Infection and Drug Resistance

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ORIGINAL RESEARCH

Emergence of *bla*$_{NDM-1}$ Harboring *Klebsiella pneumoniae* ST29 and ST11 in Veterinary Settings and Waste of Pakistan

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Introduction: Intense livestock farming practices enforcing the farmers to use antibiotics as food supplements on a routine basis. Aberrant use of antibiotics is associated with the emergence of antibiotics resistance and resistant superbugs. Keeping in view the current scenario, the present study was designed for the first time from Pakistan with a specific aim to estimate the prevalence of the carbapenem-resistant *Klebsiella pneumoniae* in veterinary settings and the waste in Pakistan.

Methods: A total of 138 samples from various veterinary sources were collected by employing a nonprobability sampling technique. Isolation and phenotypic identification of carbapenem-resistant *K. pneumoniae* were performed according to the CLSI standard. Molecular detection of various antibiotic resistance genes (ARGs) was done through PCR by using specific primers against each ARG. According to the pasture scheme, the multilocus sequence typing (MLST) was performed to characterize the *K. pneumoniae* sequence types (STs).

Results: According to the results of the study, overall 9.4% (13/138) isolates were confirmed carbapenem-resistant *K. pneumoniae*. Among various carbapenem ARGs particularly, the *bla*$_{NDM-1}$ was found in 92.3% (12/13) isolates followed by *bla*$_{OXA-48}$ 84.6% (11/13). MLST results revealed that overall 3 STs were found in the study which includes ST29, ST11, and ST258. Taking together, this is the first study to our best knowledge which demonstrated the prevalence of carbapenem-resistant *K. pneumoniae* and its various STs prevalent in veterinary settings and the waste of Pakistan.

Conclusion: Based on the above-mentioned facts, we suggested that veterinary settings and waste are the potential source and reservoir of carbapenem-resistant *K. pneumoniae*, which may be disseminated to the environment and ultimately can affect the public and companion livestock health.

Keywords: veterinary settings, carbapenem resistance, *Klebsiella pneumoniae*, Pakistan

Introduction

The modern intensive integrated livestock production systems require regular antibiotics use at farms to maintain animal health and production. Globally, it is suggested that antibiotic consumption is double in animals compared to humans. The regular and imprudent use of antibiotics in modern veterinary practices is associated with the emergence of different multidrug-resistant (MDR) bacteria.[1,2] These MDR pathogens of animal origin may be disseminated to humans via the wider environment including food products, sewage and agricultural system.[1–3]

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http://doi.org/10.2147/IDR.S248091

Klebsiella pneumoniae is the most important member of the *Klebsiella* genus of *Enterobacteriaceae*. It can transfer to humans through contaminated products of like poultry products, beef, fish, milk, etc.[4,5] Additionally, the prevalence of carbapenem-resistant *K. pneumoniae* causing community and hospital-acquired infections is increasing significantly throughout the world, especially in developing countries.[6,7] Carbapenems are a class of beta-lactam antibiotics, are regularly prescribed as a last choice in the treatment of Gram-negative bacteria infection but the emergence of carbapenem-resistant *Enterobacteriaceae* is a critical clinical problem in the world.[8,9]

The key mechanisms for carbapenem resistance include alteration in porins function or expression, efflux pumps, and acquirement of enzymes capable of antibiotic hydrolyzing called carbapenemases. These enzymes are b-lactamases of Ambler class A, class D, and class B (Metallo-β-lactamases; MBLs).[1,10,12–14] Currently, a total of 5 Carbapenemases are key public health concerns, among them, New Delhi Metallo- β-lactamase-1 (NDM-1) has attracted the most attention because $bla_{\text{NDM-1}}$ has diverse antibiotic resistance activity.[15] The bla_{NDM-1} was the first time reported in *K. pneumoniae* isolated from an Indian patient in Sweden in 2008.[8] Since then a lot of studies from various regions of the world have reported the occurrence of the bla_{NDM-1}.[8,9] Despite all these reports so far, data about the detection of bla_{NDM-1} in veterinary and animal settings is still lacking.

Pakistan is a leading livestock populated country in the world. In the last decade, intensive livestock farming increased on a large scale in Pakistan. Therefore, livestock farmers are adapting the modern intense farming protocols in which antibiotics are used as food supplements routinely. This unrestrained antibiotic application in livestock feed is a leading factor that steers the evolution of multi-drug-resistant (MDR) pathogens. On the other hand, monitoring practices of antibiotics usage in veterinary and farm settings is inadequate.[16] Recently we have reported the first bla_{KPC} harboring *K. pneumoniae* ST258 from Pakistan and we also have reported the occurrence of ESBL-producing K. pneumoniae in hospital settings and waste from Pakistan.[3,17] Here, for the first time from Pakistan, we demonstrated the prevalence of bla_{NDM-1} harboring carbapenem-resistant *K. pneumoniae* in veterinary settings and waste from Pakistan.

Materials and Methods

Ethical Approval and Study Settings and Sample Collection

The study was approved by the Ethical Review Board (ERB), Government College University, Faisalabad Pakistan (letter No. 4162 dated 23–11-2017). Sample collection and processing was done at the Department of Microbiology Government College University, Faisalabad Pakistan while, molecular investigations were conducted at the Paul G. Allen School for Global Animal Health, Washington State University, Washington, USA, and Biomedical Research Center, Northwest Minzu University, Lanzhou China. In this study, we applied a nonprobability sampling technique to collect the samples (n= 138). The detail about samples distribution is given in Table 1. Standard aseptic conditions and microbiological procedures were adopted for sample collection and transportation.

Isolation and Identification of *Klebsiella pneumoniae*

In brief, abattoir samples and fecal swabs were dipped into 1mL of PBS and then streaked on the surface of nutrient media; however, milk and wastewater samples were directly streaked on nutrient agar plates and were incubated at 37°C for 24 hours. Subsequently, Mackonkey's agar and HiChrom Klebsiella Selective agar (Himedia®) were used as selective media for the isolation of

Table 1 Description the Sample Distribution and Their Positive Results

Sample Source	No of Samples	Confirmed Isolates	Prevalence %
Abattoir wastewater	28	3	10.71
Animal farm wastewater	21	3	14
Poultry farm wastewater	16	1	6.25
Milk Sample	19	2	10.52
Fecal samples	21	2	9.52
Animal Sludge	20	1	5.0
Poultry Sludge	13	1	7.69
Total	138	13	9.4%

K. pneumoniae. API 20E Kit and VITEK identification system (bioMérieux, France) was applied for the biochemical identification of the isolates.

Molecular identification was done by amplifying and sequencing the 16S rDNA using specific primers through PCR. The reaction started with the initial melting temperature at 95°C for 3 min, a total of 35 PCR cycles were carried out with following scheme: denaturation at 95°C for 30 secs, annealing at 50°C for 25 secs, extension at 72°C for 65 sec and final extension was done at 72°C for 5 min. Afterward, agarose (ThermoFisher Scientific, USA) gel electrophoresis was performed and results were interpreted in the gel documentation system (Bio-Rad, USA).

Antibiotic Susceptibility Testing

The disc-diffusion assay was used to decipher the antibiotic resistance pattern of the isolates As described in CLSI 2018.[18] Various groups of antibiotics along with their concentration used in the antibiogram analysis of *K. pneumoniae* include Penicillins (ampicillin 10µg and piperacillin 100µg), Cephalosporins (cefuroxime 30µg, cefixime 5µg, ceftriaxone 30µg, and cefepime 30µg), Carbapenems (meropenem 10µg), Fluoroquinolones (ciprofloxacin 5µg), Tetracycline (tetracycline 30µg and minocycline 30µg), Sulfa drugs (trimethoprim-sulfamethoxazole 1.25/23.75µg), Polymyxins (colistin 10µg), and tigecycline 15µg. All the tests were performed in duplicate and a control strain of *K. pneumoniae* (ATCC® 13,883™) was used for the assay.

For further confirmation, the minimum inhibitory concentration (MIC) of the above-listed antibiotics against the 13 CRKP isolates was determined using broth microdilution assay as described previously.[12,13] Briefly, fresh isolates of *K. pneumoniae* were used to obtain 0.5 McFarland standards. Dilutions of the antibiotics were made with the concentrations range 0.06 µg/mL to 256 µg/mL. Inoculum from the wells showing the turbidity was streaked on nutrient agar plates for the confirmation of bacterial growth. The MIC of the isolates showing growth in the well having the antibiotic concentration of 256 µg/mL were considered as ≥256 µg/mL Results were interpreted according to the CLSI recommendations.

Phenotypic Detection of Carbapenem-Resistant *K. pneumoniae*

The Double Disc Synergy Test (DDST) was employed for the phenotypic characterization of ESBL producing *K. pneumonia* as described previously.[18] Briefly, a 30 µg containing cefotaxime disc alone and a cefotaxime disc in combination with clavulanic acid, having the concentration of 30:10 µg, respectively were placed at a distance of 20 mm, and the difference between discs in terms of zone of inhibition was observed, the difference of ≥5 mm was considered positive for ESBL production.

Modified Hodge Test (MHT) was employed for phenotypic confirmation of carbapenem resistance. A 0.5 McFarland standard dilution of ATCC 25,922 (*E. coli)* was prepared in broth; a lawn was made using ten-fold dilution. In the center, ertapenem (10 µg) disk was placed. From the edge of the disk, the isolate was streaked up to the edge of the plate. Plates were incubated at 37°C for 24 hours. According to CLSI guidelines, MHT Positive ATCC1705 (*K. pneumoniae*) and MHT Negative ATCC1706 (*K. pneumoniae*) were used as control. Besides, MHT positive isolates were confirmed through the CarbNP test as recommended by CLSI 2018.

Molecular Detection of Antibiotic Resistance Genes (ARGs)

Freshly grown isolates of *K. pneumoniae* were subjected to DNA extraction through the DNA Extraction kit (Qiagen, Hilden, Germany). Quantification of extracted genomic DNA was carried out by using Thermo Scientific™ Nanodrop 2000, USA and DNA ≥60ng/µL concentration were considered further for the experiment. The extracted DNA of all isolates were subjected to PCR for the amplification of ARGs listed in Table 2 by using specific primers (IDT™, USA). The composition of the reaction mixture was; 5 µL of template DNA, 2 µL 100 pM forward and reverse primers, 10 µL of PCR Master Mix (2X DreamTaq, Thermo-Scientific™). The final volume was adjusted to 25 µL with sterile dH2O/Nuclease free water (Ambion-AM9932). The annealing temperature of each gene was adjusted according to primer's Tm described in Table 2. Subsequently, PCR products were analyzed on 1.2% agarose gel electrophoresis and examined under the gel documentation system (BioRad, USA).

Multi Locus Sequence Typing

The MLST was done according to the Pasteur MLST scheme (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) by amplification of seven housekeeping genes of *K. pneumoniae* isolates. The primers for *gap*A, *inf*B, *mdh, pgi, pho*E, *rpo*B and *ton*B were manufactured from Integrated DNA Technologies Inc. (California, USA).

Table 2 Table Showing the Primer Sequence, Amplification Conditions and Distribution of Different Antibiotic-Resistance Determinants Among *K. pneumoniae* Isolates

Name of the Gene	Primers Sequence	Annealing Temp (°C)	Percentage Prevalence	Reference
16SrDNA	F-AGAGTTTGATCTGGCTCAG R- AAGGAGGTGWTCCACC	51	100	[43]
bla_{TEM}	F-TCAACATTTCCGTGTCG R-CTGACAGTTACCAATGCTTA	42	100	[44]
bla_{SHV}	F-ATGCGTTATATTCGCCTGTG R-AGATAAATCACCACAATGCGC	47	92.30	[44]
$bla_{CTX\text{-}M\text{-}1}$	F-CGTTTCCGCTATTACAAACCGTTG R-GCCCATGGTTAAAAAATCACTGC	56	84.6	[45]
$bla_{CTX\text{-}M\text{-}2}$	F-TGGAAGCCCTGGAGAAAAGT R-CTTATCGCTCTCGCTCTGTT	60	15.3	[45]
$bla_{CTX\text{-}M\text{-}9}$	F-ATGGTGACAAAGAGAGTGCA R-CCCTTCGGCGATGATTCTC	56	30.7	[45]
$bla_{CTX\text{-}M\text{-}14}$	F-GAGAGTGCAACGGATGATG R-TGCGGCTGGGTAAAATAG	56	84.6	[45]
$PAN_{CTX\text{-}M}$	F- GGATATCGTTGGTGGTGCCATA R-TTTGCGATGTGCAGTACCAGTAA	60	100	[44]
bla_{KPC}	F-TGCAGAGCCCAGTGTCAGTTT R-CGCTCTATCGGCGATACCA	52	7.6	[46]
bla_{IMP}	F-GGAATAGAGTGGCTTAATTCTC R-CCAAACCACTACGTTATC	55	0	[47]
bla_{VIM}	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG	52	0	[47]
bla_{GIM}	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACTTTGCCATGC	52	0	[47]
$bla_{NDM\text{-}1}$	F-TGCCCAATATTATGCACCCGG R-CGAAACCCGGCATGTCGAGA	60	92.30	[48]
$bla_{OXA\text{-}48}$	F-TTGGTGGCATCGATTATCGG R-GAGCACTTCTTTTGTGATGGC	56	84.6	[49]
*Qnr*A	F-ATTTCTCACGCCAGGATTTG R-GATCGGCAAAGGTTAGGTCA	52	0	[50]
*Qnr*B	F-GATCGTGAAAGCCAGAAAGG R-ACGATGCCTGGTAGTTGTCC	50	53.8	[50]
*Qnr*S	F-ACGACATTCGTCAACTGCAA R-TAAATTGGCACCCTGTAGGC	52	46.1	[50]
TetA	F-GTGAAACCCAACATACCCC R-GAAGGCAAGCAGGATGTAG	52	30.7	[51]
TetB	F-CCTTATCATGCCAGTCTTGC R-ACTGCCGTTTTTTCGCC	52	23.0	[51]

(*Continued*)

Table 2 (Continued).

Name of the Gene	Primers Sequence	Annealing Temp (°C)	Percentage Prevalence	Reference
sul1	F-CGGCGTGGGCTACCTGAACG R-GCCGATCGCGTGAAGTTCCG	67	30.7	52
sul2	F-GCGCTCAAGGCAGATGGCATT R-GCGTTTGATACCGGCACCCGT	67	30.7	52

MLST PCR cycle conditions were as follow: denaturation at 94°C for 2 minutes, followed by 35 cycles of initial denaturation at 94°C for 20 seconds, annealing for all genes at 50° expect for *gapA* (60°) and *tonB* (45°) for 30 seconds and extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes in PCR Thermal Cycler (Bio-Rad Inc., USA).[19]

Conjugation Assay

Plasmid transferability was determined by performing conjugation assay using *E. coli* (ThermoFisher™, USA) as recipient cells.[20] Initially, LB media and LB broth (HIMEDIA®, India) were prepared, autoclaved, and pour into two different sets of plates and culture tubes. In the first set of plates and tubes, only LB media and LB broth were poured without adding anything and labeled. In other sets of culture tubes, LB broth was supplemented with DAP (diaminopimelic acid, 0.3 mM), ceftriaxone (30 µg/mL) and meropenem (10 µg/mL) and labeled. A fresh *K. pneumoniae* suspension was prepared in LB broth having the antibiotics by incubating over nightly. Fresh recipients *E. coli* suspension was prepared in plain LB broth by incubating over nightly. In a tube, 50+50 µL of both cells were mixed by repeated pipetting to make a ratio of 1:1 and 100 µL of mixed cell suspension was poured on LB agar plates containing meropenem (10 µg/mL) and labeled as "conjugation plate", kept at 37 °C for 24 hours. Moreover, PBS suspension of the bacterial colony was used for further confirmation of the transconjugants via PCR using specific primers against the $bla_{\text{NDM-1}}$ gene.

Statistical Analysis

The differences in the percentage/proportions of *K. pneumonia* bacteria isolated from different sources were tested by using "prop. Test" function in R software. This test is used for testing the null that the proportions in each group are the same (R Core Team, 2017).

Results

Distribution of *K. pneumoniae* According to the Respective Sample Source

The findings of the study revealed that the overall prevalence of confirmed carbapenem-resistant *K. pneumoniae* was 9.4% (13/138). The highest prevalence of 10.7% (7/65) was recorded from abattoir/wastewater samples followed by 10% (4/40) prevalence from milk and fecal samples While 6 % (2/33) prevalence was found in veterinary sludge samples (Table 1). Statistically, the results from Pearson's chi-squared test statistic (using prop. test in R software) showed that the difference between proportions or percentage of occurrence of *K. pneumoniae* isolated from seven different sources (shown in Table 1) is non-significant ($p = 0.96$, Chi-squared statistics = 1.35, df = 7).

Resistance Profile of the Isolates

For the 13 carbapenem-resistant isolates, the disc diffusion assay showed that all the isolates 100% (13/13) of *K. pneumoniae* were resistant to all the generations of cephalosporins as well as to meropenem. The resistance to ciprofloxacin, tetracyclines, and trimethoprim-sulfamethoxazole were 76.9% and 46.1% and 38.46% respectively as shown in Table 3.

Distribution of ARGs

After phenotypic characterization, all the carbapenem-resistant *K. pneumoniae* isolates were subjected to PCR for the detection of various ARGs. Among various ESBL genes bla_{TEM} was detected in all (100%; 13/13) the isolates followed by bla_{SHV} (92.30%; 12/13) and $bla_{\text{CTXM-1}}$ (84.6%; 11/13). Among various MBL genes $bla_{\text{NDM-1}}$ was detected in 92.30% (12/13) isolates followed by $bla_{\text{OXA-48}}$ 84.61% (11/13), while bla_{KPC} was detected in 01 (7.69%) isolate only. Whereas, bla_{IMP}, bla_{VIM}, and bla_{GIM} were not detected in any isolate of the study. Other detected ARGs include, *qnr*A 0%,

Table 3 Distribution of MICs of Carbapenem-Resistant *Klebsiella pneumoniae* (KP) Isolates Belonging to Various Multilocus Sequences Typing (MLST)

AntImicrobial Agents	MIC* Interpretative Breakpoints (μg/mL)	Isolates MIC (μg/mL) and MLST Types												
		Kp-1[a]	Kp-2[b]	Kp-3[g]	Kp-4[c]	Kp-5[d]	Kp-6[e]	Kp-7[a]	Kp-8[d]	Kp-9[a]	Kp-10[b]	Kp-11[b]	Kp-12[e]	Kp-13[f]
AMP	≥32	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256
PRL	≥128	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256
CXM	≥4	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256
CFM	≥16	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256
CRO	≥4	≥256	≥256	≥256	≥256	256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256
FEP	≥16	≥256	256	≥256	256	256	≥256	256	256	256	128	≥256	≥256	≥256
MEM	≥4	64	128	128	64	32	128	64	64	128	64	128	128	128
CIP	≥1	8	32	32	1	16	16	1	8	0.5	32	0.5	16	0.5
MH	≥16	16	2	64	16	64	2	16	1	1	2	1	2	16
TE	≥16	32	4	16	16	16	4	32	2	2	2	2	4	16
TGC*	≥2	0.25	0.25	0.5	0.125	0.125	0.125	0.25	0.125	0.125	0.25	0.125	0.125	0.25
CT	≥4	0.5	0.5	0.25	0.5	0.5	0.25	0.25	0.125	0.125	0.5	0.125	0.25	0.5
SXT	≥4/76	16/304	2/38	64/1216	8/152	8/152	2/38	8/152	1/19	1/19	2/38	2/38	2/38	2/38
MLST Types		ST29	ST11	ST258	ST29	ST29	ST11	ST29	ST11	ST29	ST11	ST29	ST11	ST29

Notes: [a]Abattoir wastewater, [b]Animal farm wastewater, [c]Poultry farm wastewater, [d]Milk Sample, [e]Fecal samples, [f]Animal Sludge, [g]Poultry Sludge, *EUCAST breakpoint.

Abbreviations: *MIC, minimum inhibitory concentration; Amp, ampicillin; CIP, ciprofloxacin; CT, colistin; CFM, cefixime; CXM, cefuroxime; CRO, ceftriaxone; FEP, cefepime; MEM, meropenem; MH, minocycline; PRL, piperacillin; TE, tetracycline; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole (1:19).

*qnr*B 53.84% (7/13) and *qnr*S 46.1% (6/13), *tet*A 30.7% (4/13), *tet*B 23% (3/13), *sul*1 and *sul*2 30.7% (4/13). Details of the ARGs distribution in *K. pneumoniae* isolates along their respective PCR product size were given in Table 2. The distribution of different Beta-lactamases genes among carbapenem resistant *K. pneumoniae* isolates is shown in Table 4.

Confirmation of Transconjugants

Bacterial colonies that appeared on LB agar plates supplemented with meropenem (10 µg/mL) were considered as transconjugants. Additionally, $bla_{\text{NDM-1}}$ was detected in all the transconjugants subjected to colony PCR. All the transconjugants displayed a strong resistance pattern against meropenem, as all the transconjugants showed a MIC of 128 µg/mL, which is significantly higher than the interpretative breakpoint given by CLSI.

Prevalent Sequence Types of *K. pneumoniae* in Veterinary Sample Sources

MLST results revealed that overall, three different STs of *K. pneumoniae* were detected in the study which includes ST 29, ST11 and ST 258. Among these STs, ST 29 was found in 53.84% (7/13) isolates followed by ST11 which was found in 38.46% (5/13) isolates whereas, single isolate (7.69%; 1/13) of the study showed the allelic profile of ST258. It was found that ST29 was the most prevalent ST in abattoir wastewater, as all the isolates 100% (3/3) from abattoir wastewater displayed the allelic profile of ST29. While both isolates 100% (2/2) from fecal samples belong to ST11. The single isolate of *K. pneumoniae* which showed the allelic profile of ST258 was isolated from poultry sludge (Table 3).

Discussion

During the last few years, gram-negative bacteria displayed a significant increase in the resistance against beta-lactam antibiotics, because of different plasmid-mediated ESBL genes, present in the *Enterobacteriaceae* family especially in *E. coli* and *K. pneumoniae*. The emergence of resistant *K. pneumoniae* due to the abuse of antibiotics in livestock farms and veterinary settings is a serious public as well as livestock health concern; it may ultimately be disseminated to the human via various environmental niches.[21]

Pakistan is an agriculture-based country, 70% of people directly or indirectly are involved with agriculture particularly livestock farming. Intense modern livestock farming practices force them to use antibiotics as feed additives, which ultimately associated with the development and dissemination of antibiotic-resistant superbugs. In this context, the prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) has been well reported from various regions of the world.[3,7] However, data regarding the prevalence of CRKP from Pakistan is very limited. Here, in the present study, we demonstrated the prevalence of carbapenem-resistant *K. pneumoniae* in veterinary settings and waste from Pakistan.

Findings revealed that overall 9.4% (13/138) prevalence of CRKP was observed in various samples from veterinary settings and waste. The prevalence was higher in wastewater samples compared to the other samples source (Table 1). Yet no such study has reported the prevalence of CRKP in veterinary settings and waste from Pakistan. However, the prevalence of CRKP has been well documented in Veterinary settings and waste in the world.[4,22] Livestock farms have also been recognized as a reservoir of ARGs and their dissemination in Jiangsu Province, China.[23] Similarly, some studies from India also reported that animal milk and meat, etc. are the potential source of ESBL producing *K. pneumoniae*.[24,25] In this study, we also have isolated CRKP from milk and slaughterhouse waste. The possible explanation of this high prevalence is that antibiotics are aberrantly used in veterinary practice here in Pakistan and as a growth promoter in food animals, which ultimately steer the evolution of antibiotic-resistant bacterial strains. Several studies have documented that food-producing animals are the possible source or reservoir for the dissemination of resistant bacterial strains or ARGs to humans.[26,27] While, another possible reason for the high prevalence of CRKP in milk may be due to mastitis, as bovine mastitis is a substantial factor for the growth and colonization of bacterial pathogens including *K. pneumoniae*.[26] In the past, a study reported the detection of ESBL producing *K. pneumoniae* in the milk of healthy cows.[28]

Different ARGs (Table 2) have been detected in the isolates of the study. Particularly, a total of 12 (92.3%) isolates were positive for the $bla_{\text{NDM-1}}$ gene and 11 (84.6%) isolates were positive the $bla_{\text{OXA-48}}$. The occurrence of the $bla_{\text{NDM-1}}$ gene in food-producing animals has already been reported form different parts of the world[29] but no such report is published from Pakistan. Additionally, several studies stated that usually $bla_{\text{NDM-1}}$ gene is located on mobile a genetic element that carry few

Table 4 Co-Existence of Different β-Lactamases and Metallo-β-Lactamases Encoding Genes Among Carbapenem-Resistant *Klebsiella pneumoniae* (KP) Isolates

Carbapenem-Resistant *K. pneumoniae* Isolates	Source	MIC* of Meropenem (μg/mL)	Beta-Lactamases Genes
Kp-1	Abattoir wastewater	64	blaCTXM-1+blaCTXM-9+ blaCTXM-14+blaOXA48 +blaNDM
Kp-2	Animal farm wastewater	128	blaCTXM-1+blaCTXM-14+ blaOXA48+blaNDM
Kp-3	Poultry Sludge	128	blaCTXM-1+blaCTXM-14+ blaOXA48+ blaNDM
Kp-4	Poultry farm wastewater	64	blaCTXM-1+blaCTXM-14+ blaOXA48+blaNDM
Kp-5	Milk	32	blaCTXM-1+blaCTXM-14+ blaOXA48
Kp-6	Fecal samples	128	blaCTXM-1+blaCTXM-14+ blaOXA48+ blaNDM
Kp-7	Abattoir wastewater	64	blaCTXM-2+blaCTXM-9+blaNDM
Kp-8	Milk	64	blaCTXM-2+blaCTXM-9+blaNDM
Kp-9	Abattoir wastewater	128	blaCTXM-1+blaCTXM-14+ blaOXA48+blaNDM
Kp-10	Animal farm wastewater	64	blaCTXM-1+blaCTXM-14+ blaOXA48+ blaNDM
Kp-11	Animal farm wastewater	128	blaCTXM-1+blaCTXM-9+ blaCTXM-14+ blaOXA48+ blaNDM+blaKPC
Kp-12	Fecal samples	128	blaCTXM-1+blaCTXM-14+ blaOXA48+ blaNDM
Kp-13	Animal Sludge	128	blaCTXM-1+blaCTXM-14+ blaOXA48+ blaNDM

Abbreviation: *MIC, minimum inhibitory concentration.

additional ARGs as well which helps in the dissemination of $bla_{\text{NDM-1}}$.[30] The Indian subcontinent is recognized, as a reservoir of $bla_{\text{NDM-1}}$ harboring *Enterobacteriaceae*. Moreover, many studies have also demonstrated the distribution of carbapenem-resistant *Enterobacteriaceae* in livestock from all across the globe.[31] On the other hand, the incidence of $bla_{\text{NDM-1}}$ harboring *K. pneumoniae* has only been well documented in clinical and hospital settings from Pakistan.[32,33] It is very tough to compare our results with local findings, as according to our best knowledge yet no study has reported the prevalence of $bla_{\text{NDM-1}}$ harboring *K. pneumoniae* in veterinary settings and waste from Pakistan.

All the CRKP isolates were subjected to conjugation assay to check the transferability of the $bla_{\text{NDM-1}}$ gene. As expected, it was observed that $bla_{\text{NDM-1}}$ was transferable to the donor *E. coli* cells, indicating that irrespective of the ST, all the $bla_{\text{NDM-1}}$ was located on conjugative plasmids and a significant source of horizontal gene transfer (HGT). The range of conjugation efficiency was 1.0×10^{-6} and 3.5×10^{-6} per transconjugant which also suggested the probability of HGT. Different sample niches especially veterinary sludge and wastewater may have high bacterial density and there would be a significant chance for horizontal gene transfer of $bla_{\text{NDM-1}}$ to various bacterial strains present in these sources. The same recommendations have been made in the past that resistance genes may be disseminated through bacterial strains via mobile genetic elements and plasmids. Additionally, detailed molecular studies have been reported that *K. pneumoniae* strains may disseminate different ARGs particularly $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ via HGT.[6,34]

To know the sequence types of the isolates MLST was performed. During MLST three discrete STs of *K. pneumoniae* were observed which include ST29 (46.1%), ST11 (38.5%) and ST 258 (7.7%). In this study, ST29 in a leading sequence type which is quite interesting. The same results have been reported from Saudi Arabia, where they found that ST29 was the predominant ST of carbapenem-resistant *K. pneumoniae* in Riyadh.[35] Incidence of *K. pneumoniae* ST29 in veterinary products has been reported

from various regions, a study conducted in Ghana reported the occurrence of ESBL producing *K. pneumoniae* ST29 in local and imported poultry meat.[36,37] Several studies have described the transmission of ESBL harboring *Enterobacteriaceae* from food animals and their products to humans. Detection of similar clones of ESBL- harboring *E. coli* and *K. pneumoniae* in animals and humans provides indirect proof of such cross species-transmission.[38] In this study, we have reported ST11 from veterinary settings of Pakistan for the very first time, before that ST11 has only been documented in clinical and hospital settings in Pakistan.[33] Whereas, ST11 has been recognized as the most prevalent ST in Asia particularly in China and Taiwan.[37,39] Detection of *K. pneumoniae* ST258 in the present study is also worrisome, as ST258 is one of the most detected STs of *K. pneumoniae in* the world.[40,41] Few factors contribute substantially to the global expansion of ST258 are virulence genes, type IV secretion system, type IV pilus, and type-III restriction-modification system.[42]

Conclusion

To best of our knowledge, this is the first study from Pakistan which demonstrated that veterinary settings and waste are the potential sources of carbapenem-resistant *K. pneumoniae*. In Pakistan, sanitation facilities are inadequate, which is a considerable risk factor associated with the dissemination of CRKP to the community. Strong surveillance and monitoring policy are required to estimate the exact burden of this public and livestock health menace which would be beneficial to curtail this health concern.

Data Sharing Statement

The aggregate data supporting findings contained within this manuscript will be shared upon request submitted to the corresponding author. Identifying patient data will not be shared.

Acknowledgments

We are grateful to the HEC Pakistan for IRSIP fellowship awarded to Dr. Tamoor Hamid Chaudhry at the Paul G. Allen School for Global Animal Health, Washington State University, Washington, USA.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Funding

The study was funded by the resources available at the Department of Microbiology, Government College University without external funding.

Disclosure

The authors have no conflicts of interest to declare for this work.

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