Comparative genomic analyses of *Klebsiella pneumoniae* K57 capsule serotypes isolated from bovine mastitis in China

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**ABSTRACT**

*Klebsiella pneumoniae* can cause severe clinical mastitis in dairy cows, with *K. pneumoniae* type K57 (K57-KP) being the most common capsular serotype. To identify virulence factors and antimicrobial resistance (AMR) genes of K57-KP with varying virulence, *Galleria mellonella* (greater wax moth) larvae were infected as a screening model to characterize virulence of 90 K57-KP strains, with 10 and 11 strains defined as virulent or attenuated, respectively, based on larval survival rates. Next, virulence of these 21 isolates was subsequently confirmed in adhesion and lactate dehydrogenase release assays, using bovine mammary epithelial cells cultured in vitro. Finally, genes associated with virulence and AMR were characterized with whole-genome sequencing. These 21 K57-KP strains were designated into 16 sequence types based on multilocus sequence typing and allocated in phylogenetic analysis based on single nucleotide polymorphisms. There was great genetic diversity among isolates. In addition, adhesion-associated genes (e.g., *fimA*, *sfaA*, and *focA*) aminoglycoside-resistance genes (*aph(6)-Id*, *strAB*) were associated with virulence. This study provided new knowledge regarding virulence of K57-KP associated with bovine mastitis, which may inform development of novel diagnostic tools and prevention strategies for bovine mastitis.

Keywords: bovine mastitis, Klebsiella pneumoniae, K57 capsule serotype, virulence, antimicrobial resistance

**INTRODUCTION**

*Klebsiella pneumoniae* is an opportunistic and environmental mastitis-causing pathogen, highly prevalent among Gram-negative bacteria and causing substantial economic losses worldwide (Halasa et al., 2007; Gao et al., 2017; Jamali et al., 2018; Cheng et al., 2021a). Cows with an intramammary *K. pneumoniae* infection often have severe clinical signs: a swollen udder, fever, depression, inappetance, and a profound decrease in milk production (Verbist et al., 2011; Schukken et al., 2012).

Of the *K. pneumoniae* causing human infections, K1, K2, K5, K20, K54, and K57 capsular serotypes are considered highly virulent *K. pneumoniae* that cause meningitis, myocarditis, and other severe infections (Ko, 2017). In *K. pneumoniae* from bovine mastitis, the K57 capsular serotype had the highest prevalence (Gao et al., 2019). We reported that *K. pneumoniae* rapidly adhered to and invaded bovine mammary epithelial cells (bMECs) cultured in vitro, caused cellular damage and apoptosis, and prompted bMECs to produce key inflammatory factors (IL-8, IL-13, and TNF-α) (Cheng et al., 2020). Furthermore, K57 *K. pneumoniae* (K57-KP) induced profound mitochondrial damage and dysfunction in bMECs (Halasa et al., 2007; Gao et al., 2017; Jamali et al., 2018; Cheng et al., 2021b).

Many *K. pneumoniae* have virulence genes that enhance ability to cause infections, increase bacterial cellular fitness, and evade host immunity. For example, *K. pneumoniae* adheres to host cells using adhesins such as fimbriae and pili (Alcántar-Curiel et al., 2018). Production of a robust capsular polysaccharide confers resistance to host immune cells along with the O-antigen portion of liposaccharide (LPS) (Muraya et al., 2022). *K. pneumoniae* can also obtain iron through siderophore secretion and it can express a variety of siderophores, including enterobactin, yeriniabactin, salmochelin, and aerobactin. *K. pneumoniae* can be resistant to various compounds; some isolates contained multiple antimicrobial resistance (AMR) genes, including those for...
β-lactams (blaCTX-M, blaSHV, and blaTEM), tetracyclines (tetA, tetB), and quinolones (opxAB) (Holt et al., 2015; Ni et al., 2020; Muraya et al., 2022; Song et al., 2022).

Most studies on distribution of virulence and AMR genes in *K. pneumoniae* isolated from bovine mastitis used PCR, which is targeted in nature, and focused on specific genes, including aerobactin, entB, ybtS, kfu, intA, fimH1, mkrD, tmpA, blaTEM, blaSHV, blaCTX-M, blaKPC, tetA, tetB, mcr-1, aac-I, and aac-II (Gao et al., 2019; Carvalho et al., 2021). However, whole-genome sequencing (WGS) is preferred to assess *K. pneumoniae* isolates by detecting subtle differences in virulence and resistance. Sequences of 180 *K. pneumoniae* isolates from milk of dairy cattle with mastitis in the United States revealed much genomic diversity and identified the most prominent genes associated with ferric citrate uptake, lactose fermentation, and resistance to heavy metals (Zheng et al., 2022). Another recent study investigated prevalence of *K. pneumoniae* in milk samples from 6,301 dairy cows with clinical mastitis (CM) in northern China. All *K. pneumoniae* isolates belonged to 1 of 3 phylogroups; KpI may have a high prevalence on dairy farms due to relatively high rates of antimicrobial resistance and virulence genes (Song et al., 2022).

As WGS explained variability in resistance of *Staphylococcus aureus* from bovine mastitis (Ivanovic et al., 2023), it should provide new insights regarding K57-KP genes involved in resistance and virulence. Here we aimed to investigate virulence and AMR genes in K57-KP of varying virulence status, using a combination of infection models, cell culture and WGS. Our goal was to identify elements linked to virulence in K57-KP of bovine mastitis origin.

## MATERIALS AND METHODS

### Dairy Farm and *K. pneumoniae* Isolates

Details of the dairy farm and the CM outbreak have been described (Cheng et al., 2021b). The farm was located in Shandong Province in the north of China, with 8,230 lactating Holstein-Friesian cows fed total mixed rations and milked 3 times/day in rotary parlors. Recycled manure solids (50–60% dry matter) were used as bedding. In January 2019, a CM outbreak occurred, with *K. pneumoniae* isolated from 53% of CM cases.

A total of 328 *K. pneumoniae* isolates were obtained from the dairy farm; of those, 148 (45%) isolates were K57-KP (Cheng et al., 2021b). When stored isolates were reinstated, 21 were contaminated and 37 were not viable; therefore, 90 K57-KP were available for further analyses. From the 90 isolates, 22 were from CM milk (CM), 12 were from subclinical mastitis milk (SCM), 48 were from environmental samples (feces, teat skin, or bedding), and 8 were isolated from bulk tank milk, with these 56 designated as EB.

### Galleria mellonella Infection Model

A *Galleria mellonella* (greater wax moth) infection model was used as described (Insua et al., 2013), with slight modifications. A total of 2,790 *G. mellonella* larvae were temporarily stored at 4°C in the dark to promote dormancy. With a creamy color, well-developed larvae of similar weight and size (250–350 mg, 3.0 ± 0.5 cm) were selected and resuscitated in a 37°C incubator for at least 1 h before the experiment.

The 90 K57-KP isolates, isolated from freshly streaked lysogeny broth (LB) agar plates, were grown in 3 mL LB at 37°C on an orbital shaker (220 rpm) until the logarithmic phase was reached. Then, 1 mL bacterial solution was washed twice with sterile PBS (28 000 x g, 10 min) and bacterial concentration adjusted to 10^6 cfu/mL.

Larvae were injected with 10 μL of bacterial suspension via the penultimate proleg on the right posterior using a disposable microsyringe (30 G, 12.7 mm). For each isolate, 10 larvae were treated and placed in 9-cm Petri dishes lined with filter paper. Additionally, 10 larvae were injected with 10 μL PBS as a control. After injections, *G. mellonella* larvae were placed in a 37°C incubator in darkness for 72 h. Survival rate, activity, and melanization were recorded every 24 h, with melanization related to larval death (Loh et al., 2013; Tsai et al., 2016). Isolates with larval survival rates ≤ 20% and ≥ 80% (virulent and attenuated, respectively) were selected for further studies, with each performed as 3 independent experiments.

### Adhesion Assay and Lactate Dehydrogenase Release Assay

The bMECs (MAC-T line, Shanghai Jingma Biological Technology Co. Ltd., Shanghai, China) were grown in culture dishes containing Dulbecco’s modified Eagle’s medium with high glucose (HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 100 IU/mL penicillin (HyClone), and 100 μg/mL streptomycin (HyClone) in a 37°C incubator with 5% CO₂ on cell culture plates (Corning Inc., Corning, NY). Cells were separated and plated in 24- or 96-well plates in culture medium without antibiotics. Cells were exposed to bacteria after achieving 80 to 90% confluence, whereas control cells were grown in culture media only. Every challenge was repeated 3 times. Before testing, the 21 K57-KP isolates with high or low larval survival rates were grown in Mueller-Hinton broth (Aobox, China)
and incubated in an orbital shaker (220 rpm) at 37°C. Counts (cfu) were measured using a WGZ-2XJ bacterial turbidimeter (Shanghai Xinrui Instrument Co. Ltd., Shanghai, China).

An adhesion assay of *K. pneumoniae* to bMECs was done as described (Pöhlmann-Dietze et al., 2000; Chen et al., 2017; Cheng et al., 2020), with slight modifications. The bMECs were cultured in 24-well plates (Corning Inc.) and infected with *K. pneumoniae* at a multiplicity of infection (MOI; the ratio of *K. pneumoniae* to cells) of 50:1 for 3 h at 37°C with 5% CO₂. After incubation, the culture medium was removed, and cells were washed 3 times with PBS (pH 7.4) to remove non-adherent bacteria. Plates were treated with 500 μL of trypsin-EDTA and 500 μL of 1% Triton X-100 (0.5% vol/vol) to lyse bMECs and release adhering bacteria. Finally, Mueller-Hinton agar plates with cell lysates plated on them were serially diluted 10-fold before being incubated at 37°C for 20 h to enumerate cfu.

Cytotoxic effects of *K. pneumoniae* on bMECs were evaluated with a lactate dehydrogenase (LDH) assay kit (Beyotime Institute of Biotechnology, Beijing, China). *K. pneumoniae* was introduced to cells in 96-well plates (Corning Inc.) grown at 37°C and 5% CO₂ and challenged for 3, 6, 9, or 12 h at an MOI ratio of 5:1. Cells that were similarly cultured but not infected served as a control. Following incubation, the cell culture plate was centrifuged at 400 x g for 5 min in a multi-well plate centrifuge. A new 96-well polystyrene plate was then progressively filled with 60 μL of reaction mixture and 120 μL of supernatant. The plate was incubated on a rotary shaker (150 rpm) at room temperature in the dark for 30 min. Absorbance was read at 492 nm (680 Multipurpose Microplate Reader, Biorad, Hercules, CA).

**Antimicrobial Susceptibility Testing**

Phenotypic AMR was determined using a microdilution method following Clinical Laboratory and Standards Institute (CLSI) guidelines (CLSI, 2013). Resistance was defined according to CLSI (2013, 2017) or European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015) against the following compounds: amoxicillin/clavulanate; ceftiofur; ceftquinome; enrofloxacin; imipenem; polymyxin B; tetracycline; kanamycin. Antimicrobial concentrations ranged from 0.25 to 128 μg/mL. Resistance was determined based on the following resistance fold points: amoxi/clav (≥32; CLSI HUM), ceftquinome (≥4; CLSI HUM), ceftiofur (≥8; CLSI VET E. coli), enrofloxacin (≥4; CLSI VET E. coli from skin, soft tissue, respiratory and urinary infections), imipenem (≥4; CLSI HUM), kanamycin (≥64; CLSI HUM), polymyxin B (≥4; EUCAST), and tetracycline (≥16; CLSI HUM) (Cheng et al., 2019).

**DNA Extraction and Whole-genome Sequencing**

Genomic DNA was extracted from 21 strains with larval survival rates ≤ 20% or ≥ 80% (virulent or attenuated, respectively), using a TIANamp bacteria DNA kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The quality and concentration of the extracted genomic DNA were determined with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA). In each sample, total DNA was ≥ 0.2 μg and DNA concentration was ≥ 2.5 ng/μL. All 21 K57-KP isolates were submitted for WGS on an Illumina NovaSeq sequencing platform using the paired-end sequencing mode (2 × 150 bp) under the PE400 library by Shanghai Personalbio Biotechnology (Shanghai, China), with sequencing data quality assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Sequence reads were de novo assembled using SPAdes (Bankevich et al., 2012); resulting assemblies were evaluated, compared, and base-corrected using Pilon software (Walker et al., 2014). Genome annotation with default settings was done with Prokka Version 1.14.6 (https://github.com/tseemann/prokka). Single nucleotide polymorphisms (SNPs) were identified using Parsnp version 1.7.2 (https://github.com/marbl/parsnp) using sequence alignment files produced by Parsnp and snp-dists version 0.8.2 (http://sanger-pathogens.github.io/snp-sites/). Due to more robust development of whole-genome phylogeny constructed based on core genes (Song et al., 2021), phylogenetic trees based on core-genome SNPs were annotated using iTOL v6. The pan-genome of all 21 *K. pneumoniae* isolates was calculated (Chaudhari et al., 2016) using BPGA Version 1.3 (https://ibc.res.in/bpga/) and the USEARCH technique used to cluster orthologous gene families using faa files of local isolates created by Prokka. For BPGA analyses, a core gene was present in every genome; furthermore, an accessory gene was present in >1 genome but not in every genome, and a unique gene was present in just 1 genome. Following a comparison of sequences to clusters of orthologous groups (COGs) of proteins and Kyoto encyclopedia of genes and genomes (KEGG) (http://www.genome.ad.jp/kegg/) databases included in BPGA Version 1.3, functional annotations of core, accessory, and unique genes were produced (Kanehisa M., 1997). All Illumina sequencing data used in this study are available under BioProject ID: PRJNA919159.
Identification of Sequence Types, Virulence Factor Associated Genes and AMR Genes

Sequence types (STs) of all K57-KP isolates were determined by MLST based on 7 housekeeping genes: gapA, infB, mdh, pgi, phoE, rpoB and tonB. Genomic data of each isolate obtained by WGS were directly used; sequences of each locus were assigned an allele number, and corresponding combinations of the 7 allele numbers of each isolate were submitted to the PubMLST database (https://pubmlst.org/organisms/pseudomonas-aeruginosa) to obtain an ST for each isolate. To infer evolutionary links among STs, a minimal spanning tree was generated using the goeBURST method and presented using the PhyloViz web server (https://online.phyloviz.net/index). Virulence-associated genes and antimicrobial-resistance genes were identified using the Virulence Factors of Pathogenic Bacteria (VFDB) databases (Chen, 2004) and Kleborate (Lam et al., 2021); furthermore, these were visualized using iTOL v6 with corresponding features of each isolate. Advanced Heatmap Plots were produced using OmicStudio tools at https://www.omicstudio.cn.

Statistical Analyses

All data were analyzed using GraphPad Prism 8.0 and data reported as means ± standard deviation (SD). For all analyses, P < 0.05 was considered significant. Student’s t-tests were used to compare measured variables among isolates according to presence or absence of selected genes, as well as according to infection severity status (CM or SCM).

RESULTS

Virulence Phenotype of K57-KP

Larval survival rates for CM, SCM, and EB groups were 20, 68, and 86%, respectively, with isolates from the CM group having the lowest survival (P < 0.001) (Figure 1A), whereas melanization indices were 0.6, 2.1 and 3.4 (range of 0–4 for individual isolates). The CM group had the highest degree of melanization (P < 0.001). Interestingly, although K57-KP of SCM did not kill larvae, it caused significantly more melanization of larvae than the EB group (Figure 1B). Isolates CM7 and CM11 killed 100% of infected G. mellonella larvae on the second day. Although no SCM isolate caused 100% mortality of G. mellonella larvae, survival rate for the SCM1 isolate decreased to 20% on the third day. Some EB isolates also caused mortality. For example, EB18 killed 80% of larvae on the first day after infection, whereas EB14 reduced larval survival rate to 20% on the third day. Altogether, 10 isolates with larval survival rates ≤20% were regarded as virulent strains (VS), including CM4, CM5, CM7, CM8, CM9, CM11, CM15, SCM1, EB14, and EB18 (designated VS1-VS10, respectively), whereas 11 isolates (EB50, EB6, EB23, SCM4, SCM6, SCM8, EB39, EB40, EB41, EB51, EB55) with larval survival rates ≥80% were regarded as attenuated strains (AS; designated AS1-AS11, respectively).

To verify reliability of G. mellonella trials, all 21 isolates were evaluated for adhering to bMECs and releasing LDH. After 3 h of infection, VS isolates had higher adherence to bMECs than AS isolates (P < 0.001, Figure 2A). Regarding LDH release, there was no difference between VS and AS groups at 3, 6, or 9 h of infection, but after 12 h, LDH release was higher in the VS group than in the AS group (P < 0.001, Figure 2B). LDH release differed between VS3 and AS8 at 9 h (P < 0.01) and 12 h (P < 0.001).

The Virulence Genotype of K57-KP

The outer membrane protein-related gene ompA was identified in all isolates (Figure 3). In addition, entA, entB and fepC genes involved in siderophore enterobactin biosynthesis were identified in all isolates. Almost all isolates harbored genes associated with adhesion except AS8, a unique environmental isolate. Importantly, only AS6 harbored iron uptake system-related genes iucA, iucB, iucC, iutA, whereas these genes were absent from AS9, located in the same branch as AS6. Furthermore, AS1 and VS8 did not harbor the sfaA gene.

Copy numbers of virulence gene obtained from alignments with VFDB are in Figure 4. Nearly 40% of isolates in the VS group had >1 copy of fimA, fimC, fimD, fimG, fimI, sfaF, sfaE, focD, focC and focA genes. All of these isolates were from CM, and 3 belonged to the same ST. In addition, only AS8 lacked adhesion-associated genes and had meager adhesion rates in the bacterial adhesion assay. The copy number of iucA, iucB, iucC was 2 in AS6 but 4 in iutA. Number of adhesion-associated genes (fimA, fimC, fimD, fimG, fimI, sfaA, sfaE, sfaF, focA, focC, and focD) differed between virulent and attenuated strains.

Antimicrobial Resistance

All VS and AS isolates were phenotypically resistant to amoxicillin/clavulanate and imipenem, but all were sensitive to cefotaxime. In VS isolates, 30% of isolates were resistant to ceftiofur and 30% (3/10) to polymyxin B, tetracycline, and kanamycin. Ceftiogur resistance was 20% (2/10) and 9% (1/11) in VS and AS, respectively, whereas 1 AS isolate was resistant to
cefquinome and another to tetracycline. Finally, all AS isolates were sensitive to polymyxin B and kanamycin (Table 2). In addition to all isolates being resistant to amoxicillin/clavulanate and imipenem, 4 isolates were also resistant to 5 other antimicrobial agents; all 4 isolates were from CM and were > 80% lethal to larvae (Figure 3A).

AMR genes of all 21 isolates included, but were but not limited to, genes conferring resistance to aminoglycosides (aac(3)-IV, aph(4)-Ia, aph(6)-Id, aac(6′)-Ib-cr, aph(3′)-Ia, and strAB), β-lactams (blaOXA-10, blaTEM-1D, blaCTX-M-15 and blaCTX-M-65), tetracyclines [tet(A)], quinolones (oqxAB and qnrS1), macrolides (mphA), sulfonamides (sul1), and phenicol (floR) (Table S3). Gene oqxAB (95%, 20/21) was most frequently identified, followed by aph(6)-Id (29%, 6/21), strA (24%, 5/21) and strB (19%, 4/21). Extended-spectrum β-lactamase (ESBL)-encoding genes were identified in 3 K57-KP isolates (VS1, VS3 and AS2), including blaCTX-M-65 (5%, 1/21) and blaCTX-M-15 (10%, 2/21). Notably, aminoglycoside-resistance genes, macrolide-resistance genes and phenicol-resistance genes were not detected in the AS group. All isolates harbored oqxAB, except for VS6. However, only VS5 carried 5 aminoglycoside-resistance genes, aph(6)-Id, aac(6′)-Ib-cr, aph(3′)-Ia, and strAB. Three genes (aph(6)-Id and oqxAB) encoding aminoglycoside-resistance and quinolone-resistance had a higher prevalence in mastitis strain genomes: gene

![Figure 1](image1.png)

**Figure 1.** Mean ± SD survival and melanization index of 90 strains of K57 *K. pneumoniae* in a *Galleria mellonella* infection model. All experiments were performed as at least 3 independent experiments. ***P < 0.001; CM, clinical mastitis isolates; SCM, subclinical mastitis isolates; EB, environment sources isolates.

![Figure 2](image2.png)

**Figure 2.** Mean ± SD adhesion and lactate dehydrogenase (LDH) release of bovine mammary epithelial cells (bMECs) infected with 21 K57 *K. pneumoniae* isolates. (A) Bacterial adhesion of bMECs after being infected for 3 h. (B) LDH release after 3, 6, 9 or 12 h after isolate infection of bMECs. All experiments were repeated 3 times and each experiment was performed in triplicate. ***P < 0.001; n.s, not significant; VS, Virulence Strains; AS, Attenuated Strains.
Gene Aminoglycoside Resistance and Virulence

Two isolates had genes against 4 antimicrobials: isolate VS1 with resistance to aminoglycosides (aac(3)-IV, aph(4)-Ia, aph(6)-Id, strA), phenicol (floR), quinolones (oqxAB and qnrS1), β-lactam (blaOXA-10), and ESBL (blaCTX-M-65) gene; isolate VS3 harbored the ESBL gene, blaCTX-M-15, the aminoglycoside-resistance gene (aph(6)-Id), the quinolone-resistance genes (oqxAB and qnrS1), and the tetracycline-resistance gene [tet(A)].

Genomic Diversity of K57-KP

The WGS analysis statistics of the 21 isolates are presented in Supplementary Table S1 (10.6084/m9.figshare.22769051). The K57-KP isolate sequences ranged from 5.05 to 5.55 Mbp. The average G+C% content of the genome was 57.35% and the genomes contained 4,753 to 5,207 open reading frames.

Based on MLST, the 21 K57-KP isolates from this farm were designated into 16 distinct STs (Table 1). Despite the diversified STs, 24% (5/21) of the isolates were concentrated in ST107, of which 4 and 1 were from milk and environmental sources, respectively. Although the 5 isolates had the same clonotype, they differed in how harmful they were to larvae. There were 2 attenuated and 3 virulent strains, perhaps related to their various ecological niches or copy number (Figure 3B). Furthermore, 2 other environmental source isolates belonged to the same ST200 (AS2 and AS3). There was substantial genetic diversity among K57-KP isolates from this dairy farm, with many STs and a high number of SNPs. Notably, although VS2 and AS5 only had 104 SNPs in common and shared the same clonotype, they differed in virulence. In the case of the same copy number, the difference may have been due to isolate source. AS2 and AS3 had a mere 38 SNPs, all originating from the environment. Notably, they shared the same ST and had equivalent pathogenicity toward larvae. This alignment with the aforementioned hypothesis underscored the importance of strain source in relation to pathogenicity. Furthermore, this association held true even when considering strains with identical ST types and copy numbers, but with fewer SNPs (Table S2, 10.6084/m9.figshare.22769051).

Pan-Genome Analysis of K57-KP

The pan-genome of the 21 K57-KP characterized in this study had 7,674 genes. The core genome (shared by 100% isolates) consisted of 4,010 genes, the accessory genome (genes in > 2 isolates but not in all) was 2,033 genes, and the unique genome was 1,642 genes. The calculation by BPGA indicated that the pan-genome remained open but is predicted to close soon. Functional annotation of genes in the pan-genome revealed functional categories among 3 pan-genome sets (Figure 5).

According to blasting with KEGG databases, this distinct distribution was observed (Figure 5A, 5B). Functions categories of cellular processes, human diseases and organismal systems were enhanced in unique genes. In contrast, function categories of environmental information processing, genetic information processing
and metabolism were enhanced in core genes in KEGG functional pathways. The functions category of cellular processes was enhanced in accessory genes rather than core or unique genes. Notably, carbohydrate metabolism was enhanced in accessory genes rather than core or unique genes. Meanwhile, functions of amino acid
metabolism, energy metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, and translation were all enhanced in core genes. In contrast the functions of cancers, cell growth and death, glycan biosynthesis and metabolism, and replication and repair were enhanced in unique genes. Metabolism was identified as the most abundant COG category in the core and accessory genes. The overall percentage of metabolic functions in core genes was 46.7%, whereas that in the accessory and unique genes were 32.5 and 26.2%, respectively (Figure 5C). The COG functional categories enriched in the unique genome included information storage and processing. The functions of general function prediction only, and replication, recombination and repair were enhanced more often in unique genes than in core or accessory genes (Figure 5D).

**DISCUSSION**

In the present study, adhesion-related genes may have been the main reason for differences in virulence of K57-KP, with virulent strains harboring, in addition, more β-lactam and aminoglycoside resistance genes. Furthermore, there was great genetic diversity among isolates. Previous studies on K57-KP pathogenicity and genome mainly focused on isolates from humans (Wei et al., 2021). However, there are field epidemiological

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<tr>
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<td>SCM</td>
<td>ST107</td>
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<tr>
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<td>ST107</td>
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<td>ST133</td>
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<tr>
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<tr>
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<td>EB</td>
<td>ST2494</td>
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<td>2</td>
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</tbody>
</table>

\(^1\)ST = sequence type.
\(^2\)VS = virulence strain; AS = attenuated strain.
\(^3\)CM = clinical mastitis; SCM = subclinical mastitis; EB = environment and bulk tank milk.

**Table 2.** Resistance rates of all 21 K57 *K. pneumoniae* isolates to 8 antibiotics.*

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Breakpoint (μg/mL)</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/clavulanate</td>
<td>≥32</td>
<td>VS(^1) (n = 10): 100</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>≥8</td>
<td>20</td>
</tr>
<tr>
<td>Cefquinome</td>
<td>≥4</td>
<td>40</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>≥4</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≥4</td>
<td>100</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>≥4</td>
<td>30</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≥16</td>
<td>30</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>≥64</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^1\)VS = virulence strain; AS = attenuated strain.

*Range (μg/mL) = 0.25–128.
studies on *K. pneumoniae* mastitis in dairy cows (Song et al., 2022; Zheng et al., 2022) and virulence and AMR genes were identified by PCR (Gao et al., 2019; Carvalho et al., 2021).

K57 is the most prevalent capsular type in *K. pneumoniae* from mastitis in China. Consistent with previous studies, ST107 was the predominant ST (Cheng et al., 2021b). That ST107 was detected in environmental and cow-source isolates (1 environmental and 4 milk sources) strongly implicated environmental reservoirs of mastitis-associated K57-KP on this dairy farm. Another study similarly identified ST107 as the most prevalent Kp genotype that was common in cows with CM (Song et al., 2022). In this study, WGS generated complete genome sequences; this facilitated a direct execution of MLST analysis, conducted on the foundation of the genome sequence. This method involved the scrutiny of 7 housekeeping genes, with variations in these genes serving as the basis for establishing sequence types, and helping to classify bacterial strains. Fourteen isolates belonged to a unique ST, emphasizing the genetic diversity of K57-KP on this dairy farm. Although they had the same parentage and belonged to the same ST, VS2 (CM) and AS5 (SCM) differed by 104 SNPs and caused disparate larval mortality, emphasizing the power of WGS for strain typing.

Since all isolates originated from laboratory-preserved strains, and due to incomplete information regarding source, including severity of clinical signs, a larval assay was used to assess virulence. Although *G. mellonella* lacks an adaptive immune response, its innate immune response has similarities to that of mammals. The *G. mellonella* infection model has been commonly used for bacterial virulence testing (Tsai et al., 2016), including differences in *K. pneumoniae* (Insua et al., 2013). The melanization index evaluates the melanization response of *G. mellonella* (the lower the index, the more severe the melanization response) (González-Santoyo and Córdoba-Aguilar, 2012; Tsai et al., 2016). Larval survival was < 30% after 3 d of infection with isolates from the CM group, significantly different from the SCM and EB groups, as was the melanization index. The *G. mellonella* infection assay can be readily used to assess strain virulence, although results are not completely reliable and need to be verified by other methods. In the present study, we validated larval infection results with bacterial adhesion assays and LDH release assays;
the VS group adhered more to bMECs than the AS group and released more LDH.

In our study, we confirmed the existence or absence of virulence-related genes and based on copy numbers of virulence genes, we concluded that adhesion-related genes may account for differences in virulence of K57-KP, consistent with a previous report (Xu et al., 2021). Fimbriae are a pathogenic factor of K. pneumoniae and an essential mediator of K. pneumoniae adhesion. Fimbrial adhesins facilitate binding of bacteria to eukaryotic cells, the first step in the pathogenic process, and they may have an important role in avoiding the host immune system. In K. pneumoniae, types I and III fimbriae are major adhesion structures and considered pathogenic factors. In this study, the VS group had more type I fimbriae. Genes fimA, C, D, G, I are responsible for fimbrial protein production, forming fimbriae, and enabling E. coli to colonize the host’s mammary epithelium (Das Mitra et al., 2022). Adhesion is the first step in biofilm formation or invasion into host cells, promoting survival of microorganisms in infected tissues and facilitating development of mastitis (Boyd and Hartl, 1998; Tan et al., 2019). Constructing overexpressing fimA strains and altering E. coli biofilm formation under industrial fermentation conditions enabled fimA to regulate formation of E. coli biofilm (Liu et al., 2022). In addition, knocking out fimA reduced bacterial adhesion, implicating fimA in bacterial adhesion (Kallas et al., 2020). In contrast, type S and F1C fimbriae are typically present in E. coli; perhaps they were detected because they have a similar structural composition to type I fimbriae. The presence of more adhesion-associated genes in the group of VS may contribute to higher virulence, as they adhere to epithelial cells better than attenuated strains, thus increasing their chance of infection, resulting in a consensus in human medicine that high adhesion is the main characteristic of highly virulent K. pneumoniae. In subsequent studies, we will explore the role of these adhesion-associated genes in pathogenesis and their potential as vaccine candidates, which could reduce K57-KP-associated mastitis, enhance animal welfare, and reduce losses in the dairy industry.

In our study of antimicrobial-resistance genes and phenotypes, the VS group had higher resistance rates and more AMR genes than the AS group. Antimicrobials are commonly used for treatment and control of bovine mastitis. However, overuse promotes emergence of pan-resistant strains (Oliver et al., 2011) and increases rates of AMR among mastitis-associated pathogens in dairy cows. In this study, AMR phenotyping (8 compounds) was done on the 21 K57-KP with variable virulence. The VS group had a high resistance rate to various antimicrobials, including amoxicillin/clavulanate (100%), imipenem (100%), cefquinome (40%), tetracycline (30%), polymyxin B (30%), and kanamycin (30%). Similarly, in a 2019 study on antimicrobial resistance of clinical mastitis-derived Klebsiella on large dairy farms in China, there was resistance to amoxicillin/clavulanate (38%), followed by tetracycline (32%), and polymyxin B (24%) (Cheng et al., 2019). In Europe, resistance to tetracyclines in K. pneumoniae was high (19.5%) whereas resistance to β-lactams varied from 0% (cefotaxime) to 6.9% (cephalexin), and the MIC of fluoroquinolones and kanamycin was very low (de Jong et al., 2018). K57-KP in the AS group had high antimicrobial resistance rates only to amoxicillin/clavulanate and imipenem. All 21 isolates had high resistance rates against imipenem, the least effective antimicrobial against E. coli and Klebsiella spp. (Kurt and Esık, 2021). Furthermore, compared with another study (Song et al., 2022), there were apparently regional differences in China regarding rates of resistance against kanamycin and polymyxin B.

Kleborate enables a more targeted analysis of K. pneumoniae resistance genes. K. pneumoniae is intrinsically resistant to ampicillin due to the chromosomally encoded β-lactamase SHV. Some K. pneumoniae strains also carry acquired mobile SHV alleles that can confer additional inhibitor resistance and resistance to third-generation cephalosporins (Lam et al., 2021). VS1 and VS3 carried both aminoglycoside and β-lactam-resistance genes, and both isolates were predicted to contain SHV alleles encoding ESBLs (blaCTX-M-15 and blaCTX-M-65), explaining their multi-drug resistance (>2 antimicrobials). No aminoglycoside-resistance genes were identified in any isolates in the AS group, consistent with universal susceptibility to kanamycin. In contrast to previous studies from China, the detection rate of blaCTX-M was not high, perhaps due to limited isolates. Furthermore, more AMR genes were detected in CM and SCM versus environmental isolates, similar to previous studies. Kleborate can help to interpret susceptibility but is not a direct prediction of phenotype. Perhaps the high resistance rate to β-lactams, including carbapenems (e.g., imipenem), was related to specific β-lactamases and modifications of outer membrane proteins, but this requires further study (Poirel et al., 2004). Because our isolates came from strains stored in the lab, we lacked information on antimicrobial use on farms. Regardless, for management of K. pneumoniae-associated mastitis, dairy farms should minimize β-lactam and aminoglycoside antimicrobials to mitigate emergence of AMR strains.

Due to our limited sample size, it was not feasible to evaluate changes in functional predictions between isolates. Regardless, we can still make predictions for all K57-KP. Pan-genomic analysis of 21 K57-KP iso-
lates revealed that the core genome was more related to metabolism, whereas unique genomes were more relevant to genetic replication and repair. Therefore, perhaps core metabolism genes have potential as tools to identify K. pneumoniae in the microbiome.

This study had some limitations. First, the limited number of sequenced isolates and their single farm origin from only 1 farm limits external validity. Second, mechanisms of action of virulence factor-related genes remain undefined but will be pursued in our subsequent studies.

CONCLUSIONS

In this study, K57-KP from bovine mastitis and environmental sources on dairy farms had substantial genetic diversity. Sixteen STs were identified in 21 isolates, and nearly all isolates had significant SNP differences. This diversity emphasized the imperative for rigorous disease monitoring and environmental management on dairy farms. Adhesion-associated genes were associated with virulence differences among K57-KP isolates. Future studies will focus on pathogenesis and vaccine potential of these genes. Our findings advanced understanding of K57-KP’s bovine mastitis virulence, potentially guiding new diagnostic and preventive approaches.

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DECLARATION OF COMPETING INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Yang et al.: Comparative genomic analyses of Klebsiella pneumoniae


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