TITLE: Molecular characterization of carbapenem resistant *Klebsiella pneumoniae* clinical isolates: Preliminary experience from a tertiary care teaching hospital in the Himalayas

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Key words:

1. Molecular characterization
2. Carbapenem resistance
3. *Klebsiella pneumoniae*

42 **ABSTRACT:**

43 **Introduction:** *Klebsiella pneumoniae* is recognized as an urgent threat to human health because of the emergence of multidrug-resistant (MDR) and
44 hypervirulent strains. Development of novel highly effective and safe treatment options is the need of the hour. One of the ways of achieving this goal is by
45 conducting molecular characterization studies of antibiotic resistant bacteria.

46 **Hypothesis/Gap Statement:** The government of India is committed to generate validated AMR data from different parts of the country. Efforts are being
47 made to perform molecular characterization of MDR and potentially virulent bacteria by whole-genome sequencing (WGS). However, the data that we have
48 at present is skewed as many parts of the country remain underrepresented. Uttarakhand is one such state located in the Himalayan belt of India, with
49 relatively poor access to healthcare and a paucity of research.

50 **Aim:** This study was performed to generate WGS based preliminary data about the population structure, multi-locus sequence types (MLST), and virulence
51 factors of CRKp isolates recovered from patients in a tertiary care teaching hospital and institute of national importance, located in Rishikesh, Uttarakhand,
52 India.

53 **Methodology:** A cross-sectional study was conducted at a tertiary care teaching hospital. It included twenty-nine randomly selected and archived
54 carbapenem resistant *Klebsiella pneumoniae* (CR-Kp) isolates obtained from various clinical samples submitted in the Bacteriology laboratory for culture
55 and sensitivity testing, from July 2018 to August 2019. After preliminary identification (ID) and antibiotic susceptibility testing (AST), as per standard
56 guidelines, these isolates were sent to Central Research Laboratory (CRL), Bengaluru, India, for further characterization & WGS.

57 **Results:** Twenty-seven out of twenty-nine test isolates were CRKp. Among the 27 CRKp isolates, ST14 was the most common sequence type (8, 29.6%),
58 followed by ST231 (5, 18.5%) and ST147(3, 11.1%) respectively. KL2 (9/27, 33.3%) and KL51 (8/27, 29.6%) were dominant K loci types in this study. Out of 5
59 O antigens identified, O1 and O2 together accounted for 88.9% (n=27) CRKp isolates. Yersiniabactin and Aerobactin were identified in 88.9% (24/27) &
60 29.6% (8/27) of the CRKp isolates of the isolate. Regulatory genes rmpA2 and rmpADC were found in 14.8% (4/27) and 3.7% (1/27) isolates respectively. The
61 predominant plasmid replicons present were ColKP3 (55.5%), IncFII(K) (51.8%), IncFIB(pQiI) (44.4%), IncFIB(K) (37%), IncR (33.3%) and Col44 OI (18.5%)
62 respectively. A perfect agreement (100%) was observed between phenotypic and genotypic resistance profiles in the case of fluoroquinolones, penicillins,
63 Beta Lactam/Beta Lactam Inhibitor combinations (BL/BLI), and cephalosporins respectively. As compared to phenotypic resistance, higher genotypic
64 resistance for aminoglycosides (96.3%) and folate pathway inhibitors (92.6%) respectively, was observed.

65 **Conclusion:** This study emphasizes the need for continued genomic surveillance of emerging CRKp and other MDR bacteria in Uttarakhand and
66 neighbouring states of India. This in turn would help in generating critical information that can be used to assess the emergence, dissemination, and
67 potential impact of important variants.

68

69 Introduction:

70 *Klebsiella pneumoniae* is recognized as an urgent threat to human health because of the emergence of multidrug-resistant (MDR) and hypervirulent strains
71 associated with hospital outbreaks and severe community-acquired infections [1]. Of particular importance is carbapenem resistant *Klebsiella pneumoniae*
72 (CRKp), which apart from being multi-drug resistant, is associated with high morbidity and mortality particularly among persons with prolonged
73 hospitalization exposed to invasive devices [2].

74 Owing to paucity of treatment options for life threatening infections caused by MDR Gram negative bacteria (including CRKp), there is renewed interest in
75 using highly toxic and narrow spectrum antibiotics like polymyxins, which were discovered almost six decades ago [3]. Development of novel highly
76 effective and safe treatment options is the need of the hour. One of the ways of achieving this goal is by conducting molecular characterization studies of
77 antibiotic resistant bacteria. In a review published by Karakostas et al, it has been highlighted that determination of genetic resistance mechanisms,
78 particularly in CRKp isolates, may play a vital role in deciding novel treatment options like ceftazidime/avibactam, imipenem/relebactam etc., in hospitalised
79 patients [4].

80 India has been referred to as the “Antimicrobial resistance (AMR) capital” of the world' [5]. As per the 'scoping report on antimicrobial resistance in India
81 (2017)', under the aegis of the Department of Biotechnology, Government of India, >70% isolates of *Klebsiella pneumoniae* were resistant to broad-
82 spectrum antibiotics like fluoroquinolones and third generation cephalosporins. Increasing rates of resistance to carbapenem antibiotics and beta-
83 lactam/beta-lactamase inhibitor combination antibiotics like piperacillin-tazobactam was also observed in *K. pneumoniae*, by the investigators [6]. The
84 government of India is committed to generate validated AMR data from different parts of the country. Efforts are being made to perform molecular

characterization of MDR and potentially virulent bacteria by whole-genome sequencing (WGS). These initiatives will significantly improve our ability to analyse outbreak scenarios and characterize the transmission dynamics & mechanisms of antibiotic resistance, of important pathogens [7]. However, the data that we have at present is skewed as many parts of the country remain underrepresented. Uttarakhand is one such state located in the Himalayan belt of India, with relatively poor access to healthcare and a paucity of research.

This study was performed to generate WGS based preliminary data about the population structure, multi-locus sequence types (MLST), and virulence factors of CRKp isolates recovered from patients in a tertiary care teaching hospital and institute of national importance, located in Rishikesh, Uttarakhand, India. This institute was established almost a decade ago and consistent efforts were being made to streamline the hospital infection control and antibiotic stewardship related activities, since it's time of conception. A review of our partially documented cumulative antibiogram from January 2018 to December 2020 revealed, that 14.3% (279/1947) of MDR Gram negative bacterial isolates, were carbapenem resistant *Klebsiella pneumoniae*. Being classified as "Priority 1 pathogens" by the World Health Organisation (WHO) [8], CRKp isolates were selected to kick-start a genomic surveillance programme of MDR bacteria, at our institute. To the best of our knowledge, this study will be the first of its kind from the northern hilly terrain of India.

Methods:

Bacterial isolates and phenotypic characterization:

A cross-sectional study was conducted at a tertiary care teaching hospital, after obtaining ethical approval (AIIMS/IEC/18/477 & KIMS IEC/S12-2017). The study included twenty-nine randomly selected and archived carbapenem resistant *Klebsiella pneumoniae* (CR-Kp) isolates obtained from various clinical samples submitted in the Bacteriology laboratory for culture and sensitivity testing, from July 2018 to August 2019. After preliminary identification (ID) and antibiotic susceptibility testing (AST), as per standard guidelines [9,19], these isolates were sent to Central Research Laboratory (CRL), Bengaluru, India, for further characterization & WGS. At CRL, the test isolates were subjected to ID & AST, using the VITEK-2 compact system (bioMérieux), the results of which were interpreted as per Clinical Laboratory Standards Institute (CLSI) guidelines 2021 [11].

Molecular characterization:

Genomic DNA was extracted from bacterial isolates with Qiagen QIAamp DNA Mini kit, in accordance with the manufacturer's instructions. Double-stranded DNA libraries with 450 bp insert size were prepared and sequenced on the Illumina platform with 150 bp paired-end chemistry. The genomes passing the QC were assembled using Spades v3.14 to generate contigs and annotated with Prokka v1.5 [12]. SNPs were identified for all 29 *K. pneumoniae* isolates by mapping of reads to the NCBI reference genome, *K. pneumoniae* strain NTUH-K2044, NC_006625.1 (https://www.ncbi.nlm.nih.gov/nuccore/NC_006625.1/). MLST, K-and O-antigen types, virulence factors and AMR genes were identified using the protocols as detailed in www.protocols.io [13].

Results:

1. Baseline information of the collection: The test isolates were obtained from clinical samples of patients with an age range of 16 months to 79 years (mean age of 42.81 years; male: female=23: 6), twenty-seven of which were CRKp. Most isolates were from urine (12/29; 41.4%) and pus (11/29; 37.9%). Sequencing results revealed that all test isolates were *K. pneumoniae* sensu stricto.

2. Clonal distribution: Among the 27 CRKp isolates, ST14 was the most common sequence type (8, 29.6%), followed by ST231 (5, 18.5%) and ST147(3, 11.1%) respectively. Sequence types 11, 15, 43, 395, 437 and 3392 together constituted 37% of the CRKp isolates. One novel ST (ST16-1LV) with XDR was identified in a CRKp isolate.

KL2 (9/27, 33.3%) and KL51 (8/27, 29.6%) were dominant K loci types in this study. The phylogenetic tree of the test isolates shows the correlation between capsular locus type and sequence type, for example of KL2 mainly with ST14, and of KL51 with ST231 and ST147 respectively. (Figure 1).

Out of 5 O antigens identified, O1 and O2 together accounted for 88.9% (n=27) CRKp isolates (Table 1). Other antigens present included O4 (3.7%), OL101 (3.7%), OL103 (3.7%). O-locus (OL) types also showed ST-specific distribution. In particular, O1v1 was found in STs 14, 43, 395, 15 and 3392, O1v2 in ST231 and O2v2 in STs 147 and 11 respectively (Table 1 & Figure 1). The two carbapenem sensitive MDR *K. pneumoniae* isolates belonged to ST 147, with K & O loci being KL124 and O3b, respectively.

3. Virulence factors & plasmid repertoire: Predominant virulence factor genes and plasmid replicons found in CRKp isolates in the present study, have been summarized in Table 1. Yersiniabactin, an iron uptake locus (ybt), was identified in 88.9% (24/27) of the isolates. Aerobactin, a critical virulence factor for Hypervirulent *K. pneumoniae* (HvKP), was found in 29.6% (8/27) of the isolates, all belonging to ST231, 43, 15 & 3392 respectively. Regulatory genes rmpA2 and rmpADC were found in 14.8% (4/27) and 3.7% (1/27) isolates respectively. While Colibactin and rmpA genes were absent in all isolates, Salmochelin was found in only one isolate.

The predominant plasmid replicons present in the 27 CRKp isolates were ColKP3 (55.5%), IncFII(K) (51.8%), IncFIB(pQil) (44.4%), IncFIB(K) (37%), IncR (33.3%) and Col44 Ol (18.5%) respectively.

4. Antimicrobial resistance profile and their distribution: Table 2 depicts phenotypic and genotypic antimicrobial resistance profile of 27 CRKp test isolates. High phenotypic resistance rates to aminoglycosides (88.8%), fluoroquinolones (100%), penicillins (100%), BL/BLI (100%), cephalosporins (100%), folate pathway inhibitors (81.5%), and glycylycylines (66.7%) respectively, were observed. Resistance to polymyxins was observed in 3.7% of the isolates. A perfect agreement (100%) was observed between phenotypic and genotypic resistance profiles in the case of fluoroquinolones, penicillins, Beta Lactam/Beta Lactam Inhibitor combinations (BL/BLI), and cephalosporins respectively. As compared to phenotypic resistance, higher genotypic resistance for aminoglycosides (96.3%) and folate pathway inhibitors (92.6%) respectively, was observed. While no colistin

resistance genes were observed in 1 phenotypically resistant isolate, another isolate showed a disruption of the MgrB gene with a colistin MIC value in the intermediate range (≤ 0.5 $\mu\text{g/ml}$). None of the CRKp isolates contained tigecycline resistance genes.

All 27 CRKp isolates showed phenotypic resistance to imipenem, meropenem and ertapenem. Of these, 77.8% (21/27) carried *bla*_{OXA-48} like genes (*bla*_{OXA181} and *bla*_{OXA232}), and 55.6% (15/27) carried *bla*_{NDM-1/5}, respectively (Table 3). We observed that 71.4% (n=15/21) of the *bla*_{OXA-48} like genes carrying CRKp isolates harboured a plasmid with the ColKP3 replicon sequence (Figure 1). Notably, no *bla*KPC genes were detected.

In the present dataset, resistance to carbapenems was also conferred by ESBLs when combined with porin loss-of-function mutations. Disruption of the Ompk35 porin (n=21) and insertion mutations of TD/GD amino acids at position 115 of Ompk36 gene (n=19) were found in CRKp isolates. The disruption of major outer membrane protein genes (Ompk35/Ompk36) along with ESBLs (*bla*_{CTX-M-15}, *bla*_{SHV-11}, *bla*_{SHV-31}, *bla*_{OXA-1}, *bla*_{OXA-9}, *bla*_{TEM-1}, *bla*_{FONA-5}, *bla*_{CMY-6}, *bla*_{SFO-1}) were detected in 40.7% of the isolates (11/27). There were 15 isolates in which multiple ESBL genes were detected.

Discussion:

156 In the present study, all clinical isolates were correctly identified as *Klebsiella pneumoniae* by VITEK-2 Compact, taking whole genome sequencing results as
 157 gold standard. Keeping in mind the potential clinical and epidemiological significance of each phylogroup, accurate identification of microbial isolates is
 158 imperative. Inability to distinguish species within the *K. pneumoniae* complex using phenotypic methods has been reported in the past by Bowers et al [14].

159 The predominant sequence types observed in our study were ST14, ST231 and ST147. A noteworthy finding was the absence of ST258, the most prevalent
 160 and extensively disseminated KPC-producing *K. pneumoniae* globally [15], in our collection. These findings are in concordance with published data from
 161 India [15]. ST14 and ST147 have often been described as international high-risk clones associated with extensive drug resistance (XDR) [16,17]. ST231 is an
 162 emerging CRKP epidemic clone which was first reported in 2013 in Delhi, India. This clone has also been reported from several other southeast Asian and
 163 European countries and has frequently been found to be MDR, combining resistance to carbapenems, extended-spectrum cephalosporins, and broad-
 164 spectrum aminoglycosides [15,18,19]. Another interesting finding of our study was that ST11 was identified in 7.4% of the CRKp test isolates. The ST11
 165 clone is a single locus variant of ST258 and is associated with the spread of KPC in Asian countries like China and Taiwan [20]. Reports of presence of these
 166 clones in the Indian subcontinent [21], has made the AMR situation in the country, even more gruesome.

167 Owing to paucity of treatment options for infections caused by MDR *K. pneumoniae* strains, newer avenues in the form of lipopolysaccharide and capsule
 168 polysaccharide-based vaccines, are currently being explored [22]. Therefore, it is important to address the high diversity of surface-exposed
 169 polysaccharides, synthesized as O-antigens (lipopolysaccharide) and K antigens (capsule polysaccharide), present in *K. pneumoniae*. KL2, KL51 and O1, O2
 170 were the predominant K-loci and O serotypes respectively, among CRKp isolates in our study. These findings are in concordance with a study conducted by

171 Follador et al, in which serotypes O1, O2 and O3 were most prevalent in the sample set, accounting for approximately 80 % of all infections. In contrast, K
172 serotypes showed an order of magnitude higher diversity and differed among infection types [22].

173 In the present study, virulence factor genes like yersiniabactin, aerobactin and carbapenemase carrying ColKP3 plasmid replicons were predominantly
174 observed in ST14 & ST231 CRKp clones. Carriage of resistance markers on plasmids and virulence genes on integrative conjugative elements, is often
175 associated with international clones of CRKp such as ST11, ST14, ST15, ST63, ST147, ST231, etc. his has aided in their rapid dissemination across boundaries
176 [23].

177 Discordant phenotypic and genotypic resistance profile was observed in this study for aminoglycosides and folate pathway inhibitors, with higher genotypic
178 as compared to phenotypic resistance. The discrepancy between phenotypic and genotypic resistance is a major shortcoming for the accuracy
179 of genotypic antimicrobial resistance (AMR) prediction. Furthermore, differences in the expression of AMR genes may cause unjustified restriction of
180 therapeutic options resulting in the use of less potent and more toxic substances [24]. The colistin susceptibility test results of 2 isolates were not in
181 agreement with WGS results. Since ID/AST automated systems are known to produce variable in vitro colistin susceptibility test results, the joint Clinical and
182 Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Polymyxin Breakpoints Working Group
183 recommended broth micro dilution (BMD) method as the reference test for determining colistin susceptibility, in March 2016 [25]. Also, the phenotypic
184 susceptibility test results of tigecycline cannot be considered reliable because as per EUCAST guidelines 2021, for Enterobacterales other than *Escherichia*
185 *coli* and *Citrobacter koseri*, the activity of tigecycline varies from insufficient to variable [20]. Both CLSI and EUCAST have not defined any clinical
186 breakpoints for tigecycline in case of *K. pneumoniae* [11,26].

187 Resistance to antimicrobials in *K. pneumoniae* is primarily acquired by horizontal gene transfer mediated by plasmids and/or mobile genetic elements [27].
 188 The highly challenging MDR-Kp or CR-Kp isolates are mainly associated with the NDM or/and OXA-48-like carrying ST11, ST14, ST15, ST101, ST147, ST395
 189 and ST231 in Asia and KPC harbouring ST258 in North America and Europe, respectively [28]. In the present study, all CRKp isolates showed phenotypic
 190 resistance to imipenem, meropenem and ertapenem, explained by the presence of *bla*_{OXA-48} like genes, *bla*_{NDM} genes, and a combination of ESBLs &
 191 inactivation of porins Ompk35/36 [29]. While 77.8% of these isolates carried *bla*_{OXA-48} like genes, 55.6% carried *bla*_{NDM-1/5}, which supports the reported
 192 changing trends in carbapenem resistance [30].

193 Some of the lacunae of this study were small sample size and lack of a structured sampling framework. This study emphasizes the need for continued
 194 genomic surveillance of emerging CRKp and other MDR bacteria in Uttarakhand and neighbouring states of India. This in turn would help in generating
 195 critical information that can be used to assess the emergence, dissemination, and potential impact of important variants.

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212 **Author statements:**

Contributor role	Author 1	Author 2	Author 3	Author 4	Author 5	Author 6	Author 7	Author 8
Conceptualization	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
Methodology	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
Software	-	Yes	-	-	-	-	-	-
Validation	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
Formal analysis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Investigation	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
Resources	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Data curation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Original draft preparation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Visualisation	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
Supervision	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
Project administration	Yes	-	-	-	Yes	Yes	-	Yes
Funding	-	-	-	-	-	-	-	Yes (NIHR funded)

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214 **Conflict of interest:** None

215 **Funding information:** This is a multi-centric project being funded by NIHR, UK.

216 **Ethical approval:** Ethical approval was obtained vide letter #AIIMS/IEC/18/477 & KIMS IEC/S12-2017.

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