRO	TZP	AMC	CAZ	OFX	CPM	AK	MEM	IMP
P.aeruginosa	59%	33 %	69 %	43 %	NT	88 %	NT	52 %	61 %	58 %	86 %	100 %	100 %
K. pneumoniae	NT	38 %	77 %	NT	72 %	92 %	47 %	49 %	74 %	55 %	90 %	100 %	100 %

Abbreviations: NT= Not tested, TOB= Tobramycin (10µg), SXT=Cotrimoxazole (25µg), Cip= Ciprofloxacin (5µg), CN=Gentamycin (10µg), CRO= Ceftriaxone (30µg), TZP= Piperacillin/tazobactam (100/10µg), AMC=Amoxicillin/clavulanic acid (30µg), CAZ= Ceftazidime (30µg), OFX= Ofloxacin (5µg), CPM= Cefepime (30µg), AK= Amikacin (30µg), MEM= Meropenem (10µg), IMP= Imipenem (10µg).

Table-2: Susceptibilities of blaNDM-1 producers.

Isolate	IPM	TGC	CIP	CRO	PB	CT	Other resistance markers (Disc diffusion Method)
		MICs (µg/dL) E-test			MICs (µg/mL) Broth microdilution		
Pa-1	>32 R	>256 R	> 32	NT	8 R	16 R	ETP, MEM, SXT, TZP, AK, CN, FOS, CLR, EN, OFX, CPM, TOB, LEV, NOR, CAZ
Kp-58	16 R	2 S	> 32	>32 R	1 S	1.5 S	ETP, MEM, SXT, AMC, TZP, AK, CN, AMP, TE, CE, FOS, CLR, EN, OFX, CXM,
Kp-117	16 R	0.5 S	> 32	>32 R	16 R	8 R	ETP, MEM, SXT, AMC, TZP, AK, CN, AMP, TE, CE, FOS, CLR, EN, OFX, CXM,
Pa-152	>32 R	>256 R	> 32	NT	8 R	16 R	ETP, MEM, SXT, TZP, AK, CN, FOS, CLR, EN, OFX, CPM, TOB, LEV, NOR, CAZ
Kp-269	>32 R	0.12 S	> 32	>32 R	0.5 S	1 S	ETP, MEM, SXT, AMC, TZP, AK, CN, AMP, TE, CE, FOS, CLR, EN, OFX, CXM,
Pa-287	16 R	8 R	> 32	NT	16 R	24 R	ETP, MEM, SXT, TZP, AK, CN, FOS, CLR, EN, OFX, CPM, TOB, LEV, NOR, CAZ

Pa, Pseudomonas aeruginosa; Kp, Klebsiellapneumoniae; NT, not tested;IMP, imipenem; TGC, tigecycline; CIP, ciprofloxacin; CRO, ceftriaxone; PB, polymyxinB; CT, colistin; ETP, ertapenem; MEM, meropenem; SXT, trimethoprim/sulfamethoxazole; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; AK, amikacin; CN, gentamycin; AMP, ampicillin; TE, tetracycline; CE, cephradine; FOS, fosfomycin; CLR, clarithromycin; EN, enoxacin; OFX, ofloxacin; CXM. Cefixime; TOB, tobramycin; LEV, levofloxacin; NOR, norfloxacin; CAZ, ceftazidime.

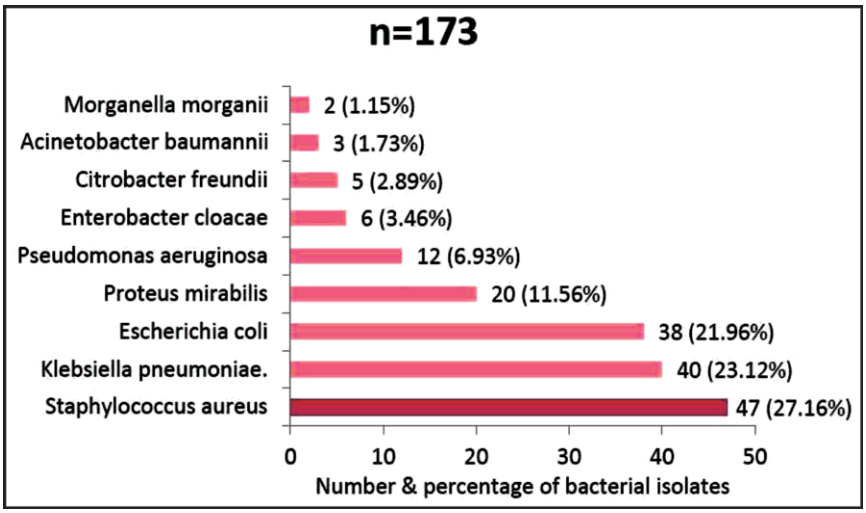

Figure-1: Abundance of bacterial isolates in pus samples.

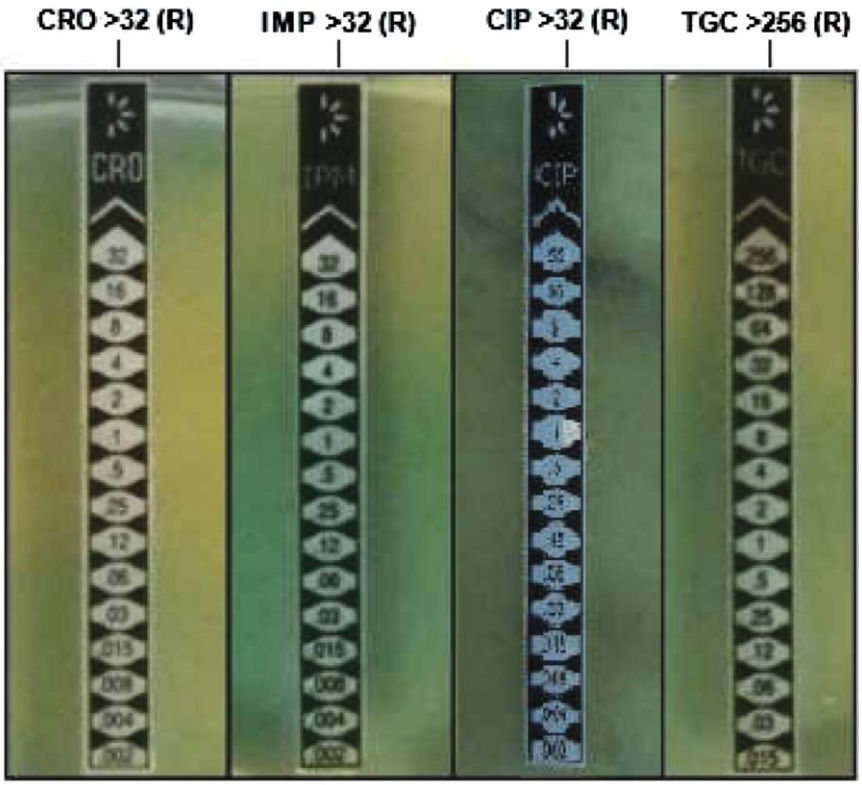

Figure-2: Minimum Inhibitory Concentrations (MICs) of Pan-Drug Resistant (PDR) Pseudomonas (P.) aeruginosa.

100% similarity to K. pneumoniae and P. aeruginosa.

Representative PCR-amplified products post-sequencing showed 99% similarity to the bla$_{NDM-1}$ genes.

Discussion

The current study isolated 6 NDM-1 positive isolates; three each of K. pneumoniae with XDR and P. aeruginosa with PDR pattern. In previous studies, NDM-1-positive isolates were predominantly K. pneumonia.[7,21] Concerning resistance pattern, previous studies depicted K. pneumoniae resistant to tigecycline and Fosfomycin, but sensitive to colistin,[22] while divergent observations were recorded in the present work where K. pneumoniae isolate117 was sensitive to tigecycline but resistant to polymyxin-B and colistin, showing probable genetic diversity of the isolate. Similarly, P. aeruginosa was reported to be extremely sensitive to carbapenems, like meropenem (69.64%) and imipenem (78.57%), while it was resistant to aztreonam (71.43%).[23] P. aeruginosa isolates 1, 152 and 287 in the current study displayed outstanding resistance against 21 antibiotics belonging to cephalosporins, monobactams, quinolones, tetracyclines, β-lactams, aminoglycosides, macrolides, phosphonic acid, penicillins, polypeptides and sulphonamides groups of antibiotics. The MICs of imipenem, tigecycline, ciprofloxacin, ceftriaxone, polymyxin-B and colistin exhibited XDR pattern for K. pneumoniae and PDR pattern for P.aeruginosa.

Second NDM-1 K.pneumoniae clone was isolated and identified after 61 days of hospital admission in the same patient.[24] In the current study, on follow-up after about 3 months of initial sample collection, a patient of Afghan origin admitted in surgical-II ward BMCH, harbouring NDM-1 positive K.pneumoniae with XDR was screened again and found with NDM-1 harbouring P.aeruginosa and PDR pattern.

Prevention of nosocomial infections can reduce propagation of MDR bacterial strains as well mortality and morbidity.[25] The current study found that the same dressing table was in use in burns units which was a playing major role in the dissemination of such resistant

superbugs.

Researchers working on enterobacteriaceae family have shown that bla_{NDM-1} encoding genes are mostly present on plasmids.[7,26] All the bla_{NDM-1} encoding genes in the current study were amplified from plasmid DNA. Our results indicated that sensitivity of Modified Hodge Test in detecting class-A carbapenemases was 83.3%.

Conclusion

Three isolates of P. aeruginosa displayed PDR against all the 21 antibiotics tested. The spread of NDM-1, antimicrobial resistance and the scarcity of new antibiotics, particularly in gram-negative bacteria, is quite alarming globally. The spread of these highly resistant bacterial pathogens can be minimised, particularly in the developing countries like Pakistan, by adopting standard waste treatment and disposal practices. Irrational use of antibiotics may also have been a key factor behind these resistant superbugs.

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Conflict of Interest: None.

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References

1. Zhu YQ, Zhao JY, Xu C, Zhao H, Jia N, Li YN. Identification of an NDM-5-producing Escherichia coli Sequence Type 167 in a Neonatal Patient in China. Sci Rep. 2016;6:29934.
2. Basak S, Singh P, Rajurkar M. Multidrug resistant and extensively drug resistant bacteria: A study. J. Pathog. 2016; 2016:4065603.
3. Karuniawati A, Saharman YR, Lestari DC. Detection of carbapenemase encoding genes in Enterobacteriace, Pseudomonas aeruginosa, and Acinetobacter baumanii isolated from patients at Intensive Care Unit Cipto Mangunkusumo Hospital in 2011. Acta Med Indones. 2013;45:101–6.
4. Nordmann P. Naas T, Poirel L. Global Spread of Carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2011;17:1791–98.
5. Queenan AM, Bush K. Carbapenemases : the Versatile beta - Lactamases. Clin Microbiol Rev. 2007;20:440–58.
6. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrob Agents Chemother. 2010;54:969–76.
7. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother. 2009;53:5046–54.
8. Kumarasamy K, Toleman MA, Walsh TR, Jay B, Fafhana B, Ravikumar B, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK. A molecular, biological, and epidemiological study. Lancet Infect Dis. 2010;10:597–602.
9. Giske CG, Monnet DL, Cars O, Carmeli Y. Clinical and Economic Impact of Common Multidrug-Resistant Gram-Negative Bacilli. Antimicrob Agents Chemother. 2008;52:813–21.
10. Khan E, Irfan S, Sultan BA, Nasir A. Dissemination and spread of New Delhi metallo-beta-lactamase-1 superbugs in hospital settings. J Pak Med Assoc. 2016;66:999–1004.
11. Jones LS, Carvalho MJ, Toleman MA, White PL, Connor TR, Mushtaq A, et al. Characterization of plasmids in extensively drug-resistant Acinetobacter strains isolated in India and Pakistan. Antimicrob Agents Chemother. 2015;59:923–9.
12. Sattar H, Toleman M, Nahid F, Zahra R. Co-existence of bla NDM-1 and bla KPC-2 in clinical isolates of Klebsiella pneumoniae from Pakistan. J Chemother. 2016;28:346–9.
13. Steel RGD, Torrie JH. Principles and Procedures of Statistics: A Biometrical Approach 2nd ed. McGraw-Hill:NewYork; 1980.
14. Harris AM, Bramley AM, Jain S, Arnold SR, Ampofo K, Self WH, et al. Influence of antibiotics on the detection of bacteria by culture-based and culture-independent diagnostic tests in patients hospitalized with community-acquired pneumonia. Open Forum Infect Dis. 2017;4: ofx014.
15. Duggal S, Khatri PK, Parihar RS, Arora R. Antibiogram of Various Bacterial Isolates from Pus Samples in a Tertiary Care Centre in Rajasthan. Int J Sci Res. 2015;4:1580–4.
16. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S Ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173:697–703.
17. CLSI. Perfor CLSI. Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute. Available from:URL:www.clsi.orgmance
18. Nicolau DP, Quintana A, Korth- Bradley JM, Wible M, Dowzicky MJ. A rationale for maintaining current tigecycline breakpoints as established by the USA food and drug administration. Arch Clin Microbiol. 2015; 6: 1–12.
19. Mushtaq S, Irfan S, Sarma J, Doumith M, Pike R, Pitout J, et al. Phylogenetic diversity of Escherichia coli strains producing NDM-type carbapenemases. J Antimicrob Chemother. 2011; 66: 2002-5.
20. Altschul SF, Gish W, Miller W, Myers EW. Lipman D.J. Basic local alignment search tool. J Mol Biol. 1990; 215: 403-410.[Online] [Cited 2018 October 08]. Available from: URL: https://https://blast.ncbi.nlm.nih.gov/Blast.cgi.
21. Dong F, Zhang Y, Yao K, Lu J, Guo L, Lyu S, et al. Epidemiology of Carbapenem-Resistant Klebsiella pneumoniae Bloodstream Infections in a Chinese Children's Hospital: Predominance of New Delhi Metallo-β-Lactamase-1. Microb Drug Resist. 2018; 24: 154-60.
22. Oteo J, Domingo-García D, Fernández-Romero S, Saez D, Guiu A, Cuevas O, et al. Abdominal abscess due to NDM-1-producing Klebsiella pneumoniae in Spain. J Med Microbiol. 2012;61:864–67.
23. Javiya VA, Ghatak SB, Patel KR, Patel JA. Antibiotic susceptibility patterns of Pseudomonas aeruginosa at a tertiary care hospital in Gujarat, India. Indian J Pharmacol. 2008;40:230–34.
24. Hernández-García M, Pérez-Viso B, León-Sampedro R, Navarro-San Francisco C, López-Fresneña N, Díaz-Agero C, et al. Outbreak of NDM-1-CTX-M-15-DHA-1 producing Klebsiella pneumoniae high-risk clone in Spain due to an undetectable colonized patient from Pakistan. Int J Antimicrob Agents 2019;54:233-9.
25. Azimi L, Alaghehbandan R, Asadian M, Alinejad F, Lari AR. Multi-drug resistant Pseudomonas aeruginosa and Klebsiella pneumoniae circulation in a burn hospital, Tehran, Iran. GMS Hyg Infect Control. 2019;14:Doc01.
26. Marquez-Ortiz RA, Haggerty L, Olarte N, Duarte C, Garza-Ramos U, Silva-Sanchez J, et al. Genomic Epidemiology of NDM-1-Encoding Plasmids in Latin American Clinical Isolates Reveals Insights into the Evolution of Multidrug Resistance. Genome Biol Evol. 2017;9:1725–41.