BMC Veterinary Research

RESEARCH

Open Access

Whole-genome sequencing of multidrug-resistant *Klebsiella pneumoniae* with capsular serotype K2 isolates from mink in China

Na Sun[1†], Yong Yang[2†], Guisheng Wang[4], Li Guo[1], Liming Liu[1], Zhihao San[1], Cuiqing Zhao[1], Lifeng Zhao[1], Mingwei Tong[2], Yuening Cheng[3] and Qiang Chen[1*]

Abstract

Background *Klebsiella pneumoniae* is a zoonotic opportunistic pathogen, and also one of the common pathogenic bacteria causing mink pneumonia. The aim of this study was to get a better understanding of the whole-genome of multi-drug resistant *Klebsiella pneumoniae* with K2 serotype in China. This study for the first time to analyze Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, resistance and virulence genes of *Klebsiella pneumoniae* in mink.

Results The isolate was *Klebsiella pneumoniae* with serotype K2 and ST6189 by PCR method. The string test was positive and showed high mucus phenotype. There was one plasmid with IncFIB replicons in the genome. The virulence factors including capsule, lipopolysaccharide, adhesin, iron uptake system, urease, secretory system, regulatory gene (*rcsA*, *rcsB*), determinants of pili adhesion, enolase and magnesium ion absorption related genes. The strain was multi-drug resistant. A total of 26 resistance genes, including beta-lactam, aminoglycosides, tetracycline, fluoroquinolones, sulfonamides, amide alcohols, macrolides, rifampicin, fosfomycin, vancomycin, diaminopyrimidines and polymyxin. Multidrug-resistant efflux protein AcrA, AcrB, TolC, were predicted in the strain.

Conclusion It was the first to identify that serotype K2 *K. pneumonia* with ST6189 isolated from mink in China. The finding indicated that hypervirulent and multi-drug resistant *K. pneumoniae* was exist in Chinese mink. The whole-genome of *K. pneumoniae* isolates have importance in mink farming practice.

Keywords *Klebsiella pneumoniae*, Mink, K2 serotype, Resistance genes, Virulence genes

†Na Sun and Yong Yang contributed equally to this work.

*Correspondence:
Qiang Chen
chenqiangdoctor17@163.com
[1]College of Animal Science and Technology, Jilin Agricultural Science and Technology University, Jilin, China
[2]School of Basic Medical Sciences, Shanxi Medical University, Taiyuan, China
[3]Key Laboratory of Special Animal Epidemic Disease, Ministry of Agriculture, Institute of Special Economic Animals and Plants, Chinese Academy of Agricultural Sciences, Changchun, China
[4]Shandong Provincial Center for Animal Disease Control and Prevention, Jinan, China

Background

Klebsiella pneumoniae (K. pneumoniae) is a major opportunistic pathogen, a member of the Enterobacteriaceae family. It is a gram-negative bacillus causing various types of community and hospital-acquired infections in debilitated or immunocompromised patients, such as enteritis, urinary tract infection, cystitis, pneumonia, meningitis, liver abscess, endogenous endophthalmitis, sepsis and other serious infectious diseases [1–3]. Emergent hypervirulent *K. pneumoniae* (hvKp) has been responsible for severe diseases, posing a severe public health threat. Several reports have shown that *K. pneumoniae* strains with serotypes K1 and K2 have been found to be the most common in hvKp. These strains are more resistant to phagocytosis and intracellular death by macrophages and neutrophils [4]. *K. pneumoniae* mainly causes mink pneumonia, which is mainly manifested as dyspnea, rapid onset and death. It mostly occurs in the warm and humid season in autumn. At present, *K. pneumoniae* has become one of the main infectious diseases that harm mink farming. In China, *K. pneumoniae* strains from minks were prevalent in Hebei, Shandong, Liaoning, Heilongjiang and other regions, causing serious economic losses to the mink farming industry. *K. pneumoniae* strains are mainly in the respiratory and intestinal tracts. They can be transmitted through aerosols and faeces and has the potential to infect other animals and humans. However, there were relatively few studies specifically focused on mink.

Antibiotics have been used to treat a wide range of bacterial infections in humans and animals [5]. In recent years, due to the selection pressure and inappropriate antibiotic use in healthcare and agriculture, the resistance of *K. pneumoniae* has become increasingly serious, showing an increasing trend year by year, mainly manifested as multi-drug resistance. Multiple factors contribute to development and maintenance of antibiotic resistance, lack of new antimicrobials, and spread of organisms or resistance genes within healthcare facilities and beyond. Therefore, the understanding of the drug resistance of *K. pneumoniae* is very significant. In recent years, the drug resistance of *K. pneumoniae* isolated from mink has become increasingly serious. A study from China found that the *K. pneumoniae* strain isolated from mink was resistant to chloramphenicol, aminoglycosides, tetracycline, sulfonamides and most cephalosporins [6]. The detection of extended-spectrum beta-lactamase-producing of *K. pneumoniae* from mink feces showed that all ESBLs fecal isolates were resistant to ampicillin, cefazolin, and cephalothin [7]. The found from the Netherlands showed that people who lived in the lowest mink density in the municipalities had a low ESBL/pAmpC-E prevalence of *E. coli* and *K. pneumoniae* [8].

Whole-genome sequencing is a powerful tool for the study of the molecular epidemiology of *K. pneumoniae*, and determine the dissemination [9–13]. In this study, the objectives of the study were to analyze the antimicrobial susceptibility of *K. pneumoniae* with capsular serotype K2 isolated from mink in China. Whole-genome sequencing was used to explore resistance and virulence genes of *K. pneumoniae* strains. They will be helpful to reveal the key role of spread of antimicrobial resistance.

Results

Identification of strain and serotype analysis

The result of detecting and sequencing the *khe* gene showed that the stain was *K. pneumonia.* The string test was positive and showed high mucus phenotype. The isolate was serotype K2 using PCR method and sequencing. High mucus phenotype and serotype K2 were the main manifestations of high virulence *K. pneumonia*. MLST results showed that the isolate were ST6189.

Antimicrobial susceptibility testing

The strain was detected determined by Kirby-Bauer disk diffusion method. The results showed in Table 1. The strain was sensitive to ceftazidime (CAZ), cefazolin (CFZ), ceftriaxone (CRO), levofloxacin (LEV), amikacin (AMK), gentamicin (GEN), imipenem (IPM) and meropenem (MPM), and resistant to ampicillin (AMP), cefoxitin (CFO), ciprofloxacin (CIP), chloramphenicol (CLO), florfenicol (FLR), tetracycline (TET), doxycycline (DOX), polymyxin (CT), streptomycin (STR), kanamycin (KAN) and trimethoprim/sulfamethoxazole (SXT). The.

Genome assembly and component analysis

The optimized assembly results showed that there were one chromosome with 5237.551 kb and one plasmid with 125.072 kb, the chromosome and plasmid were circular. The plasmid with IncFIB replicons. The genome size was 5362.623 kb, and gene number was 5111. The gene length distribution was showed in Fig. 1. There were 87 tRNA, 3 rRNA and 43 sRNA. A total of 13 GIs were detected, there were 10 GIs in the chromosome and 3 GIs in the plasmid.

Gene function analysis

The functions in the identification of 43 GO terms included 22 terms in biological process, 11 terms in cellular component and 10 terms in molecular function. The GO enrichment items were shown in a bar diagram (Fig. 2). In terms of cellular components, GO-terms were mainly enriched in the cell, cell part, macromolecular complex, organelle and other processes. GO-terms in the biological process were enriched in cellular process, metabolic process, biological regulation, establishment of localization, localization, regulation of biological process

Table 1 Results of drug sensitivity test

Antibiotics	Antimicrobial agents concentration(ug)	Antibacterial diameter range(mm)	Bacteriostatic diameter(mm)	Susceptibility
AMP	10	13–17	5	R
CAZ	30	14–18	20	S
CFO	30	14–18	8	R
CFZ	30	14–18	21	S
CRO	30	14–18	20	S
CIP	5	15–21	10	R
LEV	5	12–16	18	S
CLO	30	12–18	6	R
FLR	30	12–18	8	R
TET	30	14–19	8	R
DOX	30	12–16	10	R
CT	30	8–12	6	R
AMK	30	14–17	20	S
STR	10	11–15	8	R
GEN	10	12–15	18	S
KAN	30	13–18	10	R
SXT	1.25/23.75	10–16	6	R
IPM	10	13–16	20	S
MPM	10	16–18	20	S

and other processes. GO-terms in molecular function were enriched in binding, catalytic activity, nucleic acid binding transcription factor activity, transporter activity and others in molecular function.

A KEGG pathway enrichment analysis was performed, which identified 219 pathway terms. The terms are displayed in a bar diagram (Fig. 3). The items are associated with the cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems. There were 171 genes and 22 genes participated in cellular community-prokaryotes and cell growth and death, respectively. A total of 389 genes and 145 genes were included in membrane transport and signal transduction. In genetic information processing, there were 82 genes in translation pathway and 60 genes in replication and repair. Drug resistance: antimicrobial pathway included 67 genes. More than 100 genes were in nucleotide metabolism, metabolism of cofactors and vitamins, energy metabolism, carbohydrate metabolism and amino acid metabolism.

For COG annotation, we noticed that the genes of strain grouped to COG-G (carbohydrate transport and metabolism), COG-E (amino acid transport and metabolism) and COG-K (transcription) were significantly higher than type of other function (Fig. 4).

The whole genome sequencing data uploaded to VFDB database for virulence factor comparison, the results were in Table 2. The virulence factors including capsule (*galF*, *wcaJ*, *manC*, *manB*), lipopolysaccharide (*uge*), adhesin (*fimH*, *fimD*, *fimI*, *fimA*, *fimE*, *fimB*, *mrkA*, *mrkB*, *mrkC*, *mrkF*, *mrkI*, *mrkH*, *pilD*, *pilQ*, *pilT*, *pilW*, *pilR*), iron uptake system (*fepC*, *fepD*, *entA*, *entB*, *entE*, *entF*,*aerobacin*, *ybtA*), urease (*ureA*, *ureB*, *ureG*, *allR*), secretory system (*hcp/tssD*, *sciN/tssJ*, *icmF/tssM*, *vipB/tssC*, *vasH/clpV*), regulatory gene (*rcsA*, *rcsB*), determinants of pili adhesion (*stiB*, *stkB*, *sthC*, *sthB*), enolase (*eno*) and magnesium ion absorption related genes (*mgtB*, *mgtC*). The colibactin genes had not been detected.

The drug resistance gene annotation results were showed in Table 3. A total of 26 drug resistance genes in 12 classes were screened. There were beta-lactam (*pbp1a*, *pbp2*, bla_{SHV-2}, $bla_{OKP-A-8}$), aminoglycosides (*aadA6*), fluoroquinolones (*qnrB2*, *aac(6')-Ib-cr*), tetracycline (*tet34*, *tetA*, *tetG*), sulfonamides (*sul1*), amide alcohols (*cmle3*, *floR*), macrolides (*mpha*), rifampicin (*arr-3*), fosfomycin (*fosA5*), vancomycin (*vanA*, *vanC*), diaminopyrimidines (*dfra22*, *dfra5*, *dfrA25*) and polymyxin (*arnA*, *pmrF*, *pmrE*, *pmrB*, *pmrC*). Multidrug-resistant efflux protein AcrA, AcrB, TolC, were predicted in the strain.

Discussion

K. pneumoniae is a common opportunistic pathogen and easy to infect people and animals. It is widely distributed in the respiratory tract and intestinal tract of animals, and its infection rate is second only to *Escherichia coli*. Serotypes K1, K2 and K5 are highly virulent in experimental infection in mice and may cause severe infections in humans and animals [14]. *K. pneumoniae* is one of the main pathogens causing pneumonia in mink. However, the studied about *K. pneumoniae* isolated from mink had been less reported. In the study, the *K. pneumoniae* isolate was identifed as serotype K2 and hypermucoviscous phenotype. A study found that 14 of the

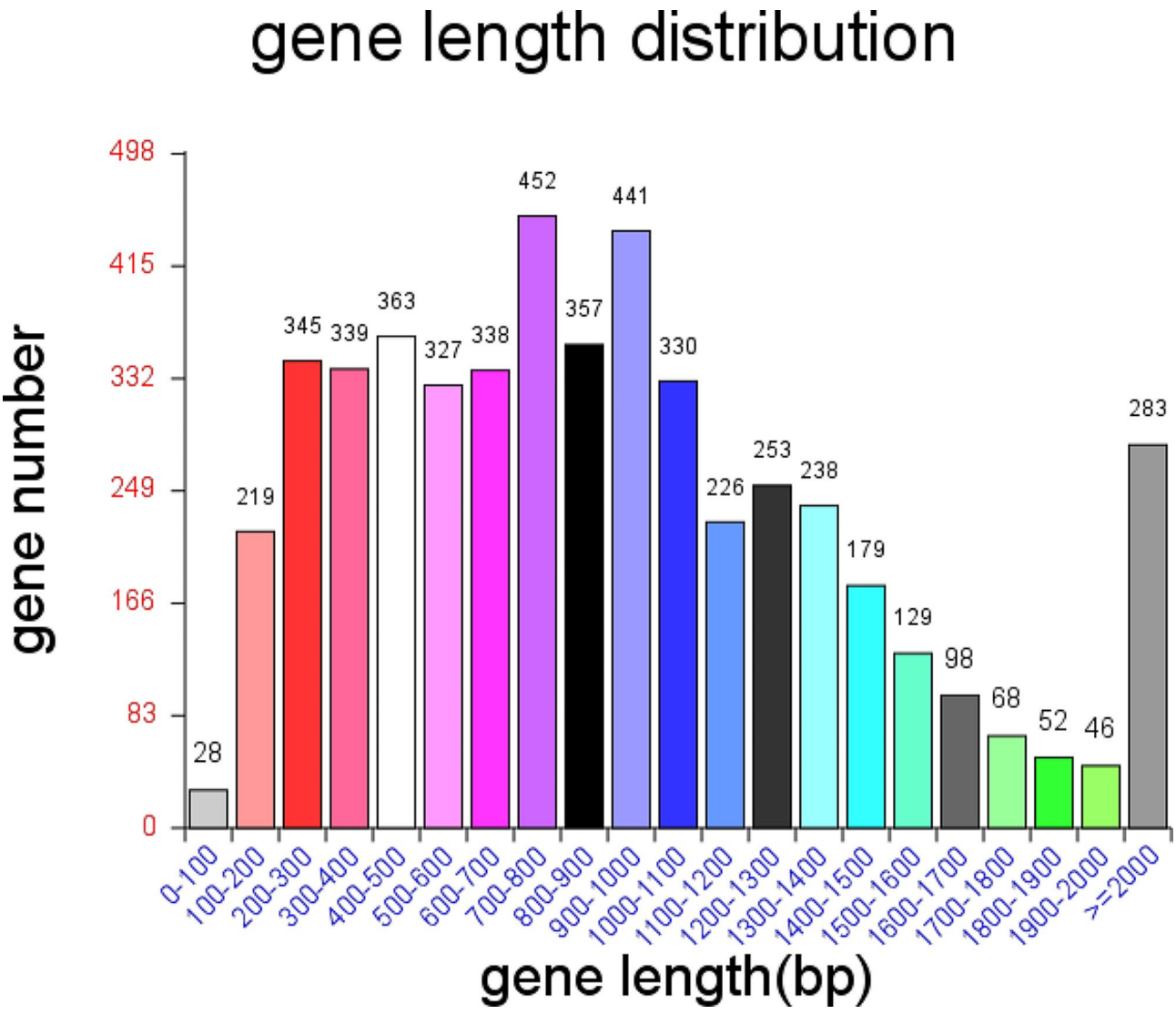

Fig. 1 The gene length distribution of genomic. The horizontal coordinate was the gene length and the vertical coordinate was the corresponding gene number

15 *K. pneumoniae* strains isolated from minks in China were identified as serotype K2 [15]. It implied that serotype K2 was prevalent in mink in China. At present, there are many ST types of *K. pneumoniae*, but ST11, ST15, ST23 and ST37 are the main epidemic ST types [16, 17]. In this study, the isolate belonged to ST6189. There were no studies of *K. pneumoniae* strains with ST6189 were reported. The strain with ST6189 isolated from mink was first reported in our study. It is suggested to strengthen and regularly monitor the epidemic ST types of *K. pneumoniae.*

Antibiotic resistance has been a potentially serious threat to global public health. Literature regarding resistant bacteria and antibiotic use in mink is scarce [18]. The sensitivity test results of this study showed that *K. pneumoniae* isolate was highly sensitive to IPM and MPM, which may be due to the fact that carbapenem drugs had been strictly prohibited for use in animals. *K. pneumoniae* isolate was resistant to AMP, which was consistent with the report that *K. pneumoniae* exhibited inherent resistance to AMP [19]. The sensitivity of this strain to cephalosporin was not exactly the same as other reports [20, 21]. This was mainly due to regional differences in drug use. Fluoroquinolones, aminoglycosides, tetracycline, sulfonamides and amide alcohols were the important class of wide-spectrum antibacterial agents and had been widely used for disease treatment and control in mink production in China. So it was highly resistant to these drugs. The Chinese government had officially banned polymyxin as an animal growth promoter, but the strain was also resistant to polymyxin. The increased prevalence of multi-resistant strains and

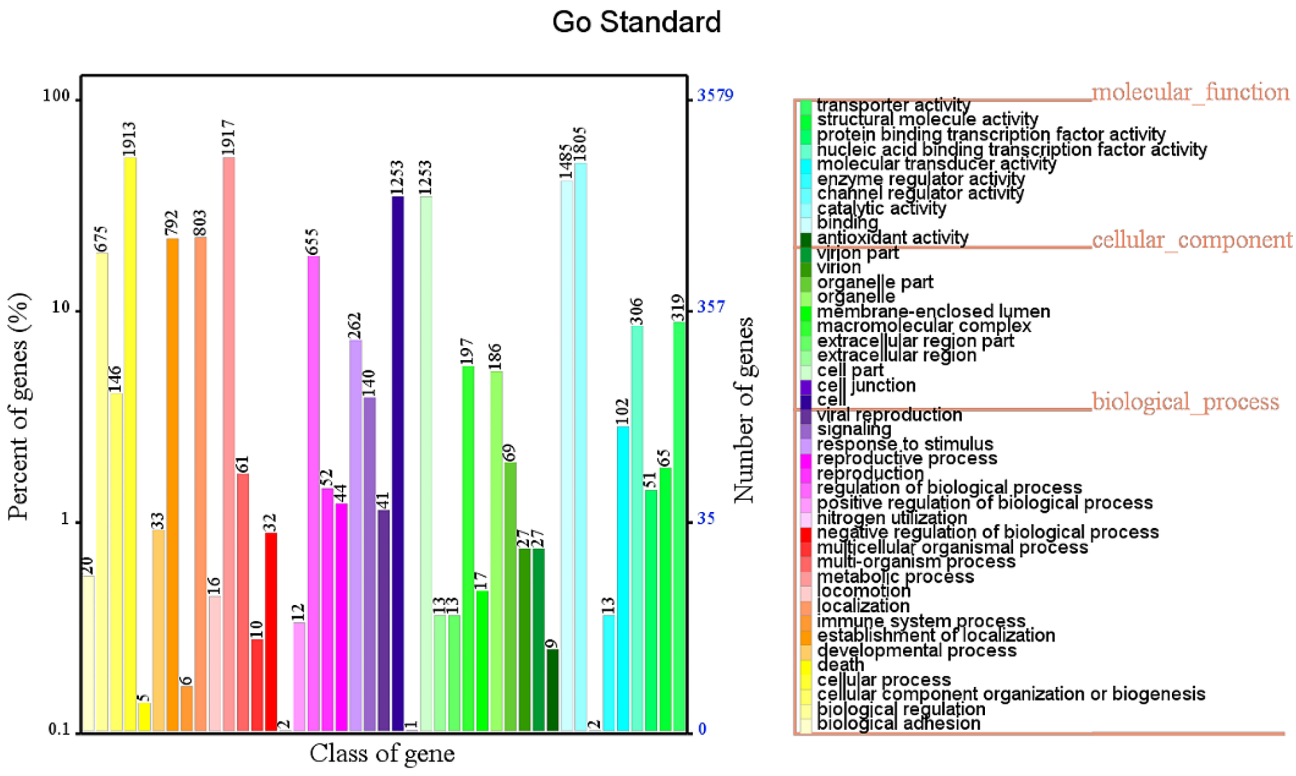

Fig. 2 GO analysis for genome. The bar diagram displaying GO terms of each category the horizontal coordinate shows significantly enriched GO terms. The right vertical coordinate shows the number of genes on the annotation and the left vertical coordinate shows number of genes annotated as a percentage of all encoded genes

pathogenic bacteria in animal populations had increased the risk of transmission of zoonotic pathogens, and also proposed a serious challenge for clinical treatment of animals.

The genome analysis showed that it had the similar base pairs with other *K. pneumoniae* strains. *K. pneumoniae* presented at least one plasmid sequence, plasmid with IncFIB, IncFIA, IncFIB, IncHI1B, and IncFII replicons [22–24]. In this study, the strain had one plasmid with IncFIB replicons. This conjugative plasmid had been associated with the dissemination of the bla_{NDM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} genes in *K. pneumoniae* [25, 26].

Meanwhile, general function analysis of the *K. pneumoniae* strain genome showed that the identified genes were primarily involved in various GO, KEGG pathways and COG, which revealing the diversity of gene functions. The function analysis showed that the genes were mainly in metabolic aspect. Bacteria were small and had a relatively large surface area. Therefore, material exchange was frequent and rapid, showing a very active metabolism. Various bacteria had different nutritional requirements, energy sources, enzyme systems and metabolites, forming a variety of metabolic types to adapt to the complex external environment. Carbohydrate were the necessary raw materials for the synthesis of bacteria and the main source of energy for bacterial metabolism. The draft genome sequence of *K. pneumoniae* isolated from a patient with chronic diarrhoea showed that a non-ribosomal peptide synthetase (NRPS), bacteriocin and thiopeptide biosynthetic gene cluster, can also be predicted. The function of genes of strain assigned were not consistent with previous studies reporting [27, 28].

The pathogenicity of *K. pneumoniae* depends on the action of virulence factors. The exact definition of hvKp remained controversial. Although the string tests were generally accepted as the method for identifying hvKp, many wire drawing positive *K. pneumoniae* did not exhibit high virulence [4]. High viscosity was not equal to high virulence. It was necessary to combine molecular biological detection methods to identify. Several virulence factors such as K1/K2 serum types, *rmpA* and *aerobactin* seemed to be closely related to the high virulence of hvKp [4]. In this study, the string test was positive and the strain belonged to K2 serotype, and 10 virulence factor categories including 48 kinds of virulence genes were detected. The detection rate of pili, iron uptake system, T6SS and capsule related genes were higher than other genes. The *rmpA* gene and the genes encoding colibactin toxin were not detected, but *aerobactin* gene was existing. Aerobactin supplementation of a defined minimal medium with transferrin markedly reduced the growth of avirulent strains but had no

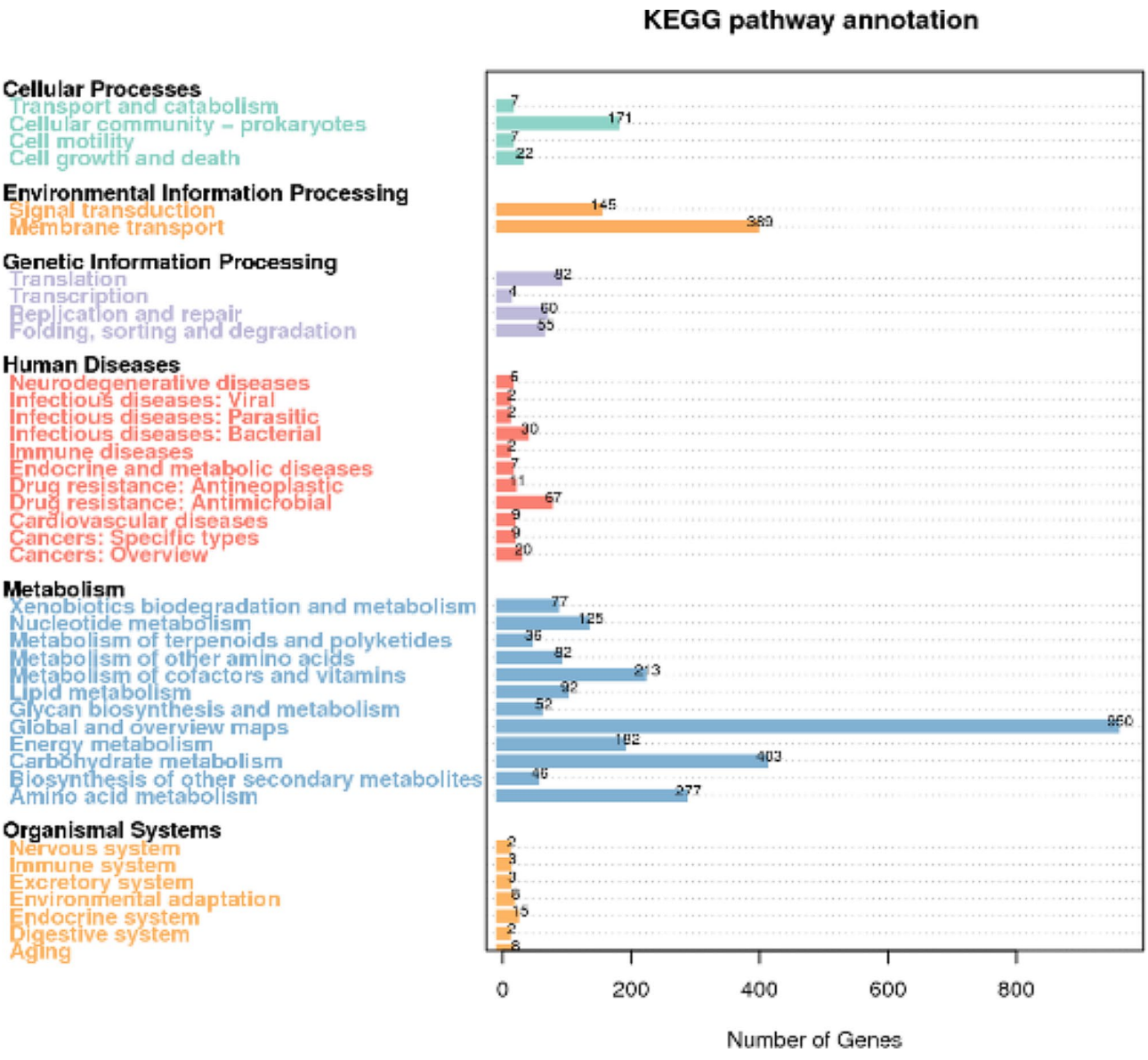

Fig. 3 KEGG pathway analysis for genome. The bar diagram displays the items, the x-axis shows the number of genes, and the y-axis shows significantly enriched pathway terms

significant effect on the growth of virulent strains, and production of aerobactin could be correlated with virulence [29]. Therefore, it is speculated that the virulence mechanism of *K. pneumoniae* isolated in this study might include: (1) Synthesizing pili and adhering to the surface of host cells formation to play a role in exerting virulence. (2) By secreting iron carriers to absorb iron from the host for reproduction and infection. (3) By using the capsular polysaccharide contained in the capsule to trigger the bacterial immune escape mechanism. (4) Lipopolysaccharide aggregates on the surface of bacteria, forming complexes to mediate bacteria to escape or resist the killing of host innate immunity [30, 31]. Combined with the above conclusions, it was confirmed that the strain was highly virulent *K. pneumonia.* The genome of *K. pneumoniae* isolated from feral swine showed that a total of 14 virulence factors related to adherence, invasion, and iron uptake were detected [32]. The virulence genes such as *yagZ/ecpA*, *yagY/ecpB*, *yagV/ecpE*, *yagX/ecpC*, *fimB*, *entA*, *entB*, *fepC*, *kdsA*, *fimH*, *fepB* and *yagW/ecpD* were detected in the genome of swine *K. pneumoniae* in Jilin Province in China [33]. Although the kinds of virulence genes were not the same in these studies, the virulence genes of the strains were diverse and mainly caused diseases in animals. Monitoring virulence of *K. pneumoniae* was as important as drug resistance to prevent. Because the virulence of the strains was a threat to animal populations and the health.

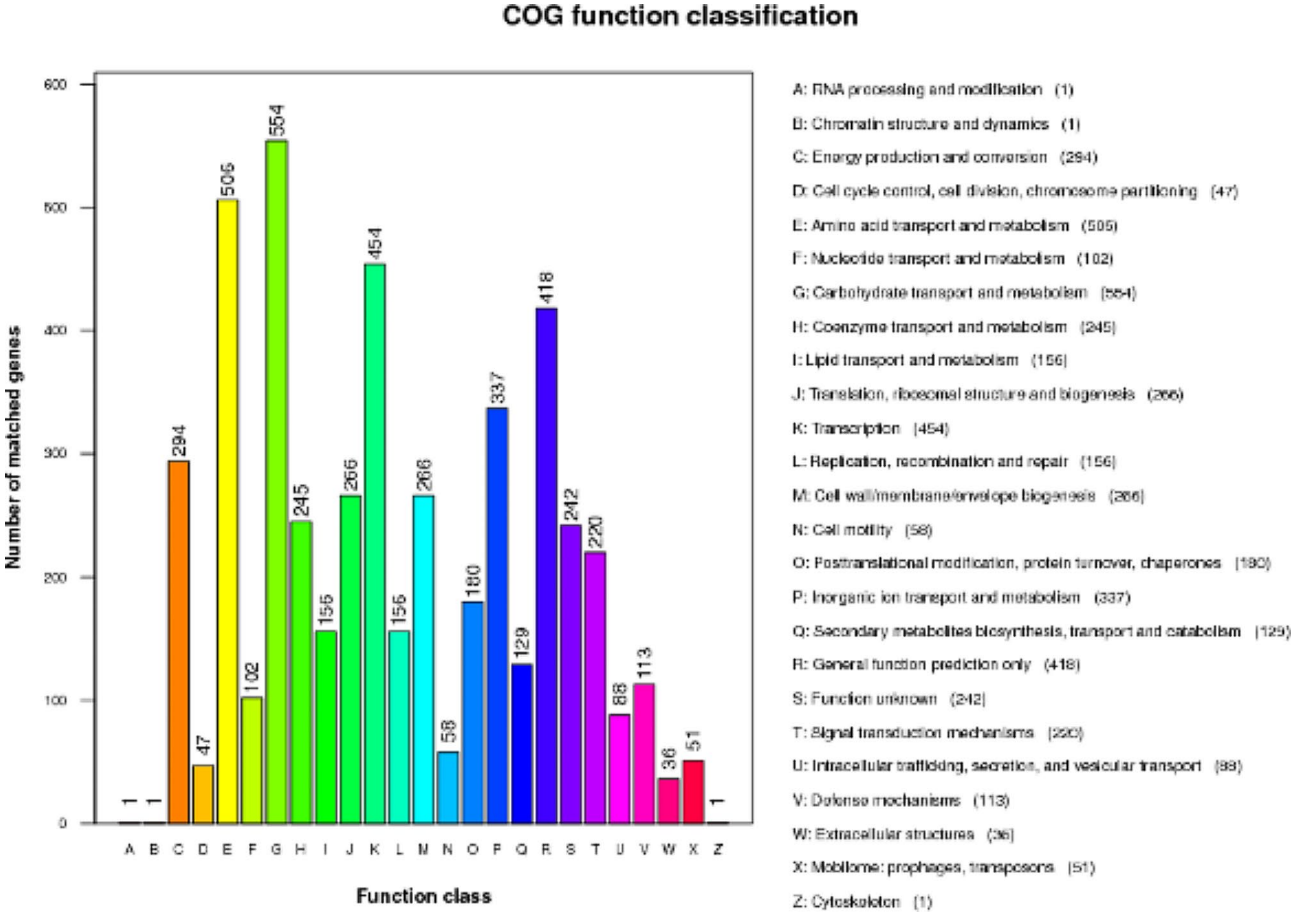

Fig. 4 COG analysis for genome. The x-axis represents COG functional type, and the y-axis represents the number of genes on the annotation

Table 2 The results of virulence genes

Virulence factor category		Virulence gene
Capsule		*galF*, *wcaJ*, *manC*, *manB*
Lipopolysaccharide		*uge*
Adhesin	Type I fimbriae	*fimH*, *fimD*, *fimI*, *fimA*, *fimE*, *fimB*
	Type 3 fimbriae	*mrkA*, *mrkB*, *mrkC*, *mrkF*, *mrkI*, *mrkH*
	Type 4 fimbriae	*pilD*, *pilQ*, *pilT*, *pilW*, *pilR*
Iron uptake system	Enterobactin	*fepC*, *fepD*, *entA*, *entB*, *entE*, *entF*
	Aerobactin	*aerobactin*
	Yersinia	*ybtA*
Urease		*ureA*, *ureB*, *ureG*, *allR*
Secretory system	T6SS	*hcp/tssD*, *sciN/tssJ*, *icmF/tssM*, *vipB/tssC*, *vasH/clpV*
Regulatory gene		*rcsA*, *rcsB*
Determinants of pili adhesion		*stiB*, *stkB*, *sthC*, *sthB*
Enolase		*eno*
Magnesium ion absorption related genes		*mgtB*, *mgtC*

Antibiotic resistance had been a potentially serious threat to global public health. In this study, β-lactam resistance genes of *pbp1a*, *pbp2*, bla_{SHV-2} and $bla_{OKP-A-8}$ were detected which hydrolyzed penicillin and cephalosporin drugs resulting in resistance to β-lactam drugs. The resistance genes of bla_{OKP} were derived from *K. pneumoniae*, and could be replicated and continuously expressed along the host bacteria chromosome. They belonged to the narrow spectrum β-lactamase genes and mediated the bacteria resistant to amoxicillin, ampicillin, carbobenzicillin, ticacillin and first-generation cephalosporin. The early family of bla_{SHV} genes were

Table 3 The results of resistance genes

Drug resistance class	Resistance gene
Beta-lactam	*pbp1a*, *pbp2*, bla_{SHV-2}, $bla_{OKP-A-8}$
Aminoglycosides	*aadA6*
Fluoroquinolones	*qnrB2*, *aac(6')-Ib-cr*
Tetracycline	*tet34*, *tetA*, *tetG*
Sulfonamides	*sul1*
Amide alcohols	*cmle3*, *floR*
Macrolides	*mpha*
Rifampicin	*arr-3*
Fosfomycin	*fosA5*
Diaminopyrimidines	*dfra22*, *dfra5*, *dfrA25*
Vancomycin	*vanA*, *vanC*
Polymyxin	*arna*, *pmrF*, *pmrE*, *pmrB*, *pmrC*

first discovered in *K. pneumoniae*, and then gradually found in other Enterorod bacteria, and several gene subtypes had evolved [34, 35]. The genes of SHV-type had been reported as the most prevalent β-lactamases genes in *K. pneumoniae.* Only one aminoglycosides resistance gene was detected which cause the strain was resistant to streptomycin. It has been reported that *tetA*, *tetG*, *floR* and *cmle3* mediate the efflux pump of tetracycline, florfenicol and chloramphenicol respectively, which could pump drugs out of cells and reduce intracellular drug concentration to develop resistance [36–38]. In this study, the resistance genes of *tetA* and *floR* were on the plasmid. *tet34* is commonly found in *K. pneumoniae*, and causes the activation of Mg^{2+}-dependent purine nucleotide synthesis, which protects the protein synthesis pathway [39]. There were two plasmid-mediated quinolone resistance (PMQR) genes *qnrB* and *aac(6')-Ib-cr* which mediated horizontal transmission of resistance genes. In this study, the strain was resistant to polymyxin and five resistance genes were detected. These genes as bifunctional enzyme that catalyzes the oxidative decarboxylation of UDP-glucuronic acid (UDP-GlcUA) to UDP-4-keto-arabinose (UDP-Ara4O) and the addition of a formyl group to UDP-4-amino-4-deoxy-L-arabinose (UDP-L-Ara4N) to form UDP-L-4-formamido-arabinose (UDP-L-Ara4FN). The modified arabinose is attached to lipid A and is required for resistance to polymyxin and cationic antimicrobial peptides. Also, vancomycin resistance genes were annotated in the strain. Vancomycin could block the synthesis of bacterial cell walls, and also affect the membrane permeability of some gram-positive and gram-negative bacteria. VanA type vancomycin resistance operon genes and vanC type vancomycin resistance operon genes, which could synthesize peptidoglycan with modified C-terminal D-Ala-D-Ala to D-alanine–D-lactate. Vancomycin belonged to the tricyclic glycopeptide class of antibiotics, which mainly had strong bactericidal activity against gram-positive bacteria. Vancomycin resistance gene clusters were commonly seen in *Enterococcus* and *Staphylococcus aureus*. This was the first time to report that *K. pneumoniae* carried vancomycin resistance genes. AcrAB-TolC was a kind of multidrug resistance efflux pump system that had been found in gram-negative bacteria. It mainly caused the resistant to aminoglycoside, glycylcycline, macrolide, beta-lactam, acriflavin and fluoroquinolones. In the presence of antibiotics or toxic compounds, efflux can be the fastest and most effective resistance mechanism of the stress response of enterobacterium [40]. In addition, AcrAB-TolC system may play a role in plasmid-mediated transmission of bacterial resistance. A study found that the acquisition of resistance to tetracycline in *Escherichia coli* depends on the AcrAB-TolC multidrug efflux pump, because it reduces the concentration of tetracycline in cells. Therefore, protein synthesis can be sustained and TetA expression can be initiated immediately after plasmid acquisition. The efflux activity of AcrAB-TolC can also maintain resistance through plasmid transfer in the presence of antibiotics and other modes of action [41]. The genome sequence analysis of multidrug-resistant *K. pneumonia* isolated from a pig farm in China showed that the resistance genes included $bla_{OKP-B-3}$, *aac(6')-Ib-cr*, *oqxA*, *oqxB*, *qnrB6*, *dfrA27*, *floR*, *aadA16*, *fosA5*, *sul1*, *arr-3* and *tetD* [42]. Compared to our study, there were some of the same resistance genes in the genome of *K. pneumonia* isolated from pig and mink. It could be seen that the *K. pneumonia* strains from animal carried a variety of drug resistance genes, and the drug resistance was very serious.

Conclusion

It was the first to identify that serotype K2 *K. pneumonia* with ST6189 isolated from mink in China. The genome analysis showed that the strain carried 10 virulence factor categories including 48 kinds of virulence genes. The pili, iron uptake system, T6SS and capsule related genes play the major role. The drug sensitivity test results showed that the strain was multi-drug. The resistance genes were diverse and mediated bacterial resistance to antibiotics. Vancomycin resistance genes were the first time to report in *K. pneumoniae*. So great attention should be paid to the etiology and epidemiological surveillance of *K. pneumoniae* in mink.

Methods

Collection of strain and serotype analysis

In 2021, a mink farm in Jilin Province of China had 2,000 minks which were feeded caged together, and more than 20 minks were dead. The dead minks were mainly young males and showing symptoms of respiratory distress. One of the dead minks was sent for testing by the farmer. The dead mink showed some clinical symptoms, such as lethargy, loss of appetite, cough, runny nose, abdominal

breathing and diarrhea. The *K. pneumoniae* strain was isolated from a lung sample and streaked onto Maconkey Agar medium. It was according to standard clinical microbiologic methods. The bacterial colony from an overnight culture was added to 300μL water and boiled for 15 min to release DNA template. The isolate was identified using PCR based on the *khe* gene, a specific target gene of *K. pneumonia* [15]. The string test was performed by touching a colony with a loop and pulling up. A test result is considered to be positive when a string of ≥5 mm is observed. The serotype of the isolate was using PCR for serotypes K1, K2, K5, K20, K54 and K57, and sequencing was performed in Kumi Biotechnology (Jilin) Co., Ltd (Jilin, China) [15].

The molecular typing of *K. pneumoniae* was detected by MLST method. 7 housekeeping gene (*gapA*, *infB*, *mdh*, *phoE*, *pgi*, *rpoB* and *tonB*) were amplified by PCR, the primers and reaction program were referenced *K. pneumoniae* MLST online database (http://www.pasteur.fr/mlst). All PCR amplified positive products of 7 housekeeping genes were sequenced by Kumi Biotechnology (Jilin) Co., Ltd (Jilin, China) and submitted to the website (http://www.pasteur.fr/mlst/Kpneumoniae. html) to obtain the allele number and ST type of each steward gene of MLST.

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were determined by Kirby-Bauer disk diffusion antibiotic testing method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2020) using 19 different antibiotic disks of all classes: ampicillin, ceftazidime, cefoxitin, cefazolin, ceftriaxone, ciprofloxacin, levofloxacin, chloramphenicol, florfenicol, tetracycline, doxycycline, polymyxin, amikacin, streptomycin, gentamicin, kanamycin, imipenem, meropenem and trimethoprim/sulfamethoxazole. The drug sensitive paper purchased from Hangzhou Microbial Reagent Co., LTD in China. The specific antimicrobial agents used for susceptibility testing were mainly used for the treatment of *K. pneumoniae* and some drugs prohibited in veterinary clinic.

Extraction of genome DNA

Genomic DNA was extracted with the FastPure® Bacteria DNA Isolation Mini Kit (Vazyme Biotech Co.,Ltd. Nanjing, China). The harvested DNA was detected by the agarose gel electrophoresis and quantified by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Library construction and sequencing

Libraries for single-molecule real-time (SMRT) sequencing was constructed with an insert size of 10 kb using the SMRT bell TM Template kit, version 1.0. Briefly, the process were that fragment and concentrate DNA, repair DNA damage and ends, prepare blunt ligation reaction, purify SMRTbell Templates with 0.45X AMPure PB Beads, size-selection using the, BluePippin System, repair DNA damage after size-selection. At last, the library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and detected the insert fragment size by Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA).

A total amount of 1 μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the DNA sample was fragmented by sonication to a size of 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR.

The whole genome of the isolate was sequenced using PacBio Sequel platform and Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd.

Genome assembly

In order to ensure the accuracy of the subsequent analysis results, the low-quality reads were filtered (less than 500 bp) to obtain Clean data. Using the automatic error correction function of SMRT portal, the long reads were selected (more than 6000 bp) as the seed sequence, and the other shorter reads were aligned to the seed sequence by Blast, so that the accuracy of the seed sequence could be improved further. After assembling we got an initial result.

By the variant Caller module of the SMRT Link software, the arrow algorithm was used to correct and count the variant sites in the preliminary assembly results. The corrected assembly result, which was used as the reference sequence, was blast with Illumina data by bwa. Futhermore the result was filtered with the base minimum mass value of was 20, the minimum read depth of 4 and the maximum read depth of 1000. Based on the overlap between the head and the tail, we confirmed whether the chromosomal sequence formed a circle or not, then corrected the initial site by blast with the DNA database. At last the chromosome and plasmid sequences were screened by blast with plasmid database.

Genome component prediction

Genome component prediction included the prediction of the coding gene, non-coding RNA and genomics

islands. The available steps were proceeded as follows: (1) For bacteria, we used the GeneMarkSprogram to retrieve the related coding gene. (2) Transfer RNA (tRNA) genes were predicted by the tRNAscan-SE. Ribosome RNA (rRNA) genes were analyzed by the rRNAmmer. Small nuclear RNAs (sRNA) were predicted by BLAST against the Rfam database. (3) The IslandPath-DIOMB program was used to predict the Genomics Islands (GIs).

Gene function

GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes) and COG (Clusters of Orthologous Groups) were used to predict gene functions. A whole genome Blast search (E-value less than 1e-5, minimal alignment length percentage larger than 40%) was performed against above the databases. For pathogenic bacteria, we added the pathogenicity and drug resistance analyses. We used the PHI (Pathogen Host Interactions), VFDB (Virulence Factors of Pathogenic Bacteria), ARDB (Antibiotic Resistance Genes Database) to perform the above analyses. In addition, targeted PCR had been used to confirm the absence of the colibactin genes (*clbA*, *clbB*, *clbN* and *clbQ*) as previously described [43].

Acknowledgements
Not applicable.

Author contributions
NS, YY, and QC designed the study; NS, GW, and LG performed the experiments; LL, ZS, and CZ analyzed the data; LZ, MT, and YC prepared the figures and tables; NS wrote the manuscript. All authors have read and approved the manuscript.

Funding
This study was funded by the Jilin Province Science and Technology Development Plan Project Assignment (20200201108JC) and Jilin Province Science and Technology Research Project of Education Department (JJKH20220401KJ), the Doctoral Foundation of Jilin Agricultural Science and Technology University (20217014).

Data availability
The sequences of the strain generated or analysed during the current study are available in the GenBank repository, the accession numbers are CP138346-CP138347.

Declarations

Ethics approval and consent to participate
Not applicable. We have obtained informed consent from the owner to use the animal in our study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 August 2023 / Accepted: 5 August 2024
Published online: 10 August 2024

References

1. Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev. 1998;11(4):589–603.
2. El Fertas-Aissani R, Messai Y, Alouache S, Bakour R. Virulence profiles and antibiotic susceptibility patterns of Klebsiella pneumoniae strains isolated from different clinical specimens. Pathol Biol (Paris). 2013;61(5):209–16.
3. Huang W, Qiao F, Zhang Y, Huang J, Deng Y, Li J, Zong Z. In-hospital medical costs of infections caused by Carbapenem-resistant Klebsiella pneumoniae. Clin Infect Dis. 2018;67(suppl2):S225–30.
4. Lee CR, Lee JH, Park KS, Jeon JH, Kim YB, Cha CJ, Jeong BC, Lee SH. Antimicrobial Resistance of Hypervirulent Klebsiella pneumoniae: Epidemiology, Hypervirulence-Associated determinants, and Resistance mechanisms. Front Cell Infect Microbiol. 2017;7:483.
5. Zinner SH. Antibiotic use: present and future. New Microbiol. 2007;30(3):321–5.
6. Han K, Lv J, Wang YYY, Wang Y, Xu H, Chen L, Feng E, Bai X, Yan X. Isolation,identification and drug susceptibility of Klebsiella pneumoniae isolated from Racoon Dog. China Anim Husb Veterinary Med. 2019;46(04):1209–16.
7. Agga GE, Silva PJ, Martin RS. Detection of extended-spectrum beta-lactamase-producing and Carbapenem-resistant Bacteria from Mink Feces and feed in the United States. Foodborne Pathog Dis. 2021;18(7):497–505.
8. Wielders CCH, van Hoek A, Hengeveld PD, Veenman C, Dierikx CM, Zomer TP, Smit LAM, van der Hoek W, Heederik DJ, de Greeff SC et al. Extended-spectrum β-lactamase- and pAmpC-producing Enterobacteriaceae among the general population in a livestock-dense area. *Clin Microbiol Infect* 2017, 23(2):120.e121-120.e128.
9. Becker L, Kaase M, Pfeifer Y, Fuchs S, Reuss A, von Laer A, Sin MA, Korte-Berwanger M, Gatermann S, Werner G. Genome-based analysis of carbapenemase-producing Klebsiella pneumoniae isolates from German hospital patients, 2008–2014. Antimicrob Resist Infect Control. 2018;7:62.
10. Becker L, Fuchs S, Pfeifer Y, Semmler T, Eckmanns T, Korr G, Sissolak D, Friedrichs M, Zill E, Tung ML, et al. Whole genome sequence analysis of CTX-M-15 producing Klebsiella isolates allowed dissecting a polyclonal outbreak scenario. Front Microbiol. 2018;9:322.
11. Brhelova E, Antonova M, Pardy F, Kocmanova I, Mayer J, Racil Z, Lengerova M. Investigation of next-generation sequencing data of Klebsiella pneumoniae using web-based tools. J Med Microbiol. 2017;66(11):1673–83.
12. Khan FA, Hellmark B, Ehricht R, Söderquist B, Jass J. Related carbapenemase-producing Klebsiella isolates detected in both a hospital and associated aquatic environment in Sweden. Eur J Clin Microbiol Infect Dis. 2018;37(12):2241–51.
13. Lu J, Zhang J, Xu L, Liu Y, Li P, Zhu T, Cheng C, Lu S, Xu T, Yi H, et al. Spread of the florfenicol resistance floR gene among clinical Klebsiella pneumoniae isolates in China. Antimicrob Resist Infect Control. 2018;7:127.
14. Turton JF, Perry C, Elgohari S, Hampton CV. PCR characterization and typing of Klebsiella pneumoniae using capsular type-specific, variable number tandem repeat and virulence gene targets. J Med Microbiol. 2010;59(Pt 5):541–7.
15. Jian-Li W, Yuan-Yuan S, Shou-Yu G, Fei-Fei D, Jia-Yu Y, Xue-Hua W, Yong-Feng Z, Shi-Jin J, Zhi-Jing X. Serotype and virulence genes of Klebsiella pneumoniae isolated from mink and its pathogenesis in mice and mink. Sci Rep. 2017;7(1):17291.
16. Yan JJ, Wang MC, Zheng PX, Tsai LH, Wu JJ. Associations of the major international high-risk resistant clones and virulent clones with specific ompK36 allele groups in Klebsiella pneumoniae in Taiwan. New Microbes New Infect. 2015;5:1–4.
17. Ma Y, Bao C, Liu J, Hao X, Cao J, Ye L, Yang J. Microbiological characterisation of Klebsiella pneumoniae isolates causing bloodstream infections from five tertiary hospitals in Beijing, China. J Glob Antimicrob Resist. 2018;12:162–6.
18. Nikolaisen NK, Fertner M, Lassen DCK, Chehabi CN, Ronaghinia AA, Chriél M, Jensen VF, Jensen LB, Pedersen K, Struve T. Association between Antibiotic Consumption and Resistance in Mink Production. Antibiot (Basel) 2022, 11(7).
19. Fu Y, Zhang F, Zhang W, Chen X, Zhao Y, Ma J, Bao L, Song W, Ohsugi T, Urano T, et al. Differential expression of Bla(SHV) related to susceptibility to ampicillin in Klebsiella pneumoniae. Int J Antimicrob Agents. 2007;29(3):344–7.
20. Aracil-García B, Oteo-Iglesias J, Cuevas-Lobato Ó, Lara-Fuella N, Pérez-Grajera I, Fernández-Romero S, Pérez-Vázquez M, Campos J. Rapid increase in resistance to third generation cephalosporins, imipenem and co-resistance in Klebsiella pneumoniae from isolated from 7,140 blood-cultures (2010–2014) using EARS-Net data in Spain. Enferm Infecc Microbiol Clin. 2017;35(8):480–6.

21. Moghnieh RA, Musharrafieh UM, Husni RN, Abboud E, Haidar M, Abboud E, Abou Shakra D. E. Coli, K. pneumoniae and K. Oxytoca community-acquired infections susceptibility to cephalosporins and other antimicrobials in Lebanon. J Med Liban. 2014;62(2):107–12.
22. Shankar C, Muthuirulandi Sethuvel DP, Neeravi AR, Venkatesan M, Devanga Ragupathi NK, Anandan S, Veeraraghavan B. Identification of plasmids by PCR based replicon typing in bacteremic Klebsiella pneumoniae. Microb Pathog. 2020;148:104429.
23. Long J, Zhang J, Xi Y, Zhao J, Jin Y, Yang H, Chen S, Duan G. Genomic insights into CRISPR-Harboring plasmids in the Klebsiella Genus: distribution, backbone structures, Antibiotic Resistance, and virulence determinant profiles. Antimicrob Agents Chemother. 2023;67(3):e0118922.
24. Richter L, du Plessis EM, Duvenage S, Allam M, Ismail A, Korsten L. Whole genome sequencing of extended-spectrum- and AmpC- β-Lactamase-positive enterobacterales isolated from spinach production in Gauteng Province, South Africa. Front Microbiol. 2021;12:734649.
25. Oliveira ÉM, Beltrão EMB, Scavuzzi AML, Barros JF, Lopes ACS. High plasmid variability, and the presence of IncFIB, IncQ, IncA/C, IncHI1B, and IncL/M in clinical isolates of Klebsiella pneumoniae with bla KPC and bla NDM from patients at a public hospital in Brazil. Rev Soc Bras Med Trop. 2020;53:e20200397.
26. Rimoldi SG, Gentile B, Pagani C, Di Gregorio A, Anselmo A, Palozzi AM, Fortunato A, Pittiglio V, Ridolfo AL, Gismondo MR, et al. Whole genome sequencing for the molecular characterization of carbapenem-resistant Klebsiella pneumoniae strains isolated at the Italian ASST Fatebenefratelli Sacco Hospital, 2012–2014. BMC Infect Dis. 2017;17(1):666.
27. Zhou H, Guo W, Zhang J, Li Y, Zheng P, Zhang H. Draft genome sequence of a metallo-β-lactamase (Bla(AIM-1))-producing Klebsiella pneumoniae ST1916 isolated from a patient with chronic diarrhoea. J Glob Antimicrob Resist. 2019;16:165–7.
28. Zhang R, Xu Z, Xue G, Feng J, Du B, Gan L, Fan Z, Fu T, Feng Y, Zhao H et al. Combined methylation and transcriptome analysis of Liver Injury of non-alcoholic fatty liver Disease Induced by High Alcohol-producing Klebsiella pneumoniae. Microbiol Spectr 2023:e0532322.
29. Russo TA, Gulick AM. Aerobactin Synthesis Proteins as Antivirulence targets in Hypervirulent Klebsiella pneumoniae. ACS Infect Dis. 2019;5(7):1052–4.
30. Li B, Zhao Y, Liu C, Chen Z, Zhou D. Molecular pathogenesis of Klebsiella pneumoniae. Future Microbiol. 2014;9(9):1071–81.
31. Liu Y, Bai J, Kang J, Song Y, Yin D, Wang J, Li H, Duan J. Three Novel sequence types Carbapenem-resistant Klebsiella pneumoniae strains ST5365, ST5587, ST5647 isolated from two tertiary teaching General hospitals in Shanxi Province, in North China: molecular characteristics, resistance and virulence factors. Infect Drug Resist. 2022;15:2551–63.
32. Liu H, Zhai Y, Liu T, Fan P, Boughton R, Jeong KC. Draft genome sequence of NDM-Encoding Klebsiella pneumoniae isolated from feral swine. Microbiol Resour Announc. 2021;10(41):e0080821.
33. Qi Y, Xue JZ, Li SS, Elken EM, Haqmal MA, Li XS, Xu GY, Kong LC, Ma HX. Analysis of an IncR plasmid carried by carbapenem-resistant Klebsiella pneumoniae: a survey of swine Klebsiella pneumoniae in Jilin Province. J Glob Antimicrob Resist. 2023;34:83–90.
34. Stoesser N, Batty EM, Eyre DW, Morgan M, Wyllie DH, Del Ojo Elias C, Johnson JR, Walker AS, Peto TE, Crook DW. Predicting antimicrobial susceptibilities for Escherichia coli and Klebsiella pneumoniae isolates using whole genomic sequence data. J Antimicrob Chemother. 2013;68(10):2234–44.
35. Zou LK, Wang HN, Zeng B, Zhang AY, Li JN, Li XT, Tian GB, Wei K, Zhou YS, Xu CW, et al. Phenotypic and genotypic characterization of β-lactam resistance in Klebsiella pneumoniae isolated from swine. Vet Microbiol. 2011;149(1–2):139–46.
36. Acosta-Pérez G, Ibáñez-Cervantes G, Bello-López JM, Hernández JM, Hernández-Montañez Z, Giono-Cerezo S, León-García G, León-Avila G. Structural diversity of class 1 Integrons in multiresistant strains of Escherichia coli isolated from patients in a hospital in Mexico City. Curr Microbiol. 2015;71(4):501–8.
37. Græsbøll K, Larsen I, Clasen J, Birkegård AC, Nielsen JP, Christiansen LE, Olsen JE, Angen Ø, Folkesson A. Effect of tetracycline treatment regimens on antibiotic resistance gene selection over time in nursery pigs. BMC Microbiol. 2019;19(1):269.
38. Wang YH, Li XN, Chen C, Zhang J, Wang GQ. Detection of floR gene and active efflux mechanism of Escherichia coli in Ningxia, China. Microb Pathog. 2018;117:310–4.
39. Lv F, Cai J, He Q, Wang W, Luo Y, Wang X, Mi N, Zhao Z, Li G, Luo W. Overexpression of Efflux Pumps Mediate Pan Resistance of Klebsiella pneumoniae sequence type 11. Microb Drug Resist. 2021;27(10):1405–11.
40. Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJV, Luisi BF. Multidrug efflux pumps: structure, function and regulation. Nat Rev Microbiol. 2018;16(9):523–39.
41. Nolivos S, Cayron J, Dedieu A, Page A, Delolme F, Lesterlin C. Role of AcrAB-TolC multidrug efflux pump in drug-resistance acquisition by plasmid transfer. Science. 2019;364(6442):778–82.
42. Zhao Y, Liu L, Wang S, Tian M, Qi J, Li T, Yu S. Draft genome sequence analysis of a novel MLST (ST5028) and multidrug-resistant Klebsiella quasipneumoniae subsp. similipneumoniae (Kp4) strain 456S1 isolated from a pig farm in China. J Glob Antimicrob Resist. 2021;24:275–7.
43. Lan Y, Zhou M, Jian Z, Yan Q, Wang S, Liu W. Prevalence of pks gene cluster and characteristics of Klebsiella pneumoniae-induced bloodstream infections. J Clin Lab Anal. 2019;33(4):e22838.

Publisher's Note