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# *Klebsiella pneumoniae* clinical strains of hypervirulent capsular serotypes K1 and K2 demonstrating resistance against human serum bactericidal activity and virulence in *Galleria Mellonella* model

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**Abstract:** Hypervirulent *Klebsiella pneumoniae* (hvKp) is a variant that has been increasingly linked to severe, life threatening infections including pyogenic liver abscess and bloodstream infections. HvKp belonging to the capsular serotypes K1 and K2 have been reported worldwide, however very scarce studies are available on the their genomics and virulence. In the current study we report four hypermucoviscous hvKp ESBL clinical strains of capsular serotype K1 and K2 isolated from pus and urine of critically ill patients in tertiary care hospitals in Oman. These strains belong to diverse sequence types (STs) namely ST-23(K1), ST-231(K2), ST-881(K2), and ST-14(K2). To study their virulence, *Galleria Mellonella* model and resistance to human serum killing were used. The *G. mellonella* model revealed that the K1/ST-23 isolate is the most virulent as 50% of the larvae died in the first day, followed by isolate K2/ST-231 for which 75% of the larvae died in the second day and isolate K2/ST-14 for which 50% of the larvae died in the second day. Resistance to human serum killing showed there was complete inhibition of bacterial growth of all four isolates by the end of the first hour and up to the third hour. For ST-23(K1), ST-231(K2), ST-881(K2), and ST-14(K2), there was a gradual decrease in the number of colonies up to the second to third hour, which indicates neutralization of bacterial cells by the serum components. However, this was followed by sudden increase of bacterial growth, indicating possible resistance of bacteria against human serum bactericidal activity. Whole genome sequencing (WGS) revealed that hvKp strains display unique genetic arrangement of *k-loci*. Whole genome SNP-based phylogenetic analysis revealed that these hvKp isolates are phylogenetically distinct, belonging to diverse clades and belong to different STs in comparison to global isolates. This is the first report from Oman detailing the WGS of hvKp clinical isolates and assessing their resistance and virulence genomics, which reinforce our understanding of their epidemiology and dissemination in clinical settings.

**Keywords:** hypermucoviscous[1]; *Klebsiella pneumoniae*[2]; hypervirulent[3]; mobile genetic elements[4]; capsule serotypes[5]; virulence[6]; Whole genome sequencing[7]; sequence types[8]

## 1. Introduction

*Klebsiella pneumoniae* is considered to be the most common cause of hospital-acquired infections (HAIs) accounting for 10% of all nosocomial infections worldwide [1]. Immunocompromised patients are at higher risk of HAIs comprising 8% to 12% of hospitalized patients particularly ventilator-associated pneumonia [2]. This can result in life-threatening illnesses with mortality rates ranging from 50% to 100% [3,4]. *K. pneumoniae* have evolved over the years from the classical type into a hypervirulent *K. pneumoniae* (hvKp), which has been observed in many countries around the globe [4]. However, hypervirulent *K. pneumoniae* strains (hvKp) can also be acquired from the community and have been shown to infect otherwise healthy and immunocompetent individuals [5]. There has been an increase trend in emerging of new clonal lineages of hypervirulent strains with co-occurrence of multidrug resistance genotype, known as MDR- hvKp. The most common circulating hvKp serotypes include K1, K2, K20, K54 and K57, with K1 and K2 being the most virulent and accounting for 70% of hvKp isolates [7]. HvKp strains producing hyper-mucoviscous exopolysaccharide coating capsule which contributes significantly to the pathogenicity and virulence of the bacteria and have been associated with dissemination of severe infections such as pyogenic liver abscess mainly in K1 and ST-23 serotypes [8,9]. Moreover, hvKp can cause other invasive infections particularly serotypes K2 and ST-65 [4]. However, it is well established that the hypermucoviscosity and hypervirulence are two distinct phenotypes and do not necessarily co-exist in virulent strains [10].

In addition to the capsule, other virulence factors in *K. pneumoniae* can increase the virulence including the mucoid phenotype regulators (*rmpA* and *rmpA2*), siderophores including: yersiniabactin *irp2, ybt* and Aerobactin *iucA*, outer membrane porin (KpnO), and phospholipase D family protein (PLD1) [11]. Other virulence factors which have not been thoroughly characterized including efflux pumps, iron transport systems and genes involved in allantoin metabolism [12–14]. Recent studies have shown possible contribution of allantoin related genes to progression of colorectal cancer [15]. In addition, some studies have demonstrated that the virulome of the same species could change depending on the host factors [16]. The *host factors that expose hospitalized individuals to colonization and infection including the admission into the intensive care unit (ICU), poor adherence to infection control strategies, prolonged use of invasive devices and broad-spectrum antibiotics as well as immunocompromised state especially diabetics and alcoholics* [17]. *However, as discussed earlier, K. pneumoniae infection can also be acquired from the community especially hypervirulent strains that proved to infect people who are healthy and immunocompetent* [14].

Apart from the intrinsic resistance to ampicillin in hvKp, antibiotic resistance phenotype in hvKp is still not very prevalent and the reason is still unclear [4]. However, there has been an increasing trend in resistance in hvKp isolates due to dissemination via mobile genetic elements such as types of NDM, OXA-48 and *K. pneumoniae* carbapenamase (KPC). Recently, the hvKp serotypes K1 and K2 have been detected from clinical isolates in Oman. Therefore, this study aims to characterize their virulence and antibiotic resistance genomically. We aimed to study host responses against infection with *K. pneumoniae* K1 and K2 serotypes circulating in Oman.

## 2. Results

### 2.1. ST-231 and ST-395 are the two prominent Sequence Types

Initial PCR screening of MDR *K. pneumoniae* was performed for the purpose of assessing the prevalence of hvKp capsular serotypes [Supplementary figure 1]. This screening revealed that only four out of 129 *K. pneumoniae* isolates were hypervirulent capsular strains: Kp124 (K1/ST-23), Kp 125 (K2/ST-231), Kp 126 (K2/ST-881) and Kp 83 (K2/ST-14) [Figure 1].
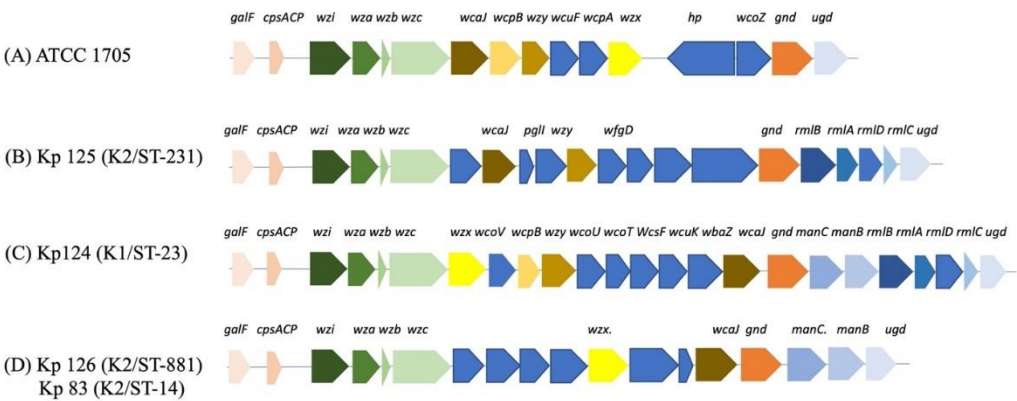
**Figure 1. *k-loci* gene arrangement of hvKP isolates.** (A) ATCC 1705 (B) Kp 125 (K2/ST-231), (C) Kp124 (K1/ST-23, (D) Kp 126 (K2/ST-881) and Kp 83 (K2/ST-14).   Gene names are indicated above the colored arrows and each group of genes with shared function or operon are colored with same colors. Chromosomal genes are conserved in all isolates including capsular synthesis, capsule translocation and surface assembly genes *galF, ORF2, wzi, wza, wzb, wzc,* and *gnd. wcoZ* is uniquely found in *K. pneumoniae* ATCC 1705 control strain and one additional isolate from our study (Kp102) Supplementary [Figure 5]. GDP-D-mannose synthesis genes *manC* and *manB* (C and D), and glucose conversion genes *rmlA, rmlB, rmlC and rmlD* (B and C).

Three of these isolates were invasive *K. pneumoniae* strains with hypermucoviscous phenotype (HMV *K. pneumoniae*) and one of these (K2/ST-881) was carbapenemase-producing (CRE) isolate. WGS analysis was performed for these four isolates in addition to the other isolates for detrmining the ST and k-loci composition. MLST was performed on the isolates from WGS data to determine their sequence type (ST) using the CGE website [Table 1] [18]. One isolate from a pyogenic infection, belonged to the ST-23, similar to global trends associating these features [Lee et al., 2017]. On the other hand, the three other isolates belonged to three different STs including ST-14, ST-231 and ST-881 [Table 1]. One third (32%, n=10) of the isolates belonged to ST-231 and 19% of the isolates (n=6) belonged to ST-395 [Table 1]. Moreover, 6% of the isolates (n=2) had ST-23 and another 6% of the isolates (n=2) had ST-405.

**Table 1.** Sequence types of local *K. pneumoniae* clinical isolates (n=31).

| Isolate (Kp) | Sequence type | Percentage (%) |
|---|---|---|
| 102, 106, 110, 112, 113, 114, 115, 116, 118, 125 | 231 | 32 |
| 103, 104, 108, 111,121, 123 | 395 | 19 |
| 124 and 128 | 23 | 6 |
| 105 and 117 | 405 | 6 |
| 100 | 13 | 1 |
| 101 | 45 | 1 |
| 107 | 1714 | 1 |
| 109 | 280 | 1 |
| 119 | 1710 | 1 |
| 120 | 147 | 1 |
| 122 | 37 | 1 |
| 126 | 881 | 1 |
| 127 | 11 | 1 |
| 83 | 14 | 1 |
| 129 | 86 | 1 |

*2.2. K-loci analysis shows unique genetic arrangement for STs with hvKp*

Initial screening of MDR *K. pneumoniae* revealed that four isolates displayed hvKp phenotype. These four isolates were isolated mainly from urine and one from wound swab. We conducted a comparison of the k-loci in these isolates to known *K. pneumoniae* references in the database using the Kaptive online tool to analyze similarities and variabilities [19]. The genetic arrangement of different *k-loci* in each isolate as well as percentage of similarity between the isolate to known references showed that most of the screened *K. pneumoniae* clinical isolates have similar genetic arrangement [Supplementary figure 3-30]. On the other hand, it was shown that the isolates with unique *k-loci* genetic arrangements belong to the hvKp isolates with serotypes K1/ST-23, K2/ST-231, K2/ST-881 and K2/ST-14 [Table 2].

**Table 2.** Source and molecular genotype of clinical hypervirulent *Klebsiella pneumoniae* isolates.

| Isolate | Serotype | ST | Source | Cluster |
|---|---|---|---|---|
| Kp 83 | K2 | 14 | Urine | D |
| Kp 124 | K1 | 23 | Pus | C |
| Kp 125 | K2 | 231 | Urine | B |
| Kp 126 | K2 | 881 | Urine | A |

The phenotypic antimicrobial susceptibility profiles of the hvKp isolates were performed using disk diffusion [Table 3 and 4]. All hvKp isolates in this study (Kp 83, Kp 125 and Kp 126) that are K2 positive and Kp 124 that is K1 positive are ESBL producers. Kp 83 (K2/ST-14) showed resistance to AMP, TZP, FEP, CTX, IMP, CAZ and CIP. Kp 126 (K2/ST-881) was resistant to AMP, FEP, CTX, IMP, CAZ, CIP. Whereas Kp124 (K1/ST-23) was resistant to AMP, TZP, FEP, CTX, FOX, CAZ. On the other hand, Kp 125 (K2/ST-231), is KPC producer with resistance to AMP, TZP, FEP, CTX, CAZ.

**Table 3.** Hypervirulent *Klebsiella pneumoniae* genotype and phenotype.

| Strain name, serotype, and ST | Plasmid | Resistance Phenotype | Resistance genotype | Resistance Mechanism | Drug Class |
|---|---|---|---|---|---|
| Kp 83 K2/ST-14 | IncFIA (HI1) 204,189 | *ESBL,* | *tet (B), tetR* | Antibiotic efflux, target alteration | Tetracycline |
| | | AMP, TZP, FEP, CTX, IMP, CAZ, CIP | | | |
| | IncFIB(K) 208,191 bp | | *catI* | Antibiotic inactivation | Phenicol |
| | | | *drfA12* | Antibiotic target replacement | Folate pathway antagonist |
| | | | *aadA2* | Antibiotic inactivation | Aminoglycoside |
| | | | *qacEdelta1* | Efflux | Disinfecting agents and intercalating dyes |
| | | | *sul1* | Target replacement | Sulfonamides |
| | | | *mphA* | Antibiotic inactivation | Macrolides |
| | IncFIB(p NDM-Mar) | | *OXA-1* | Antibiotic inactivation | Carbapenem, Cephalosporin |
| | | | *NDM-1* | Antibiotic inactivation | Carbapenem, Cephalosporin |

| | | | | Target protection | Quinolones |
|---|---|---|---|---|---|
| | 372 826 bp | | QnrB1 | Target protection | Quinolones |
| | | | catI | Antibiotic inactivation | Phenicol |
| | | | CTX-M-15 | Antibiotic inactivation | Cephalosporin |
| | | | AAC(6')-lb-cr6 | Antibiotic inactivation | Quinolones, Aminoglycoside |
| | | | tet(D) | Efflux | Tetracycline |
| | | | sul2 | Target replacement | Sulfonamides |
| | IncR 68 649 bp | | drfA14 | Target replacement | Folate pathway antagonist |
| | | | SHV-2 | Inactivation | Carbapenem, Cephalosporin |
| | | | QnrS1 | Target protection | Quinolones |
| | | | APH(6)-Id | Inactivation | Aminoglycoside |
| | ColKP3 5095 bp | KPC | OXA-181 | Antibiotic Inactivation | Carbapenem, Cephalosporin |
| | IncFIB(pQil) 115.300 bp | AMP, TZP, FEP, CTX, CAZ | KPC-3 | Antibiotic Inactivation | Carbapenem, Cephalosporin |
| | | | TEM-1 | Antibiotic Inactivation | Carbapenem, Cephalosporin |
| | | | APH(3')-la | Antibiotic Inactivation | Aminoglycoside |
| | IncFII(pAMA1167-NDM-5) 175,879 bp | | sul1 | Target replacement | Sulfonamide |
| Kp125 K2/ST-231 | | | qacEdelta1 | Efflux | Disinfecting agents and intercalating dyes |
| | | | aadA5 | Antibiotic Inactivation | Aminoglycoside |
| | | | NDM-5 | Antibiotic Inactivation | Carbapenem, Cephalosporin |
| | | | mphA | Antibiotic Inactivation | Macrolides |
| | | | drA17 | Target replacement | Folate pathway antagonist |
| | | | tetR | Antibiotic efflux, target alteration | Tetracylines |
| Kp124 K1/ST-23 | | ESBL/KPC | KPC-3 | | |

| | | | | | |
|---|---|---|---|---|---|
| | IncFIB(pQil) 115.300 bp | AMP, TZP, FEP, CTX, FOX, CAZ | | Antibiotic Inactivation | Carbapenem, Cephalosporin |
| | IncFIB(K) 208,191 bp | *ESBL,* | *catI* | Antibiotic inactivation | Phenicol |
| | | AMP, FEP, CTX, IMP, CAZ, CIP | *drfA12* | Antibiotic target replacement | Folate pathway antagonist |
| Kp126 K2/ST-881 | | | *aadA2* | Antibiotic inactivation | Aminoglycoside |
| | | | *qacEdelta1* | Efflux | Disinfecting agents and intercalating dyes |
| | | | *sul1* | Target replacement | Sulfonamides |
| | | | *mphA* | Antibiotic inactivation | Macrolides |

*2.3. Plasmid compositions of hvKp K1/ST-23 (Kp124), K2/ST-231 (Kp125), K2/ST-881 (Kp126) and K2/ST-14 (Kp83)*

The four isolates belonging to K1/ST-23, K2/ST-231, K2/ST-881and K2/ST-14 were collected during possible cross-transmission episodes in SQUH, Muscat, Oman between 2019 to 2020. Whole genome sequences of these isolates comprised 5569468 (Kp125 K2/ST-231) bases, 5572070 (K1/ST-23) bases, 5638661 (Kp126 K2/ST-881) bases, and 5447756 (Kp83 K2/ST-14) bases with 57% GC content in all strains [Supplementary data 3]. There has been an increase in reporting MDR in hvKp clinical strains over the last few years, therefore designated as MDR- hvKp. This was attributed to the horizontal gene transfer mediated by plasmids (type I and type II) [5]. For example, type I plasmids were reported to carry *bla*NDM-1 and *bla*KPC-3 [20–23]. *In Silico* detection of plasmids using plasmidFinder demonstrated that the plasmids in these strains carry mobilizable pathogenicity and resistance-conferring genes [24,25] [Table 3]. The isolate Kp 125 belonging to K1/ST-231 contain seven plasmids; pColKP3 which carries one copy of *OXA-181 and KPC-3 c*onferring carbapenem and cephalosporin resistance by mechanism of antibiotic inactivation. The other plasmids are pIncFII(pAMA11 67-NDM- 5), carrying New Delhi metallo-β-lactamase (NDM)-producing enzymes, and pIncFIB(pQil). Isolate belonging to K2/ST-23 carries three plasmids, including pIncFIB(pQil), containing KPC-3, which has been attributed to the global spread of *bla-*KPC.

In K1/ST-23 Col440II shared 100% with the reference plasmid (CP023921). On the other hand, pIncFIB(K) shares 98% identity with the reference plasmids. Isolate belonging to K2/ST-231 has three plasmids which are: IncFIB(pQil), IncFII(pAMA1167-NDM-5) and RepB. pRepB is a megaplasmid (244,152 bp) associated with the IncFIB family and has been known to integrate massive accessory modules. In ST-231 pRepB carries a very large composite transposon (15,832 bp) and insertion sequences (ISEc27) and IS102-like among other mobile elements like integrons, and MDR conferring genes [26].

Isolate Kp 83 (K2/ST-14) has four plasmids including, pIncFIA (HI1) that carries resistance genes for several antibiotic categories such as tetracyclines, phenicols, folate pathway antagonists and aminoglycosides. pIncFIB(K) harbors resistance genes against sulfonamides, macrolides and beta lactams. pIncFIB(pNDM-Mar) carries antimicrobial genes against phenicols, beta lactams, quinolones and aminoglycosides. Finally, pIncR carries antibiotic genes conferring resistance to beta lactams, quinolones and aminoglycosides [Table 3]. While most of these plasmids share common sequences,

there are wide areas of mosaic genetic structures which might indicate multiple recombination events [Supplementary figures 31 and 32].

### 2.4. The pathogenicity genes in hvKp strains

WGS of the K124 (K1/ST-23), Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14), were compared to the reference genomes of *K. pneumoniae* strain RJF293 (accession number CP014008.1 ) which belongs to serotype K2 assigned to ST-374. WGS analysis revealed that genes encoding for pathogenicity are conserved in the analyzed strains at the same relative chromosomal position. There are four main virulence classes in *K. pneumoniae* including the capsule, lipopolysaccharide (LPS), siderophores and fimbriae [supplementary file 3]. Aerobactin, but not Yersiniabactin, Salmochelin, or Enterobactin, enables the growth and survival of hvKp Ex-vivo and In-vivo [4]. Enterobactin (*entB*) is detected in all the four hvKp isolates, whereas Aerobactin (*iucA*) is found in Kp 124 (K1/ST-23), Kp 125 (K2/ST-231), Kp 126 (K2/ST-881), but not Kp83 (K2/ST-14). yersiniabactin (*irp2* and *ybt*) and salmochelin (*iroB*) were missing in all isolates. Capsule biosynthesis gene *rcsB* was detected in all isolates but not the regulators *rmpA* and *rmpA2*. LPS, adhesins and type 3 fimbriae were detected in all isolates. These findings indicate that our hvKp are well equipped with the virulence machinery which could under the right conditions enable hypervirulent phenotype.

### 2.5. Clinical hvKp were susceptible to human serum and associated with increased virulence in G. mellonella model

The isolates K124 (K1/ST-23), Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14), were tested for survival in normal human serum in comparison to the ATCC 1705 *K. pneumoniae* control, [27]. In isolate K124 (K1/ST-23) [Figure 2], there is a slight decrease in the growth indicating the death of bacterial cells followed by a slight increase in the bacterial growth in at T=3. In isolate Kp125 (K2/ST-231), in the first hour there is a decrease in the number of colonies, however, in the second and third hours there is a significant increase in bacterial growth. In isolate Kp126 (K2/ST-881), there is a decrease in growth of bacteria in the first hour but in the second and third hour there is a noticable increase in the number of colonies, which is higher compared to the previous two isolates. In Kp83 (K2/ST-14), is a slight decrease in the number of colonies in the first and second hours. However, after the second hour and by the third hour, there is a dramatic increase in the bacterial growth. In all replicates of the four tested isolates, p value showed statistical significance (*p*-value of 0.0007).
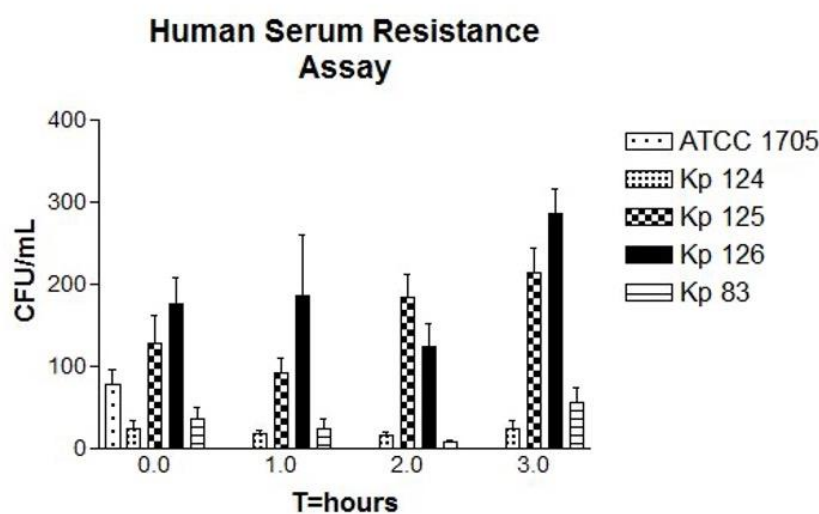
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**Figure 2. Bactericidal activity of human serum samples against hvKp clinical strains.** The CFU counts of all strains including the control were tested at four time points, T=0, T=1, T=2 and T=3 (time in hours). Each strain represents mean for three replicates, *K. pneumoniae* ATCC 1705 (control), K1/ST-23(Kp 124), K2/ST-231 (Kp125), K2/ST-881 (Kp126) and K2/ST-14 (Kp83). P-value of 0.0007 for CFU counts which is statistically significant as shown by One-way ANOVA. Error bars represents SEM. A slight dip in the bacterial growth at T=2 is followed by a slight to significant increase in the bacterial growth in all three replicates at T=3, For Kp125, Kp 126 and Kp 38.

To correlate between resistance to serum killing and virulence, the same strains were injected into *G. mellonella* larvae. The survival curve of the three replicates separately, are shown for comparison [Figure 3]. Isolates K124 (K1/ST-23) and Kp83 (K2/ST-14) seem to be the most virulent isolates as most larvae died in the first two days. However, isolates K125 (K2/ST-231) and Kp126 (K2/ST-881) seem to be less virulent as the number of larvae gradually decreased throughout the experiment. In the three replicates [Figure 3 (A)], the Control 1 larvae, consisted of the untouched larvae. In one replicate of the control group, there was a gradual decline in the survival of the larvae from day 2 to 5 as the first larva died in the second day.
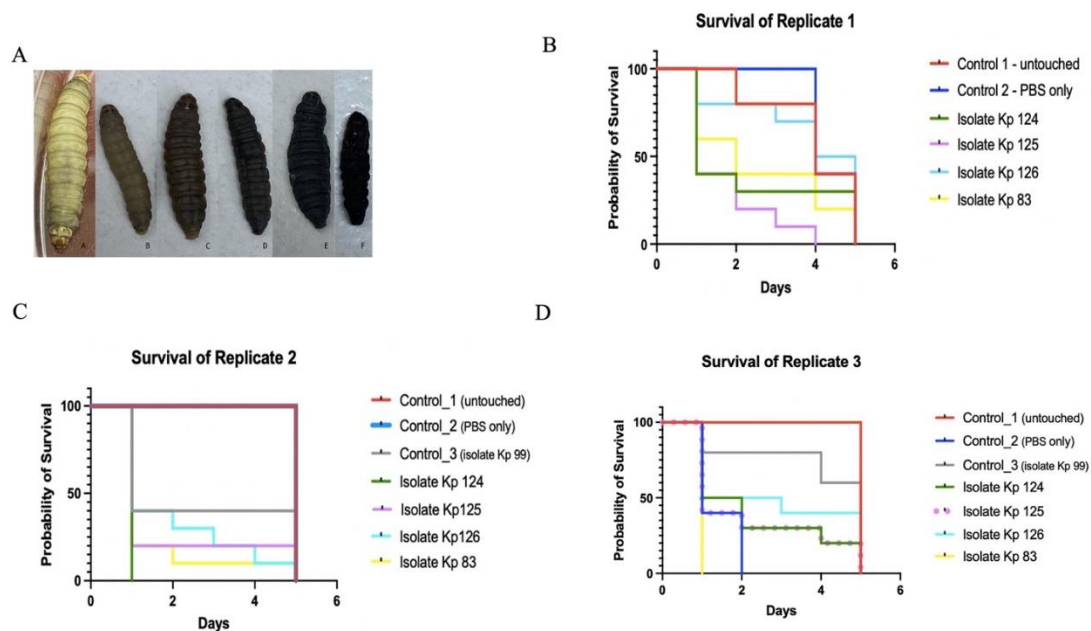


**Figure 3. Kaplan-Meier survival curve of *K. pneumoniae* clinical isolates. (A)** Phenotypic characteristics of *G. mellonella* larvae pre and post injection and throughout the 5 observation days. (B, C and D) Survival of *G. mellonella* larvae infected with K1/ST-23(Kp 124), K2/ST-231 (Kp125), K2/ST-881 (Kp126) and K2/ST-14 (Kp83) in comparison to controls 1 and 2 over the course of 5 days. Control 1 – untouched, uninfected larvae is represented in red. Control 2 – larvae injected with PBS is represented in blue. A third control, Kp 99 in replicate (C) and (D), was used which was a non-hypervirulent *K. pneumoniae* isolate just to compare the rate of survival to the hypervirulent strains. .

Isolate Kp 124 is represented in green, isolate Kp 125 in purple straight line in (C) and dotted in panel (D) for clarity, isolate Kp 126 in cyan, and isolate Kp 83 in yellow as indicated in the colour legend in the right side of each image.

However, in the other two replicates, there were no deaths throughout the 5 observation days but by the fifth day the larvae slow down as cocoon formation commenced. In Control 2 larvae, consisted of PBS only infected larvae, there is also a gradual decline in the survival of the larvae throughout the 5 days in replicates one, however no death was noticed in the second replicate until the larvae slowed down in motility as cocoon formation commenced. However, in the third replicate, there was 100% death, which could suggest the poor quality of that batch of control larvae. In Control 3 larvae, consisting of larvae injected with isolate Kp 99 that is negative for all hypervirulent capsular

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serotypes, in the first day about 20- 50% of the larvae died with no death in the remaining 50-60% of larvae until the end of the experiment as observed in the three replicates. In strain K124 (K1/ST-23) test group, more than 50% in two replicates and 100% in the other replicate of the larvae died in the first day of the experiment. The remainder of larvae were dead by the fourth day. For strain K125 (K2/ST-231) test group, about 50-75% of the larvae died in the second day and by the 4$^{th}$ day all larvae were dead, in almost all three replicates. In isolate Kp 126 (K2/ST-881) test group, the death ranged between 25% to 60% of the larvae in the first day but overall, the survival rate declines gradually throughout the 5 observation days, in the three replicates. In isolate Kp 83 (K2/ST-14), more than 50%-100% of larvae died by the second day of the experiment in the three replicates.

### 2.6. Phylogenetic analysis shows diverse sequence types

A whole-genome single nucleotide polymorphisms (SNPs) calling phylogenetic tree of 131 isolates including 30 strains in this study (from CPHL and Kp 83, represented in blue) as well as 101 strains from Genbank global strains (represented in black) [27] were plotted to compare genetic relatedness (Figure 4). The hypervirulent isolates are marked with differently colored arrows to highlight their position in the tree amongst other isolates.
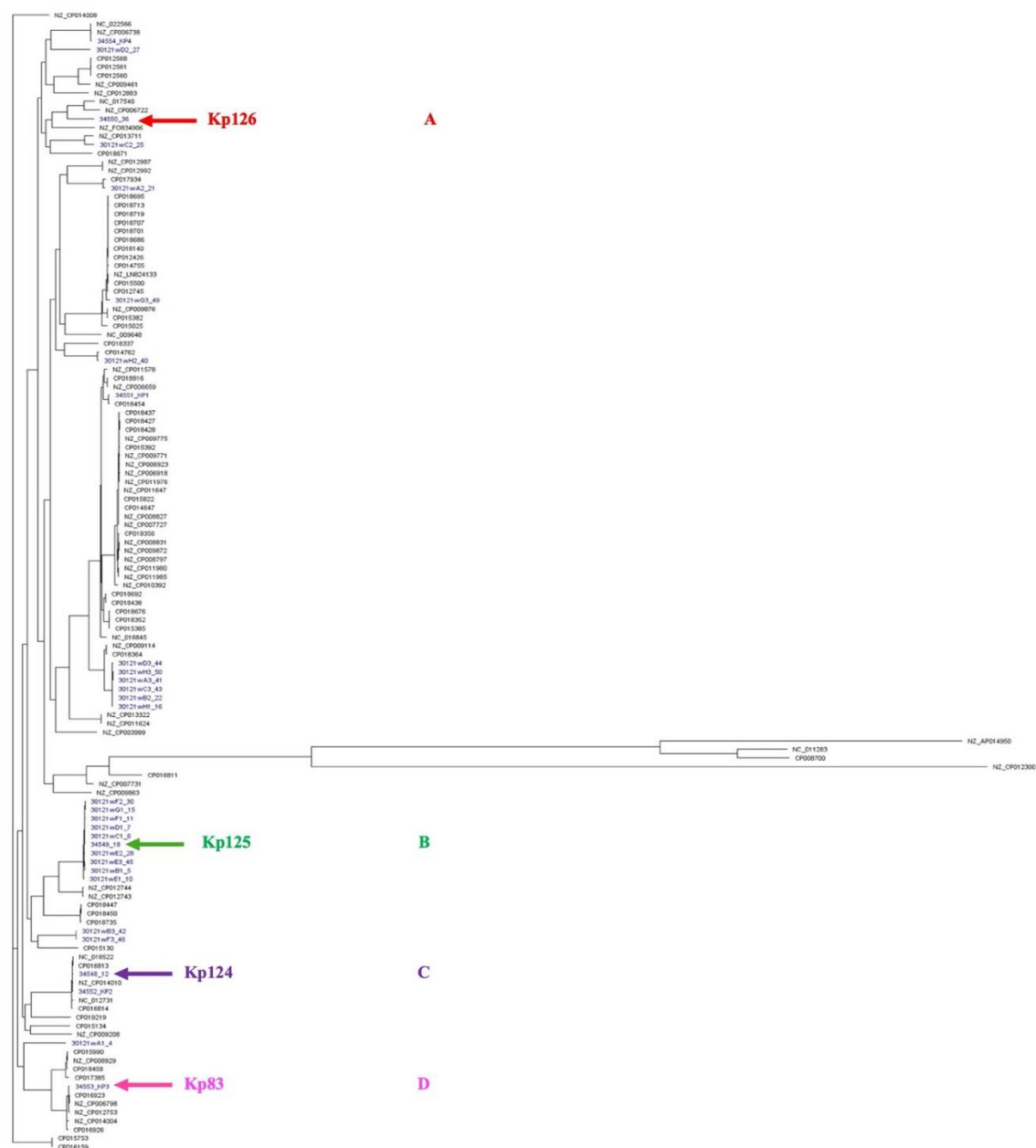
**Figure 4.** A whole-genome single nucleotide polymorphisms (SNPs) calling phylogenetic tree of *K. pneumoniae* isolates (A) (n= 131). A maximum-likelihood tree showing genetic relatedness between *K. pneumoniae* strains based on WGS data. The strains are labelled with serial numbers and grouped according to their STs. *K. pneumoniae* RJF293 (GenBank accession number CP014008) was used as a reference. The isolates starting with NZ and CP (in black) are global published isolates retrieved from Genbank, were used for comparison. The isolates in blue are strains from this study. The hypervirulent isolates are pointed at with arrows; red for K2/ST-881 (Kp126), green for K2/ST-231 (Kp125), purple for K1/ST-23(Kp 124) and pink for K2/ST-14 (Kp83). iTOL was used to visualize and annotate the tree. Clades are indicated in A, B, C and D. This figure compares the local strains in this study (represented in blue) as well as global strains, which clearly demonstrates the diverse clonality of clinical isolates from Oman, belonging to various STs.

A separate phylogenetic tree of 31 isolates for a close-up inspection, including the hypervirulent isolates of this study Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14), as well as 27 other non-hypervirulent *K. pneumoniae* local clinical isolates [Figure 5]. The unique STs and are highlighted in different colors. Group A in blue is representing isolates with ST-395, group B in yellow belong to ST-405, group C in green belong to ST-23, group D in red has ST-231 and finally the unhighlighted isolates belong to miscellaneous STs. The hypervirulent isolates Kp124 (K1/ST-23), Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14) are indicated with magenta arrows. Hypervirulent Kp83 (K2/ST-14) and Kp124 (K1/ST-23), are branching from the same clade but belong to two different clusters, C and D, respectively. Following constructing the *k loci* in Kaptive, it was evident from the phylogenetic tree that the clinical strains with similar STs have high similarity in the genetic arrangement of *k-loci* [Figure 1] and [supplementary figures 3-30]. The hypervirulent serotypes positive for K2, namely K2/ST-231 (Kp125), K2/ST-881 (Kp126), and K2/ST-14 (Kp83), exhibit diversity in their STs, resulting in distinct k-loci gene compositions.
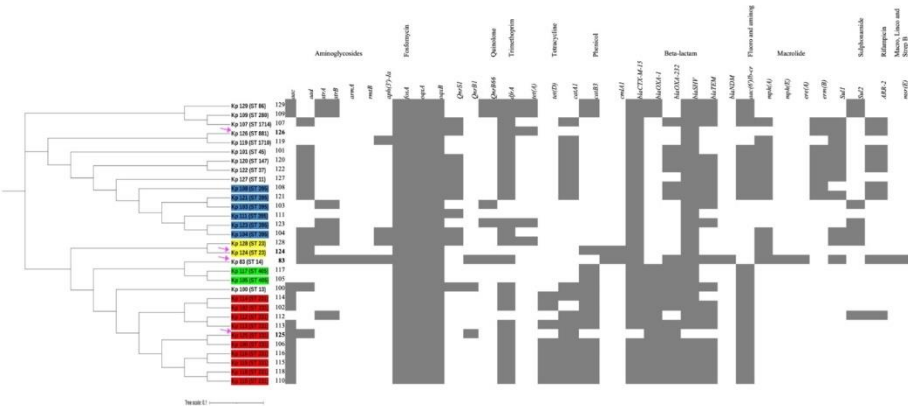


**Figure 5.** A whole-genome single nucleotide polymorphisms (SNPs) calling phylogenetic tree of *K. pneumoniae* isolates (n= 31) and heat map of resistance-encoding genes distribution and their antimicrobial classes. A maximum-likelihood tree showing genetic relatedness between *K. pneumoniae* strains based on WGS results. The isolates are grouped and highlighted according to their STs (Blue = ST 395, Red= ST 231, Green= ST 405, Yellow= ST 23, Unhighlighted= miscellaneous STs). The hypervirulent isolates K1/ST-23(Kp 124, K2/ST-231 (Kp125), K2/ST-881 (Kp126) and K2/ST-14 (Kp83) are pointed at using magenta arrows. *K. pneumoniae* RJF293 (GenBank accession number CP014008) reference was used. iTOL was used to visualize and annotate the tree. Hypervirulent Kp83 (K2/ST-14) and Kp124 (K1/ST-23), are branching from the same clade but belong to two different clusters, C and D, respectively. The relation between their STs and resistance phenotype indicates the diversity in the acquired antimicrobial genes in these hypervirulent strains.

The *K. pneumoniae* capsular synthesis loci, *k-loci*, in all our isolates generally consists of the following conserved genes including *galF, ORF2 (cpsACP), wzi, wza, wzb, wzc,* and *gnd* which are chromosomally encoded core and similar to what has been reported in the literature [28,29] [Figure 1] as these are necessary for capsular synthesis along with other genes. Moreover, *galF, ORF2* and *gnd*

are involved in carbohydrates metabolism, *wzi (orfX), wza, wzb,* and *wzc* are responsible for the capsule translocation and surface assembly [30,31]. In our isolates ICEfinder showed that Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14), have 7, 3, 2, and 1 putative Type 4 secretion system with putative ICEs in, respectively [Supplementary figure 33].

The hypervirulent strains carry a wide range of acquired antimicrobial genes [Figure 5]. Kp83 (K2/ST-14) carries a variety of resistance-conferring genes as compared to the other isolates except for *qnrS1, blaOXA-2, blaAP* and *tet(A)* genes.   Kp 124 (K1/ST-23) carries the least number of resistance-conferring genes and harbors no resistance genes for some antibiotic classes including folate pathway antagonist, quaternary ammonium compound, streptogramin b, rifamycin, aminocyclitol, macrolide, lincosamide and tetracycline. Moreover, isolate Kp125 (K2/ST-231), carries resistance genes for all antibiotic classes in [Figure 4] except glycosamide and tetracycline. In addition, Kp 126 (K2/ST-881), seems to lack resistance-conferring genes to aminoglycosides, amphenicol, streptogramin b, rifamycin, aminocyclitol, macrolide and lincosamide.

## 3. Discussion

This study aims to investigate the capsular serotypes in hvKp K1 and K2 high risk clones of ST-23, ST-231, ST-881 and ST14 clinical isolates from Oman from 2016-2021. These clones were compared to the non-outbreak clones and less common STs. This comparison facilitated further exploration of genomic landscape of resistance and virulence co-existence in a wide *K. pnuemoniae* population. Virulence markers in addition to their spread and resistome was also investigated. The hvKp are known to be associated with increased virulence as well as their association with severe human infections [32]. Only 3% of the clinical isolates tested positive for K1 and K2 during the same period in 2019 at SQUH, from urine and pus samples, and one K2 case was reported in 2017 from a urine specimen. This finding is consistent with several studies which showed that the most commonly circulating hypervirulent capsular serotypes are K1 and K2 and has been attributed to disruption of epithelial and mucosal barriers such as endotracheal tubes, catheters, and surgical wounds in hospital settings [21,27,33–35]. Phenotypically, all the hypervirulent isolates in this study are ESBLs, which are known to be associated with increased length of stay and mortality rates. In contrast to previous studies, the prevalence rate of ESBLs were higher in non-hypermucoviscous *K. pneumoniae* isolates [37]. The possibility of an opportunistic infection in these patients cannot be excluded as the majority are immunocompromised [37,38].

The *k-loci* of the capsular operon share similar genetic organization, in which major operons are conserved in most isolates with few exceptions. MLST analysis showed that most of the isolates with similar STs have similar gene arrangements with a similar reference genome in Kaptive. Moreover, these strains were in the same cluster of in the Whole Genome SNP calling phylogenetic tree. The remaining strains belonged to miscellaneous STs and therefore, variable *k-loci* genetic arrangement figures were observed. To the best of our knowledge, this observation has not been previously reported, making this study the first to indicate a correlation between genetic k-loci and their STs.

In addition to the conserved genes in the *k-loci*, there are other genes that are commonly present in the including *manC* and *manB* that are involved in the synthesis of GDP-D-mannose as well as RmlA, RmlB, RmlC and RmlD proteins involved in the conversion of glucose 1-phosphate to dTDP-L-rhamnose [40]. Previous studies proved that hypervirulent *K. pneumoniae* strains are associated with increased antibiotic resistance, therefore, recognizing their global dissemination is an urgent priority [5]. According to ResFinder and CARD online tools findings, the hypervirulent strains in this study including Kp124 (K1/ST-23), Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14) are in fact highly resistant as they carry a wide variety of antibiotic resistance genes for multiple antimicrobial classes. All these strains carry incompatibility group (Inc) FIB plasmids that are known to encode both virulence and antimicrobial resistance genes in a wide variety of intestinal bacteria including *E. coli, Salmonella* and *Klebsiella spp.* including IncFIA and IncFIB that represent one of the most common plasmid types that play a major role in the dissemination of AMR in *Enterobacteriaceae* [39–41]. Furthermore, IncFIB (K) is carried by both Kp 83 (K2/ST-14) and Kp 126 (K2/ST-881) which suggests likelihood of HGT of these plasmids between different bacteria [42]. Moreover, Kp 125 (K2/ST-231)

carries ColKP3 plasmid that is known to harbor OXA-232 carbapenemase responsible for the rapidly increasing carbapenem resistance in *K. pneumoniae* strains worldwide [43]. Moreover, Kp 125 (K2/ST-231) and Kp 124 (K1/ST-23) both carry IncFIB(pQil) that is known to be harbored by *K. pneumoniae* and other pathogens responsible for nosocomial infections [44]. This finding suggests the likelihood of HGT of this plasmid amongst *K. pneumoniae* bacterial isolates since both Kp 125 (K2/ST-231) and Kp 124 (K1/ST-23) were isolated in the same period in 2019 in SQUH.

SNP-calling phylogenetic trees performed on WGS data shows that *K. pneumoniae* strains isolated from different geographical regions (n=131) are arranged with distinct clustering [27]. The strains in our study were found to be phylogenetically diverse belonging to various STs. The hvKp Kp124 (K1/ST-23), Kp125 (K2/ST-231) are present in a clade A, whereas Kp126 (K2/ST-881) is in clade B. The close-up tree showed that our strains are grouped in four main clusters highlighted with different colors indicating the diverse group of strains circulating in our local hospitals. However, there are clustering in these four main groups indicating a possibility of dissemination at possibly larger scale [45,46]. Isolates belonging to ST-231 have been associated with MDR spread in Europe with the potential to cause a future epidemic possessing a public threat. These strains produce OXA-232 as well as RmtF which are associated with high-level carbapenem resistance [47,48]. Furthermore, *K. pneumoniae* belonging to ST-395 are associated with the production of OXA-48 which is the most common enzyme causing healthcare associated infections globally. OXA-48 enzyme hydrolyses carbapenems and shows weak activity against extended spectrum cephalosporins such as cefepime and ceftazidime, making it difficult to treat these infections [49,50]. This clone of *K. pneumoniae* has been detected in many countries of the Middle east including Oman, Yemen and Saudi Arabia [51–53]. Furthermore, Kp 117 and Kp 105 belong to ST 405 (blue cluster) that have also been associated with the production of OXA-48 just like clones belonging to ST 395 and thus has been responsible for many hospital- associated outbreaks and war-related outbreaks (in Germany) and some of which are resistant to colistin [54–56].

Several studies have shown that infection of *G. mellonella* with *K. pneumoniae* has resulted in host responses similar to the innate immune responses in murine models including cell death, inhibition of phagocytosis and antimicrobial peptide production. Furthermore, *G. mellonella* can differentiate between pathogenic and non-pathogenic *Klebsiella* strains and showed that the more virulent strains were associated with increased survival and host cellular damage as compared to avirulent strains [59]

In the present study, it was shown that Kp 124 (K1/ST-23) is the most virulent as 50% of the larvae died in the first day followed by K2/ST-231where 75% of the larvae died in the second day and Kp 83 (K2/ST-14) where 50% of the larvae died in the second day. Similarly in Europe, K1 positive *K. pneumoniae* belonging to ST-23 has been associated with severe, and fatal infections associated with liver abscesses[60–62]

However, in Kp 126 (K2/ST-881) there was a gradual decline in the number of larvae over the course of 5 days perhaps making it the least virulent as the death was slower and steadier. It was expected for Kp 126 (K2/ST-881), although it was a hypervirulent strain, it belongs to ST-881 that according to the literature has not been associated with severe human infections as compared to the other STs of Kp 83 (K2/ST-14), Kp 124 (K1/ST-23) and Kp 125 (K2/ST-231). The *G. mellonella* experiment was repeated three times to allow for comparison and to check how the quality of larvae might affect the results. The results of the experiments though all indicate the higher virulence of hypervirulent K1 and K2 capsular serotype isolates as compared to non-hypervirulent strains [61,62]. However, the main limitation in this assay was due to shipping and delivery restrictions caused by the COVID-19 pandemic as the larvae used in this study were not ideal.

Human serum resistance experiments were performed for the hypervirulent strains Kp124 (K1/ST-23), Kp125 (K2/ST-231), Kp126 (K2/ST-881) and Kp83 (K2/ST-14). For each isolate, 3 replicates were done for accurate comparison and reproducibility. There was complete inhibition of bacterial growth in all four isolates by the end of the first hour and up to the third hour. This is due to the presence of complement proteins and lysozyme which have a bactericidal effect [63]. Furthermore, unexpected pattern of bacterial growth was observed in all replicas for all four strains. For Kp124 (K1/ST-23), there was a gradual decrease in the number of colonies up to the second hour which

indicates neutralization of bacterial cells by the serum components, however, in the third hour there was a sudden splurge of bacterial growth. For Kp125 (K2/ST-231), the same decrease in bacterial growth was noticed by the end of the first hour but the dramatic increase in the bacterial growth starting from the second hour unlike in Kp124 (K1/ST-23). Furthermore, for Kp126 (K2/ST-881) and Kp83 (K2/ST-14) similar patterns of sudden growth of bacterial cells commenced in the third hour. Therefore, the sudden increase in the bacterial growth despite the unfavorable serum environment for growth indicates possible resistance of bacteria against human serum bactericidal activity [64]. Human serum, unlike blood, contains antibodies and complement proteins that contribute to the neutralization of bacteria but does not contain immune cells such as phagocytes. Previous studies discuss the bactericidal activity that human serum possesses against Gram-negative bacteria including *K. pneumoniae.* Moreover, they highlight that the resistance against human serum may be a significant virulence factor. Therefore, the strains used in this assay might have acquired the resistance against human serum as one of their virulence factors [65]. Furthermore, earlier studies have shown that *K. pneumoniae* LPS O antigen and the capsule polysaccharide contributes strongly to the resistance against human serum antibodies and complement proteins. The isolates tested in this experiment are hypervirulent and possess a thick capsule therefore, their resistance to human serum could be explained by this theory [66,67]. In future studies it would be worthy to test the hypervirulent strains of this study in human blood that contains immune cells such as phagocytes to investigates how it compares to human serum. In previous studies *K. pneumoniae* has shown its ability to resist killing by phagocytosis in human blood killing assays [68].

## 4. Materials and Methods

### 4.1. Bacterial isolates

The study was commenced after obtaining approval by the Medical Ethics Research committee (MREC#1896), College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman. Initially, we screened 129 *K. pneumoniae* clinical isolates for K1, K2, K20, K54 and K57 from various samples (urine, wound, tracheal aspirate, blood, sputum), from patients from Central Public Health Laboratories (CPHL) representing various areas from Oman collected between 2015 to 2020. Another 30 isolates were screened from Sultan Qaboos University Hospital (SQUH), Muscat Oman collected between 2019 to 2021 (Supplementary Table 1). PCR preliminary data is presented in supplementary table 5 and 6 including PCR cycling conditions and primers. Colonies were collected from purity plates of CLED agar (Oxoid, Basingstoke Hampshire, UK) were used to make frozen stock of our samples in cryotubes containing beads according to the manufacturer's instructions (Mast Diagnostics, Derby, UK). The samples were frozen at −80 °C for future use.

### 4.2. String test for hypermucoviscosity

An inoculation loop was used to stretch a colony on a Cystine Lactose Electrolyte Deficient (Oxoid, Basingstoke Hampshire, UK) agar plate and the immediate formation of a viscous string of > 5mm in length was indicative of a hypermucoviscous strain of *K. pneumoniae* [69].

### 4.3. Antibiotic Susceptibility testing

Antimicrobial susceptibility profiles of the isolates were carried out using disk diffusion method [72]. Firstly, from an overnight pure culture, three to five colonies were suspended in 5 mL of normal saline (Fisher Scientific, United Kingdom). The suspension was adjusted to 0.5 McFarland (approximately $1–2 × 10^8$ CFU/mL) using CrystalSpec nephlometer (BD Diagnostics, USA) following the manufacturer's protocol. The antibiotic disks include ampicillin (AMP 10 mg), piperacillin/tazobactam (TZP 110 mg), cefepime (FEP 30 mg), cefotaxime (CTX 30 mg), cefoxitin (FOX 30 mg), ceftazidime (CAZ 30 mg), imipenem (IPM 10 mg), meropenem (MEM 10mg), amikacin (AK 30 mg), gentamicin (CN 10 mg), and ciprofloxacin (CIP 5 mg), (Liofilchem, Italy and BioMérieux, France. The susceptibility assay was carried out according to the CLSI standards [70] were placed on

the nutrient agar plates (Oxoid, United Kingdom) and inoculated using sterile forceps. The plates were then incubated for 18-24 hours at 37°C. *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used as susceptible control strains. The readings were taken after 18-24 hours and the interpretive categories and zone diameter breakpoints are listed in Table 4.

**Table 4.** Interpretive categories and zone diameter breakpoints according to CLSI guidelines.

| Antibiotic | Abbreviation | Disk content (μg) | Zone diameter breakpoints (mm) | | |
|---|---|---|---|---|---|
| | | | Susceptible | Intermediate | Resistant |
| **Ampicilin** | AMP | 10 | ≥ 17 | 14-16 | ≤ 13 |
| Piperacillin-tazobactam | TZP | 110 | ≥ 21 | 18-20 | ≤ 17 |
| Cefepime | FEP | 30 | ≥ 25 | 19-24 | ≤ 18 |
| Cefotaxime | CTX | 30 | ≥ 26 | 23-25 | ≤ 22 |
| Cefoxitin | FOX | 30 | ≥18 | 15-17 | ≤ 14 |
| Ceftazidime | CAZ | 30 | ≥ 21 | 18-20 | ≤ 17 |
| Imipenem | IMP | 10 | ≥ 23 | 20-22 | ≤ 19 |
| Meropenem | MEM | 10 | ≥ 23 | 20-22 | ≤ 18 |
| Gentamicin | CN | 30 | ≥ 15 | 13-14 | ≤ 12 |
| Amikacin | AK | 10 | ≥ 17 | 15-16 | ≤ 14 |
| Ciprofloxacin | CIP | 5 | ≥ 31 | 21-30 | ≤ 20 |

*4.4. DNA extraction and WGS*

DNA was extracted from an overnight culture using QIAamp DNA Mini Kit (Qiagen, Germany) for whole genome sequencing. The manufacturer's protocol was followed with slight modifications. For highly mucoid *K. pneumoniae* isolates, a pre-lysis step was performed which includes the preparation of a pre-lysis buffer constituting of 100 μl of Tris//EDTA (TE) buffer (ThermoFisher Scientific, USA), 1.00 μl lysozyme 10mg/mL (final concentration 0.1mg/mL) (Sigma-Aldrich, USA), 0.20 μl lysostaphin 10mg/mL (final concentration 0.02mg/mL) (ThermoFisher Scientific, USA), 0.10 μl RNAse A 100mg/mL (final concentration 0.1mg/mL) (Qiagen, Germany). The bacterial suspension from an overnight culture was centrifuged at 8000 x g for 2 minutes and the pelleted bacterial cells were resuspended in 100 μl of the pre-lysis buffer by pipetting up and down several times with a P200 pipette. The suspension was then incubated for 60 minutes. After incubation, 1.00 μl of Proteinase K (Sigma-Aldrich, USA) was added as a final step in the pre-lysis stage. The sample was then ready for the first step of the Qiagen DNA extraction protocol as discussed in the previous section.

After DNA quantification by NanoDrop (ThermoFisher Scientific 1000 NanoDrop Spectrophotometer), the samples were sent to microbesNG in the United Kingdom for WGS by Illumina next-generation sequencing (https://microbesng.co.uk, Birmingham, United Kingdom) [71]. The extracted DNA was prepared following the manufacturer's protocol as described in the company's website as follow: ILLUMINA SEQUENCING (SGS and EGS) Genomic DNA libraries are prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: input DNA is increased 2-fold, and PCR elongation time is increased to 45 s. DNA quantification and library preparation are carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Pooled libraries are quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Libraries are sequenced using Illumina sequencers (HiSeq/NovaSeq) using a 250bp paired end protocol. Reads are adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [72].De novo assembly is performed on samples using SPAdes version 3.7 [76], and contigs are annotated using Prokka 1.11[77].

### 4.5. Bioinformatics analysis

For visualization of the *K. pneumoniae* contigs as well as the investigation of main virulence genes, Artemis [75] and SnapGene Viewer (https://snapgene.com) software were used. Moreover, Artemis was used to identify Klebsiella capsule synthesis loci in the different isolates.  Basic Local Alignment Search Tool (BLAST) was used to identify the similarities between the loci against a reference K. pneumoniae RJF293 (GenBank accession number CP014008) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [76]. In addition, Genbank was used to search for similar DNA sequences or genes from the DNA database to the sequenced genomes of this study [77]. Kaptive was used for locus typing and variant analysis on the pre-assembled sequences against a K. pneumoniae database (https://kaptive-web.erc.monash.edu/) [78]. The Klebsiella capsule synthesis loci (k-loci) of each of the sequenced genomes were generated and were used for comparison between the different isolates (Supplementary figures 3-30). Kleborate v2.1.0 was used to investigate other virulence factors including yersiniabactin, colibactin, aerobactin and salmochelin [79]. ICEfinder online tool was used to detect integrative and conjugative elements (https://bioinfo-mml.sjtu.edu.cn/ICEfinder/ICEfinder.html) [80]. The multilocus sequence typing (MLST) was determined using the MLST tool in the Center for genomic epidemiology (CGE) (http://cge.cbs.dtu.dk/services) [81]. PlasmidFinder and ResFinder in the CGE website were used to identify plasmids and acquired antimicrobial resistance genes, respectively [82]. Additionally, Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/) was used to detect putative antimicrobial resistance genes using Resistance Gene Identifier (RGI) tool. This tool identifies the antibiotic resistome(s) as well as point mutations within the resistance-conferring genes [83]. Furthermore, the phylogeny tool in the CGE server was used to investigate the genetic relatedness between the different sequences by identifying Single Nucleotide Polymorphisms (SNPs) [84]. The tree files were visualized using Mega7 that performs automatic sequence alignment and infers phylogenetic trees [ 88 ] .In addition, a combined phylogenetic tree of 131 isolates that includes the isolates in this study as well as published global isolates was created using R, a statistical programming software for comparison and visualization of genetic similarities or differences between the project's isolates and the published ones [86]. iTOL; an online tool, was used to display, annotate, and manage the phylogenetic tree [90]. A whole genome SNP alignment was generated using Snippy v4.4.5 [Seemann 2015] and the AE015929 genome as a reference. Then, iqtree v1.6.12, using model finder and ultrafast bootstrap [92], was used to produce a maximum likelihood phylogenetic tree. A phylogenetic tree of the whole genome SNP was constructed and linked to the gene analysis heat map using the R platform [ 93,94 ]. List of Clinical isolate serial numbers of phylogenetic trees in Figures 4 and 5 are presented in supplementary table 4. For statistical analysis, two-way ANOVA was used to analyze variations in bactericidal activity of human serum against *K. pneumoniae* clinical isolates using GraphPap Prism software (10.0.3). *P*-value of $< 0.05$ is considered as statistically significant. Observation tables are presented in Supplementary table 3.

### 4.6. G. mellonella Virulence assays

Virulence assay was performed using the larvae of *G. mellonella* as a model of infection. The protocol was modified from [92]. To ensure quality, the larvae were manually picked through local beekeepers in Oman and were kept at room temperature (25 °C) in the dark and wood shavings were provided as a food source. Stage 6 larvae, approximately 2-3 cm in size were selected for the purpose of the experiment. For each isolate and the controls, a replicate of 10 and 5 larvae were used, respectively. The larvae were distributed in Petri dishes lined with wood shavings before injecting them with bacteria the next day.   In day 1, the selected isolates for the experiment were sub-cultured into CLED agar plates (Oxoid, Basingstoke Hampshire, UK) from cryo-beads. In day 2, three to five colonies were picked up and resuspended in 10 mL of liquid broth then incubated overnight at 37 °C. In day 3, a 1:100 dilution (1 mL bacterial suspension + 9 mL liquid broth) was grown for 3-4 hours. The bacterial suspension was then centrifuged, the supernatant discarded, and the pelleted bacteria was washed three times with sterile 1x Phosphate buffered saline (PBS) to remove excess salts (Invitrogen, USA). The bacterial pellet was then resuspended in PBS to an absorbance of 0.2 ($OD_{600}$)

which was repeated for each isolate. The absorbance was measured using a spectrophotometer (Eppendorf BioPhotometer Spectrophotometer UV/VIS, USA) and the colony-forming unit (CFU) counts were determined for each sample. For the test larvae, 10 μL of each sample (undiluted 0.2 $OD_{600}$ suspension) was injected into the larvae. For Control 1 larvae, PBS only was injected and for Control 2 larvae, they were kept untouched, for Control 3 larvae, Kp 99 non-hypermucovirulent (negative for any capsular serotype) bacterial suspension was injected. All larvae were injected through the haemocoel via the rear left pro-leg using an insulin syringe. Post injection, the larvae were incubated at 37 °C and their mortality was assessed every 24 hours for 5 days. Over the course of 5 days, the larvae were examined with forceps by flipping them into their backs and checking if there are signs of motility. Live larvae will quickly flip back whereas sick larvae will be slow, and the exterior will be hardened and darkened, and they will usually die within the coming 1-2 days. Dead larvae will have a hard, dry, and very dark exterior and will shrink in size. The results were observed and noted, and survival graphs were generated using GraphPad prism 10.0.3 software (La Jolla California USA, www.graphpad.com). Observation data is presented in Supplementary table 3.

*4.7. Serum Resistance Assay*

The susceptibility of bacteria to human serum was investigated using the method of [93] with some modifications. In day 1, the selected isolates for the experiment were sub-cultured into agar plates from cryo-beads as well as *K. pneumoniae* ATCC 1705 that was used as a positive control in this experiment. In day 2, 3-5 colonies were picked up and resuspended in 10 mL of liquid broth then incubated overnight at 37 °C. In day 3, the bacterial suspension was diluted to $2 \times 10^{6}$ CFU/mL. The Then 25 μL of bacterial suspension and 75 μL of human serum (Sigma- Aldrich, USA) were dispensed in microtitration trays and mixed then incubated at 37 °C. To assess viability, the samples were streaked onto agar plates immediately at time zero, after 1 hour, after 2 hours and finally after 3 hours. Each isolate was tested 3 times. The streaked agar plates were incubated overnight. In day 4, CFU counts of each sample and the control at 4 different time points were recorded and graphs were made using Microsoft Excel, 2010. (Supplementary tables 6-10)

## 5. Conclusions

In summary, to our knowledge this is the first study in Oman to depict the molecular and genomic characteristics of hypervirulent *K. pneumoniae* isolates collected from all around the country, where analysis of WGS of the isolates showed distinct pattern of *k-loci* depending on the particular STs. The hvKp strains in this study are diverse in the phylogenetic origin but the likelihood of transmission and future outbreaks is key to detect possible spread of hvKp isolates in health-care settings via active surveillance.

## Appendix A

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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