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Molecular Characterization Of Carbapenem-Resistant *Klebsiella Pneumoniae* Isolated From Wound Infections

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Abstract
Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates can increase the mortality among hospitalized patients as a result of serious infections. Molecular studies provide valuable information about the mechanisms of resistance among the resistant isolates. The aim of this study was to investigate the phenotypic and molecular characteristics of the carbapenem-resistance *K. pneumoniae* isolates and detect the Metallo-Beta-Lactamases (MBLs) encoding genes in these isolates which obtained from patients with wound infections hospitalized in two hospitals in Baghdad, Iraq. In this study**,** 55 *K. pneumoniae* isolates were collected from wound swabs of patients, who referred to Al-Kindy and Al-Yarmook hospitals in Baghdad during January to October 2018. Resistance to carbapenems was investigated by disc-diffusion and E-test methods. Ethylenediaminetetraacetic acid (EDTA)-combine disc (ECD) methods were performed to determine carbapenemases. Carbapenemase-encoding genes including *bla*NDM-1, *bla*OXA-48, and *bla*KPC were investigated by polymerase chain reaction (PCR). Among the 55 *K. pneumoniae* isolates, 18 (32.7%) were resistant to Imipenem, whereas 11 (20%) were resistant to Meropenem. All carbapenems-resistant *K. pneumoniae* isolates (n=18) were positive with MBL production. Also, the resistances to third and fourth generation cephalosporins were taken into consideration. The *bla*OXA-48, *bla*NDM-1 and *bla*KPC were found in 83.3% (n = 15), 55.5% (n = 10) and 16.6% (n = 3), of the resistant isolates, respectively. Phylogenetic analysis showed very less variation in *bla*NDM-1 gene with respect to *bla*NDM-1 possessing *K. pneumoniae* isolates from other parts of Iraq and abroad. In conclusion**,** the present study revealed a high frequency of MBL production and the presence of *bla*OXA-48 among local *K. pneumoniae* strains, this study indicated that resistance to carbapenems and isolation of bacteria producing *bla*OXA-48 and *bla*NDM-1 is increasing.

Keywords: Carbapenems Resistance, *Klebsiella pneumoniae*, MBL genes.

INTRODUCTION

One of the most important emerging carbapenem-resistant bacteria is *Klebsiella pneumoniae* [1] which is an opportunistic pathogen which accounts for many of nosocomial infections including urinary tract infections, blood stream infections, and pneumonia [2]. Three main classes of carbapenemases including *K. pneumoniae* carbapenemase (KPC) that was mostly identified in *K. pneumoniae*. KPC is able to hydrolyze penicillins, carbapenems, cephalosporins and aztreonam. Class B metallo-beta-lactamase (MBL) is sensitive to metallic ion chelator like ethylenediaminetetraacetic acid (EDTA) because it is zinc dependent. New Delhi metallo-b-lactamase (NDM) and Imipenemase (IMP) are the most important enzymes in this class. NDM-1 and Class D, oxacillinase (OXA), have spread widely in the world and have many variations in hydrolyzing penicillins and carbapenems [3, 4, 5].The gene *bla*OXA-48 had first been identified in *K. pneumoniae* in Turkey and also recorded in Europe and the Middle East [6]. According to the Center for Disease Control and Prevention (CDC) in the USA, approximately 8.7% of *Klebsiella* nosocomial isolates in 2006-2007 were carbapenem-resistant compared to less than 1% in 2000 [7]. It was found that the expression of MBLs genes is considered among the main reasons of dominating resistance strains of Enterobacteriaceae pathogens and thus spreading nosocomial infections in Iraqi clinical centers [8].

Due to increasing risk of wound infections caused by carbapenem resistant *K. pneumoniae* strains and lack of local information about the frequency of resistant genes which responsible for carbapenems antibiotic resistance. The present study aimed to investigate the antibiotic susceptibility pattern of *K. pneumoniae* isolates and determine the frequency of MBLs producing *K. pneumoniae* obtained from patients with wound infections in Baghdad hospitals by phenotypic and genotypic methods.

MATERIALS AND METHODS

Isolation and identification of *K. pneumoniae*

This study was performed at Al-Kindy and Al-Yarmook hospitals in Baghdad during January to October 2018. Out of 400 wounds swab samples, a total of 55 isolates were collected from wound infections. Blood agar and McConkey agar were used for isolation *K. pneumoniae* and CHROM agar Orientation medium for rapid detection of these bacteria. The isolates were identified according to the biochemical tests, with an API 20E system (bioMerieux, France).

Antibiotic Susceptibility Test

This test was conducted using Kirby Bauer method by agar diffusion test. *K. pneumoniae* colonies were took from overnight growth on Blood agar and resuspended in Mueller-Hinton broth (HiMedia, India). This suspension was adjusted to an equivalent 0.5 McFarland and used for testing the susceptibility of bacterial isolates on Mueller-Hinton agar (HiMedia, India). Meropenem (MRP 10 μg), Imipenem

(IMP 10 μg), Ceftazidime (CZX 30 μg), Cefotaxime (CTX 30 μg), Cephalexin (CN 30 μg), Ceftriaxone (CTR 30 μg), Cefixime (CFM 5 μg), Cefepime (FEP 30 μg) (MAST, UK) were placed on the Mueller-Hinton agar plates, the incubation was at 35 °C for 18 h. and then the inhibition zone was measured and the data were compared according to the test cultures by CLSI. The results were interpreted by CLSI breakpoint interpretative Criteria [9].

Screening MBLs producing isolates by phenotypic method

For the CD assay, a bacterial suspension equivalent to 0.5 McFarland was inoculated onto a Muller-Hinton's (MH) agar plate (HiMeda, India). Two 10 μg disks of each carbapenem (IMI and MEM) were initially placed on the MH plates and 10 μL of MBL inhibitor solution (0.5 M EDTA) was added to one of them to obtain the 750 μg concentration. After a 24 hour incubation period at 35°C, inhibition zone diameter of each disk was measured and compared with each other. If the difference of inhibition zone between carbapenem disk and carbapenem-EDTA disk was ≥ 7mm, the isolate was considered as a MBL-producer. MBL E-test (imipenem (IMI) [4-256 μg/mL] and imipenem/EDTA (IMD) [1-64 μg/mL]) was performed according to the manufacturer's recommendations. Briefly, the strips were placed onto MH plates that had been inoculated with a bacterial suspension equivalent to 0.5 McFarland and incubated at 35°C. The test was considered positive when the IMI/IMD ratio was ≥ 8 μg/mL [10].

DNA extraction and identification of MBL genes by Polymerase Chain Reaction (PCR)

Bacterial DNA was extracted from cells by using DNA extraction Kit (Promega, USA) according to the procedure of the manufacture. The DNA concentration was estimated by spectrophotometer. The Primer sequences, which were used for detection of MBL genes in this study, were as in Table 1.

Table 1. Primer sequences for PCR detection of MBLs genes in *K. pneumoniae.*

Target gene	Oligonucleotide primer sequence 5' to 3'	Amplicon size (bp)	Reference
*bla*KPC F *bla*KPC R	5'-CGTCTAGTTCTGCTGTCTTG-3' 5'-CTTGTCATCCTTGTTAGGCG-3'	798	[11]
*bla*NDM-1F *bla*NDM-1R	5′- CAACTGGATCAAGCAGGAGA-3′ 5′- TCGATCCCAACGGTGATATT-3′	621	[12]
*bla*OXA-48 F *bla*OXA-48 R	5'-TGTTTTTGGTGGCATCGAT-3' 5'-GATCGCGATTCCAAGTGG-3'	300	[11]

PCR conditions for amplification of MBLs gene was carried out by the thermocycler (Applied Biosystems, Malaysia) as the references in table 1. Amplification steps include a 5 min denaturation at 95°C, followed by 36 cycles of 94°C for 45 sec, 53°C for 45 sec, and 72°C for 1 minute. Final extension was at 72°C for 6 min (for the genes *bla*OXA-48 and *bla*KPC) while the gene *bla*NDM-1 the conditions as follows: initial denaturation at 94°C for 10 min, denaturation at 94°C for 30 sec, annealing at 56°C for 40 sec, and extension at 72°C for 50 sec, was repeated for 36 cycles; a final extension at 72°C for 5 min.

Agarose gel electrophoresis was done a 2% agarose gel at 80V for 2 hours. After electrophoresis fragments were stained by Ethidium Bromide, and then visualized with ultraviolet light.

Phylogenetic analysis

For phylogenetic analysis, the neighbour-joining method was used to construct phylogenetic trees for the nucleotide sequences, using the program MEGA 6.

RESULTS AND DISCUSSION

In this study, Out of the 400 wound infections samples, 55 *K. pneumoniae* isolates (13.7%) were obtained from wound swabs of patients who referred to Al-Kindy and Al-Yarmook hospitals in Baghdad during January to October 2018. Biochemical tests and API 20E system (bioMerieux, France) were used for the identification of *K. pneumoniae* isolates.

Among *K. pneumoniae*, 18 (32.7 %) isolates were found to Metallo-Beta-Lactamase (MBL) positive, while 37 were MBL negative. The detection of MBL enzymes by *K. pneumoniae* isolates was conducted by CD method as shown in figure 1.

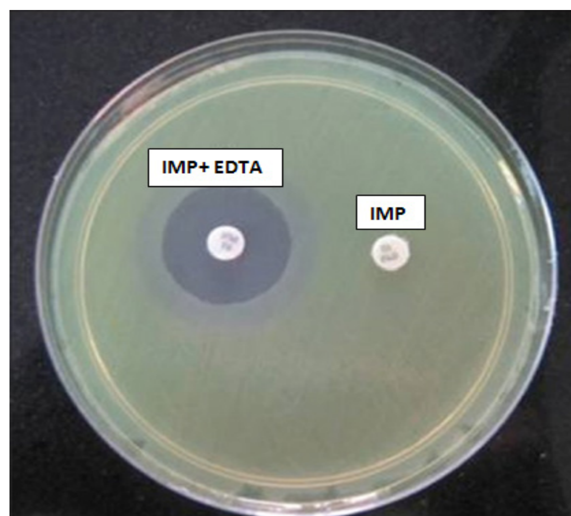

Figure 1. Phenotypic Detection of Metallo-Beta-Lactamase (MBL) in *K. pneumoniae* by the Combined Disk Test (CD Test). Two Imipenem disks (IMP 10 μg), plus 750 μg EDTA on the Left disk; IMP/EDTA-IMP = 8.6 mm (MBL positive if ≥6 mm).

Our study revealed that 32.7 % of *K. pneumoniae* isolates to be MBL producers. The study conducted in Greece, 2014 to 2016, indicated that Carbapenemase production was confirmed in 389 isolates (98.7%) with a positive combination disk test, including 262 (66.5%), 54 (13.7%), 34 (8.6%) and 14 (3.6%) cases of *bla*KPC,

*bla*NDM, *bla*VIM and *bla*OXA-48- like genes, respectively [13]. In another study, the screening for carbapenemase production in *K. pneumoniae* isolated from different clinical specimens by MHT was positive in 90 out of 370 isolates. Of the total isolates, KPC screening test using ertapenem and boronic acid was showed positive in 14 isolates [14]. Oberoi *et al*. (2013) [15] showed that the major ESBL and AmpC producer was *Escherichia coli*, while *K. pneumonia* was the predominant MBL producer. The co production of the ESBL/MBL/ AmpC β- lactamases was observed in 19.04% of strains and a multidrug resistance to the fluoroquinolones and the aminoglycosides was also observed in the β-lactamase producing organisms.

Table 2. Outlines patterns of susceptibility and resistance among MBL and non-MBL pathogens to 8 commonly used beta-lactam antibiotics. It was obvious that the most of MBL producers *K. pneumoniae* isolates were resistant to carbapenemes (Imipenem and Meropenem) with 100% resistance for Imipenem (n=18) and 61.1% for Meropenem (n=11). Also, the results revealed that the most isolates were resist to Cefotaxime (100%), Cephalexin (100%) and Ceftriaxone (94.4%). All antibiotics had a high resistance among MBL producers when compared to non-MBL resistant isolates. Among non-MBL producers, moderate resistance was recorded for Cefotaxime, Cephalexin and Ceftriaxone and high sensitivity for other antibiotics.

Table 2. Percentages of antimicrobial susceptibility rate of *K. pneumoniae* isolates against 8 antimicrobial agents.

Antibiotic*	Positive MBL isolates (N= 18)			Negative MBL isolates(N= 37)
	Resistant	Intermediate	Sensitive	Resistant
IPM	18 (100 %)	0(0.0 %)	0(0.0 %)	3 (8.1 %)
MEM	11 (61.1 %)	1 (5.5 %)	6(33.3 %)	0 (0.0 %)
CN	18 (100 %)	0 (0 %)	0 (0 %)	23 (62.1 %)
CTX	16 (100 %)	0 (0 %)	2 (11.1 %)	20 (54.0 %)
CTR	17 (94.4 %)	0 (0.0 %)	1 (5.5 %)	16 (43.2 %)
CAZ	14 (77.7 %)	2 (11.1 %)	2 (11.1 %)	14 (37.8 %)
CFM	10 (55.5 %)	2 (11.1 %)	6 (33.3 %)	9 (24.3 %)
FEP	10 (55.5 %)	0 (0.0 %)	8(44.4 %)	11 (29.7 %)

Meropenem: MEM, Imipenem: IMP, Ceftazidime: CAZ, Cefotaxime: CTX, Cephalexin: CN, Ceftriaxone: CTR, Cefixime CFM, Cefepime: FEP.

The study of Agha-Seyed Hosseini *et al.* (2016) [16] in Kashan, Iran, indicated that among 181 *K. pneumoniae* isolates, 26.5% of the isolates were Imipenem-resistant and the isolates showed high resistance to Ampicillin, Cefalotin, and Cefotaxime. It was found that nearly 50% of *K. pneumoniae* that first colonized the patient's wounds was resistant to third-generation cephalosporins, fluoroquinolones and aminoglycosides. Approximately two-thirds of *Klebsiella* septicaemias are caused by multidrug-resistant strains [17]. Also there was a resistance to penicillin and third generation cephalosporins, mediated by the production of ESBL, AmpC β-lactamase and carbapenemase for *K. pneumoniae*. Blood stream infections caused by antibiotic-resistant *K. pneumoniae* are associated with a mortality rate of 24% to 72% [18, 19].

The phenotypically identified MBL-producing *K. pneumoniae* were subjected to PCR using *bla*OXA-48 and *bla*KPC and *bla*NDM-1 specific primers. Of the 18 phenotypically MBL-producing *K. pneumoniae* isolates, the *bla*OXA-48, *bla*NDM-1 and *bla*KPC were found in 83.3% (n = 15), 55.5% (n = 10) and 16.6% (n = 3), of the resistant isolates, respectively. The results of PCR-products electrophoresis showed genomic patterns related to *bla*NDM-1 (621 bp), *bla*KPC (798 bp)and *bla*OXA-48 (300bp) (Figures 2, 3 and 4).

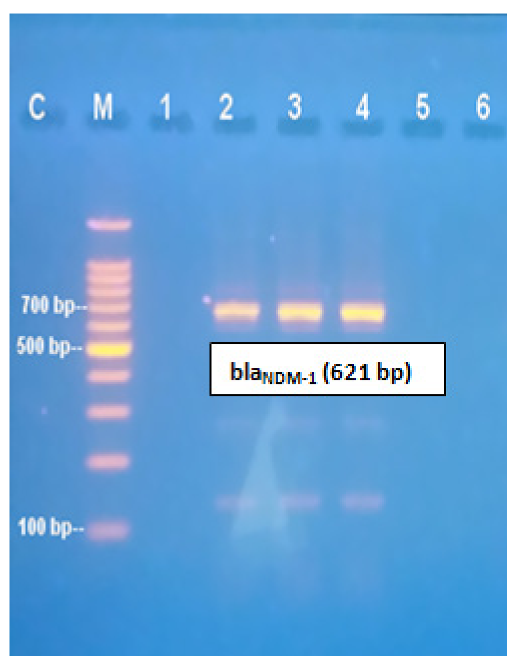

Figure 2. Electrophoresis of the amplified products of *bla*NDM-1 (621 bp) genes by a PCR in a 2 % agarose gel. Lane 2 to 4; positive result of gene detection in *K. pneumoniae* isolates. Lane C, Negative control. Lane M, 100 bp DNA ladder.

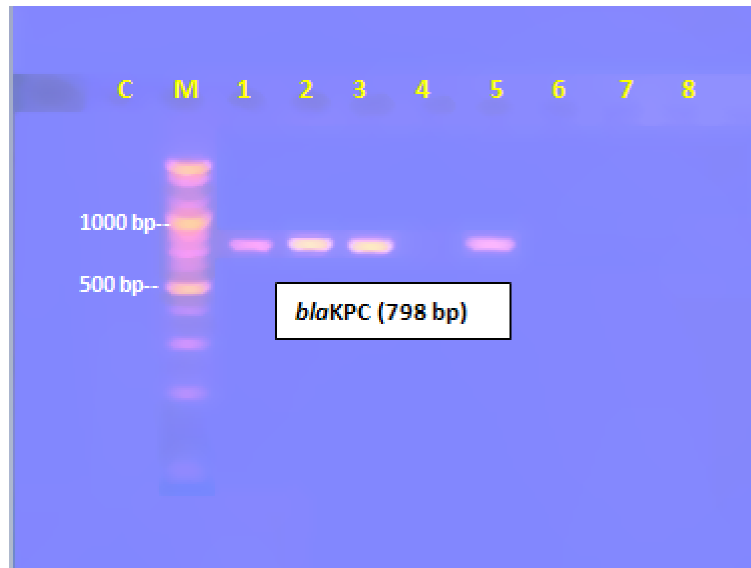

Figure 3. Electrophoresis of the amplified products of *bla*KPC (798 bp) genes by a PCR in a 2 % agarose gel. Lane 1,2,3 and 5; positive result of gene detection in *K. pneumoniae* isolates. Lane C, Negative control. Lane M, 100 bp DNA ladder.

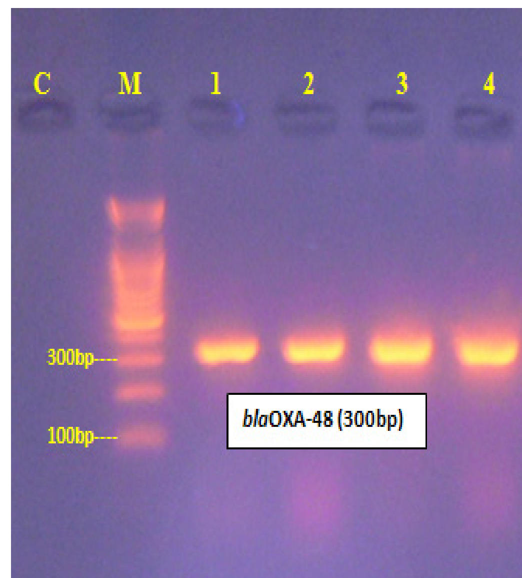

Figure 4. Electrophoresis of the amplified products of *bla*OXA-48(300 bp) genes by a PCR in a 2 % agarose gel. Lanes 1,2,3 and 4; positive result of gene detection in *K. pneumoniae* isolates. Lane C, Negative control. Lane M, 100 bp DNA ladder.

Sakkas *et al*. (2019) [20] demonstrated that 19 out of 24 *K. pneumoniae* isolates were resistant to carbapenems (79.2%), Nineteen *K. pneumoniae* isolates positive for class A (*bla*KPC) and class B (*bla*NDM and *bla*VIM) genes by immunochromatographic test (IT) were screened against *bla*KPC, *bla*NDM, *bla*OXA-48 and *bla*VIM genes using the appropriate primers. The multiplex PCR reaction produced amplicons with the expected length of 798bp, 621bp, 438bp and 390bp respectively. The study on *K. pneumoniae* isolated from Lebanon revealed that all of the isolates were resistant to Ertapenem together with Imipenem and/or Meropenem. Phenotypic resistance was due to bla_{OXA-48}, bla_{NDM-1}, bla_{NDM-7}, or the coupling of ESBLs and outer membrane porin modifications [21].

The Carbapenem-resistant Enterobacteriaceae (CRE) in the university-associated hospitals in Ahvaz city, Iran, 9.9% were resistant to carbapenems. Combined-disk test (CDT) was positive in 62 (86.1%) of the 72 carbapenem-resistant isolates suggesting Metallo-β-lactamase production, of which. *E. coli* had highest rate (45.2%) followed by *K. pneumoniae* (30.7%). None of MBL producer Enterobacteriaceae was positive for the NDM-1 gene by PCR assay [22]. Our study was in agreement with the results of Al-Zahrani *et al*. (2018) [11] who found that the major type of carbapenemases was *bla*OXA-48 with 81.5% and it seems to reach an endemic level. New Delhi metallo-ß-lactamas (NDM) was the second most frequent carbapenemase among *K. pneumoniae* isolated from 2 largest hospitals in Abha city in Saudi Arabia. *Klebsiella pneumoniae* isolates producing *bla*OXA-48 carbapenemase was first identified in the Middle-East (in Turkey) and has rapidly spread globally, and is considered the most common

carbapenemase in the Middle-Eastern countries [23]. The bla_{OXA-48} gene was detected in 27/28 of *K. pneumoniae* isolates were obtained from clinical specimens taken from patients hospitalized in a burn unit from Motahari Hospital, Teheran, Iran [24].

The phylogenetic relationships among 2 *K. pneumoniae* from Baghdad and other sequences obtained from GenBank were investigated. Figure 5 showed the phylogenetic tree of 8 closely related nucleotide sequences of $bla_{\text{NDM-1}}$ genes of *K. pneumoniae* isolates.

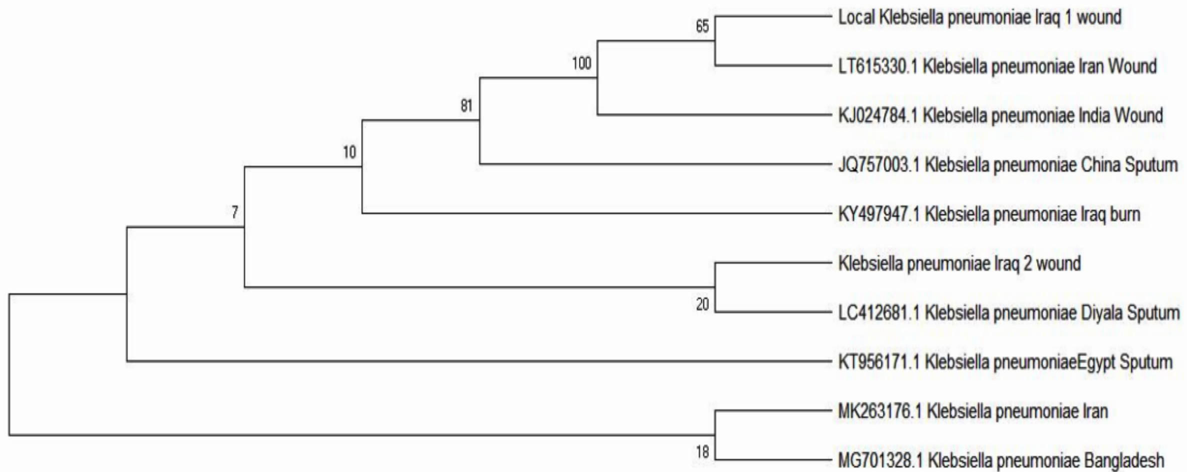

Figure 5. Phylogenetic relationships based on partial nucleotide sequence of the *bla*NDM-1 genes of *K. pneumoniae* local isolates (K1 and K5). Cluster analysis was based upon the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method.

The phylogenetic tree based on the *K. pneumoniae* gene sequence homologies was divided into many branches. The majority of *K. pneumoniae* isolates formed an independent lineage distinct from other *K. pneumoniae* isolates. There were 100% identities between the local isolate and *K. pneumoniae* strain 327 isolated from wound infection in Iran (accession number LT615330) (figure 6). The progressive multiple sequence alignment identity among the sequences in this lineage was between 99.8% and 100%. The results of multiple sequence alignment revealed very high identity (more than 99 %) of *bla*NDM-*1* gene of local isolates with the most global isolates and strains from Asian countries such as Iran, China and India especially with isolates from wound infections.

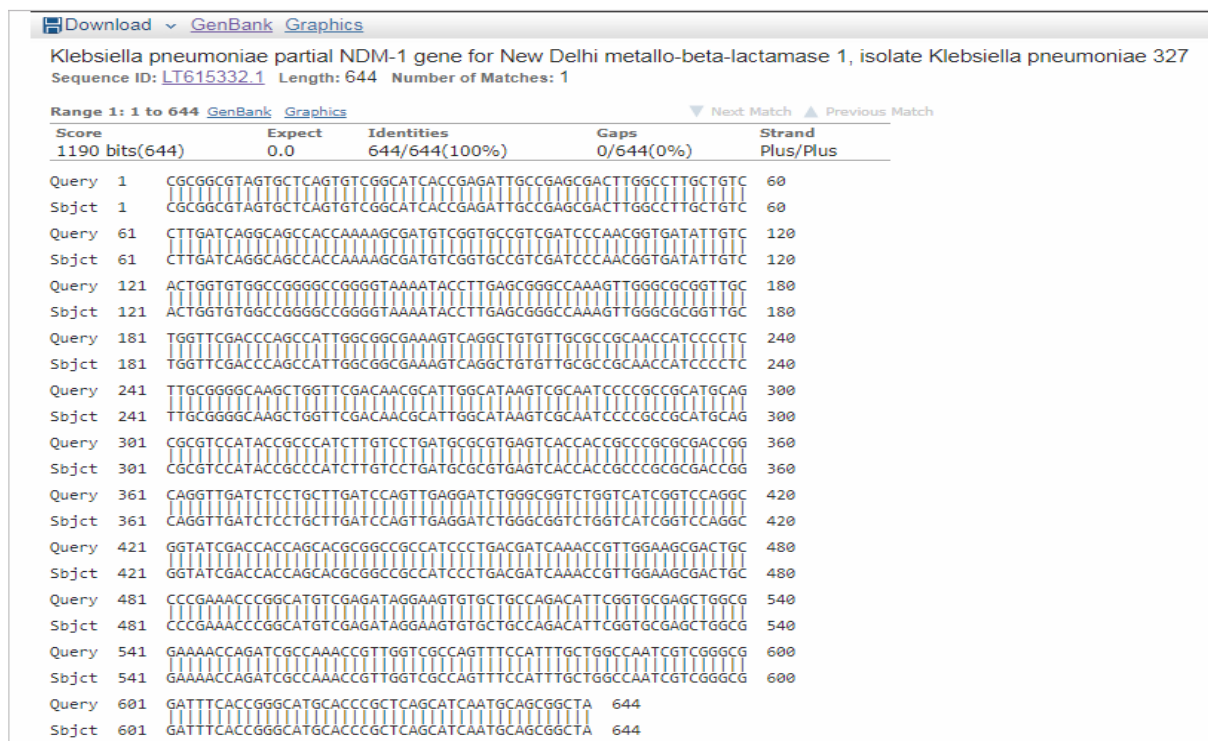

Figure 6. Alignment of *K. pneumoniae bla*NDM-1 gene sequence from this study with reference strain *K. pneumoniae* 327 available in GenBank.

The rapid emergence of antibiotic resistance among nosocomial pathogens is a major concern for both developed and developing countries. The high prevalence of NDM-1 producing bacteria may be facilitated by the conditions like overcrowding, misusing of antibiotics, low level of hygiene, and weak local antibiotic policies. Transmission of NDM-1 producing bacteria mainly takes place by faecal-oral route and inadequate sewage system further intensifies the problem [25]. Carbapenemase-producing bacteria have been expanding and are endemic in several European countries, the Middle East, South America and Asia that causes a serious challenge in the diagnosis and treatment of nosocomial infections. On the one hand, choosing the drug for treatment of these infections are inherently limited [26].

CONCLUSION

Carbapenem resistant *K. pneumoniae* have been considered as one of the greatest threats to the global health care. The high prevalence of *bla*NDM-1 producing *K. pneumoniae* have heightened this threat. Development of quick, effective molecular diagnostic techniques for identification of MBL resistance genes can significantly improve treatment protocols. The presence of genes involved in the development of multi-drug resistance and antibiotic therapy should be evaluated for facilitating efficient infection control and reducing the escalation of resistance.

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