Emergence of multidrug-resistant ST11 bla\textsubscript{KPC-2} producing \textit{Klebsiella pneumoniae} cohaboring \textit{bla\textsubscript{CTX-M}} and \textit{bla\textsubscript{SHV}} in Pakistan

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Abstract

Introduction: Carbapenemases are primarily responsible for the intensified spread of multidrug-resistant (MDR) \textit{K. pneumoniae} by virtue of antibiotics overuse. Therefore, frequent investigation of high-risk clones especially from developing world is crucial to curtail global spread.

Methodology: In this observational study, 107 \textit{K. pneumoniae} were retrieved and confirmed genotypically from April 2018 to March 2020 from tertiary care hospitals in Lahore, Pakistan. Carbapenemases and extended-spectrum \(\beta\)-lactamases were verified by Polymerase Chain Reaction and Sanger sequencing. Multilocus sequence typing and plasmid replicon typing were used to assign clonal lineages and plasmid replications.

Results: Among the \textit{K. pneumoniae}, 72.9% (78/107) strains were carbapenem resistant (CR) with 65.4% (51/78) exhibiting carbapenemase producing phenotype. Among CR \textit{K. pneumoniae} 38.5% (30/78) strains exhibited the following carbapenemase genotypes: \textit{bla\textsubscript{NDM-1}} (26.7%, 8/30), \textit{bla\textsubscript{OXA-48}} (26.7%, 8/30), \textit{bla\textsubscript{KPC-2}} (20.0%, 6/30), \textit{bla\textsubscript{VIM}} (10.0%, 3/30), \textit{bla\textsubscript{NDM-1}}/\textit{bla\textsubscript{OXA-48}} (10.0%, 3/30), \textit{bla\textsubscript{OXA-48}}/\textit{bla\textsubscript{VIM}} (3.3%, 1/30), and \textit{bla\textsubscript{OXA-48}}/\textit{bla\textsubscript{IMP}} (3.3%, 1/30). Tigecycline and polymyxin-B retained susceptible profile. \(\beta\)-lactam drugs showed intermediate to high resistance. The occurrence of CR \textit{K. pneumoniae} infections was significantly associated with wound (39.7%, \(p=0.0007\)), pus (38.5%, \(p=0.009\)), general surgery (34.6%, \(p=0.002\)) and intensive-care unit (30.0%, \(p=0.04\)).

\textit{bla\textsubscript{KPC-2}} producing \textit{K. pneumoniae} cohaboring \textit{bla\textsubscript{CTX-M}}/\textit{bla\textsubscript{SHV}} (66.7%) and \textit{bla\textsubscript{CTX-M}} (33.3%) exhibited sequence type (ST) 258 (\(n=4\)) and ST11 (\(n=2\)) sequence types with IncFII, IncN, IncFIKA, IncL/M and IncFIIK plasmids.

Conclusions: This is the first report describing the emergence of MDR \textit{bla\textsubscript{KPC-2}} producing \textit{K. pneumoniae} ST11 cohaboring \textit{bla\textsubscript{CTX-M}} and \textit{bla\textsubscript{SHV}} in Pakistan.

Key words: ST11; \textit{K. pneumoniae}; Multilocus sequence typing; ST147.

Introduction

Over the years, evolutionary changes have resulted in the emergence of successful epidemic bacterial clones that ultimately lead to the development of multidrug resistance (MDR) attributable to selection pressure and misuse of antibiotics. Clinically, the Enterobacteriaceae family accommodates many such high-risk MDR clones, thereby posing one of the most frightening issues of deteriorating efficacy of antibiotics. As a result, life expectancy is dramatically reduced compared to the pre-antibiotic era [1]. Carbapenem-resistant \textit{K. pneumoniae} (CRKP) has turned out to be an exceptionally diverse MDR species associated with a variety of healthcare and community-based infections and features successful accumulation of survival and resistant determinants. Such high-risk clones are of great clinical concern due to the presence of ever evolving \(\beta\)-lactamases that present the hardest challenge to all the \(\beta\)-lactams by hydrolyzing the \(\beta\)-lactam bond, thus rendering them non-functional [2].

\textit{K. pneumoniae} carbapenemases (KPCs) present one of the most critical genetic mechanisms of acquired carbapenem resistance among the clinically important transmissible carbapenemases including \textit{bla\textsubscript{NDM-1}}, \textit{bla\textsubscript{OXA-48}}, \textit{bla\textsubscript{VIM}} and \textit{bla\textsubscript{IMP}}. Since the first identification of KPC-producing \textit{K. pneumoniae} in the United States, its spread has been reported in several countries like Israel, India, China, several European countries,
Vietnam, Taiwan, China, and Pakistan demonstrating its remarkable potential for universal distribution [3-9]. However, distinct resistance patterns exist in different regions depicting the mixing of resistance clones due to globalization, traveling and different regional infection control measures. This is evidenced from reports mentioning the coexistence of blaKPC-2 with other resistance genes in K. pneumoniae such as blaKPC/blaOXA-48 from Taiwan, blaKPC/blaNDM-1/blaSHV/blaCTX-M from Turkey, blaKPC/blaSHV/blaCTX-M from India and China, blaKPC/blaGES from Iran, blaKPC/blaNDM-1 from China and Greece and the coexistence of blaKPC and blaNDM-1 has been reported in Pakistan [4,10].

This multidimensional spread of blaKPC-harboring CRKP strains is facilitated through several mechanisms. However clonal spread is considered as one of the important modes of transmission responsible for the growing pervasiveness of CRKP strains globally. Among CRKP, the prominent clonal group is CC258 with varying diversity of sequence types (ST) geographically. These include ST258 which is the most prevalent clone in the US and ST11 in China. Sporadic reports of other STs among blaKPC-2 bearing CRKP strains has been described such as ST15, ST660, ST48 from China, ST15 from Turkey, ST48, ST340 from Thailand, ST307, ST48 from South Korea and ST101 from India [11]. However, only ST258 has previously been identified in Pakistan demonstrating the transmission capacity of selected clonal groups in relation to different regions [12]. Therefore, it is critical to understand the molecular mechanisms that ascertain the emergence and distribution of clinically fit lineages of blaKPC-2 harboring CRKP variants worldwide.

There has been worrisome rise in antimicrobial resistance globally and there is only limited information available regarding the prevalence and underlying population structure of blaKPC-2 harboring CRKP strains in Pakistan [4,12]. In view thereof, the present study was intended to encompass the prevalence, genetic variability and clonal expansion of MDR blaKPC-2 harboring K. pneumoniae in Pakistan.

Methodology
Identification of clinical isolates
In this observational study, 107 MDR K. pneumoniae strains were collected from April 2018 to March 2020, from the Pathology sections of tertiary care hospitals in Lahore, Pakistan. Phenotypic and biochemical characterization of the strains were carried out by analyzing colony morphology, Gram staining and API-20E (bioMerieux, Marcy-l’Etoile, France).

Antimicrobial susceptibility testing
Standard disc diffusion technique was used for antimicrobial susceptibility testing (AST) as per the Clinical and Laboratory Standards Institute, USA (CLSI) guidelines. The following antibiotic discs were used; imipenem (IPM; 10 µg), ceftazidime (CAZ; 30 µg), polymyxin-B (PB; 300 U), ampicillin (AMP; 10 µg), piperacillin-tazobactam (TZP; 100/10 µg), amoxicillin-clavulanic acid (AMC; 20/10 µg), cefepime (FEP; 30 µg), ceftaroline (CIP; 30 µg), gentamicin (CN; 10 µg), meropenem (MEM; 10 µg), amikacin (AK; 30 µg), ertapenem (ETP; 10 µg), ciprofloxacin (CIP; 5 µg), doxycycline (DO; 30 µg), tigecycline (TGC; 15 µg), aztreonam (ATM; 30 µg) cefotaxime (CTX; 30 µg) and trimethoprim-sulfamethoxazole (SXT; 1.25/23.75 µg) (Oxoid, Basingstoke, UK). P. aeruginosa ATCC 27853 and E. coli ATCC 25922 were used as quality control strains. Standard broth microdilution technique was used to determine the Minimum Inhibitory Concentration (MIC) as per CLSI guidelines. Susceptibility of antibiotics was interpreted as per the CLSI M100 clinical breakpoints whereas European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for colistin and tigecycline. Strains were grouped as carbapenem resistant (CR) when non-susceptible to at least one of the used carbapenems as per zone diameter breakpoints according to CLSI supplement 28. Strains were categorized as MDR if they remained non-susceptible to at least one antibiotic in more than three and less than six antimicrobial classes and extensively drug-resistant (XDR) if they exhibited non-susceptibility to more than six antimicrobial classes.

Phenotypic identification of β-lactamases
The frequency of extended spectrum β-lactamases (ESBLs) among all CRKP strains was determined by double-disc synergy test using AMC alone and in combination with CAZ in accordance with CLSI guidelines. Carbapenem inactivation method (CIM) was performed for the phenotypic screening of carbapenemase production as per CLSI guidelines.

Molecular identification of strains and carbapenemases
Genomic DNA was isolated from bacterial cultures using the heat lysis technique. Briefly, 2 to 3 colonies of bacterial culture were mixed with 500 µL sterile distilled water and heated at 98 °C for 10 min at 300 rpm (ThermoMixer, Fischer Scientific, Waltham, MA, USA). Tubes were centrifuged at 1000 rpm for 10 min.
and the supernatant collected in newly labeled tube. DNA was stored at -80 °C. Selected carbapenemase encoding genes such as \( \text{bla} \text{KPC}-2 \), \( \text{bla} \text{NDM-1} \), \( \text{bla} \text{VIM} \), \( \text{bla} \text{IMP} \) and \( \text{bla} \text{CTX-M} \) selected ESBLs including \( \text{bla} \text{SHV} \), \( \text{bla} \text{TEM} \) and \( \text{bla} \text{OXA-48} \) were amplified and molecular identification of \textit{Klebsiella} were performed by standard Polymerase Chain Reaction (PCR). The PCR reaction mixture contained 25 μL of 2x PCR Master Mix (Cat # K0171, ThermoScientific, Waltham, MA, USA), 1 μL of each primer, 2 μL of DNA and dH₂O upto 50 μL in a thermal cycler (Proflex, Applied Biosystems, ThermoFischer Scientific, Waltham, MA, USA). Amplicons were resolved by agarose gel electrophoresis (1-1.5%). The details of the primer sequences are listed in Table 1.

**Sequencing of carbapenemase genes**

Carbapenemases were further analyzed by gold standard Sanger’s sequencing technique at Advance Research Center for Biomedical Sciences, King Edward Medical University, Lahore. Cycle sequencing was performed by BigDye Terminator v3.1 kit with 10

<table>
<thead>
<tr>
<th>Table 1. Primer sequences used for PCR and Sequencing.</th>
<th>Primer Sequences</th>
<th>Tm (°C) / Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbapenemases</strong> [4,10]**</td>
<td>( \text{F: } ) GCT ACA CCT AGC TCC ACC TTC ( \text{R: } ) ACA GTG GTT GTG AAT CCA TGC</td>
<td>55 / 989</td>
</tr>
<tr>
<td>( \text{blaKPC-2} )</td>
<td>( \text{F: } ) GGG CAG TCG CTG CTA CAC ACC GA</td>
<td>53 / 476</td>
</tr>
<tr>
<td>( \text{blaNDM-1} )</td>
<td>( \text{F: } ) GAT GTT CTC ATG GTC GGC AT</td>
<td>52 / 390</td>
</tr>
<tr>
<td>( \text{blaVIM} )</td>
<td>( \text{F: } ) GCG TGG TTA AGG ATG AAC AC</td>
<td>52 / 438</td>
</tr>
<tr>
<td>( \text{blaCTX-M} )</td>
<td>( \text{F: } ) GGA ATA GAG TGG CTT AAY TCT C</td>
<td>52 / 232</td>
</tr>
<tr>
<td><strong>ESBLs</strong> [9]**</td>
<td>( \text{F: } ) CTT TAT CGG CCC TCA CTC AA</td>
<td>55 / 237</td>
</tr>
<tr>
<td>( \text{blaSHV} )</td>
<td>( \text{F: } ) CGG CGC ATA CAC TAT TCG CAG ATG GA ( \text{R: } ) ACG CTC ACC GGC TCC AGA TTT AT</td>
<td>55 / 445</td>
</tr>
<tr>
<td>( \text{blaTEM} )</td>
<td>( \text{F: } ) ATG TGC AGY ACC AGT AAR GTK ATG GC</td>
<td>55 / 593</td>
</tr>
<tr>
<td>( \text{blaOXA-48} )</td>
<td>( \text{F: } ) CAA CGG TGT GGT TAC TGA CG ( \text{R: } ) TCT ACG AAC TGG CTT TTG TC</td>
<td>55 / 108</td>
</tr>
<tr>
<td><strong>Species identification</strong> [8]**</td>
<td>( \text{F: } ) GAT ACG GAG TAT GCC TTT ACG GT</td>
<td>55 / 343</td>
</tr>
<tr>
<td>( \text{rpoB} )</td>
<td>( \text{F: } ) CGC GTA CTA TAC GCC ATG AAC GTA ( \text{R: } ) ACC GTT GAT CAC TTC GGT CAG C</td>
<td>55 / 441</td>
</tr>
<tr>
<td><strong>Multi Locus Sequence Typing (MLST)</strong> [11]**</td>
<td>( \text{F: } ) TGA AGT ATG ACT CCA CTC ACG G ( \text{R: } ) AAC GCC TTT CAT TGC GGC TGC TGA A</td>
<td>60 / 662</td>
</tr>
<tr>
<td>( \text{gapA} )</td>
<td>( \text{F: } ) CTT CTC GGT GAT GGC TAG GCC TAG C</td>
<td>52 / 566</td>
</tr>
<tr>
<td>( \text{infB} )</td>
<td>( \text{F: } ) GAT TGG TTT CAG TCA AGC CTA G</td>
<td>52 / 602</td>
</tr>
<tr>
<td>( \text{mdh} )</td>
<td>( \text{F: } ) ACC TGA AAT CAA GGC TGG CTT C</td>
<td>52 / 1075</td>
</tr>
<tr>
<td>( \text{pgi} )</td>
<td>( \text{F: } ) TTC ATG TGG ATG TGG TAA TCC AC</td>
<td>48 / 539</td>
</tr>
<tr>
<td>( \text{phoE} )</td>
<td>( \text{F: } ) GAG AAA AAC CTA CCG GTG GTG CGT CC</td>
<td>52 / 662</td>
</tr>
<tr>
<td>( \text{rpoB} )</td>
<td>( \text{F: } ) CTC TCT TCT ATG TCA ATG T</td>
<td>52 / 462</td>
</tr>
<tr>
<td>( \text{tonB} )</td>
<td>( \text{F: } ) TGG TGG TGG ATG TAT GAA AAC CA</td>
<td>52 / 756</td>
</tr>
</tbody>
</table>

* References. \( \text{blaGAC} \): \textit{klebsiella pneumonia} carbapenemase gene; \( \text{blaNDM} \): New Delhi metallo beta lactamase; \( \text{blaOXA-48} \): beta-lactamase oxacillinase 48 gene; \( \text{blaCTX-M} \): beta-lactamase cefotaxime munich gene; \( \text{blaSHV} \): beta-lactamase sulfhydryl reagent variable gene; \( \text{blaVIM} \): metallo-beta-lactamase verona integron gene; \( \text{blaTEM} \): beta-lactamase temoneira gene; \( \text{rpoB} \): RNA polymerase beta-subunit gene; \( \text{pehX} \): polygalacturonase gene; \( \text{gyrA} \): DNA gyrase subunit A gene; \( \text{gapA} \): glyceraldehyde-3-phosphate dehydrogenase A gene; \( \text{infB} \): translation initiation factor IF-2 gene; \( \text{mdh} \): malate dehydrogenase gene; \( \text{pgi} \): phosphoglucone isomerase gene; \( \text{phoE} \): phosphoprotein E gene; \( \text{tonB} \): periplasmic energy transducer gene; \( \text{rpoB} \): beta-subunit of RNA polymerase gene.
μL PCR reaction mixture containing BigDye terminator 3.1 Ready Reaction Mix 4 μL, forward primer (3.2 pmol) 0.5 μL, purified DNA template (5–20 ng) 2 μL and dH2O 3.5 μL. PCR cycling conditions were 96 °C for 1 min, 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 2 min (35 cycles) on Proflex thermal cycler (Proflex, Applied Biosystems, ThermoFischer Scientific, Waltham, MA, USA). The PCR product was purified by using BigDye X Terminator purification kit as per kit instructions and capillary electrophoresis was conducted by Genetic Analyzer (ABI-3500, Thermo Fischer, Waltham, MA, USA). Allelic discrimination of carbapenemases was carried out by sequencing. Data sets were analyzed and interpreted through sequencing analysis software v 6.1 and basic local alignment (BLAST) at National Center for Biotechnology Information (NCBI) to identify allelic profiles. The sequences were annotated and submitted to Genbank (NCBI, Bethesda, USA) with the following accession numbers: MW581777, MW581778, MW581779, MW581780, MW581781, MW581782, MW581783, MW581784, MW581785, MW581786, MW581787, MW581788, MW581789, MW581790, MW581791, MW581792, MW581793, MW581794, MW585068, MW585069, MW585070, MW585071, MW585072, MW585073. *K. pneumoniae* ATCC BAA-1705 was used as KPC positive control and nuclease free water as negative control. The details of primer sequences used in this experiment are listed in Table 1.

**Identification of clonal lineage**

The clonal relatedness of selected CRKP strains was determined by Multilocus Sequence Typing (MLST), based on allelic discrimination of seven housekeeping genes i.e., glyceraldehyde-3-phosphate dehydrogenase A gene (*mdh*), translation initiation factor IF-2 gene (*infB*), malate dehydrogenase gene (*mdh*), phosphoglucone isomerase gene (*pgi*), phosphoprin E gene (*phoE*), periplasmic energy transducer gene (*tonB*), beta-subunit of RNA polymerase gene (*rpoB*). The Sequence Types (STs) were assigned using the online MLST database (http://bigd.db.pasteur.fr/klebsiella/klebsiella.html). Plasmid typing was done based on their incompatibility group using the PCR based replicon typing as described before [13].

**Statistical Analysis**

Statistical analysis was performed on SPSS 24.0 (Chicago, IL, USA). Normality of the data were analyzed by the Kolmogorov–Smirnov test. Categorical data were evaluated by χ² test or Fisher’s exact test where applicable. The p value of < 0.05 was considered statistically significant.

**Ethics approval and informed Consent**

The study was approved by institutional review board of the University of Health Sciences, Lahore, Pakistan and King Edward Medical University, Lahore, Pakistan. Informed consent was obtained from the participants for inclusion in study.

**Results**

Out of 107 clinical strains of *K. pneumoniae*, 51.4% (n = 55) and 48.6% (n = 52) were isolated from male and female patient samples, respectively. These strains originated from wound (31.8%, n = 34), pus (30.8%, n = 33), pleural fluid (8.4%, n = 9), endotracheal tube (ETT) (7.5%, n = 8), drain (6.5%, n = 7), sputum (5.6%, n = 6), tip cells (5.6%, n = 6) and blood (3.7%, n = 4). The strains were distributed in general surgery (29.0%, n = 31), ICU (28.0%, n = 30), general medicine (15.0%, n = 16), dermatology (13.1%, n = 14), pediatric medicine (5.6%, n = 6), chest medicine (3.7%, n = 4), orthopedic surgery (3.7%, n = 4) and neurology (1.9%, n = 2).

Of the total *K. pneumoniae*, 72.9% (n = 78) were carbapenem resistant (CRKP) with 65.4% (n = 51) being carbapenemase producers (CPKP) phenotypically. Among the CRKP, 39.7% (n = 31) and 38.5% (n = 30) were collected from wound and pus, respectively. The occurrence of CRKP infection was significantly associated with wound (p = 0.0007) and pus (p = 0.009). The CRKP were stemmed from different clinical departments predominantly from general surgery (34.6%, n = 27), intensive-care unit (ICU) (26.9%, n = 21), dermatology (12.8%, n = 10) and general medicine (11.5%, n = 9). General surgery was found to be significantly associated with the CRKP infections (p = 0.04). Moreover, CRKP strains from wound/pus samples were found significantly associated with general surgery (p = 0.002) and ICU (p = 0.04).

The strains were mainly non-susceptible to carbapenems, fluoroquinolones, aminoglycosides and β-lactam combination agents, and susceptible to TGC and PB. The analyzed antimicrobial resistance was MEM (94.5%), IMP (99.2%), ETP (91.7%), AMC (97.4%), CAZ (92.3%), FEP (94.9%), CTX (96.1%), ATM (66.7%), CIP (93.6%), AK (80.8%), SXT (43.6%), DO (55.1%), TGC (39.7%) and PB (2.6%). As such, 74.9% of the clinical isolates were classified as MDR and 25.1% as XDR.

Of all the clinical strains, 87.9% (n = 94) were detected as ESBL producers with 66.0% resistant to all
Table 2. Molecular and genetic profile of multi-drug resistant K. pneumoniae.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Carbapenemases</th>
<th>ESBLs</th>
<th>MLST</th>
<th>Replicon type</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP-47, KP-51</td>
<td>blaKPC-2</td>
<td>blaCTX-M, blaSHV</td>
<td>ST11</td>
<td>IncFII, IncN, IncFIA, IncFIK</td>
</tr>
<tr>
<td>KP-48, KP-52</td>
<td>blaKPC-2</td>
<td>blaCTX-M, blaSHV</td>
<td>ST258</td>
<td>IncFII, IncL/M, IncFIB, IncN, IncFIA</td>
</tr>
<tr>
<td>KP-49, KP-50</td>
<td>blaKPC-2</td>
<td>blaCTX-M, blaSHV</td>
<td>ST258</td>
<td>IncFII, IncN, IncFIA</td>
</tr>
<tr>
<td>KP-29, KP-32</td>
<td>blaNDM-1, blaOXA-48</td>
<td>blaCTX-M, blaSHV</td>
<td>ST147</td>
<td>IncL/M, IncFII, IncA/C</td>
</tr>
<tr>
<td>KP-33</td>
<td>blaNDM-1, blaOXA-48</td>
<td>blaCTX-M, blaSHV</td>
<td>ST147</td>
<td>IncL/M, IncFII, IncA/C</td>
</tr>
<tr>
<td>KP-34, KP-35, KP-37</td>
<td>blaNDM-1</td>
<td>blaCTX-M, blaSHV, blatem</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KP-36, KP-38, KP-39, KP-40,</td>
<td>blaNDM-1</td>
<td>blaCTX-M, blaSHV</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KP-43, KP-44</td>
<td>blaNDM-1</td>
<td>blaSHV, blatem</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KP-42, KP-45, KP-46</td>
<td>blaNDM-1</td>
<td>blaSHV, blatem</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KP-30, KP-31, KP-41</td>
<td>blaNDM-1</td>
<td>blaCTX-M</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ESBLs: extended-spectrum β-lactamases; MLST: Multi-locus Sequence Typing; ST: Sequence type; KP: Klebsiella pneumoniae.

Table 3. Resistance pattern of carbapenemase gene positive K. pneumoniae isolates.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Antibiotics (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 1 2 4 8 16 32 64 128 256 512 1024</td>
</tr>
<tr>
<td></td>
<td>IMI ETP CTP CAZ CIP AK DO PB</td>
</tr>
<tr>
<td>blisterKPC-2</td>
<td>- - - - 1 - - - 1 - - 1</td>
</tr>
<tr>
<td>blanLDM-1</td>
<td>- - - - 1 - - - 1 - - 1</td>
</tr>
<tr>
<td>blanOXA-48 / blanLDM-1</td>
<td>- - - - 1 - - - 1 - - 1</td>
</tr>
</tbody>
</table>

IMI: imipenem; ETP: ertapenem; CTP: ceftaroline; CAZ: ceftazidime; CIP: ciprofloxacin; AK: amikacin; DO: doxycycline; PB: polymyxin-B.

the third generation cephalosporins (3GCs), 28.7% were resistant against two 3GCs, while 5.3% strains were resistant to one of the 3GCs. Association analysis established that 69.1% ESBL producers were surfaced from the wound/pus samples.

Among the CRKP strains (n = 78), 38.5% (n = 30) expressed carbapenemase encoding genes encompassed by 32.1% (n = 25) as single-gene consisting of blaNDM-1 (26.7%, n = 8), blaOXA-48 (26.7%, n = 8), blaKPC-2 (20.0%, n = 6) and blaVIM (10.0%, n = 3). The frequency of CRKP coharboring carbapenemase genes was 6.4% (n = 5); blanNDM-1/blanOXA-48 (10.0%, n = 3), blanOXA-48/blanVIM (3.3%, n = 1) and blanOXA-48/blanIMP (3.3%, n = 1), blanNDM-1 and blanKPC-2 genes were confirmed by DNA sequencing. The detected ESBL genes were blanCTX-M (n = 11), blasHIV (n = 7), blatem (n = 4), blanCTX-M/blasHIV (n = 35), blasHIV/blatem (n = 13), blanCTX-M/blatem (n = 19) and blanCTX-M blasHIV/blatem (n = 5). CRKP coharboring blanNDM-1 and blanOXA-48 genes were positive for blanCTX-M/blasHIV (n = 3) and blanKPC-2 positive CRKP were positive for blanCTX-M/blasHIV (n = 4) and blanCTX-M (n = 2). The results are shown in Table 2.

CRKP harboring carbapenemase genes were obtained from wound (n = 15), pus (n = 9), blood (n = 2), sputum (n = 2) and ETI (n = 2). General surgery (n = 13) and ICU (n = 8) were the main clinical wards responsible for the spread of CRKP. Most of the carbapenemase-gene positive CRKP (n = 30) exhibited non-susceptible profile for the tested antimicrobials. The MIC values of β-lactams for carbapenemase-gene positive CRKP are shown in Table 3.
MLST typing demonstrated that carbapenemase-genes harboring CRKP strains (bla<sub>KPC-2</sub> and bla<sub>NDM-1</sub>/bla<sub>OXA-48</sub>) exhibited distinct sequence types. bla<sub>KPC-2</sub>-harboring CRKP belonged to ST258 (n = 4) and ST11 (n = 2) with IncFII, IncN, IncFIIA, IncL/M, IncA/C and IncFIIK plasmids. While ST1147 (n = 3) was observed among bla<sub>NDM-1</sub>/bla<sub>OXA-48</sub> co-harboring CRKP with IncL/M, IncFII and IncA/C replicon types.

Discussion
The unregulated use of antibiotics has resulted in the dispersal of high-risk CRKP clinical strains worldwide, thus narrowing the available treatment options leading to a staggering burden on healthcare facilities. It is critical to trail the spread of resistant species in recalcitrant infections in order to prevent them. This challenge is amplified due to the insufficient available data, especially from developing countries.

In the current study, we detected high prevalence of carbapenem resistance with 72.8% CRKP and 65.4% CPKP strains highlighting resistance up trends compared to previous reports from Pakistan that documented up to 82.8% resistance and other reports form South Asia [14-17]. This clearly indicates that carbapenem resistance has increased in the study population over the years due to the lack of stewardship and unrestrained use of antimicrobials. The occurrence of CRKP infection was found significantly associated with wound (39.7%, n = 31, p value = 0.0007), pus (38.5%, n = 30, p value = 0.009), general surgery (34.6%, n = 27, p < 0.002) and ICU (26.9%, n = 21, p < 0.04). This is the first study reporting such associations in Pakistan; however, these were consistently observed worldwide [14,18]. Therefore, the emergence of CRKP in general surgery and ICU underscores their possible contribution in the successful survival of high-risk MDR <i>K. pneumoniae</i>.

We reported higher antimicrobial resistance for carbapenems (99.2%), 3GC (> 90%) and AK (80.8%) that were derived from <i>K. pneumoniae</i> only. Comparatively, other studies from Pakistan reported variable antimicrobial resistance against carbapenems 1.71%, 35.1%, 38.0%, 39.1% and 11.0% [15,16,19,20] by taking into account isolate total isolated strains. On the other hand, we observed higher susceptibility against PB (97.4%) and TGC (60.3%); however, another study reported higher TGC resistance (70.5%) [19]. The observance of variable antimicrobial resistance patterns with predominance of MDR-CRKP clinical strains in Pakistan indicated that resistance has increased over the years.

The hardest challenge that antimicrobials face is the genetic versatility of bacterial populations featuring the presence of carbapenemases with the frequent detection of bla<sub>KPC-2</sub>, bla<sub>NDM-1</sub>, bla<sub>OXA-48</sub> and bla<sub>VIM</sub> among CRKP isolates [21]. We identified carbapenemases in 38.5% of CRKP with the frequency of single detected genes: 26.7.0% for bla<sub>NDM-1</sub>, 26.7% for bla<sub>OXA-48</sub>, 20.0% for bla<sub>KPC-2</sub>, 10.0% for bla<sub>VIM</sub> and coharbored genes: 10.0% for bla<sub>NDM-1</sub>/bla<sub>OXA-48</sub>, 3.3% for bla<sub>OXA-48</sub>/bla<sub>NDM-1</sub>, 3.3% for bla<sub>OXA-48</sub>/bla<sub>IMP</sub>. Other studies from Pakistan reported that bla<sub>NDM-1</sub> is detected more frequently supervened by bla<sub>OXA-48</sub> and bla<sub>VIM</sub> [12,16,20,22-25]. However, the first report of bla<sub>KPC-2</sub> emergence surfaced from Pakistan in 2016 followed by two recent studies that described 1.8% and 11.8% of KPC-2-producing CRKP [4,12,16]. Our results showed 20.0% KPC-2-producing <i>K. pneumoniae</i> coharboring bla<sub>CTX-M</sub> and bla<sub>SIV</sub> indicating that the prevalence of bla<sub>KPC-2</sub> is on the verge of expansion in Pakistan. On the other hand, data from other countries showed higher emergence of bla<sub>KPC-2</sub> CRKP such as from China 88.1%, Italy 79%, France 84.1%, Iran 29.3% and Algeria 21.4% [26]. However, in contrast to other studies from Pakistan, the high detection rate and presence of multiple resistance genes in our study indicated that more successful lineages have emerged pointing towards the urgent need of proper surveillance and infection control measures to halt its spread in the study population.

The successful dissemination of KPCs has been attributed to ST258 <i>K. pneumoniae</i> and its single locus variant ST11. It has been reported that ST258 was identified mainly from Europe and US while ST11 was commonly spotted in the Asian region [11]. In the current study, we identified novel bla<sub>KPC-2</sub>-producing <i>K. pneumoniae</i> ST11 coharboring bla<sub>SIV</sub> and bla<sub>CTX-M</sub> which to the best of our knowledge has not been previously reported in Pakistan. ST11 was linked to bla<sub>NDM-1</sub> and bla<sub>OXA-48</sub> CRKP in Pakistan and several other countries such as Europe, China, USA, Thailand, Australia, UAE, Malaysia and Iran depicting the potential of ST11 to retain resistance with the acquisition of different carbapenemases [11,20]. While ST11 harboring bla<sub>KPC-2</sub> CRKP has been shown to become a dominant strain in China as a result of progressive evolutionary changes in the genetic makeup [27]. On the other hand, ST258 among bla<sub>KPC-2</sub> CRKP has been described from Pakistan [12] but we observed ST258 CRKP isolates coharbored bla<sub>KPC-2</sub>/bla<sub>SIV</sub> and bla<sub>CTX-M</sub>. Determination of ST11 bearing bla<sub>KPC-2</sub>/bla<sub>SIV</sub> and bla<sub>CTX-M</sub> in our study demonstrated that the heterogeneous ST11 genome has started emerging in Pakistan.
Thereafter, we identified IncFII, IncN, IncFIAA, IncL/M and IncFIK replicon types among blaKPC-2 CRKP isolates. It has been shown that IncFII-like plasmids are largely responsible for the dissemination of blaKPC-2 among K. pneumoniae especially in ST11 due to the presence of Tn1721 transposon. Other reported replicon types among K. pneumoniae isolates responsible for the spread of carbapenemases included IncR, IncFIB, IncX3, IncA/C and IncL/M IncFIK [11].

Conclusions
We report the emergence of blaKPC-2 producing K. pneumoniae ST11 coharboring blashv and blactx-m in Pakistan. The emergence of such high-risk clones with several resistance genes due to misuse and selection pressure of antimicrobials is quite alarming and warrants immediate attention. Therefore, we need to understand resistance patterns and the uniqueness of such high-risk clones in order to decipher their inexorable fate.

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**Conflict of interests:** No conflict of interests is declared.