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*bla*KPC, *bla*NDM AND *bla*OXA-48 *Carbapenemases* AMONG *Klebsiella pneumoniae* IN HOSPITALIZED PATIENTS: FIRST REPORT FROM NORTHWEST PAKISTAN

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ABSTRACT

Klebsiella pneumoniae causes infections especially in hospital environment. Emergence of antibiotics resistance among *K. pneumoniae* is a global concern. Resistance through Carbapenemase production reported in *K. pneumoniae.* The purpose of the study is to find the frequency of *K. pneumoniae*, related antibiogram, and phenotypic and molecular detection of carbapenemases. A total of 5475 samples were processed for culture, in which 1140 exhibited bacterial growth. Among 1140 culture positive samples, 110 isolates were *K. pneumoniae.* Phenotypic and molecular methods were used for detection of carbapenemases. All the isolates were resistant to Fosfomycin, cefotaxime, gentamicin and cloxacillin. 65.4% (n=72) isolates were resistant to meropenem and 57.2% (n=63) to imipenem. *bla*NDM gene was most prevalent (22.7%) followed by *bla*KPC (13.6%); however, *bla*OXA-48 was detected in one isolate. In the undertaken study, antibiotic resistance was increasingly observed in *K. pneumoniae* through carbapenemases. The current work will help to devise an appropriate antibiotic hospital policy to reduce K. *pneumoniae* resistance burden.

KEYWORDS:
Klebsiella pneumoniae, Hospitalized, Carbapenemase procuction, *bla*KPC, *bla*NDM, *bla*OXA-48

INTRODUCTION

Klebsiella pneumoniae is bacterial pathogen causing nosocomial infections. Aminoglycosides and third-generation cephalosporin are used to treat *K. pneumoniae* infections. The prevalence rate of *K. pneumoniae* among urinary tract infections is 90% with a resistance of 70% against cephalosporins [1]. Among health care facilities, resistance becomes higher up to 80-95% against cephalosporins and quinolones [2]. The emergence of antibiotics resistance limits the choice of available antibiotics to treat *K. pneumoniae* infections. *K. pneumoniae* resistance to cephalosporin and carbapenem is reported due to the production of carbapenemase [3]. Three classes of carbapenemase were detected, involving the emergence of class A *Klebsiella Pneumoniae* Carbapenemase, class B Metallo Beta-Lactamase and class D Oxacillinases [4]. The prevalence rate of carbapenemase-producing *K. pneumoniae* is causing global health threats against mankind [2]. Carbapenemase-producing *K. pneumoniae* were reported in Pakistan, throughout which the presence of NDM was explicitly high while the existence of KPC among *K. pneumoniae* was also reported [5]. The recent biochemical and molecular studies documented the presence of these mutative genes of *bla*KPC and *bla*MBL. Resistance against extended-spectrum cephalosporins are also found in *K. pneumoniae* either plasmid-mediated or chromosomally encoded [6]. There is ultimate concern regarding the high prevalence of carbapenemases in different parts of the world [7]. Limited data found on carbapenemase detection in *K. pneumoniae* from Pakistan. The undertaken study reported the prevalence of *blaK*PC, *bla*NDM and *bla*OXA-48 types of carbapenemase detection in *K. pneumoniae*. The finding of the current work may be helpful for further characterization of molecular drug resistance in *K. pneumoniae*.

MATERIALS AND METHODS

Sampling. The samples of urine, pus, sputum, and blood were collected from different wards and then processed for culture identification at Tertiary Care Hospital. The study duration was from January 2017 to December 2019.

Isolation and identification of *K. pneumoniae.* Samples were inoculated on MacConkey agar (Oxoid, UK). Biochemical identification of *K.*

pneumoniae was performed on API 20E (BioMerieux, USA).

Antibiotics susceptibility test. Antibiotics susceptibility evaluation were performed by Kirby Bauer disc diffusion method [8]. Isolates of *K. pneumoniae* were inoculated on Muller Hinton Agar (Oxoid, UK) while antibiotic disks were placed on plates aseptically. Antibiotic disks (Oxoid, UK) used in the study were ampicillin (AMP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cloxacillin (OB), ampicillin-sulbactam (SAM), aztreonam (ATM), cephradine (CE), cefixime (CFM), cefotaxime (CTX), ceftriaxone (CRO), cefepime (FEP), cefoperazone-sulbactam (SCF), imipenem (IMI), doripenem (DR), ertapenem (ETP), meropenem (MEM), doxycycline (DO), amikacin (AK), gentamicin (CN), tigecycline (TG), ciprofloxacin (CIP), norfloxacin (NOR), levofloxacin (LEV), , moxifloxacin (MOX), nitrofurantoin (F), fosfomycin (FOS) and colistin (CT). The result was interpreted according CLSI (2018).

Phenotypic detection of carbapenemase production. Modified carbapenem inactivation method (MCIM) was done as described earlier [9]. All carbapenem-resistant *K. pneumoniae* isolates were subjected to carbapenemase production. Briefly, *K. pneumonia* isolates were suspended in 1ml of tryptic soya broth (Oxoid, UK) along with meropenem disc (10 µg) and incubated at 35°C. Meropenem disc was set on MHA inoculated with 0.5 McFarland of *Escherichia coli* (ATCC 25922) and incubated overnight at 37°C. In the case of *K. pneumoniae,* the result was interpreted as the presence of pinpoint colony in the range of 6-18mm zone of inhibition was considered as carbapenemase producer [10].

Phenotypic detection of KPC, NDM, and OXA-48 among *K. pneumoniae.* A combined disc method was used for KPC, NDM, and OXA-48 carbapenemase detection. These phenotypic tests were performed on MHA plates with imipenem discs having carbapenemase inhibitors. For *blaKPC* production, a single imipenem disc (10µg) and the second disc containing imipenem (10µg) with APBA (Acetyl phenylboronic acid, 600µg) on MHA streaked plate with *K. pneumoniae* [11]. For NDM production, *K. pneumoniae* was streaked on MHA plates. A combination of imipenem (10µg) with 0.5 M dehydrated EDTA while another disc containing single imipenem (10µg) was placed on MHA [12]. Oxacillinase (OXA) production was checked with a disc carrying temocillin (30µg) [13].

Molecular detection of *bla*KPC, *bla*NDM and *bla*OXA-48 carbapenemase. Boiling method was used for DNA extraction [14]. PCR amplification was performed using *bla*KPC*, bla*NDM *and bla*OXA-48 specific genes primers as described [15]. PCR amplification condition involved the first round of initial denaturation for 10 min at 94°C and then 36 cycles of denaturation at 94°C, annealing at 52°C and extension at 72°C. PCR product was resolved on agarose gel electrophoresis and visualized under UV light [16, 17].

RESULTS

A total of 1146 (20.9%) samples exhibited bacterial growth while remaining 4329 (79.1%) showed no growth. Among the culture positive samples, 110 (9.6%) isolates were positive for *K. pneumoniae.*

In sample demographics, *K. pneumoniae* isolates from female were 41 (37.2%) while from male were 69 (62.8%). It was observed that age group of 19-50 years encountered the most (n=60; 54.5%) with *K. pneumoniae* infections while the less prevalent (n=5; 4.5%) in 5-12 years of age.

K. pneumoniae (n=110) isolates exhibited increased susceptibility towards cefoperazone/sulbactam (n=105; 95.4%) and cefixime (n=99; 90%). Similarly, more susceptibility was observed against cephradine (83.5%) and amoxicillin-clavulanic acid (72.7%). The resistance towards gentamicin, cloxacillin and cefotaxime was significantly high (100%) (Table 1).

Modified carbapenem inactivation method showed that 31 *K. pneumoniae* isolates were carbapenemase producer while 79 (71.9%) were carbapenemase non-producer. Phenotypic detection showed that 10 (13.6%) *K. pneumoniae* isolates possessed KPC carbapenemase while 21 (22.7%) isolates of *K. pneumoniae* possess NDM carbapenemase. OXA was only detected in one (0.9%) sample (Figure 1, Table 2).

Molecular detection of carbapenemase through PCR showed that *bla*KPC was detected among 15 (13.6%) isolates while *bla*NDM was detected among 25 (22.7%) isolates. Simultaneously the occurrences of *bla*OXA-48 were detected in one isolate. The prevalence rate of *bla*KPC is remarkably low as compared to *bla*NDM both on phenotypic and molecular methods (Figure 2, Table 2).

DISCUSSION

Antibiotic resistance in *K. pneumoniae* is a major health threat that limits the treatment options. This study reported the increased resistance towards diverse classes of antibiotics. *K. pneumoniae* was 100% resistant to ampicillin, cloxacillin, cefotaxime and fosfomycin. cefotaxime was frequently used antibiotics among respiratory infections while fosfomycin was the drug of choice in UTIs. *K. pneumoniae* was susceptible > 90% to few antibiotics such as cefoperazone/sulbactam, ertapenem,

TABLE 1
Antibiotic susceptibility pattern of *K. pneumoniae*.

Antibiotics	Resistant N (%)	Sensitive N (%)
1. Bacterial cell wall inhibitors		
a. Beta-lactam drugs		
Cloxacillin	110 (100)	0 (0)
Ampicillin	110 (100)	0 (0)
Amoxycillin-clavulanic acid	20 (27.3)	80 (72.7)
Ampicillin-sulbactam	55 (50)	55 (50)
Piperacillin-tazobactam	27 (24.6)	83 (75.4)
b. Monobactam		
Aztreonam	11 (10)	99 (90)
c. Cephalosporins		
Cefixime	11 (10)	99 (90)
Cephradine	17 (16.55)	93 (83.5)
Ceftriaxone	93 (83.5)	17 (16.5)
Cefotaxime	110(100)	0 (0)
Cefepime	51 (46.3)	59 (53.7)
Cefoperazone-sulbactam	5 (4.6)	105 (95.4)
d. Carbapenem		
Imipenem	63 (57.6)	37 (42.4)
Ertapenem	10 (7.3)	100 (92.7)
Meropenem	72 (65.4)	38 (34.6)
Doripenem	0 (0)	110 (100)
2. Cell membrane inhibitors		
Colistin	11 (10)	99 (90)
3. Protein synthesis inhibitors		
a. Aminoglycosides		
Gentamicin	99 (90)	11 (10)
Amikacin	44 (40)	66 (60)
b. Tetracycline		
Doxycycline	94 (85.5)	16 (14.5)
4. DNA gyrase inhibitors		
Ciprofloxacin	60 (54.5)	50 (44.5)
Levofloxacin	70 (63.6)	40 (36.4)
Moxifloxacin	40 (36.4)	70 (63.6)
Norfloxacin	38 (34.6)	72 (65.4)
5. Others antibiotics		
Nitrofurantoin	55 (50)	55 (50)
Tigecycline	66 (60)	44 (40)
Fosfomycin	110 (100)	0 (0)

Note: The Resistance/Sensitive was calculated and interpreted as per criteria given in CLSI 2018.

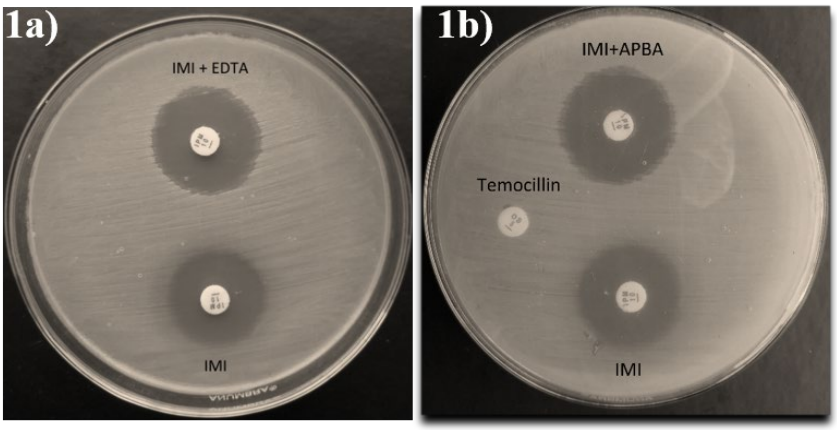


FIGURE 1
Phenotypic detection of KPC, NDM and OXA carbapenemase.
(1a) Shows MHA plate containing two discs IMI+EDTA combined and single IMI, combined disc showed slightly greater zone of inhibition (> 4 mm). (1b) shows MHA plate containing two discs IMI+APBA combined and single IMI, combined disc showed significantly greater zone of inhibition (>4 mm) than IMI alone. In the case of temocillin disc showed no zone of inhibition (< 10 mm).

TABLE 2
Detection of *bla*KPC, *bla*NDM and *bla*OXA-48 in *K. pneumoniae* isolates.

Methods	Carbapenemases								
	KPC N (%)		P value	NDM N (%)		P value	OXA-48 N (%)		P value
	Positive	Negative		Positive	Negative		Positive	Negative	
Phenotypic	10 (9.0)	100 (91.0)	0.288	21 (19.0)	89 (81.0)	0.507	1 (0.9)	109 (99.1)	1
Molecular	15 (13.6)	95 (86.4)	0.288	25 (22.7)	85 (77.3)	0.507	1 (0.9)	109 (99.1)	1

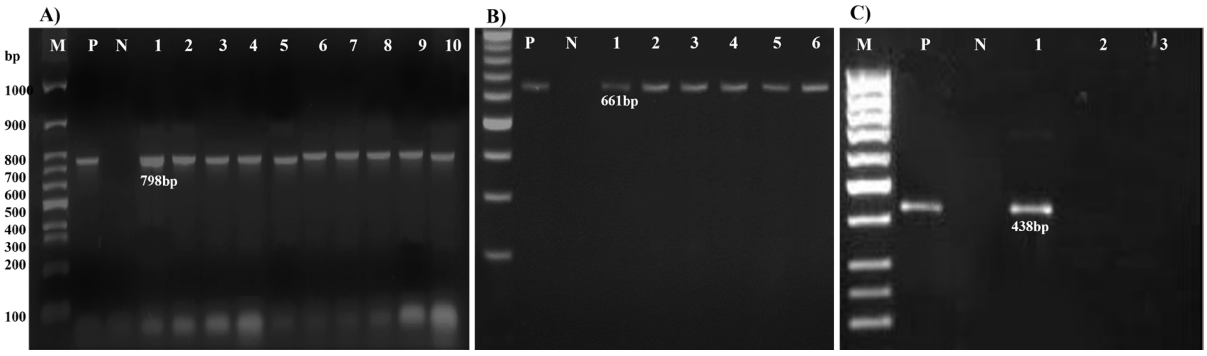


FIGURE 2
Molecular detection of *bla*KPC, *bla*NDM and *bla*OXA-48
A) Detection of blaKPC (798bp), B) Detection of blaNDM (621bp), C) Detection of blaOXA-48 (438bp) M: DNA marker, P: Positive control, N: Negative control, 1-10: Sample of *K. pneumoniae*.

aztreonam, cefixime and colistin. Many reports are published about the resistance of *K. pneumoniae* towards antimicrobial agents [18, 19].

The resistance is because of the production of carbapenemase which causes inactivation of beta-lactam by hydrolysis mechanism [20]. The reason for spreading resistance in developing countries is the misuse of antibiotics such as in Pakistan. However, resistance of *K. pneumoniae* against quinolone was relatively low as compared to cephalosporin ranging from 17-53%, according to surveillance report 10-40% of the resistance of *K. pneumoniae* towards quinolone [21]. As discussed before excessive and frequent use of cephalosporin leads to resistance which developed a trend to switch towards alternative therapy, aminoglycosides, and quinolone [22, 23]. Judicious use and structure dissimilarities are the reasons which overcome the resistance problem. Amoxicillin/clavulanic acid showed a lower picture of resistance rate as compared to other studies [24]. In some cases, carbapenem has excellent efficacy against *K. pneumoniae* infection. These antibiotics are not commonly used so least resistance is observed in *K. pneumoniae*. The *K. pneumoniae* in hospitalized patients was found highly resistant than non-hospitalized [2].

The *K. pneumoniae* isolates were proved resistant and significantly carbapenemase producers by confirmation on a phenotypic test [25, 26, 27]. MCIM was considered accurate for the detection of carbapenemase. The current study showed 28.1% of *K pneumoniae* carbapenemase producer which is in line with other study performed at Rawalpindi, which showed 29% of carbapenemase producer [10]. It is to mention that even one study reported 63% carbapenemase producing *K pneumoniae* [28]. Combined disk test confirmed KPC (n=16; 9%) and NDM (n=21; 19%) which considerably higher than the study performed in 2016 [5]. Another study phenotypically detected that the presence of Metallo beta-lactamase (MBL) was high in Pakistan [29, 30]. MBL is an emerging carbapenemase among the Indian and Pakistan subcontinent [31].

Carbapenemase among *K. pneumoniae* are reported in various region of Pakistan [31]. Molecular study detected KPC 13.6% (n=15) which is in line with previous work [5]. The prevalence of NDM was 22.7% (n=25) that agreed with the study conducted at Karachi [28]. NDM become an emerging threat for European countries due to active tourism causes carriers of NDM [31]. The presence of OXA-48 was recorded low in this region. Although previous study suggested the existence of OXA-48 in Pakistan [32]. Most of the patients visited to Pakistan were found with OXA-48 while hospitalized due to some infection [33].

CONCLUSIONS

The production of carbapenemase among *K. pneumoniae* is an emerging threat. Which causes difficult to treat life-threatening infections caused by *K. pneumoniae.* In the undertaken study resistance in *K. pneumoniae* was highly prevalent through NDM and KPC production. The PCR method detected more carbapenemase-producing isolates as compared to the phenotypic method. The findings of the study will help to understand the mechanism of antibiotics resistance among *K. pneumoniae*.

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