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Analysis of Antibiotic Resistance and Virulence Traits (Genetic and Phenotypic) in *Klebsiella pneumoniae* Clinical Isolates from Pakistan: Identification of Significant Levels of Carbapenem and Colistin Resistance

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Background: The emergence of carbapenem-resistant and hypervirulent hypermucoviscous *Klebsiella pneumoniae* strains poses a significant public health challenge. We determined the MDR profiles, antibiotic resistance factors, virulence gene complement, and hypermucoviscous features of 200 clinical *K. pneumoniae* isolates from two major tertiary care hospitals in Islamabad and Rawalpindi, Pakistan.

Methods: Susceptibility profiling and phenotypic analysis were performed according to the CLSI guidelines. Genetic determinants of antibiotic resistance and virulence were detected by PCR. Biofilm formation analysis was performed by microtiter plate assay.

Results: The isolates displayed a high degree of antibiotic resistance: 36% MDR-CRKP; 38% carbapenem resistance; 55% gentamicin resistance; 53% ciprofloxacin resistance; and 59% aztreonam resistance. In particular, the level of resistance against fosfomycin (22%) and colistin (15%) is consistent with previous reports of increased resistance levels. Combined resistance to carbapenem and colistin was 7%. Genetic factors associated with colistin resistance (*mcr-1* and *mcr-2* genes) were detected in 12 and 9% of the isolates, respectively. Significant differences in resistance to gentamicin and levofloxacin were observed between the 200 isolates. Many of the isolates harbored genes specifying extended-spectrum and/or carbapenem-resistant β-lactamases: $bla_{\text{CTX-M-15}}$ (46%), $bla_{\text{NDM-1}}$ (39%), and $bla_{\text{OXA-48}}$ (24%). The prevalence of the hypermucoviscous phenotype was 22% and 13% of the MDR isolates carried the *rmpA* gene (regulator for mucoid phenotype). Key virulence factor genes detected include those encoding: porins (*ompK35* and *ompK36*; at 56 and 55% prevalence, respectively); adhesins (*fimH, mrkD,* and *ycfM*; at 19, 18, and 22% prevalence, respectively); and the polysaccharide regulator, *bss*, at 16% prevalence.

Conclusion: This report highlights carbapenem-resistant *K. pneumoniae* (CRKP) prevalence, emerging resistance to fosfomycin, and the presence of *mcr-1* and *mcr-2* in colistin-resistant isolates. Further, the detection of *rmpA* signifies the prevalence of the hypermucoviscous trait in CRKP clinical isolates from Pakistan.

Keywords: *Klebsiella pneumoniae*, multidrug resistance, carbapenemases, colistin resistance, hypermucoviscous *K. pneumoniae*

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Introduction

Klebsiella pneumoniae (KP) is often associated with hospital and community acquired infections including pneumonia, septicemia, and urinary tract infections (UTIs). Emergence of multidrug-resistant (MDR) KP reduces treatment options and is associated with higher mortality rates (up to 40–50%), particularly in organ transplant patients.[1] In the last decade, increased resistance against third-generation cephalosporins resulted in the greater use of carbapenems which subsequently contributed to the emergence of carbapenem-resistant Enterobacteriaceae (CRE) strains.[2] Resistance against carbapenems is mainly conferred by plasmids and transposons encoding carbapenemases although other factors such as AmpC β-lactamase over-production, porin defects, and efflux pumps also contribute significantly to resistance.[3] In Europe, the high frequency of sporadic CRE infections has resulted in CRE emergence being considered an epidemic.[4] Carbapenem-resistant *K. pneumoniae* (CRKP) is a prominent member of CRE strain set and it has become a substantial threat in both hospital and community health settings. Its combined resistance to almost all frontline antibiotics, including penicillins, β-lactams, carbapenems, fluoroquinolones, and aminoglycosides poses an unprecedented challenge. CRKP strains carry class A *K. pneumoniae* carbapenemase (KPC), and class B New-Delhi (NDM-1) and Verona integron-encoded metallo-β-lactamases (VIM).[5] MDR-CRKP strains have been implicated in transfer of resistance to other closely related pathogens such as *Enterobacter* spp. and *Escherichia coli*. Because of severe and life-threatening infections in healthy hosts, the recent emergence of hyper-virulent (hypermucoviscous) *K. pneumoniae* (hvKP) in the Asian Pacific region has attracted much attention. Such strains cause critical pneumonia, endophthalmitis, liver abscesses, and meningitis, and they have the unusual and concerning characteristic of metastatic spread to different organs in otherwise healthy adults.[6]

The first reported emergence of CRKP from Pakistan was in 2010.[7] Subsequently, the presence of $bla_{\text{NDM-1}}$ (conferring carbapenem resistance) was confirmed in clinical strains.[8] Recent developments suggest that CRKP strains display increased resistance to colistin and fosfomycin (some of the few antibiotics still effective against NDM-1 producing strains).[9,10] The current study determines the MDR profile, antibiotic resistance genes, virulence profile, and hypermucoviscous genotypes of clinical *K. pneumoniae* strains in Pakistan.

Materials and Methods

Sample Collection and Antibiotic Susceptibility Profiling

Two hundred clinical isolates of *K. pneumoniae* were collected from two tertiary-care hospitals located in the twin cities of Rawalpindi and Islamabad. The study protocol was approved by the Institutional Review Board of the Armed Forces Institute of Pathology and informed consent of patients was acquired. Ethical approval was granted by Bio-Ethical Committee (BEC) of Quaid-i-Azam University Islamabad. Experimental work was conducted at Quaid-i-Azam University (Islamabad) in compliance with the recommendations of the Institutional Ethical Committee. The isolates were identified using Gram-staining, colony morphology, and biochemical testing using the API 10S kit (Biomerieux). Antibiotic susceptibility to ampicillin (30 μg), ceftriaxone (30 μg), aztreonam (30 μg), gentamicin (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), fosfomycin (50 μg), polymyxin B (300 U), colistin (10 μg), imipenem (10 μg), and meropenem (10 μg) was determined by disc diffusion assays. Data were interpreted according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2017). *E. coli* ATCC 25922 was used as a control strain throughout the experimental procedures.

Phenotypic Detection of ESBLs and Carbapenemases

The ESBL phenotypes were confirmed via double-disc synergy testing, using discs of amoxicillin-clavulanic acid (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), and aztreonam (30 μg). ESBL production was inferred by any distortion or ≥5 mm augmentation of an inhibition zone of a cephalosporin disc towards the amoxicillin-clavulanate disc.[11] Carbapenemase production was assessed by the Modified Hodge test (MHT). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as control strains. The formation of cloverleaf indentations on a lawn of *E. coli* ATCC 25922 characterized the tested isolates as carbapenemase producers (CLSI M100, 2017).

Determination of MICs of *K. pneumoniae* Isolates

The broth micro-dilution method was used to quantify antibacterial resistance against gentamicin, levofloxacin, and colistin. Briefly, adjusted cultures (to give a final

CFU/mL of ~5 x 10^5) of the test organisms were inoculated into Muller-Hinton broth (Oxoid, UK) in microtiter plates containing two-fold serial dilutions of an initial antibiotic stock solution giving final concentrations ranging from 0.125 to 128 µg/mL. The bacterial cultures were incubated for 18 h at 37°C before growth was assessed; the lowest concentrations of antibiotic where inhibition occurred were designated as the MIC, as per CLSI guidelines. The reference breakpoints for the interpretation of MICs against gentamicin, levofloxacin, and colistin were set as ≥16, and ≥2 µg/mL, respectively.[12]

DNA Extraction and Amplification of Genes

DNA was extracted by the phenol-chloroform method.[13] PCR amplifications were performed for the genes encoding carbapenemases (bla_{NDM}, bla_{OXA-48}, and bla_{KPC}), ESBLs (bla_{TEM}, bla_{SHV}, $bla_{CTXM-14}$, $bla_{CTXM-15}$, bla_{OXA}), and efflux pump proteins (*acrAB, tolC*). Plasmid-encoded genes specifying colistin resistance (*mcr-1, mcr-2*), the virulence gene for hypermucoviscosity (*rmpA)*, iron acquisition genes (*iucA, kfu*), biofilm regulator (*bss*), adhesion genes (*fimH, mrkD, ycfM*), the serum resistance factor gene (*traT*), and porins genes (*ompK35, ompK36*) were amplified as described elsewhere.[14] Plasmid DNA was extracted using a Thermo Scientific Gene JET plasmid miniprep kit.

Detection of Virulence Phenotypes in *K. pneumoniae* Isolates

Hypermucoviscous phenotypes were tested by using the string test through the formation of a ≥5 mm mucoviscous string from a single bacterial colony. Nigrosine staining was performed for visualization of *K. pneumoniae* capsule under light microscopy. The isolates were tested for haemagglutination, and for gelatinase and lipase production. The biofilm potential of the isolates was quantified using a microtiter plate assay by comparing the "cut-off" optical densities (ODc) with negative controls at 492 nm.[11]

Results

Sample Distributions and Antibiotic Resistance

Two hundred clinical *K. pneumoniae* isolates were collected from nine different sample sites (Table 1; see Table S1 for full data set). The overall resistance level for carbapenems (imipenem and meropenem) was 38%. The greatest frequency of resistant was to ampicillin (94%), followed by ceftriaxone (71%). The frequencies of resistance against gentamicin (55%), ciprofloxacin (53%), colistin (15%), aztreonam (59%), and fosfomycin (22%) were above the threshold levels at which these antibiotics should no longer be used in empirical treatment of *K. pneumoniae* infections[15] (Table 2). Resistance against polymyxins B and E was 16 and 15%, respectively. The prevalence of MDR within the 200 isolates was 63% (125), of which 58% (72) were carbapenem-resistant (MDR-CRKP) (Table 1). Of the 72 MDR-CRKP strains, 28% (20) were ESBL producers. The prevalence of MDR-KP within *K. pneumoniae* isolates was similar in female and male patients, although there was a more than twofold higher prevalence of the combined MDR with HMV traits in isolates from males than from females. Also, a high proportion of the MDR (38%) and combined MDR/HMV (60%) strains were isolated from urine whereas much lower proportions were found in tissue, bile, swab, and catheter tip samples (Table 1).

MIC Values for Gentamicin, Levofloxacin and Colistin

MICs for gentamicin, levofloxacin, and colistin were determined for the 63 carbapenemase producers and 137 non-producers. For gentamicin, 38% of the carbapenemase producers exhibited MICs >256 µg/mL whereas only 9% of the non-producers showed resistance to such gentamicin concentrations. For levofloxacin, the MIC observed for the carbapenemase producers was twofold higher (128 µg/mL) than the non-producers (64 µg/mL). For the majority of the carbapenem-sensitive isolates, colistin MIC values were between 4 and 8 µg/mL. The differences in MICs for levofloxacin, gentamicin, and colistin between carbapenem-resistant and -sensitive isolates were statistically significant ($p < 0.01$).

Genetic Factors Specifying Antibiotic Resistance

The presence of genes encoding ESBL factors (bla_{TEM}, bla_{SHV}, bla_{CTXM}, bla_{OXA}), carbapenemases (bla_{KPC}, bla_{NDM-1}, bla_{OXA-48} type), efflux pump (*acrAB, tolC)* and colistin markers (*mcr-1, mcr-2)* was investigated. Among the carbapenemase genes, bla_{NDM-1} was the most predominant (39%) and its occurrence was significantly higher in comparison to bla_{KPC} (5%). The frequency of occurrence for the carbapenemase-encoding bla_{OXA-48} gene was

Table 1 Genetic and Phenotypic Traits of MDR *Klebsiella pneumoniae* Isolates

Total n=200 (%)		MDR n=125 (%)	Non-MDR n=75 (%)	MDR ESBLs n=42 (%)	MDR CRKP n=72 (%)	MDR HMV n=30 (%)
Specimen source						
Blood	31 (16)	21 (17)	10 (13)	9 (21)	13 (18)	3 (10)
Urine	86 (43)	47 (38)	39 (52)	15 (36)	15 (21)	18 (60)
Pus	41 (21)	28 (22)	13 (17)	8 (19)	20 (28)	5 (17)
Catheter tip	9 (5)	8 (6)	1 (1)	4 (10)	5 (7)	1 (3)
Body fluid	16 (8)	12 (10)	4 (5)	2 (5)	10 (14)	2 (7)
Bile	2 (1)	2 (2)	0 (0)	0 (0)	2 (3)	1 (3)
Nasal/Tracheal swab	8 (4)	6 (5)	2 (3)	3 (7)	6 (8)	0 (0)
Sputum	5(3)	0 (0)	5 (7)	0 (0)	0 (0)	0 (0)
Tissue	2(1)	1 (1)	1 (1)	1 (2)	1 (1)	0 (0)
Genetic determinants for:						
ESBLs						
*bla_{TEM}	60 (30)	47 (38)	13 (17)	34 (81)	23 (32)	19 (63)
*bla_{SHV}	69 (35)	49 (39)	20 (27)	32 (76)	24 (33)	24 (80)
*$bla_{CTXM-14}$	30 (15)	26 (21)	4 (5)	14 (33)	12 (17)	14 (47)
*$bla_{CTXM-15}$	91 (46)	67 (54)	24 (32)	36 (86)	32 (44)	29 (97)
*bla_{OXA}	39 (20)	34 (27)	5 (7)	21 (50)	15 (21)	13 (43)
Carbapenemases						
bla_{NDM-1}	78 (39)	69 (55)	9 (12)	22 (52)	52 (72)	26 (87)
bla_{KPC}	9 (5)	7 (6)	2 (3)	4 (10)	7 (10)	1(3)
$bla_{OXA\ 48}$ *type*	48(24)	40 (32)	8 (11)	11 (26)	30 (42)	16 (53)
Colistin resistance						
mcr-1	24 (12)	19 (15)	5 (7)	7 (17)	11 (15)	6 (20)
mcr-2	17 (9)	13 (10)	4 (5)	4 (10)	7 (10)	5 (17)
Efflux pumps						
acrAB	97 (49)	62 (50)	35 (47)	25 (60)	31 (43)	20 (67)
tolC	62 (31)	48 (38)	14 (19)	20 (48)	24 (33)	13 (43)
Porins						
ompK35	111 (56)	75 (60)	36 (48)	29 (69)	44 (61)	17 (57)
ompK36	110 (55)	65 (52)	45 (60)	20 (48)	35 (49)	11 (37)
Virulence factors						
*rmpA	21(11)	16 (13)	5 (7)	6 (14)	7 (10)	16 (53)
iucA	16 (8)	13 (10)	3 (4)	7 (16)	6 (8)	7 (23)
Kfu	6 (3)	4 (3)	2 (3)	0 (0)	1 (1)	3 (10)
Bss	31 (16)	23 (18)	8 (11)	10 (24)	10 (14)	12 (40)
fimH	38 (19)	28 (22)	10 (13)	10 (24)	20 (28)	12 (40)
mrkD	35 (18)	21 (17)	14 (19)	8 (19)	18 (25)	9 (30)
ycfM	43 (22)	26 (21)	17 (23)	7 (17)	14 (19)	4 (13)
traT	7 (4)	5 (4)	2 (3)	1 (2)	3 (4)	1 (3)

(*Continued*)

Table 1 (Continued).

Total n=200 (%)		MDR n=125 (%)	Non-MDR n=75 (%)	MDR ESBLs n=42 (%)	MDR CRKP n=72 (%)	MDR HMV n=30 (%)
Virulence phenotypes						
Capsule	198 (99)	123 (98)	75 (100)	42 (100)	70 (97)	30 (100)
Gelatinase	24 (12)	19 (15)	5 (7)	6 (14)	7 (10)	6 (20)
Blood hemolysis	15 (8)	10 (8)	5 (7)	2 (5)	3 (4)	3 (10)
Biofilm potential	187(94)	118 (94)	69 (92)	42 (100)	68 (94)	30 (100)
Haemagglutination	200(100)	125 (100)	75 (100)	42(100)	72(100)	30(100)
Hypermucoviscosity	43 (22)	30 (24)	13 (17)	12 (29)	15 (21)	30 (100)

Notes: Numbers (and percentage with respect to value in the first row of the corresponding column) of MDR isolates displaying the corresponding phenotype or genetic locus are shown. All MDR strains are included in this category (including strains with ESBL, CRKP and HMV phenotypes). A significant *p*-value indicates strong association of a factor within the four population subgroups (MDR, MDR ESBLs, MDR CRKP and MDR HMV). The significant p-value of association calculated for ESBL factors (bla_{TEM}, bla_{SHV}, $bla_{CTXM-14}$, $bla_{CTXM-15}$) within the four population groups: MDR, MDR ESBLs, MDR CRKP and MDR HMV was found to be <0.00001*; for bla_{OXA} the significant value was 0.003541*. [1]Beta-lactamase genes (bla_{TEM}, bla_{SHV}, $bla_{CTXM-14}$, $bla_{CTXM-15}$, bla_{OXA}); carbapenemase determinants: (bla_{NDM-1}, bla_{KPC}, bla_{OXA48}-*type*); colistin resistance genes : (*mcr-1, mcr-2*); efflux pump genes: (*acrAB, tolC*) porin genes: (*ompK35, ompK36*) virulence determinants: (*rmpA iucA, Kfu, Bss, fimH, mrkD ycfM, traT*). **Abbreviations:** MDR, multidrug-resistant; ESBL, extended spectrum beta-lactamase; CRKP, carbapenem resistant *Klebsiella pneumoniae*; HMV, Hypermucoviscous; MDR ESBLs, multidrug-resistant extended spectrum beta-lactamase; MDR CRKP, multidrug-mesistant carbapenem resistant *Klebsiella pneumoniae*; MDR HMV, multidrug-resistant hypermucoviscous.

24%, and the colistin-resistance *mcr-1* and *mcr-2* genes were detected in 12 and 9% of isolates, respectively. Carriage rates for bla_{TEM}, bla_{SHV}, $bla_{CTXM-14}$, $bla_{CTXM-15}$, and bla_{OXA} were ~3 fold higher in MDR-ESBL strains than in MDR (non-ESBL) strains (Table 1).

Prevalence Virulence Factors and hvKP

For the fimbrial and non-fimbrial adhesins, the occurrence rates amongst the 200 isolates were as follows: *fimH*, 19%; *mrkD*, 18%; and *ycfM*, 22%. The polysaccharide regulator gene (*bss*) was detected in 16% of the isolates. Overall, 22% of the isolates displayed a mucoviscous phenotype (string-test positive), while a similar 24% of the MDR isolates displayed this phenotype (Table 1). The *rmpA* gene encoding the "regulator of mucoid phenotype" was found in 49% of the hypervirulent phenotypes, but was not found in any of the non-HMV strains (Table 2). Isolates demonstrating an HMV phenotype showed higher resistance against fosfomycin and colistin, but a lower resistance to polymyxin B (Table 2). The frequency of occurrence of the virulence determinants *iucA, kfu, bss, fimH,* and *mrkD* was higher in the hypermucoviscous MDR isolates (Table 1). The porin genes, *ompK35* and *ompK36*, were found in 56 and 55% of isolates, respectively, and had slightly higher occurrence in the non-hypermucoviscous strains compared to the HMV strains (Table 2). Furthermore, haemagglutination (100%), capsule (99%), and biofilm formation (94%) were observed at high frequency in 200 *K. pneumoniae* isolates. Gelatinase production and blood hemolysis were observed in 12 and 8% of the isolates.

Discussion

Over the past decade, preferential use of carbapenems has generated significant selective pressure leading to the emergence of MDR-CRKP[16] and such strains are increasingly associated with life-threatening infections.[17] Likewise, emergence of hyper-virulent *Klebsiella* strains is causing an increasing challenge, particularly in countries lacking central surveillance systems for infectious diseases.[18] However, little is known about the prevalence of hvKP strains in Pakistan. In addition, very limited data are available on colistin and fosfomycin resistant CRKP. In this study, we observed that 38% of the *K. pneumoniae* isolates collected from patients were CRKP, which indicates an alarming increase of CRKP frequency in Pakistan with respect to that reported in 2010.[7] Similar increases in CRKP have been observed in India and China.[19] The resistance levels recorded for aztreonam (59%), gentamicin (55%), ciprofloxacin (53%), and imipenem (38%) were higher than the recent antimicrobial surveillance study in South Korea reporting 13.5, 24.4, 24, and 3.1% resistance, respectively.[20] Our findings align with the resistance pattern of multidrug-resistance in *K. pneumoniae* isolates from Iran with 36% aztreonam, 15.9% gentamicin, 19.4% levofloxacin, and 11.8% imipenem resistance.[21] The highest acquired resistance observed here (after the broad-spectrum cephalosporin, ceftriaxone) was for aztreonam, indicating the frequent usage of this drug against *K. pneumoniae* infections within South Asian hospital settings. This is concerning as aztreonam is the only mono-cyclic β-lactam antibiotic (monobactam) that is effective against IMP-,

Table 2 Genotypic and Phenotypic Comparison Between Hypermucoviscous and Non-Hypermucoviscous *K. pneumoniae* Isolates

Total n=200		HMV K.P n=43 (%)	Non-HMV K.P n=157 (%)	*p*-value
Antibiotic resistance				
Ampicillin	188 (94)	42 (98)	146 (93)	0.252
Ceftriaxone	142 (71)	34 (79)	108 (69)	0.188
Aztreonam	118 (59)	25 (58)	93 (59)	0.896
Gentamicin	109 (55)	27 (63)	82 (52)	0.217
Ciprofloxacin	107 (53)	23(53)	84 (54)	0.998
Levofloxacin	82 (41)	16 (37)	66 (42)	0.568
Fosfomycin	43 (22)	16 (37)	27 (17)	0.005
Polymyxin B	32 (16)	4 (9)	28 (18)	<0.00001*
Colistin	30 (15)	11 (26)	19 (12)	0.226
Imipenem	75 (38)	15(35)	60 (38)	0.689
Meropenem	63 (32)	13 (30)	50 (32)	0.839
Genetic determinants[a]				
bla_{TEM}	60 (30)	24 (56)	36 (23)	0.0005*
bla_{SHV}	69 (35)	36 (84)	33 (21)	< 0.00001*
$bla_{CTXM-14}$	30 (15)	16 (37)	14 (9)	0.000
$bla_{CTXM-15}$	91 (46)	40 (93)	51 (32)	0.000
bla_{OXA}	39 (20)	16 (37)	23 (15)	0.004
bla_{NDM-1}	78 (39)	32 (74)	46 (29)	0.000
bla_{KPC}	9 (5)	2 (5)	7 (4)	0.926
$bla_{OXA\ 48}$ type	48(24)	21 (49)	27 (17)	0.000
mcr-1	24 (12)	8 (19)	16 (10)	0.132
mcr-2	17 (9)	6 (14)	11 (7)	0.231
acrAB	97 (49)	26 (60)	71 (45)	0.076
tolC	62 (31)	20 (47)	42 (27)	0.050
ompK35	111 (56)	22 (51)	89 (57)	0.170
ompK36	110 (55)	18 (42)	92 (59)	0.008
rmpA	21(11)	21 (49)	0 (0)	<0.00001*
iucA	16 (8)	9 (21)	7 (4)	0.001*
kfu	6 (3)	5 (12)	1 (1)	0.000
Bss	31 (16)	19 (44)	12 (8)	0.000
fimH	38 (19)	20 (47)	18 (11)	0.000
mrkD	35 (18)	20 (47)	15 (10)	0.000
ycfM	43 (22)	7 (16)	36 (23)	0.208
traT	7 (4)	2 (5)	5 (3)	0.748

Notes: *A significant p-value indicates strong association of a factor within the two subgroups: HMV and non-HMV KP. [a]Genetic determinants: Beta-lactamase (bla_{TEM}, bla_{SHV}, $bla_{CTXM-14}$, $bla_{CTXM-15}$, bla_{OXA}); carbapenemase (bla_{NDM-1}, bla_{KPC}, bla_{OXA48}.*type*); colistin (*mcr-1, mcr-2*); efflux pumps (*acrAB, tolC*); porins (*ompK35, ompK36*); and virulence factors (*rmpA iucA, kfu, bss, fimH, mrkD ycfM, traT*).

Abbreviations: HMV K.P, hypermucoviscous *Klebsiella pneumoniae;* Non-HMV K.P, non-hypermucoviscous *Klebsiella pneumoniae*.

VIM-, NDM–type carbapenemase-producing *K. pneumoniae*.[22] The resistance levels recorded for colistin (15%), polymyxin B (16%), and fosfomycin (22%) were comparatively low with respect to other antibiotics tested, possibly because of their limited administration due to their associated toxicity and/or to their stringent dose administration which is based on the principle of "highest safe" rather than "minimum effective dosage"; such prescription practices would limit the emergence and spread of resistance.[22]

The 22% fosfomycin and 15% colistin resistance observed here highlight a significant increase in the resistance against these antibiotics in recent years. The resistance level recorded for colistin (15%) is approximately five times that reported in 2019 and 2020, i.e. 2% and 2.9%, respectively.[23,24] The recent surge in colistin resistance is alarming[25] since polymyxins, particularly colistin methanesulfonate (CMS), are routinely used to treat CRKP infection.[26] Colistin resistance is mainly associated with

Table 3 Colistin Resistance in CRKP and HMV *K. pneumoniae* Strains

Isolates	Colistin MIC (μg/mL)[a]	Carbapenemases[c]	Colistin Resistance Genes[b]	HMV P (n=11)	*rmpA* (n=5)
KP104j	0.5	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	–	–
KP194j	0.5	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*	*mcr-1*	HMV	*rmpA*
KP100j	1	–	–	–	–
KP124j	1	$bla_{OXA\ 48}$ *type*	*mcr-1*	–	–
KP166j	1	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	HMV	*rmpA*
KP39j	2	bla_{NDM-1}	*mcr-1*	–	–
KP64j	2	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*; bla_{KPC}	*mcr-1*	–	–
KP73j	2	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*; bla_{KPC}	*mcr-1*	–	–
KP97j	2	$bla_{OXA\ 48}$ *type*	-	HMV	*rmpA*
KP5j	**4**	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	HMV	*rmpA*
KP44j	**4**	$bla_{OXA\ 48}$ *type*	*mcr-1*	–	–
KP56j	**4**	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*; bla_{KPC}	*mcr-1, mcr-2*	–	–
KP109j	**4**	bla_{NDM-1}	*mcr-2*	–	–
KP42w	**4**	-	*mcr-1, mcr-2*	–	–
KP53w	**4**	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	–	–
KP36j	**8**	bla_{NDM-1}	-	HMV	–
KP50j	**8**	bla_{NDM-1}	-	HMV	–
KP59j	**8**	$bla_{OXA\ 48}$ *type*; bla_{KPC}	*mcr-1, mcr-2*	–	–
KP39w	**8**	bla_{NDM-1}	*mcr-1*	HMV	–
KP52w	**8**	–	*mcr-1, mcr-2*	–	–
KP77w	**8**	$bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	–	–
KP82w	**8**	$bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	–	–
KP32j	**16**	bla_{NDM-1}; $bla_{OXA\ 48}$ *type*; bla_{KPC}	*mcr-2*	–	–
KP135j	**16**	bla_{NDM-1}	*mcr-2*	HMV	*rmpA*
KP20j	**64**	bla_{NDM-1}	*mcr-1, mcr-2*	HMV	–
KP111j	**64**	bla_{NDM-1}	*mcr-1, mcr-2*	HMV	–
KP114j	**64**	bla_{NDM-1}	*mcr-1, mcr-2*	HMV	–
KP83j	**128**	bla_{NDM-1}; bla_{KPC}	–	–	–
KP38j	**>128**	bla_{NDM-1}	*mcr-1*	–	–
KP94w	**>128**	$bla_{OXA\ 48}$ *type*	*mcr-1, mcr-2*	–	–

Notes: [a]All these strains showed resistance to colistin by the disc diffusion assay, but only those with MIC values of 4 μg/mL or higher (indicated in bold) are designated as colistin resistant as per the specified CLSI breakpoint.[30] [b]*mcr* genes (*mcr-1* or *mcr-2*, or both) were detected in 25 out of 30 colistin-resistant isolates with 23 (77%) isolates exhibiting co-carriage with at least one carbapenemase gene (bla_{NDM-1}, $bla_{OXA\ 48}$ type or bla_{KPC}). Isolates have been arranged according to their degree of colistin resistance. [c]Carbapenemases (bla_{NDM-1}, bla_{KPC}, $bla_{OXA\ 48}$ *type*).

Abbreviations: MIC, minimum inhibitory concentration; HMV P, hypermucoviscous phenotype; *rmpA*, regulator of mucoid phenotype gene.

genetic mutations in housekeeping genes such as *mgr* and *phoAB*; however, the plasmid-mediated *mcr-1* and *mcr-2* factors pose a threat for rapid global spread of such resistance.[27] Plasmid-mediated resistance determinants elevate the MICs of antibiotics, resulting in higher antibiotic doses for effective treatment, which in turn leads to an increased probability that chromosomal mutations will arise that confer a further increase in resistance.[28] Our data match previous findings[29] demonstrating that horizontal transfer of *mcr-1* contributes to an elevated MIC value against colistin and that some *mcr* positive isolates remain within the susceptible range (Table 3). For majority of the *mcr-1* and *mcr-2* positive isolates, the MIC for colistin ranged from 4 to 8 μg/mL and can thus be defined as colistin resistant.[30] This is the first report from Pakistan that indicates an association of the plasmid-borne *mcr* genes with elevated colistin resistance as determined by the microbroth dilution method. Although a report in 2017 indicated colistin resistance in CRE stains from Pakistan, no attempt was made to determine the presence of the *mcr* genes.[9] However, here we find a clear co-association of *mcr* and carbapenemase loci with 92% (23/25) of *mcr* isolates carrying at least one carbapenemase gene (Table 3). Although *mcr-1* does not play a direct role in resistance to other antibiotics, the results reported here showing a coexistence of *mcr-1* with ESBL and bla_{NDM} determinants reflect a major development in the emergence of resistance in *K. pneumoniae*, where efficacy of carbapenems and colistin is threatened.[31]

In this study, the MICs determined for fluoroquinolone, aminoglycoside, and polymyxin were notably higher for the carbapenemase-producing *K. pneumoniae*. CRKP frequently harbors a combination of resistance mechanisms including carbapenemases, drug efflux, and loss of porin function[32,33] leading to higher levels of resistance to antibiotics, as observed here in the carbapenemase-producing isolates. Molecular screening for the resistance markers in 200 *K. pneumoniae* isolates in this study confirmed bla_{NDM-1} as the predominant (39% occurrence) carbapenemase-encoding gene followed by bla_{OXA-48} (24%) and bla_{KPC},(5%). Very high occurrence of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ carbapenemases suggests that they have a predominant role in the recent emergence of CRKP phenotypes in Pakistan.[34,35]

Hypervirulent *K. pneumoniae* strains are endemic in the Asian Pacific region,[36] but data are extremely scarce on prevalence of such strains in Pakistan.[37] In this study, 13% of the MDR isolates carried *rmpA* which is the first report for the presence of the *rmpA* gene in hvKP isolates, with resistance to frontline antibiotics, from Pakistan.[37] Overall, 22% of the isolates exhibited a positive string test and 11% of the isolates carried the *rmpA* gene. Up to 100% *rmpA* prevalence in hypermucoviscous isolates has been reported previously.[38] Another study showed 41% *rmpA* carriage in non-hypermucoviscous strains indicating additional underlying factors required for the hvKP phenotype.[39] Contrary to other studies that observed lower antimicrobial resistance in hvKP isolates,[6] here a higher resistance against cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems were found in hvKP strains. Until 2015, hypervirulent and the multidrug-resistant *K. pneumoniae* isolates were reported to belong to distinct clones with no reports of MDR and virulence convergence.[40] However, a recent combination of these traits is evident from a report of convergent multidrug-resistant hypervirulent strains from South and Southeast Asia in 2020.[41]

Conclusion

This study highlights the prevalence of CRKP clinical isolates with notable virulence potential. The results support previous studies indicating that the $bla_{\text{NDM-1}}$- and $bla_{\text{OXA-48}}$-encoded carbapenemases are the most prevalent *K. pneumoniae* carbapenemases in Pakistan. The rise in colistin and fosfomycin resistant strains globally points to significant treatment constraints in the near future. In general, the *K. pneumoniae* isolates studied here showed a high prevalence of resistance gene carriage but a relatively low virulence gene carriage; interestingly, the hypermucoviscous isolates exhibited increased resistance potential, a trait not frequently associated with the hypermucoviscous *K. pneumoniae* isolates.

Abbreviations

MDR, multidrug-resistant; ESBLs, extended-spectrum beta-lactamases; MICs, minimum inhibitory concentration; UTIs, urinary tract infections; ECDC, European Center for Disease Prevention and Control; AmpC, Ampicillinase C; CRE, carbapenem-resistant Enterobacteriaceae; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; PCR, polymerase chain reaction; HMVKP, hypermucoviscous *Klebsiella pneumoniae.*

Data Sharing Statement

The data generated from the research work is presented in this article. Any additional information required can be

requested from the corresponding author as per ethical guidelines.

Ethics Approval and Consent to Participate

The study was reviewed and granted approval by the Bio-Ethical Committee (BEC) of Quaid-i-Azam University, Islamabad; under the protocol number #BEC-FBS-QAU2019-148. The clinical samples were part of the routine hospital laboratory procedure.

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Author Contributions

All authors contributed significantly in conception, study design experimental work, data acquisition analysis and interpretation or in all aspects. Furthermore, all authors took part in drafting, revising and critically reviewing the article and gave final approval of the version being published and agreed on the journal to which article has been submitted and remain accountable for all aspects of the work reported.

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Disclosure

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

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