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ORIGINAL RESEARCH

Occurrence and Genomic Characteristics of Hypervirulent *Klebsiella pneumoniae* in a Tertiary Care Hospital, Eastern India

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Purpose: This study was conducted to find out the occurrence of hypervirulent *Klebsiella pneumoniae* (hvKP) isolates from different clinical specimens in a tertiary care hospital of eastern India and investigate the distribution of virulence factors, capsular serotypes and antibiogram profile. The distribution of carbapenemase-encoding genes in convergent (hvKP and carbapenem-resistant) isolates was also studied.

Materials and methods: A total of 1004 *K. pneumoniae* isolates were obtained from different clinical specimens from August 2019 to June 2021 and hvKP isolates were identified using the string test. Genes of capsular serotypes K1, K2, K5, K20, K54 and K57, virulence-associated genes, *rmpA, rmpA2, mrkD, allS, iroN, iutA, iuc, kfuB* and *ybtS*, and carbapenemase-encoding genes, NDM-1, OXA-48, OXA-181, and KPC, were evaluated by polymerase chain reaction. Antimicrobial susceptibility was determined primarily by the VITEK-2 Compact automated platform (bioMérieux, Marcy-l'Étoile, France) and supplemented by disc-diffusion/EzyMIC (HiMedia, Mumbai, India) wherever needed.

Results: Out of 1004 isolates, 33 (3.3%) were hvKP. Most frequent capsular serotype was K2 in 11 (33.3%). Amongst virulence genes, *mrkD, iutA* and *kfuB* were detected most frequently in 93.9%, 84.8% and 63.6% isolates respectively. Classical *Klebsiella pneumoniae* isolates were significantly more resistant than hvKP to cephalosporins, amoxicillin-clavulanic acid, and fluoroquinolones ($p < 0.05$). Carbapenem resistance was seen in 10 hvKP convergent isolates with the most prevalent carbapenemase-encoding gene being OXA-48 and OXA-181 in 50% isolates.

Conclusion: There is a need for continued surveillance of hvKP strains in view of the impending threat of a global spread of convergent strains.

Keywords: antimicrobial resistance, carbapenem-resistant*K. pneumoniae*, hypervirulent *Klebsiella pneumoniae*, *Klebsiella pneumoniae*, virulence gene

Introduction

Klebsiella pneumoniae (KP), a major species within the *K. pneumoniae* complex of the genus *Klebsiella* and Order Enterobacterales, is a Gram-negative, encapsulated, non-motile bacterium, that is one of the most important and medically significant pathogen of humans causing a vast majority of infections in hospitals and communities throughout the world, including those of urinary tract, soft tissue, respiratory tract, and bacteremia.[1–3] Recently, the taxonomy of the *K. pneumoniae* complex has expanded with inclusion of several species and subspecies comprising of *K. pneumoniae, K. quasipneumoniae* subsp. *quasipneumoniae, K. quasipneumoniae* subsp. *similipneumoniae, K. variicola* subsp. *variicola, K. variicola* subsp. *tropicalensis* and *K. africanensis*.[4,5] Other members of the genus *Klebsiella* (apart from the *K. pneumoniae* complex) include *K. aerogenes, K. oxytoca, K. grimontii, K. huaxiensis, K. michiganensis, K. pasteurii, K. spallanzanii and K. granulomatis*.[2,4,5] Though this nomenclatural change has been attained with the aid of numerous molecular, genomic and proteomic tools available nowadays for better differentiation amongst the species within the *K. pneumoniae* complex, however, all taxa of this complex are still assigned as *K. pneumoniae* as the phenotypic and

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biochemical characteristics of all the members are extremely similar under standard microbiological conditions.[4,5] Classical *K. pneumoniae* (cKP) species ranks ninth among common agents of bloodstream infections and 2nd–4th among both hospital-acquired as well as community-acquired urinary tract infections (UTIs).[3,6] The organism was noted amongst the top ten pathogens (second only to *Escherichia coli*) in a cohort of community-onset bloodstream infections (BSIs) in a population-based surveillance study in rural provinces of Thailand over a period of 7 years from 2007–2014.[7] In community acquired UTIs, it has accounted for 5–13.6% of isolates and in hospital-acquired UTIs, up to 20–58% of isolates.[6,8,9] In skin and soft-tissue infections, *K. pneumoniae* has accounted for 3.3%–8.1% cases.[10,11] Other less common infections include meningitis and enteritis in infants.[2,3]

Hypervirulent *Klebsiella pneumoniae* (hvKP), a newly emergent modified KP strain, first evident as a significant pathogen in 1986 in Taiwan, differs from cKP strains in a lot of clinical and microbiological aspects.[12] Unlike cKP, which cause innumerable infections in hospitals as well as long-term health care facilities, hvKP are notorious as a cause of community-acquired infections in healthy and young individuals with an inclination for disseminated spread to faraway and distant sites.[12–15] The hvKP isolates produce considerable amounts of *exopolysaccharide* capsular material resulting in a hypermucoviscous phenotype (determined by a positive string test). In addition, most of these strains belong to capsular serotypes K1 and K2.[16–19] Other prevalent capsular serotypes have been noted to be K5, K20, K54 and K57.[20,21] Several putative virulence factors, such as mucoviscosity associated geneA (encoded by *magA*), regulator of mucoid phenotypeA (*rmpA* and *rmpA2*), adhesion type 3 fimbriae (*mrkD*), allantoin metabolism (*allS*) and various siderophores such as aerobactin (*iuc*), siderophore iutA *(iutA*), salmochelin (*iroN*), yersiniabactin (y*btS*), and iron transport/phosphotransferase systems (*kfuB*) have been found to be associated with hvKP.[17,18,22–24] Further, a notable difference is observed in their sensitivity profile to various antimicrobial agents, the hvKP strains being generally susceptible to most antibiotics in contrast to cKP which have been notorious for their increased propensity for acquiring antimicrobial resistance determinants. Especially, the occurrence of carbapenem-resistant *K. pneumoniae* strains characterized by acquired carbapenemase genes consisting of New Delhi metallo-ß-lactamase (NDM), oxacillinase (OXA), *Klebsiella pneumoniae* carbapenemase (KPC), imipenemase (IMP), Verona integron-encoded metallo-ß-lactamase (VIM) has made the therapy of cKP infections very taxing and extremely challenging.[25,26] In this scenario, the recent emergence of convergent hvKP strains in certain clinical settings with combined characteristics of high amounts of virulence and carbapenem resistance, is being viewed as an impending public health threat and an upcoming clinical crisis in recent times.[27–30]

Increasing isolation of hvKP strains from other parts of the Asia Pacific Rim (apart from Taiwan) as well as from various regions of North and South America, Europe, Australia, and Africa, has truly made it a worldwide public health problem.[19,30–33] However, extremely limited information is available from India regarding the same.[31–34] Hence, we aimed to study the occurrence, antimicrobial susceptibility, and virulence characteristics of hvKP isolates from various clinical infections in a tertiary care hospital of eastern India. This information will help us in devising effective strategies for prevention, diagnosis, and treatment of hvKP infections as well as enhanced surveillance methods for rapid identification and monitoring of convergent *K. pneumoniae* strains.

Methods

This single-institutional cross-sectional study, approved by the Institutional Ethics Committee of All India Institute of Medical Sciences, Bhubaneswar, Odisha, India (Ref Number: IEC/AIIMS BBSR/PG Thesis/2019-20/05 dated 15th July 2019), and in compliance with the Declaration of Helsinki was conducted from August 2019 to June 2021 in a tertiary-care research, referral and teaching hospital in eastern India.

Isolate Identification and Susceptibility Testing

Consecutive non-repeat, discrete and clinically significant isolates of *Klebsiella pneumoniae* recovered from clinical specimens (blood, urine, pus, exudates, body fluid, respiratory samples, and any other significant source) of patients, receiving medical care, collected in a prospective manner and identified by VITEK-2 Compact automated platform (bioMérieux, Marcy-l'Étoile, France) were included in the study. Sample processing and interpretation were performed as per standard microbiological techniques, taking care to adhere to appropriate specimen collection procedures and other

pre-analytical parameters.[35,36] Isolates from catheter tips and gastrointestinal tract were excluded to rule out colonizing isolates. For identification of hvKP, all confirmed *K. pneumoniae* isolates were subjected to the string test utilizing a metallic loop made sterile by flaming till it became red-hot and allowed to cool.[15,22] The loop was touched to the bacterial colony from a blood agar plate and stretched, with the test being considered positive upon generation of a viscous string >5 mm in length.[15,22]

Antimicrobial susceptibility testing and interpretation was carried out as per the Clinical and Laboratory Standards Institute guidelines, primarily by the VITEK-2 Compact and supplemented by disc-diffusion/EzyMIC on Mueller-Hinton agar (HiMedia, Mumbai, India) wherever needed.[37] Colistin susceptibility testing was performed by the reference broth microdilution method.[37] Quality assurance of all the procedures, media, biochemical tests and antimicrobial susceptibility testing was maintained as per standard guidelines using control strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 along with *K. pneumoniae* ATCC 700603 (SHV-18, Extended spectrum beta-lactamase positive), *K. pneumoniae* ATCC BAA-2146 (NDM-1, carbapenem-resistant) and *K. pneumoniae* ATCC BAA-1705 (bla_{KPC} positive).[37] Organisms showing intermediate resistance were included in the percentage of resistant organisms and those displaying non-susceptibility to at least one agent in three or more antimicrobial categories were defined as multi-drug resistant (MDR).[38]

Molecular Investigations

Isolates testing positive by the string test (considered as hvKP) were subjected to genotypic characterization for detection of virulence-associated genes and capsular typing by polymerase chain reaction (PCR) using a panel of previously published oligonucleotide primer pairs (Sigma-Aldrich Ltd, St. Louis, Missouri) with their expected amplicon sizes as listed in Table 1.[18–21,23,24] All carbapenem-resistant hvKP were further investigated for carbapenemase-encoding genes based on the four most prevalent carbapenemase types in India (NDM-1, OXA-48, OXA-181, KPC) by PCR (Table 1).[25] All the PCRs were performed as single-plex reactions.

Table 1 List of Primers for Virulence Gene Detection, Capsular Typing and Carbapenemase Gene Detection in Hypervirulent *Klebsiella pneumoniae* Strains

Gene	Target	Oligonucleotide Sequence (5'to 3')	Amplicon Size (bp)	Annealing Temperature	Ref.
Primers for virulence gene detection					
magA	Mucoviscosity-associated gene A	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	1282	60°C	18
rmpA2	Regulator of mucoid phenotype-A2	F: CTTTATGTGCAATAAGGATGTT R: CCTCCTGGAGAGTAAGCATT	450	54°C	19
rmpA	Regulator of mucoid phenotype-A	F: ACTGGGCTACCTCTGCTTCA R: CTTGCATGAGCCATCTTTCA	516	59°C	23
iuc	Aerobactin	F: GCATAGGCGGATACGAACAT R: CACAGGGCAATTGCTTACCT	556	60°C	24
iutA	Siderophore	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	300	67°C	24
iroN	Salmochelin	F: GGCTACTGATACTTGACTATTC R: CAGGATACAATAGCCCATAG	992	55°C	24
kfuB	Iron transport and phosphotransferase function	F: GAAGTGACGCTGTTTCTGGC R: TTTCGTGTGGCCAGTGACTC	960	60°C	24
YbtS	Yersiniabactin	F: CACCGCAAACGCAATCTG R: GCCATAGACGCTGTTGTTGA	782	60°C	24
allS	Allantoin metabolism	F: CCGAAACATTACGCACCTTT R: ATCACGAAGAGCCAGGTCAC	508	54°C	24
mrkD	Adhesion type 3 fimbriae	F: TTCTGCACAGCGGTCCC R: GATACCCGGCGTTTTCGTTAC	340	60°C	18

(*Continued*)

Table 1 (Continued).

Gene	Target	Oligonucleotide Sequence (5'to 3')	Amplicon Size (bp)	Annealing Temperature	Ref.
Primers for capsular typing					
K1 (*mag*A)	K1 serotype (mucoviscosity-associated gene A)	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	1282	64°C	20
K2	K2 serotype	F: GACCCGATATTCATACTTGACAGAG R: CCTGAAGTAAAATCGTAAATAGATGGC	641	60°C	21
K5	K5 serotype	F: TGGTAGTGATGCTCGCGA R: CCTGAACCCACCCCAATC	280	60°C	21
K20	K20 serotype	F: GTGAGGACACTTTCGAAAGC R: TCATTTACATTCCTTCTTCC	1229	54°C	20
K54	K54 serotype	F: TTACCTCAGAGCGTTGCATTG R: TTAGGTATGACAATTGAGCTC	1014	56°C	20
K57	K57 serotype	F: CTCAGGGCTAGAAGTGTCAT R: CACTAACCCAGAAAGTCGAG	1037	54°C	20
Primers for carbapenemase gene detection					
bla_{NDM-1}	NDM-1	F: CGACGATTGGCCAGCAAATG R: ACTTGGCCTTGCTGTCCTTG	551	62°C	25
bla_{OXA-48}	OXA-48	F: GCGTGTATTAGCTTATC R: CGCGGTTCGGTAGTGTGTTT	760	60°C	25
$bla_{OXA-181}$	OXA-181	F: ATGCGTGTATTAGCCTTATCG R: AACTACAAGCGCATCGAGCA	898	58°C	25
bla_{KPC}	KPC	F: ATGTCACTGTATCGCCGTCT R: TTTTCAGAGCCTTACTGCCC	893	57°C	25

Briefly, genomic DNA was extracted by the HiPurA Bacterial Genomic DNA purification kit (HiMedia, Mumbai, India) as per manufacturer's instructions and PCR was performed using DreamTaq Green PCR Master Mix (Thermo fisher Scientific, USA). Each 25 µL PCR reaction mixture was composed of 12.5 µL of the master mix, 1.25 µL of each forward and reverse primer solution (in a final concentration of 200 nM), 5 µL of template DNA with concentration of 100 ng/µL and 5 µL nuclease-free water to complete the final volume. Amplification was carried out in Veriti Dx 96-well Thermal Cycler (Thermo Fisher Scientific, USA) with cycling conditions consisting of initial denaturation at 94 °C for 5 mins, 35 cycles of denaturation (94 °C, 30 s), annealing (respective temperatures, 30 s) and extension (72 °C, 45 s), followed by a final extension for 8 mins at 72 °C. Amplified products of PCR were loaded onto 1% agarose gel stained with ethidium bromide and observed in an automated UV trans-illumination system (Syngene G: BOX, Synoptics, Cambridge, UK) for visualization of bands.

Data Extraction and Statistical Analysis

Patient-related information with relevant clinical and laboratory data were extracted from the medical records of patient case sheets, requisition slips, and admission charts. Microbiology test results were noted down as per the investigations conducted. Categorization as community- or healthcare-associated infection was based on the World Health Organization guidelines.[39] All data were entered in Microsoft Excel and analyzed using simple descriptive statistics consisting of ratios, proportions and frequencies. Chi-square analysis of contingency tables was used for categorical data and *t* test for continuous data wherever required. A p value < 0.05 was considered statistically significant.

Results

A total of 1004 *K. pneumoniae* were isolated during the study period with sample-wise distribution as follows: respiratory specimens consisting of sputum, endotracheal aspirates, and broncho-alveolar lavage (345, 34.3%), urine

(326, 32.5%), pus and wound swabs (184,18.3%), blood (96, 9.6%), and body fluids consisting of cerebrospinal fluid, bile, peritoneal fluid, and pleural fluid (53, 5.3%). Eight hundred sixty-nine (86.5%) isolates were from patients admitted in the in-patient departments (IPDs) including intensive care units (ICUs), whereas 135 (13.4%) were from the outpatient departments (OPDs). Six hundred and twenty (61.7%) were from male patients, and 384 (38.2%) from females. The lowest and highest age at which *K. pneumoniae* was isolated was from the blood sample of a 2-day-old male child and from the urine sample of a 92-year-old male elderly patient, respectively.

Thirty-three isolates were identified to be hvKP with a resultant frequency of 3.3%. The demographic and clinical characteristics as well as the capsular serotypes, virulence-gene determinants and associated carbapenemase genes of these 33 hvKP isolates have been detailed in Table 2. Sample-wise occurrence of hvKP isolates was observed to be: respiratory specimens (15, 45.4%), followed by pus (8, 24.2%), urine (4, 12.1%), blood (3, 9.1%) and peritoneal fluid (3, 9.1%), while proportion-wise occurrence among *K. pneumoniae* isolates from various specimens were: body fluid, 5.6% (3/53); pus, 4.3% (8/184); respiratory specimens, 4.3% (15/345); blood, 3.1% (3/96), and urine, 1.2% (4/326). Eighteen (54.5%) hvKP were from patients admitted in various IPDs, eight (24.2%) from patients visiting the OPDs, and seven (21.2%) from patients admitted in the ICUs. Infection with hvKP was seen predominantly in male patients (25, 75.7%; male: female ratio, 3.1:1) and in adults in the age group of 18–60 years (22, 66.7%). Six (18.1%) hvKP infections were in elderly patients more than 60 years, and five (15.1%) among young patients less than eighteen years of age. One or more associated co-morbidities were noted in 22 (66.7%) patients, with underlying diabetes mellitus in 16 (45.7%) and a past history of pulmonary tuberculosis infection in eight (24.2%) (Table 2). Nineteen (57.6%) of the infections were healthcare-associated, while 14 (42.4%) were community-associated. Death was observed in 10 (30.3%) patients.

Analysis of capsular serotypes revealed that K2 was the predominant serotype detected in 11 (33.3%) isolates followed by K1 in 9 (27.3%) (Table 2). Other serotypes were, K54 in five (15.1%) and K5, K20, and K57 in two (6.1%) isolates each. Two (6.1%) isolates were non-typeable. As regards the virulence-encoding genes, adhesion type 3 fimbriae (*mrkD*) was the most prevalent virulence factor detected in thirty-one (93.9%), followed by siderophore (*iutA*) in twenty-eight (84.8%), and yersiniabactin (*ybtS*) in twenty-four (72.7%) isolates. Other virulence genes consisted of iron transport and phosphotransferase function (*kfuB*) in 22 (66.6%), aerobactin (*iuc*) in 21 (63.6%), regulator of mucoid phenotype A2 (*rmpA2*) in 19 (57.5%), *rmpA* in 16 (48.4%) and salmochelin (*iroN*) in 9 (27.2%) isolates (Table 2).

Overall, 216 (21.5%) KP isolates were susceptible to all the tested antimicrobial agents, whereas 788 (78.4%) were resistant to one or more agents. Six hundred forty three (64.0%) isolates were MDR. Table 3 shows the overall resistance of the isolates to the antibiotics tested as well as the comparative antibiogram analysis between the cKP and hvKP isolates, revealing significantly higher resistance in the former compared to latter for cephalosporins, amoxicillin-clavulanic acid, and fluoroquinolones ($p < 0.05$). Resistance to other antibiotics such as, aminoglycosides, co-trimoxazole, and carbapenems was also higher in the cKP isolates, though not statistically significant (Table 3). A total of 10 (30.3%) hvKP isolates were resistant to carbapenems indicating the occurrence of convergent hvKP strains with combined hypervirulence and carbapenem-resistance. Amongst the convergent hvKP strains, four (40%) were obtained from wound infections, two each (20%) from respiratory specimens and urine, and one each (10%) from blood and peritoneal fluid. All the ten carbapenem-resistant hvKP isolates were observed in hospitalized patients, with nine (90%) suggestive of hospital-acquired infection. Death rate was higher in patients harbouring an infection with carbapenem-resistant (convergent) hvKP strains compared to carbapenem-susceptible hvKP strains, though not statistically significant (4/10, 40% vs 6/23, 26.1%; $p > 0.05$). Genes encoding for carbapenemase-enzymes were detected in 6 (60%) of the ten carbapenem-resistant hvKP isolates distributed as $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$ (3 isolates), $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ (1 isolate), $bla_{\text{OXA-181}}$ alone (1 isolate), and $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$ and $bla_{\text{NDM-1}}$ (1 isolate). Thus, overall oxacillinases ($bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$) were found in 5 (50.0%) and NDM ($bla_{\text{NDM-1}}$) in 2 (20.0%) isolates.

Discussion

This observational, cross-sectional study presents comprehensive data on *K. pneumoniae* infections, with special emphasis on hvKP isolates, in a tertiary care hospital in the eastern part of India, which receives patients not only from local areas on a primary basis but also large referrals from adjoining districts and states. During the study period, a total of 1004 consecutive, non-repeat, discrete KP isolates were obtained from various clinical samples received in our

Table 2 Demographic Details and Genomic Characteristics of the 33 Hypervirulent *Klebsiella pneumoniae* Isolates, Eastern India

Strain	Source	Age/ Sex	CA/ HA	30-day Outcome	Associated co-Morbidities				Capsule Type	Carba-Penem Status	Carba-Penemase Genes	Virulence Gene Determinants				
					DM	CKD	CLD	Past H/O TB				Siderophores	Mucoid Phenotype Regulator Genes	Fimbriae	Allantoin Meta-Bolism	Iron Uptake
KP103	Sputum	25y/ F	CA	Alive	-	-	-	-	K2	S	-	*iuc, iutA*	-	*mrkD*	-	-
KP144	Sputum	23y/ F	CA	Alive	-	-	-	-	*magA*/K1	S	-	*iuc, iutA, YbtS*	*rmpA*	*mrkD*	*allS*	*kfuB*
KP207	Pus	32y/ M	CA	Alive	-	-	-	+	*magA*/K1	S	-	*iuc, iutA, YbtS, iroN*	*rmpA*	*mrkD*	*allS*	*kfuB*
KP239	Sputum	63y/ F	HA	Deceased	+	+	-	-	K2	S	-	*YbtS, iron*	*rmpA2*	*mrkD*	-	*kfuB*
KP241	Wound swab	35y/M	HA	Deceased	-	-	-	-	K57	R	bla_{OXA-48} $bla_{OXA-181}$	*iuc, iutA, YbtS*	*rmpA2*	*mrkD*	-	*kfuB*
KP284	Peritoneal fluid	45y/ M	HA	Alive	+	-	-	-	K2	S	-	*iuc, iutA, YbtS, iroN*	*rmpA, rmpA2*	*mrkD*	-	*kfuB*
KP303	Wound swab	31y/ M	HA	Alive	+	-	-	-	K2	R	bla_{OXA-48} $bla_{OXA-181}$	*iutA, YbtS*	-	*mrkD*	-	-
KP334	Sputum	21y/ M	CA	Alive	-	-	-	+	K2	S	-	*iuc, iutA*	*rmpA2*	*mrkD*	-	-
KP336	Urine	69y/ F	HA	Alive	+	-	-	-	K2	S	-	*iuc, iutA*	*rmpA, rmpA2*	*mrkD*	-	-
KP347	Blood	18y/ M	HA	Alive	-	-	-	-	*magA*/K1	R	-	*iuc, iutA, YbtS*	*rmpA, rmpA2*	*mrkD*	*allS*	*kfuB*
KP382	Sputum	26y/ M	CA	Alive	+	-	-	-	*magA*/K1	S	-	*iuc, iutA, YbtS*	*rmpA*	*mrkD*	*allS*	*kfuB*
KP420	Urine	67y/ M	HA	Alive	+	-	-	-	K5	S	-	*YbtS*	-	*mrkD*	-	-
KP449	Blood	2d/ M	HA	Deceased	-	-	-	-	*magA*/K1	S	-	*iuc, iutA, YbtS*	*rmpA2*	*mrkD*	*allS*	*kfuB*
KP459	Pus	47y/ F	CA	Alive	-	-	-	-	*magA*/K1	S	-	*iuc, iutA, YbtS, iroN*	*rmpA, rmpA2*	*mrkD*	*allS*	*kfuB*
KP466	Peritoneal fluid	40y/ M	HA	Alive	+	-	-	-	K2	S	-	*iuc, iutA, YbtS, iroN*	*rmpA, rmpA2*	*mrkD*	-	*kfuB*
KP487	Sputum	82y/ M	CA	Alive	-	-	-	+	*magA*/K1	S	-	*iuc, iutA, YbtS*	*rmpA, rmpA2*	*mrkD*	*allS*	-
KP490	Urine	45y/ M	HA	Alive	-	-	-	+	K54	R	bla_{OXA-48} bla_{NDM-1}	*iutA, YbtS, iroN*	*rmpA2*	*mrkD*	-	*kfuB*
KP515	Urine	32y/ F	CA	Alive	+	-	-	-	K54	R	-	*iutA*	-	*mrkD*	-	*kfuB*
KP585	Sputum	30y/ M	CA	Alive	+	-	-	-	K2	S		*iuc, iutA, iroN*	*rmpA*	*mrkD*	-	*kfuB*
KP618	Sputum	34y/ M	CA	Alive	-	-	-	+	K2	S	-	*iuc, iutA, YbtS, iroN*	*rmpA, rmpA2*	*mrkD*	-	*kfuB*
KP638	EA	16y/ M	HA	Alive	-	-	-	+	K20	R	$bla_{OXA-181}$	*iroN, YbtS*	-	*mrkD*	-	*kfuB*
KP647	Peritoneal fluid	57y/ M	HA	Deceased	+	-	+	-	K2	R	-	*iuc, iutA, YbtS*	*rmpA, rmpA2*	*mrkD*	-	-
KP653	Sputum	57y/ M	HA	Deceased	-	-	-	-	K54	S	-	*iuc, iutA, YbtS, iroN*	*rmpA2*	*mrkD*	*allS*	*kfuB*
KP658	BAL	52y/ M	HA	Deceased	+	-	-	-	*magA*/K1	S	-	*iuc, iutA, YbtS*	*rmpA*	*mrkD*	*allS*	-

KP667	Wound swab	51y/ M	CA	Deceased	+	-	-	-	K20	R	-	*iutA, iroN*	-	*mrkD*	-	-
KP676	Sputum	76y/ F	CA	Deceased	+	-	-	+	K54	S	-	-	-	*mrkD*	-	*kfuB*
KP677	Pus	64y/ M	CA	Alive	+	-	-	+	-	S	-	*iuc, iutA, YbtS*	*rmpA, rmpA2*	*mrkD*	*allS*	-
KP733	Sputum	53y/ M	HA	Alive	-	-	-	-	-	S	-	*iuc, iroN*	*rmpA, rmpA2*	-	-	*kfuB*
KP798	EA	9y/ M	HA	Alive	-	-	-	-	K57	S	-	*iutA, YbtS*	-	-	-	-
KP818	Wound swab	45y/ F	HA	Alive	+	-	-	-	K54	R	bla_{OXA-48} bla$_{OXA-181}$ bla_{NDM-1}	*iutA, YbtS*	-	*mrkD*	-	-
KP852	Blood	1mth/ M	HA	Deceased	-	-	-	-	K2	S	-	*iuc, iutA*	*rmpA2*	*mrkD*	-	*kfuB*
KP898	EA	60y/ M	HA	Deceased	-	-	-	-	K5	R	bla_{OXA-48} $bla_{OXA-181}$	*iutA, YbtS, iroN*	*rmpA2*	*mrkD*	-	*kfuB*
KP947	Pus	45y/ M	CA	Alive	+	-	-	-	*magA*/K1	S	-	*iuc, iutA, YbtS, iroN*	*rmpA, rmpA2*	*mrkD*	*allS*	*kfuB*

Abbreviations: M, male; F, female; y, years; d, days; mth, month, CA, community-associated; HA, healthcare-associated; DM, diabetes mellitus; CKD, chronic kidney disease; CLD, chronic liver disease; H/O TB, history of tuberculosis; EA, endotracheal aspirate; BAL, bronchoalveolar lavage; S, susceptible; R, resistant; +, present; -, absent; *magA*, mucoviscosity associated gene A; *iuc*, aerobactin; *iutA*, siderophore; *YbtS*, yersiniabactin; *iroN*, salmochelin; *rmpA*, regulator of mucoid phenotype A; *rmpA2*, regulator of mucoid phenotype A2; *mrkD*, adhesion type 3 fimbriae; *allS*, allantoin metabolism; *kfuB*, iron transport and phosphotransferase system; bla_{NDM-1}, New Delhi metallo-β-lactamase; bla_{OXA-48}, oxacillinase-48; $bla_{OXA-181}$, oxacillinase-181.

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Table 3 Comparative Antimicrobial Susceptibility Profile of Classical and Hypervirulent *Klebsiella pneumoniae* Isolates to Various Antimicrobials

Antimicrobial Agents	n (%) of Resistant Isolates Among		
	cKP (n=971)	hvKP (n=33)	Total (n=1004)
Cefazolin	718 (73.9)	16 (48.4)*	734 (73.1)
Cefuroxime	708 (72.9)	16 (48.4)*	724 (72.1)
Ceftazidime	682 (70.2)	13 (39.3)*	695 (69.2)
Ceftriaxone	675 (69.5)	16 (48.4)*	691 (68.8)
Cefixime	687 (70.7)	15 (45.4)*	702 (69.9)
Cefepime	668 (68.7)	15 (45.4)*	683 (68.0)
Amoxicillin – clavulanate	674 (69.4)	14 (42.4)*	688 (68.5)
Piperacillin – tazobactam	481 (49.5)	12 (36.3)	493 (49.1)
Ciprofloxacin	557 (57.3)	12 (36.3)*	569 (56.6)
Levofloxacin	525 (54.1)	11 (33.3)*	536 (53.4)
Amikacin	387 (39.8)	12 (36.3)	399 (39.7)
Gentamicin	432 (44.4)	13 (39.3)	445 (44.3)
Netilmicin	398 (40.9)	11 (33.3)	409 (40.7)
Ertapenem	449 (46.2)	10 (30.3)	459 (45.7)
Meropenem	435 (44.8)	10 (30.3)	445 (44.3)
Imipenem	431 (44.3)	10 (30.3)	441 (43.9)
Doripenem	435 (44.8)	10 (30.3)	445 (44.3)
Co-trimoxazole	533 (54.8)	15 (45.4)	548 (54.6)
Colistin	10 (1.0)	0	10 (1.0)
Nitrofurantoin[a]	136/322 (42.2)	1/4 (25)	137/326 (42.0)
Tigecycline[b]	23/556 (4.1)	1/26 (3.8)	25/582 (4.3)

Notes: *$p < 0.05$ (significant) for difference in resistance between cKP and hvKP by Chi-square test. [a]Tested in urinary isolates only. [b]Tested in respiratory and pyogenic isolates only.

laboratory, with maximum recovery from respiratory tract specimens, followed by urine and pus. Previous studies from India, have shown maximum isolation of KP from urine samples, followed by sputum or exudative specimens.[31,40] Studies from China observed maximum isolation from blood followed by sputum and ascitic fluid[17] or from pus followed by blood.[18] *Klebsiella pneumoniae* in the current study were isolated mostly from hospitalized patients and less from OPD patients. Other studies in the past have also shown KP infections to be more common in hospitalized patients.[2,3,41] Overall frequency of hvKP in our study amongst the KP isolates was 3.3% similar to a previous study from south India reporting a low prevalence of 2.4%.[31] In an Indian ICU, however, a higher rate of 19.4% (32/165) has been observed.[42] Studies from southeast Asia report a moderate to very high prevalence of hvKP infections ranging from 12.3% to 42.4%.[17,18,43–46] Other regions of the world such as, Canada, Iran, Mexico and France report a widely variable rate of 8.2%, 15.1%, 17.9% and 20.3%, respectively.[19,47–49] The difference in prevalence of hvKP in studies from across various continents may be due to the ethnicity of population, genetic susceptibility or local strain variation and needs to be studied in more detail. Demographic characteristics associated with hvKP infection in the current study such as male predominance and/or diabetes mellitus as an important underlying co-morbidity has also been ascertained in previous studies from India and outside.[17,18,31,42] Apart from diabetes, chronic diseases of the kidney, liver, and pancreas and underlying malignancies have also been found to be associated with hvKP infection.[17,18,31,42,46] However, unlike our finding, past history of tuberculosis has not been looked into in any of the previous studies. Further, in congruence to our observation, the study from Indian ICU observed a majority (68.8%) of hvKP infections to be hospital-acquired, although mortality was much higher at 56.2% in that study.[42]

The most frequent capsular serotype in the current study was K2 followed by K1, which is similar to various studies from China, South Korea and India with a predominance of K2 serotype.[18,31,43,45,50] However, a study on whole genome analysis of seven hvKP isolates by Shankar et al in another study from India reported K1 capsular serotype in five isolates (71.4%) with no isolate belonging to K2 capsular serotype.[32] Further, we found two hvKP isolates that were non-

typeable. Such strains have also been reported from Iran and China where non-typeable capsular serotypes were found in 41% and 59.1% of their hvKP isolates, respectively.[17,47] Thus, there is a need to delineate newer capsular serotypes as these may be crucial in mapping hvKP infections. Information on capsular serotypes in our study will provide a baseline data in India which may be required in future to define the susceptibility status of hvKP isolates specifically in relation to specific capsular serotypes. A knowledge of capsular serotype will also help in designing vaccines in the potential scenarios of a future outbreak of antibiotic-resistant hvKP isolates.

Analysis of virulence-encoding genes revealed that *mrkD* was detected in thirty-one (93.9%) followed by *iutA* in twenty-eight (84.8%), *ybtS* in twenty-four (72.7%), and *kfuB*, and *iuc* in twenty-one (63.6%) hvKP isolates each. Similarly, a study from Vellore showed predominance of *mrkD* in all seven isolates which was followed by *kfuB* and *alls* in four (4/7, 57.1%).[32] Mucoid phenotype regulator, *rmpA2* was more frequently detected than *rmpA* in the current study. In a similar manner, *rmpA2* was present in 8 isolates while 1 isolate harbored the *rmpA* gene in a study from north India.[33] Further, iron uptake encoding genes (fyuA/kfuA, kfuB) were predominantly observed in 7 isolates while that for allantoin metabolism (allB) was seen in a single isolate.[33] Siderophore-encoding genes (*iroC, ybt, irp, iucA*) were present in all the isolates[33] as observed in the current study. In China, *ybtS* (95.2%, 20/21), *iutA* (90.5%, 19/21) and *iuc* (57.1%, 12/21) were the most prevalent virulence factors,[24] while in Sudan, *entB* was the most predominant virulence gene (93.3%), followed by *mrkD* (78.3%), and *kfu* (60%).[30] In another very recently concluded study in China on 94 carbapenem-resistant KP, 89 (94.7%), 89 (94.7%), 87 (92.6%), and 13 (13.8%) isolates carried the *wabG, fimH, uge*, and *kfu* virulence genes, respectively, while, *rmpA* and *magA* were not detected.[51] A finding of interest in the current study was that, virulence gene *allS* was found to be almost exclusively associated with the K1 serotype. This is in concordance with the results of Guo Y et al who state significantly higher positivity rates of *ybtS* and *alls* among K1 than in K2 isolates.[18]

The antimicrobial susceptibility profile of *K. pneumoniae* in the current study revealed high resistance rates to various antimicrobials (except colistin and tigecycline), comparable to published literature from different geographical locations of the world.[16,19] As expected, a higher resistance was observed amongst cKP compared to hvKP, statistically significant for cephalosporins, amoxicillin-clavulanic acid, and fluoroquinolones. Frequency of antimicrobial resistance in hvKP isolates was also high ranging from 25.0% to 48.4% (excluding colistin and tigecycline). Specifically, the frequency of convergent *K. pneumoniae* isolates was 1.0% (10/1004) amongst all *K. pneumoniae* strains or 30.3% amongst the hvKP (10/33) strains. These findings are extremely similar to a multicentric study in China with an overall prevalence of CR-hvKP of 1.1% (21/1838) among all *K. pneumoniae* isolates[24] as well as to the study by Bhardwaj et al[41] and Chen et al[52] with a carbapenem-resistance rate of 37.5% (12/32) and 23.8% (10/42) among hvKP strains respectively. However, the prevalent carbapenemase gene in the study by Zhan et al[24] was observed to be $bla_{\text{KPC-2}}$in contrast to $bla_{\text{OXA-48-like}}$, $bla_{\text{OXA-181}}$ and $bla_{\text{NDM-1}}$found in our study. A study from south India had reported both bla_{NDM} and $bla_{\text{OXA-48}}$ in two K2 serotype strains.[31] Reports of CR- hvKP infections are also emerging from several other countries across the globe such as, United States of America, United Kingdom, Singapore, Russia, Iran and Japan.

Conclusion

The frequency of both hvKP and convergent hvKP infections in a tertiary care health-care setting in eastern India, appear to be low at present. However, continuous surveillance is necessary in order to detect any potential outbreak due to these entities in the future, as well as to study the outcomes in more detail so as to institute proper therapeutic strategies and appropriate infection control measures. Further, the present study will contribute to the existing but scarce data on the occurrence and genomic analysis of hypervirulent *Klebsiella pneumoniae* infections in India and would enable the clinical microbiology laboratory to strengthen its concept of hvKP and develop algorithms for routine screening and reporting of hypervirulent phenotypes. Information on capsular serotypes in our study will provide a baseline data which may be required in future to define the susceptibility status in relation to specific capsular serotypes and help in designing vaccines in the potential scenario of a future outbreak of antibiotic-resistant hvKP isolates.

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Disclosure

There are no potential conflicts of interest in this work.

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