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ORIGINAL RESEARCH

Novel Carbapenem-Resistant *Klebsiella pneumoniae* ST147 Coharboring bla_{NDM-1}, bla_{OXA-48} and Extended-Spectrum β-Lactamases from Pakistan

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Purpose: The emergence of multidrug-resistant *Klebsiella pneumoniae (K. pneumoniae)* is associated with the acquisition of multiple carbapenemases. Their clonal spread is a worldwide concern due to their critical role in nosocomial infections. Therefore, the identification of high-risk clones with antibiotic resistance genes is very crucial for controlling its global spread.

Materials and Methods: A total of 227 *K. pneumoniae* strains collected during April 2018 to November 2019 were confirmed by PCR. Carbapenemases and extended-spectrum β-lactamases (ESBL) were detected phenotypically. Confirmation of carbapenemases was carried out by PCR and Sanger sequencing. The clonal lineages were assigned to selected isolates by multilocus sequence typing (MLST), and the plasmid analysis was done by PCR-based detection of the plasmid replicon typing.

Results: Of the total *K. pneumoniae*, 117 (51.5%) were carbapenem resistant (CRKP) and 140 (61.7%) were identified as ESBL producers. Intermediate to high resistance was detected in the tested β-lactam drugs while polymyxin-B and tigecycline were found to be susceptible. Among CRKP, 91 (77.8%) isolates were detected as carbapenemase producing, while 55 (47%) were positive for bla_{NDM-1} 23.9% (n=28), bla_{OXA-48} 22.2% (n=26) and bla_{VIM} 0.85% (n=1) while 12.7% (n=7) carried both bla_{NDM-1} and bla_{OXA-48} genes. The CRKP coharboring bla_{NDM-1} and bla_{OXA-48} genes (n=7) were positive for bla_{CTX-M} bla_{SHV} (n=3), bla_{SHV} (n=1) and bla_{CTX-M} (n=3). The novel CRKP with the coexistence of bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M} and bla_{SHV} genes were associated with the high-risk clone ST147 (n=5) and ST11 (n=2). The assigned replicon types were IncL/M, IncFII, IncA/C and IncH1.

Conclusion: This is the first report of the coexistence of bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M} and bla_{SHV} genes on a high-risk lineage ST147 from Pakistan. This study highlights the successful dissemination of carbapenemase resistance genes in the high-risk clones that emphasizes the importance of monitoring and controlling the spread of these diverse clones globally.

Keywords: high-risk clone, New Delhi metallo-β-lactamase, MLST, *K. pneumoniae*, carbapenem resistance

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Introduction

Accelerated emergence and effective propagation of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) across the world have become a prominent public health challenge due to high mortality rate in healthcare-associated nosocomial

infections.[1,2] The CRKP has a unique ability to acquire multiple resistance encoding genes through horizontal gene transfer interceded by broad-host-range plasmids, transposons and insertion sequences thereby turning out as one of the most successful nosocomial pathogen.[3] Lack of stewardship and irrational use of carbapenems for the infections of ESBL producing *K. pneumoniae* has led to the evolution of transmissible plasmid-encoding resistance genes that supported the selection of high-risk clones of CRKP involving diverse geographic regions and populations.[4] During 2014, the World Health Organization declared CRKP as the third most critical nosocomial pathogen for future concern.[5] As carbapenemases and ESBL/AmpC β-lactamases are critical in the acquisition of multidrug resistance, the identification of such broad-spectrum resistance genes is required for the development of new intervention strategies.[6]

Clinically important carbapenemase genes encompass $bla_{\text{KPC-2}}$, bla_{VIM}, bla_{IMP}, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$.[7] Global dissemination of such plasmid-encoded carbapenemases has increased alarmingly yet their geographic prevalence varies significantly.[8,9] Clinical literature remains expressive about the linkage of NDM-1, most common MBL-type carbapenemase and its transmission to other parts of the world from the endemic areas of Indian subcontinent since the first isolation of $bla_{\text{NDM-1}}$ producing *K. pneumoniae* ST14 and *Escherichia coli* (*E. coli*) in a patient treated in India and later shifted to Sweden in 2009.[10] Frequent reports of NDM-1, KPC-2 and OXA-48 type carbapenemase are available from Pakistan during the past decade.[11,12] Similarly, the OXA-48 is endemic in several countries since its first identification from Turkey in 2001.[13] CRKP co-harboring at least two carbapenemases were reported globally such as KPC-3 and VIM-2 in Italy, NDM-1 and KPC-2 from Brazil[9] and Pakistan,[12] NDM-1 and OXA-48 in Morocco,[14] Switzerland,[15] China[16] and Sultanate of Oman.[17] However, the understanding of molecular and genetic context of the carbapenemases is scarce especially in the developing countries.

Carbapenemases have spread worldwide through evolution of high-risk clones by acquiring, retaining and efficiently transmitting resistance genes. Such globally identified high-risk *K. pneumoniae* clones for the dissemination of carbapenemases include ST258, ST11 and ST147 co-harboring broad range of plasmids. Several STs were found to be associated with $bla_{\text{NDM-1}}$ producing *K. pneumoniae* such as ST258, ST340, ST512 and ST147 along with different plasmids IncF, IncA/C and IncL/M.[9] Therefore, careful detection and treatment strategies are required especially in developing countries where carbapenemase-producing strains have diverse opportunities.

However, insufficient data are available from Pakistan that describes molecular versatility of resistance genes in relation to genetic analysis and prevalence of high-risk clones. Hence, it is imperative to promptly detect and examine these successful clones to get insights into the global spread of antimicrobial drug resistance. Therefore, the current study aimed to ascertain the prevalence of carbapenemases and to analyze their clonal relatedness.

Materials and Methods

Bacterial Collection and Identification

Clinical strains were collected during the course of routine diagnostic bacterial cultures from tertiary care hospitals of Lahore, Pakistan. A total of 227 clinical strains of *K. pneumoniae* were included from different sample types from April 2018 to November 2019. The isolates were characterized phenotypically by colony morphology, Gram's staining and biochemical characteristics by using API-20E according to the manufacturer's instructions (BioMerieux, France). The study was sanctioned by institutional review board of the University of Health Sciences, Lahore, Pakistan.

Antimicrobial Susceptibility Testing (AST)

AST was carried out by standard disc diffusion method according to the CLSI guidelines using the following antibiotic discs: imipenem (IPM), meropenem (MEM), ertapenem (ETP), ceftazidime (CAZ), ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefepime (FEP), ceftaroline (CTP), aztreonam (ATM), gentamicin (CN), amikacin (AK), ciprofloxacin (CIP), doxycycline (DO), polymyxin-B (PB), tigecycline (TGC), cefotaxime (CTX), trimethoprim-sulfamethoxazole (SXT) and piperacillin-tazobactam (TZP) (Oxoid, UK). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.[18]

Minimal Inhibitory Concentrations (MICs)

MIC of antibiotics was determined by standard broth microdilution method using cation-adjusted Mueller-Hinton broth in accordance with CLSI guidelines with antibiotic concentrations ranging from 0.5 to 1024 μg/mL.[18]

Phenotypic Characterization

Carbapenemases were identified phenotypically by carbapenem inactivation method (CIM) while the detection of ESBLs was carried out by double-disc synergy test (DDST) using amoxicillin-clavulanic acid alone and in combination with ceftazidime as per the guidelines of CLSI.[18]

DNA Isolation from Bacterial Strains

Genomic DNA was prepared from pure bacterial culture plates by heat lysis method as reported previously and stored at −20°C for onward processing.[19]

Molecular Profile Analysis by Polymerase Chain Reaction (PCR)

Klebsiella species, carbapenemase resistance genes ($bla_{\text{NDM-1}}$, bla_{VIM}, bla_{IMP} and $bla_{\text{OXA-48}}$) and ESBL encoding genes (bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{SHV}) were detected through PCR using specific primers as given in Table 1. PCR reaction mixture of 50 µL consisting of 25 µL of 2x PCR Master Mix, 1 µL of each primer, 2 µL of DNA and dH_2O upto 50 µL. Amplification was carried out in thermal cycler (Proflex, ABI) with different annealing temperatures given in Table 1. Agarose gel (1–1.5%) was used to resolve and analyze the PCR products. $bla_{\text{NDM-1}}$ genes were further analyzed for allelic discrimination by Sanger's sequencing method. *K. pneumoniae* ATCC BAA-2146 was used as NDM positive control.

Multilocus Sequence Typing (MLST) of Klebsiella Species

MLST of CRKP strains coharboring $bla_{\text{NDM-1}}$ and $bla_{\text{Oxa-48}}$ was performed using seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB* and *tonB*) as described by *K. pneumoniae* MLST website.[20] The mutation analysis of NDM-1 was carried out by cycle sequencing using specific primers given in Table 1. The cycle sequencing was performed by BigDye terminator v3.1 kit on Proflex thermal cycler while the sequencing products were analyzed through capillary electrophoresis on Genetic Analyzer AB-3500 (Life Technologies by Thermofisher, USA) as per the kit instructions. Data were analyzed by using the sequencing analysis software v6.1 and checked on basic local alignment (BLAST) at NCBI for allele identification. CRKP STs were assigned using the MLST database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Plasmid Analysis

CRKP coharboring $bla_{\text{NDM-1}}$ and $bla_{\text{Oxa-48}}$ were further analyzed for the presence of plasmids. Plasmid DNA was extracted from single colony of CRKP by using the plasmid isolation kit (Thermofisher Scientific) and DNA was stored at −20°C. The samples were run on 0.8% agarose gel for the detection of plasmids. Plasmids were classified according to their incompatibility groups by using the PCR-based replicon typing method as described before.[21]

Results

During the 19-month study period, 227 clinical strains of Klebsiella were identified by phenotypic and genotypic methods. Of the total, 129 (56.8%) were isolated from males while remaining 98 (43.2%) were from females. These isolates were collected from wound 29.5% (n=67), pus 17.6% (n=40), blood 15.5% (n=35), tracheal secretion 13.2% (n=30), sputum 11% (n=25), urine 7.04% (n=16) and tissue 6.16% (n=14). Isolates originated from different sections of the hospital such as general surgery 26.4% (n=60), SICU 18.5% (n=42), general medicine 15.8% (n=36), dermatology 5.72% (n=13), nephrology 4.40% (n=10), chest medicine 8.37% (n=19), cardiology 3.96% (n=9), pediatric medicine 7.04% (n=16), oncology 5.28% (n=12) and orthopedic surgery 4.40% (n=10).

Antimicrobial Susceptibility Testing and Phenotypic Confirmatory Tests

As high as 51.5% (n=117) clinical strains of *K. pneumoniae* were carbapenem resistant (CRKP) while remaining 48.5% (n=110) were susceptible (CS). Out of 117 CRKP, 77.7% (n=91) were detected as carbapenemase-producing strains (CPKP). Most of the CRKP exhibited resistant to intermediate resistant profile for the β-lactam combination agents, carbapenems, fluoroquinolones, aminoglycosides and trimethoprim/sulfamethoxazole. Antimicrobial resistance pattern of CRKP strains was as follows: meropenem (96.9%), imipenem (98%), ertapenem (90%), amoxicillin/clavulanic acid (93.5%), ceftazidime (91.2%), ceftriaxone (96%), cefotaxime (95%), aztreonam (90.3%), ciprofloxacin (87%), amikacin (37.1%), tigecycline (21.1%) and polymyxin-B (13.7%). The MIC values of the tested β-lactam antibiotics were as follows: 4 to >1024 mg/L for ertapenem, 8 to >1024 mg/L for meropenem, 8 to >1024 mg/L for imipenem in all tested strains. All of the isolates were recognized as MDR (72%) or XDR (28%). The MIC results of the selected strains are given in Table 2.

Table 1 Primer Sequences Used for PCR

Gene	Primer Sequence (5′–3′)	Annealing (°C)	Amplicon Size (bp)	Reference
rpoB (*K. pneumoniae*)	F: CAA CGG TGT GGT TAC TGA CG R: TCT ACG AAG TGG CCG TTT TC	55	108	[53]
pehX (K. oxytoca)	F: GAT ACG GAG TAT GCC TTT ACG GTG R: TAG CCT TTA TCA AGC GGA TAC TGG	55	343	[53]
gyrA (*Klebsiella* genus)	F: CGC GTA CTA TAC GCC ATG AAC GTA R: ACC GTT GAT CAC TTC GGT CAG G	55	441	[53]
bla_{SHV}	F: CTT TAT CGG CCC TCA CTC AA R: AGG TGC TCA TCA TGG GAA AG	55	237	[54]
bla_{TEM}	F: CGC CGC ATA CAC TAT TCT CAG AAT GA R: ACG CTC ACC GGC TCC AGA TTT AT	55	445	[54]
bla_{CTX-M}	F: ATG TGC AGY ACC AGT AAR GTK ATG GC R: TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	55	593	[54]
bla_{NDM-1}	F: ATG GAA TTG CCC AAT ATT ATG CAC R: TCA GCG CAG CTT GTC GGC	52	813	[55]
bla_{VIM}	F: GAT GGT GTT TGG TCG CAT A R: CGA ATG CGC AGC ACC AG	52	390	[56]
bla_{OXA-48}	F: GCG TGG TTA AGG ATG AAC AC R: CAT CAA GTT CAA CCC AAC CG	52	438	[56]
bla_{IMP}	F: GGA ATA GAG TGG CTT AAY TCT C R: GGT TTA AYA AAA CAA CCA CC	52	232	[56]
bla_{NDM-1}	F: TGGCTTTTGAAACTGTCGCACC R: CTGTCACATCGAAATCGCGCGA	60	1000	[57]
gapA	F: TGA AGT ATG ACT CCA CTC ACG G R: AAC GCC TTT CAT TGC GCC TTC GGA A	60	662	[20]
infB	F: CTC TCT GCT GGA CTA CAT TCG R: CGC TTT CAG CTC CAG AAC TTC	52	462	[20]
mdh	F: CCC AAC TGC CTT CAG GTT CAG R: CCT TCC ACG TAG GCG CAT TCC	52	756	[20]
pgi	F: GAG AAA AAC CTG CCG GTG CTG CTG R: CGG TTA ATC AGG CCG TTA GTG GAG C	52	566	[20]
phoE	F: ACC TGG CGC AAC ACC GAT TTC TTC R: TTC AGC TGG TTG ATT TTG TAA TCC AC	52	602	[20]
rpoB	F: GGC GAA ATG GCG GAA AAC CA R: GAG TCT TCG AAG TTG TAA CC	52	1075	[20]
tonB	F: CTC TAT ACT TCG GTA CAT CAG GTT R: CCT GTT TGG CGG CCA GCA CCT GGT	48	539	[20]

Abbreviations: *rpoB*, RNA polymerase beta-subunit gene; *pehX*, polygalacturonase gene; *gyrA*, DNA gyrase subunit A gene; *bla*SHV, beta-lactamase sulfhydryl reagent variable gene; *bla*NDM, New Delhi metallo-beta-lactamase gene; *bla*VIM, metallo-beta-lactamase verona integron gene; *bla*TEM, beta-lacatmase temoneira gene; *bla*OXA-48, beta-lactamase oxacillinase 48 gene; *bla*CTX-M, beta-lactamase cefotaxime munich gene; *bla*IMP, beta-lactamase imipenemase gene; *gapA*, glyceraldehyde-3-phosphate dehydrogenase A gene; *infB*, translation initiation factor IF-2 gene; *mdh*, malate dehydrogenase gene; *pgi*, phosphoglucose isomerase gene; *phoE*, phosphoporin E gene; *tonB*, periplasmic energy transducer gene; *rpoB*, beta-subunit of RNA polymerase gene.

Table 2 MIC Values of Selected Carbapenem-Resistant *K. pneumoniae* Strains

Strain ID	Resistance Profile	MIC (μg/mL)											
		MEM	CAZ	SXT	AMP	AMC	CPT	CIP	ATM	AK	PB	DO	CTX
KP-17	MDR	4	8	2/38	64	32/16	0.5	1	16	16	2	4	1
KP-97	XDR	512	32	8/152	8	8/4	16	8	32	8	8	16	8
KP-104	MDR	64	32	2/38	8	16/8	0.5	1	4	64	2	4	8
KP-188	MDR	32	64	2/38	16	32/16	0.5	1	4	16	2	4	32
KP-191	MDR	16	4	16/304	8	32/16	0.5	1	8	16	2	2	16
KP-194	MDR	16	4	4/76	16	8/4	0.5	1	4	32	2	2	8
KP-199	XDR	>1024	128	32/608	512	256/128	0.5	64	128	128	8	32	256
KP-222	MDR	256	128	2/38	64	32/16	0.5	1	4	8	2	4	32
KP-246	XDR	128	256	8/152	32	64/32	16	1	16	64	16	64	64
KP-268	XDR	512	256	16/304	128	16/8	16	64	64	1024	4	128	256
KP-272	MDR	256	64	2/38	8	8/4	0.5	1	32	64	2	4	32
KP-284	MDR	64	32	2/38	32	8/4	0.5	16	4	16	2	4	32
KP-289	XDR	64	16	8/152	32	256/128	32	128	128	512	8	512	8
KP-315	XDR	>1024	256	8/152	64	128/64	16	4	512	512	8	256	4
KP-326	MDR	128	64	2/38	16	8/4	0.5	0.5	32	16	2	4	16
KP-333	MDR	32	16	8/152	8	8/4	0.5	0.5	16	16	2	4	2
KP-426	MDR	8	4	2/38	16	16/8	0.5	1	16	32	2	4	0.5
KP-443	MDR	64	32	4/76	8	128/64	0.5	1	16	8	2	4	0.5
KP-465	MDR	128	128	2/38	16	8/4	0.5	1	128	16	32	4	0.5
KP-494	MDR	64	128	2/38	8	8/4	4	32	64	16	2	4	0.5
KP-544	XDR	32	64	32/608	32	128/64	0.5	1	256	512	16	128	256
KP-562	MDR	64	256	2/38	64	8/4	0.5	1	32	128	2	4	0.5
KP-611	XDR	256	64	64/1216	64	128/64	0.5	16	16	64	2	4	64
KP-663	MDR	128	512	32/608	64	8/4	0.5	1	64	8	2	4	0.5
KP-675	MDR	32	32	2/38	256	8/4	0.5	1	64	8	8	4	0.5
KP-668	MDR	64	32	2/38	8	64/32	2	1	128	16	1	4	0.5
KP-687	MDR	32	64	2/38	128	8/4	8	1	32	16	1	4	0.5
KP-704	XDR	512	128	64/1216	128	256/128	0.5	256	32	1024	16	512	32

Abbreviations: MDR, multidrug resistant; XDR, extensively drug resistant.

Significantly higher frequency of CPKP was observed in wound samples 49.4% (n=45; p=0.002), pus samples 27.4% (n=25; p<0.026) and tracheal secretion samples 23.2% (n=21; p=0.029). Clinical strains of CRKP from wound and pus samples were significantly associated with the general surgery (p<0.001) while those from tracheal secretion samples were significantly associated with the SICU (p=0.008) as compared to the other samples obtained from the general surgery and SICU. Out of 227 *K. pneumoniae* strains, 61.6% (n=140) were ESBL producers and 38.3% (n=87) were non-ESBL producers. Among the 140 strains of ESBL producing *K. pneumoniae*, 9.28% (n=13) isolates were resistant to one of the third-generation cephalosporins (3GCs), 28.5% (n=40) were resistant against 2 of the 3GCs and 62.1% (n=87) were resistant to all the 3GCs. Association analysis demonstrated that 80% (n=112) ESBL producers were collected from the samples of wound, pus and tracheal secretions (p=0.003).

Antibiotic Resistance Genes

Out of 117 CRKP, 47% (n=55) were positive for the carbapenemase resistance genes by PCR including $bla_{\text{NDM-1}}$ 23.9% (n=28), $bla_{\text{OXA-48}}$ 22.2% (n=26) and bla_{VIM} 0.85% (n=1); bla_{IMP} was not detected. However, 12.7% (n=7) of CPKP coharbored $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes. $bla_{\text{NDM-1}}$ positive strains were further confirmed by DNA sequencing. The presence of the β-lactamase-encoding genes $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} was detected in the ESBL-producing *K. pneumoniae* strains (n=140). Single ESBL gene was detected in 38.5% (n=54): $bla_{\text{CTX-M}}$ 10.7% (n=15), bla_{SHV} 22.8% (n=32), bla_{TEM} 5% (n=7) and double ESBL genes were detected in 61.4% (n=86): $bla_{\text{CTX-M}}$, bla_{SHV} 32.1% (n=45), bla_{TEM}, bla_{SHV} 12.1% (n=17), bla_{TEM}, $bla_{\text{CTX-M}}$

Table 3 Resistance Profile of Carbapenem-Resistant *K. pneumoniae*

Strain ID	Ward	Sample Type	MIC (μg/mL) Imipenem	Resistance Profile	Profile of Resistance Genes	Replicon and Sequence Type
KP-17	SICU	Wound	256	MDR	bla_{NDM-1} (MT312213)*, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV}	ST147, IncL/M, IncFII, IncA/C, IncFH1
KP-97	SICU	Pus	64	XDR	bla_{NDM-1} (MT320894)*, bla_{CTX-M}, bla_{SHV}	-
KP-104	OPD	Wound	32	MDR	bla_{NDM-1} (MT320895)*, bla_{CTX-M}, bla_{SHV}	-
KP-188	SICU	Tracheal secretion	64	MDR	bla_{NDM-1} (MT320896)*, bla_{SHV}	-
KP-191	GS	Wound	16	MDR	bla_{NDM-1} (MT320897)*, bla_{CTX-M}	-
KP-194	GS	Wound		MDR	bla_{NDM-1} (MT320898)*, bla_{SHV}	-
KP-199	OPD	Pus	>1024	XDR	bla_{NDM-1} (MT320899)*, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV}	ST147, IncL/M, IncFII, IncA/C
KP-222	GS	Sputum	128	MDR	bla_{NDM-1} (MT320900)*, bla_{OXA-48}, bla_{CTX-M}	ST147, IncL/M, IncFII, IncA/C, IncFH1
KP-246	GS	Wound	32	XDR	bla_{NDM-1} (MT320901)*, bla_{CTX-M}	-
KP-268	SICU	Tip cells	64	XDR	bla_{NDM-1} (MT320902)*, bla_{TEM}, bla_{CTX-M}, bla_{SHV}	-
KP-272	GS	Wound	32	MDR	bla_{NDM-1} (MT320903)*, bla_{CTX-M}, bla_{SHV}	-
KP-284	GS	Wound	8	MDR	bla_{NDM-1} (MT320904)*, bla_{CTX-M}, bla_{SHV}	-
KP-289	GS	Wound	16	XDR	bla_{NDM-1} (MT320905)*, bla_{TEM}	-
KP-315	SICU	Tracheal secretion	32	XDR	bla_{NDM-1} (MT320906)*, bla_{SHV}	-
KP-326	OPD	Blood	128	MDR	bla_{NDM-1} (MT320907)*, bla_{OXA-48}, bla_{CTX-M}	ST147, IncL/M, IncFII, IncA/C, IncFH1
KP-333	GS	Pus	32	MDR	bla_{NDM-1} (MT320908)*, bla_{SHV}	-
KP-426	BURN	Tracheal secretion	64	MDR	bla_{NDM-1} (MT320909)*, bla_{CTX-M}	-
KP-443	GS	Wound	64	MDR	bla_{NDM-1} (MT320910)*	-
KP-465	WSW	Wound	128	MDR	bla_{NDM-1} (MT320911)*, bla_{OXA-48}, bla_{SHV}	ST11, IncL/M, IncFII, IncA/C, IncH1
KP-494	SICU	Tracheal secretion	64	MDR	bla_{NDM-1} (MT320912)*, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV}	ST147, IncL/M, IncFII, IncA/C
KP-544	GS	Blood	32	XDR	bla_{NDM-1} (MT320913)*	-
KP-562	SICU	Tracheal secretion	64	MDR	bla_{NDM-1} (MT320914)*, bla_{CTX-M}	-
KP-611	PS	Pus	128	XDR	bla_{NDM-1} (MT320915)*, bla_{CTX-M}	-
KP-663	GS	Wound	64	MDR	bla_{NDM-1} (MT320916)*, bla_{SHV}, bla_{CTX-M}	-
KP-675	SICU	Tracheal secretion	128	MDR	bla_{NDM-1} (MT320917)*, bla_{OXA-48}, bla_{CTX-M}	ST11, IncL/M, IncFII, IncA/C

(*Continued*)

Table 3 (Continued).

Strain ID	Ward	Sample Type	MIC (μg/mL) Imipenem	Resistance Profile	Profile of Resistance Genes	Replicon and Sequence Type
KP-668	GS	Pus	64	MDR	bla_{NDM-1} (MT320918)*, bla_{TEM}, bla_{SHV}	-
KP-687	SICU	Tracheal secretion	32	MDR	bla_{NDM-1} (MT320919)*, bla_{SHV}	-
KP-704	CM	Sputum	32	XDR	bla_{NDM-1} (MT320920)*, bla_{CTX-M}	-

Note: *GenBank Accession Number.
Abbreviations: GS, general surgery; PS, plastic surgery; SICU, surgical ICU; CM, chest medicine.

15% (n=21) and bla_{TEM}, bla_{SHV}, bla_{CTX-M} 2.14% (n=3). The CRKP strains coharboring bla_{NDM-1} and bla_{OXA-48} genes (n=7) were positive for bla_{CTX-M}, bla_{SHV} (n=3), bla_{SHV} (n=1) and bla_{CTX-M} (n=3). The results are shown in Table 3.

Sequence Type Analysis and Plasmid Detection of NDM-1 Producing Isolates

CRKP coharboring bla_{NDM-1} and bla_{OXA-48} (n=7) were further analyzed for sequence typing. High-risk *K. pneumoniae* clones ST147 coharbored bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV} (n=3), bla_{NDM-1}, bla_{OXA-48} bla_{CTX-M} (n=2), while ST11 coharbored bla_{NDM-1}, bla_{OXA-48}, bla_{SHV} (n=1) and bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M} (n=1). Plasmid analysis of CRKP coharboring bla_{NDM-1} and bla_{OXA-48} identified the following replicon types: IncL/M, IncFII, IncA/C and IncH1.[21]

Discussion

The emergence of CRKP has resulted in limited effective treatment strategies thus posing a major healthcare threat worldwide.[1] Global dissemination of transmissible carbapenemases by virtue of horizontal gene transfer involving certain high-risk clones[9] has become alarming especially in developing countries in the backdrop of inconsistent antibiotic policies. *K. pneumoniae* is among one of the most commonly detected multidrug-resistant member of the Enterobacteriaceae family.[22]

In the present study, we identified 51.1% CRKP strains that consisted of 77.7% carbapenemase producers. A large-scale study conducted in Turkey has detected only 3.1% (n=45/1452) CRKP isolates,[2] while from the European cohort study, 55% (n=944/1717) isolates were carbapenem resistant and 39.84% (n=684/1717) were carbapenemase producers.[23] Similarly, 10.69% (n=247/2310) CRKP strains were reported previously.[24] However, the highest percentages of carbapenem resistant and carbapenemase producers are reported from Pakistan such as another study identified 88% carbapenemase producers.[25] Due to the presence of high carbapenem resistance among *K. pneumoniae* in Pakistan, it is tempting to speculate that *K. pneumoniae* strains have the ability to retain diverse resistance determinants especially in a situation of uncontrolled use of high amounts of antibiotics. Wound samples (49.4%) were the major source of the CRKP infection that were significantly associated with the general surgery ward. In line with our study, 40% of wound samples with carbapenemase production were reported recently in association with the emergency department.[26] However, blood, urine, sputum, tracheal secretion and pus were the major source of CRKP in other studies.[11,24,25,27–29] The identification of CRKP strains from different anatomical sites highlights the importance of diverse set of sampling sites for the surveillance studies.

In consistent with the previous studies,[2,11,30–32] the most effective antibiotics against the isolates were polymyxin-B (13.7%) and tigecycline (21.1%). However, intermediate to high resistance levels were observed against carbapenemases (meropenem, imipenem and ertapenem) 90% to 98%, cephalosporins 86% to 92%, aztreonam 90.3%, ciprofloxacin 87% and amikacin 37.1% that counts for 72% MDR and 28% XDR isolates. Sattar et al[12] have reported 45% MDR *K. pneumoniae* strains with 85% to 90% resistance to cephalosporins and 30% resistance to imipenem. Another study from Pakistan reported 22.5% MDR *K. pneumoniae* strains among the study population in 2013.[33] The detailed analysis of antibiotic resistance among the *K. pneumoniae* from Pakistan suggested that the resistance has been increasing.

The most frequently detected carbapenemases among *K. pneumoniae* are bla_{KPC} enzymes followed by bla_{NDM-1}, $bla_{OXA-48-like}$ and bla_{VIM} in *K. pneumoniae*.[22] In our study, the detailed resistome analysis revealed the presence of carbapenemase resistance genes in 55 out of 117 CRKP strains. The most commonly detected carbapenemase

genes were bla_{NDM-1} (23.9%; n=28/55) and bla_{OXA-48} (22.2%; n=26/55) while bla_{VIM} was identified in only 1 isolate and bla_{IMP} was not detected. In consistent with our results, several studies from Pakistan reported that the most prevalent carbapenemase genes in Enterobacteriaceae/*K. pneumoniae* are bla_{NDM-1} 83.3% (n=30/37),[25] 70% (n=10),[28] 14.6% (n=13/82)[34] followed by bla_{OXA-48} 86% (n=49/57),[35] 50% (n=5/10)[28] and bla_{VIM} 13.4% (n=11/82),[34] 3.5% (n=2/57).[35] The results of our study are also in line with the observations that India, Bangladesh and Pakistan are the major reservoir countries for the widespread dissemination of carbapenemase genes such as bla_{NDM-1} and bla_{OXA-48}.[36] Since the first report of bla_{NDM-1} detection in Pakistan in 2010,[37] carbapenemase genes have spread significantly. Moreover, in the present study, the bla_{NDM-1} and bla_{OXA-48} coproduction was detected in 7 out of 55 CRKP. The co-occurrence of bla_{NDM-1} and bla_{OXA-48} has been reported previously in Asian and European countries.[16,24,38–40] However, in Pakistan the coexistence of carbapenem-resistant genes is not commonly detected in *K. pneumoniae*. In clinical isolates of *K. pneumoniae,* bla_{KPC-2}, bla_{NDM-1} (n=2/20),[12] bla_{NDM-1}, bla_{OXA-48} (n=2/10)[28] and bla_{VIM}, bla_{NDM-1} (n=4/28) encoding genes in community-based *E. coli* isolates[41] have been identified.

The CRKP strains coharboring bla_{NDM-1} and bla_{OXA-48} examined in our study belonged to either ST11; single locus variant of ST258[42] or emerging ST147 high-risk CRKP clone with resistance genes located on different plasmids.[9] NDM-type carbapenemases have been described associated with ST11, ST14, ST147, ST340, ST149 and ST231.[2] The ST11 is typically associated with the acquisition of multidrug resistance due to its ability to capture multiple plasmids[42] and *K. pneumoniae* strains with multiple resistance genes have been reported previously.[43] In concurrence with this study, our data also revealed that different antimicrobial resistance and replicon type exist within the identified ST11 isolates. One of the ST11 isolate was polymyxin-B resistant while other was susceptible. Our results are in line with the previously reported study from Pakistan where out of 3 ST11 strains, 2 strains were colistin resistant and 1 strain was colistin susceptible.[28] The ST11 isolates identified in our study coharbored bla_{NDM-1} and bla_{OXA-48} genes, whereas ST11 isolates positive for bla_{NDM-1} (n=7), bla_{NDM-7} (n=2) and bla_{NDM-5} (n=1) were recently reported from Pakistan.[25]

Previously, the pandemic lineage ST147 in *K. pneumoniae* has been correlated with the spread of carbapenemase resistant genes such as bla_{CTX-M}, bla_{VIM}, bla_{OXA-48}, bla_{KPC} and bla_{NDM-1}.[44,45] ST147 has also been associated with bla_{NDM-1}, bla_{CTX-M}, bla_{SHV},[30] bla_{CMY-4}, bla_{OXA-48},[46] bla_{NDM-1}, bla_{OXA-48},[38] bla_{NDM-1}[47] and bla_{OXA-48}.[24] Antecedently, two studies are available from Pakistan that reported the existence of ST147 *K. pneumoniae* with bla_{OXA181} resistant gene[11] and ST147 *K. pneumoniae* isolate with bla_{NDM-1}, bla_{NDM-5}.[25] The coexistence of bla_{NDM-1}, bla_{OXA-48} has also been detected in ST307 from China[16] and bla_{NDM-1}, $bla_{OXA-232}$ in ST231 from Pakistan.[27] However, in our study we have identified the co-emergence of bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV} among ST147 (n=5), a globally spread high-risk clone. The identification of S147 with bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV} is alarming as it indicates that strong selection has occurred towards the resistance in these clinical isolates from Pakistan.

Subsequently, four replicon types IncL/M, IncFII, IncA/C and IncH1 were detected in the present study. Previous studies have shown that IncL/M-type plasmid was related to the OXA-48-type carbapenemases and responsible for the bla_{OXA-48} gene dissemination.[48] The molecular studies have reported that the most frequent replicon type identified in *K. pneumoniae* species is IncFII replicon[9] while IncA/C type replicons are responsible for the horizontal spread of NDM-type carbapenemase along with IncFIIK, IncL/M and IncH1.[49] Moreover, among the typed resistant plasmids, IncL/M and IncF11 plasmids may be regarded as epidemic as they have been detected in different countries with different origins and sources.[50] On the other hand, IncR, IncFIIK-type and IncA/C type replicons have been identified in OXA-48-type carbapenemases and IncR type replicons in NDM-type carbapenemases.[51] Previously reported replicon types among the NDM-producing *K. pneumoniae* from Pakistan include IncN, IncA/C[52] and IncFII, IncR.[11] In our study, the identified replicon types (IncL/M, IncFII, IncA/C and IncH1) are reported to be responsible for OXA-48-type and NDM-type carbapenemases dissemination.

Conclusion

We reported the first identification of high-risk CRKP clone ST147 coharboring several carbapenem resistance genes bla_{NDM-1}, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV} from Pakistan. Taken into account the presence of highest genetic diversity among *K. pneumoniae* worldwide, the identification of high-risk clone with multidrug resistance and coexistence of different classes of β-lactamases in the same strain highlight the severity of health challenges

posed by *K. pneumoniae* worldwide. Our findings suggested that the high antimicrobial resistance existed among study isolates that can also be associated with the presence of several β-lactams genes in a high-risk clone. Therefore, the continuous monitoring of carbapenemases is necessary to prevent the national and transnational spread of these powerful isolates especially in case when the healthcare facilities are inadequate.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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