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The *Yersinia* high-pathogenicity island (HPI) carried by a new integrative and conjugative element (ICE) in a multidrug-resistant and hypervirulent *Klebsiella pneumoniae* strain SCsl1

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ABSTRACT

Multidrug-resistant and hypervirulent *Klebsiella pneumoniae* (hvKP) poses a significant risk to public health. To better understand the molecular characteristics of multidrug-resistant and hypervirulent *K. pneumoniae* of animal origin, fifteen *K. pneumoniae* strains from the liver, blood of sick pigs and chicken feces were collected. All *K. pneumoniae* isolates were subjected to antimicrobial susceptibility testing, string test, multi-locus sequence typing and whole genome sequencing. Seven *K. pneumoniae* isolates were found carrying the *mcr-1.1* gene. Among them, a multidrug-resistant and hypervirulent *K. pneumoniae* strain SCsl1 isolated from the liver of a diseased pig was found to harbor 16 resistance genes (e.g., *mcr-1.1*) and 16 virulence genes including aerobactin. Moreover, a novel integrative and conjugative element, named ICE*KpSL1*, was identified in SCsl1, which contains a full *Yersinia* high-pathogenicity island (HPI). This element could be excised from the chromosome to form a circular intermediate, indicating potential transmission of the *Yersinia* pathogenicity island. The emergence of multidrug-resistance and hypervirulence in *K. pneumoniae* from animals warrants further surveillance.

1. Introduction

Klebsiella pneumoniae is a Gram-negative bacterium and an important pathogen that causes infections to animals and humans. Such infections often lead to invasive diseases, for example, mastitis in dairy cows, and nosocomial wound infections and pneumonia in humans (Schukken et al., 2012; Siu et al., 2012). It is commonly recognized that the *Yersinia* high-pathogenicity island (HPI) plays a vital role in the pathogenicity of *K. pneumoniae* (Schubert et al., 2003). The *Yersinia* HPI was originally identified in *Yersinia pestis* and was reported to be associated with siderophore-mediated iron acquisition system (Scott and Bearden, 1997). Recent studies indicate that *Yersinia* HPI is widely present in microbes of Enterobacteriaceae and behaves as a key factor which is responsible for the lethal phenotype in mice (Magistrali et al., 2015; Petermann, 2008). Another virulence factor gene aerobactin, which can increase siderophore production, is also a defining trait for hypervirulent *K. pneumoniae* (hvKP) (Russo et al., 2015, 2014; Zhang et al., 2016b). Despite its significance, our knowledge about hvKP strains from animal origin remains elusive.

hvKP strains are not commonly resistant to clinically important antibiotics (Zhang et al., 2016b), but studies about the co-occurrence of virulence and resistance genes in clinical *K. pneumoniae* isolates were reported in recent years (Liu et al., 2014; Zhang et al., 2016a). More importantly, resistance genes to some last-resort antibiotics (e.g., carbapenem and colistin) were recently found in clinical hvKP strains (Gu et al., 2016; Lee et al., 2016), which poses direct threat to the public health. Thus, characterizations of clinically relevant antibiotic resistance genes in hvKP pathogens are of significance.

Mobile genetic elements (MGEs), which include plasmids, transposable elements, prophages and integrative and conjugative elements (ICEs), can transfer antibiotics resistance genes and virulence genes across bacterial species (Wozniak and Waldor, 2010). The ICEs were featured by their ability to intergrate into host chromosomes and are capable of excision, circulation and transfer via conjugation (Johnson and Grossman, 2015). ICEs including Tn*916* and ICE*Kp1* family elements have been reported in *K. pneumoniae* (Lin et al., 2008; Soge et al.,

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2008). *Yersinia* HPI was found to be part of ICE*Kp1* family elements, but this only occurred in few cases of hypervirulent *K. pneumoniae* and *Escherichia coli* isolates from humans (Breurec et al., 2016; Paauw et al., 2010; Putze et al., 2009). Till now, *Yersinia* HPI-carrying ICEs in *K. pneumoniae* of animal origin has not been reported. Understanding the mechanism of ICE-dependent transmission of *Yersinia* HPI could help lower the risk of future outbreak of pathogens.

In this study, a ST107 multidrug-resistant and hypervirulent *K. pneumoniae* strain SCsl1 was identified from sick pig, this isolate possesses 16 resistance genes, 16 virulence genes, and a novel *Yersinia* HPI-carrying ICE. The combination of multidrug-resistance and hypervirulence among *K. pneumoniae* strains poses an urgent threat to the health of both livestock and people.

2. Materials and methods

2.1. Bacterial strains

During 2017 to 2018, a routine surveillance project on antimicrobial resistance in Gram-negative bacteria from livestock farms in Sichuan province (China) was carried out. A total of 15 *K. pneumoniae* strains was collected from liver, blood of sick pigs and chicken feces samples. A single bacterial strain was collected per sample on blood agar plates without adding any selective antibiotics. All the tested strains were confirmed by 16S rRNA sequencing for species identity. Specifically, seven strains (SCsl1, SCsl6, SCsl8, SCsl10, SCsl11, SCsl12, and SCsl3) were isolated from the liver and blood samples of sick pigs, which exhibited clinical symptoms of high fever, nasal discharge, diarrhea, dyspnea, conjunctivitis, and skin congestion. And eight strains (HeB85, SCcd66, SCcd67, SCcd68, SCcd69, SCcd70, SCcd71, and SCcd72) were isolated form faecal samples of chickens (Table 1). All bacterial stocks were kept at -80℃ in 25% glycerol for further analysis.

2.2. Antimicrobial susceptibility and string tests

Antimicrobial susceptibility tests were performed by using a disk diffusion (Kirby-Bauer) method with Mueller-Hinton Agar according to the standards of the Clinical and Laboratory Standards Institute. Thirteen antimicrobial agents were included: ampicillin (AM, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftizoxime (CZ, 30 μg), chloramphenicol (C, 30 μg), meropenem (MEM, 30 μg), aztreonam (ATM, 30 μg), ciprofloxacin (CIP, 5 μg), gentamicin (GM, 10 μg), fosfomycin (FOS, 200 μg), tetracycline (TE, 30 μg), amikacin (AN, 30 μg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg), and rifampin (RIF, 5 μg). Antibiotic susceptibility of all *K. pneumoniae* isolates to colistin was determined by using broth microdilution according to the standards of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 6.0. *K. pneumoniae* ATCC 700,603 was used as quality control.

The string test was performed to assess the hypermucoviscous phenotype by stretching a colony grown overnight on a blood agar plate at 37℃ with a bacteriology inoculation loop and pulling up. Strains generating a viscous string with length of 5 mm or longer were defined as hypermucoviscous *K. pneumoniae* strains (Shon et al., 2013).

2.3. Multilocus sequence typing and phylogenetic analysis of K. pneumoniae

Multilocus sequence typing (MLST) was performed by amplifying and sequencing seven housekeeping genes of *K. pneumoniae*, including *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* (Diancourt et al., 2005). Alleles and sequence types (STs) were determined based on the MLST database (www.pasteur.fr/mlst/Kpneumoniae.html). The phylogenetic relationships of collected *K. pneumoniae* isolates (n = 15) were determined. A minimum spanning tree of *K. pneumoniae* strains was constructed with the clustering method using BioNumerics v7.6.3 software (Applied Maths, Saint-Martens-Latem, Belgium) based on the numbers of seven allele in each isolate.

2.4. Whole genome sequencing and analysis

Genomic DNA was extracted from all 15 *K. pneumoniae* isolates using the Tiangen genomic DNA kit (Tian gen, China). Whole genome sequencing (WGS) was then performed on the Illumina Hiseq X Ten platform (150PE, Illumina) at a commercial sequencing company (Genebang, Chengdu, China). The paired-end reads were *de novo*-assembled using the SPAdes genome assembler version 3.9.0 (https://cge.cbs.dtu.dk/services/SPAdes/). Genome annotation was done using the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/). Antimicrobial resistance genes were identified using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/). The virulence genes also were predicted (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

2.5. Identification of integrative and conjugative elements

The presence of ICEs in the genome of *K. pneumoniae* was determined by comparing the assembled contigs to the ICEberg database which include 384 available ICEs seqeunces (http://db-mml.sjtu.edu.cn/ICEberg/) using BLASTn. Boundaries and insertion sites of the predicted ICEs were checked manually. Gaps in the predicted ICEs were amplified by PCR (Table 2) and the amplified DNA was then sequenced. PCR amplification reactions were carried out in 25 μl volumes containing 2 × PCR Master Mix (Tsingke, China), 1.0 μl template DNA and 0.5 μM of each primer. Conditions for amplification were as follows: one cycle at 95 °C (5 min), 30 cycles at 95 °C (30 s), 50 °C (30 s), 72 °C (1 or 2 min), and a final cycle at 72 °C (10 min). Open reading frames (ORFs) were predicted using ORF Finder (http://ncbi.nlm.nih.gov/gorf/gorf.html). Protein-coding genes were identified and annotated using RAST (http://rast.nmpdr.org/rast.cgi).

To detect the circular form of the integrative element ICE*KpSL1*, inverse PCR was performed with two primer sets (Table 2) that targeted on the two terminal sequences of ICE. Primers NP1 and NP4 were used to detect the *att*P site after excision and primers NP2 and NP3 were used to detect the *att*I site in the extrachromosomal circular form. The PCR amplifications were carried out in 25 μl volumes containing 2 × PCR Master Mix (Tsingke, China), 1.0 μl template DNA and 0.5 μM of each primer. Conditions for amplification were as follows: one cycle at 95 °C (5 min), 30 cycles at 95 °C (30 s), 50 °C (30 s), 72 °C (1 min), and a final cycle at 72 °C (10 min). The amplicons were sequenced by Shanghai Sangon Bioengineering Co., Ltd using an ABI 3730 sequencer (Applied Biosystems,Foster City, USA)

2.6. Mouse lethality assay

To further confirm the virulence of the *K. pneumoniae* SCsl1 strain, twelve five-week-old female BALB/c mice were used to test the lethality of this strain. Overnight culture of *K. pneumoniae* SCsl1 was inoculated into fresh LB broth at a dilution of 1:100 and grew for another 3 h at 37℃ shaking incubator. The bacterial cells were then harvested, washed once and resuspended in saline (0.9%) to OD600 of 1.0 (approximately 10^9 CFU/mL). Serial dilution was performed to the concentrations of 10^3 CFU/mL, 10^4 CFU/ mL and 10^5 CFU/mL. One hundred microliters of the bacterial suspension was injected into the mice intraperitoneally. Six mice were involved in each group, with 4 per group used for the survival analysis. The hvKP strain NTUH-K2044 was used as a control. All mice were monitored daily for survival.

Table 1
Information of fifteen *Klebsiella pneumoniae* strains.

Strain	sample of sites	sample	ST	Resistance gene	Virulence genes
SCsl1	Sichuan	pig liver	ST107	*aadA1*, *aph(6)-Id*, *aph(4)-Ia*, *aadA2*, bla_{SHV-26}, *mcr-1.1*, *qnrB4*, *oqxA*, *oqxB*, *fosA*, *cmlA1*, *sul1*, *sul3*, *sul2*, *tet*(A), *dfrA12*	*iucA*, *iucB*, *iucC*, *iucD*, *iutA*, *kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCsl6	Sichuan	Pig liver	unknown	*ant(2")-Ia*, *aac(6')-Ib-cr*, *aadA16*, *armA*, *aph(6)-Id*, *aph(3")-Ib*, bla_{DHA-1}, bla_{TEM-1A}, bla_{SHV-67}, *mcr-1.1*, *oqxA*, *oqxB*, *qnrB2*, *qnrB4*, *fosA*, *mph*(E), *catA2*, *floR*, *arr-3*, *sul2*, *sul1*, *tet*(A),*dfrA27*	*mrkA*, *mrkB*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCsl8	Sichuan	Pig liver	ST15	*armA*, *aph(3')-Ia*, *aac(6')-Ib-cr*, *aadA16*, *aac(3)-IId*, *aph(6)-Id*, *aph(3")-Ib*, bla_{DHA-1}, $bla_{CTX-M-3}$, bla_{TEM-1B}, *mcr-1.1*, *qnrS1*, *qnrB4*, *oqxA*, *oqxB*, *fosA*, *mph*(A), *msr*(E), *mph*(E), *floR*, *arr-3*, *sul2*, *sul1*, *tet*(A), *tet*(B), *dfrA27*	*kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCsl10	Sichuan	Pig blood	unknown	*aac(3)-IId,v*, *aac(6')-Ib-cr*, *aph(6)-Id*, *aph(3')-Ia*, *aph(3")-Ib*, $bla_{CTX-M-14}$, $bla_{SHV-182}$, bla_{TEM-1B}, *mcr-1.1*, *oqxA*, *oqxB*, *qnrB52*, *fosA*, *floR*,*arr-3*, *sul1*, *sul2*, *tet*(A),*dfrA27*	*mrkA*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCsl11	Sichuan	Pig liver	ST462	*aac(6')-Ib-cr*, *aac(3)-IId*, *aadA16*, bla_{SHV-2}, bla_{TEM-1A}, *mcr-1.1*, *oqxA*, *oqxB*, *qnrB6*, *fosA5*, *floR*,*arr-3*, *sul1*, *sul2*, *tet*(D),*dfrA27*	*mrkA*, *mrkB*, *mrkD*, *mrkF*, *mrkI*, *mrkJ*
SCsl12	Sichuan	Pig liver	ST462	*aadA16*, *aac(6')-Ib-cr*, *aac(3)-IId*, *aph(6)-Id*, bla_{SHV-2}, bla_{TEM-1A}, *mcr-1.1*, *oqxA*, *oqxB*, *qnrB6*, *fosA5*, *floR*,*arr-3*, *sul1*, *sul2*, *tet*(D), *dfrA27*	*mrkA*, *mrkB*, *mrkD*, *mrkF*, *mrkI*, *mrkJ*
SCsl13	Sichuan	Pig liver	unknown	*ant(2")-Ia*, *aac(6')-Ib-cr*, *aadA16*, *aac(3)-IId*, *armA*, *aph(6)-Id*, *aph(3")-Ib*, bla_{DHA-1}, bla_{SHV-67},$bla_{TEM-116}$, *mcr-1.1*, *oqxB*, *oqxA*, *qnrB2*, *qnrB4*, *fosA*, *mph*(E), *msr*(E), *catA2*, *floR*, *arr-3*, *sul2*, *sul1*, *tet*(A),*dfrA27*, *dfrA1*	*mrkA*, *mrkB*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
HeB85	Hebei	Chicken feces	ST15	*aph(4)-Ia*, *aac(3)-Iva*, *armA*, *aadA1*, *aph(3')-Ia*, bla_{SHV-28}, bla_{TEM-1B}, $bla_{CTX-M55}$, *oqxA*, *oqxB*, *qnrB2*, *fosA*, *mph*(A), *catA1*, *cml*, *floR*, *sul3*, *sul1*, *tet*(A),*dfrA12*	*kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCcd67	Sichuan	Chicken feces	ST15	*aph(4)-Ia*, *aac(3)-Iva*, *aph(3")-Ib*, *aph(3')-Ia*, *armA*, *aadA1*, *aadA2*, *aph(6)-Id*, bla_{SHV-28}, bla_{DHA-1}, $bla_{CTX-M-55}$, *oqxA*, *oqxB*, *qnrB4*, *fosA*, *mph*(E), *mph*(A), *cmlA1*, *sul1*, *sul2*, *sul3*, *tet*(A), *dfrA12*	*kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCcd66	Sichuan	Chicken feces	ST534	*aph(4)-Ia*, *armA*, *aac(3)-Iva*, *aac(3)-IId*, *aph(3')-Ia*, *aac(6')-Ib-cr*, *aadA2*, *aph(6)-Id*, bla_{TEM-1B}, bla_{DHA-1}, bla_{SHV-13}, bla_{OXA-1}, *oqxB*, *oqxA*, *qnrB4*, *fosA*, *mph*(A), *msr*(E), *mph*(E), *catB4*, *cmlA1*, *floR*, *arr-3*, *sul1*, *sul2*, *sul3*, *tet*(A),*tet(M)*, *dfrA12*	*mrkA*, *mrkB*, *mrkD*, *mrkF*, *mrkH*, *mrkJ*, *mrkI*
SCcd68	Sichuan	Chicken feces	ST11	*aph(4)-Ia*, *aac(6')-Ib-cr*, *aac(3)-Iva*, *aadA16*, *aph(3')-Ia*, *aph(3')-IIa*, *armA*, *aadA1*, *aph(3")-Ib*, *aadA2*, *aph(6)-Id*, $bla_{CTX-M-14}$, bla_{DHA-1}, bla_{TEM-1B}, $bla_{SHV-182}$, $bla_{CTX-M-55}$, *oqxA*, *qnrD1*, *oqxB*, *qnrB4*, *qnrS2*, *fosA*, *msr*(E), *mph*(E), *floR*, *cmlA1*, *arr-3*, *sul3*, *sul1*, *tet*(D), *dfrA27*	*mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*
SCcd69	Sichuan	Chicken feces	ST15	*aph(4)-Ia*, *aac(3)-Iva*, *armA*, *aph(6)-Id*, *aadA1*, bla_{DHA-1}, bla_{TEM-1B}, $bla_{CTX-M-55}$, bla_{SHV-28}, *oqxB*, *oqxA*, *qnrB4*, *fosA*, *mph*(A), *mph*(E), *floR*, *cml*, *catA1*, *sul1*, *sul3*, *tet*(A),*dfrA12*	*kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCcd71	Sichuan	Chicken feces	ST15	*aph*(4)-Ia, *aac*(3)-Iva, *aph*(3')-Ia, *armA*, *aph*(3")-Ib, *aph*(6)-Id, *aad*A1, bla_{DHA-1}, $bla_{CTX-M-55}$, bla_{SHV-28}, *oqxA*, *oqxB*, *qnrB4*, *fosA*, *mph*(A), *mph*(E), *floR*, *catA1*, *sul1*, *sul3*, *tet*(A),*dfrA12*	*kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCcd70	Sichuan	Chicken feces	ST534	*aph(4)-Ia*, *aac(3)-IId*, *aac(3)-Iva*, *armA*, *aph(6)-Id*, *aph(3")-Ib*, bla_{TEM-1B}, bla_{DHA-1}, bla_{SHV-13}, *oqxB*, *oqxA*, *qnrB4*, *fosA*, *mph*(A), *msr*(E), *mph*(E), *cmlA1*, *floR*, *sul3*, *sul2*, *tet*(A),*tet(M)*, *dfrA12*	*mrkA*, *mrkB*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*
SCcd72	Sichuan	Chicken feces	ST15	*aph(4)-Ia*, *aac(3)-Iva*, *armA*, *aph(6)-Id*, *aadA1*, bla_{DHA-1}, $bla_{CTX-M-55}$, bla_{SHV-28}, *oqxA*, *oqxB*, *qnrB4*, *fosA*, *mph*(A), *mph*(E), *floR*, *catA1*, *sul1*, *sul3*, *tet*(A),*dfrA12*	*kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI*, *mrkJ*

Table 2
Principal oligonucleotide primers used in the study.

Gene	Primer		Source or reference
	Designation	Sequence(5, -3,)	
inverse PCR	NP1	GCGACAAGAGGAGAACAC	In this study
	NP2	TTGACCAGCAGATACAGAC	
	NP3	CACGATTCCTCTGTAGTTCA	In this study
	NP4	CTGCTGTTGCTGTTGTCT	
Close the gap of ICE	ICE1-1 F	GAACCTACCTGTATCAGAACT	In this study
	ICE1-1 R	TGCCTTATTGACCGTGTT	
	ICE1-2 F	CTTCCTTGCGAATCCTTATC	
	ICE1-2 R	TAGTTCTGCGTTCCGTATT	

3. Results

3.1. Antibiotic resistance phenotypes and string tests of K. pneumoniae strains

Of the 15 *K. pneumoniae* isolates tested, all of them (100%, 15 isolates) showed resistance to trimethoprim/sulfamethoxazole, ampicillin, fosfomycin, gentamicin, chloramphenicol, ciprofloxacin, and tetracycline. The resistance rates to other antibiotics are as follows: amoxicillin/clavulanic acid (93.3%, 14 isolates), ceftizoxime (80%, 12 isolates) rifampin (73.3%, 11 isolates), amikacin (66.7%, 10 isolates) and aztreonam (53.3%, 8 isolates). Seven *K. pneumoniae* isolates were resistant to colistin (MIC ≥ 8 μg/mL) and were susceptible to meropenem.

Fourteen isolates were found to be negative in the string test. Only *K. pneumoniae* SCsl1 strain was positive, which was isolated from the liver of a sick pig in Sichuan province. The mouse lethality assay showed that all the tested mice died before or on the fifth day after injected with the SCsl1 (ST107)strain, which further confirmed that the SCsl1 strain is hypervirulent.

3.2. Multilocus sequence types and phylogenetic analysis of K. pneumoniae strains

Seven sequence types were identified among the 15 *K. pneumoniae* isolates. The most prevalent ST among the *K. pneumoniae* isolates was ST15 (n = 6), followed by ST462 (n = 2), and ST534 (n = 2). However, the sequence types of three *K. pneumoniae* strains (SCsl10, SCsl13, and SCsl6) are unknown, which might be new ST types for *K. pneumoniae* (Table 1). It is worth noting that the string test positive and *mcr-1.1* gene-carrying strain SCsl1 was identified as ST107.

A minimal spanning tree was constructed based on the seven allele numbers for each isolate (Fig. 1). These sequences were grouped into four clusters. Cluster A included eight strains, belonging to MLST type 15, 11, and an unknown ST; Cluster B included four strains of ST462 and ST534; Cluster C included two isolates, which belonged to two unknown STs. The SCsl1 strain was found distantly related to the other strains and was classified into a single cluster D (Fig. 1).

3.3. Resistance genes and virulence factors of K. pneumoniae strains

Resistance genes found in each tested strains are listed in Table 1. The colistin resistance gene *mcr-1.1* was identified in seven *K. pneumoniae* strains isolated from pig liver or blood samples. These *mcr*-positive *K. pneumoniae* strains belong to ST15, ST107, ST462 and two unknown MLST types. Thirteen genes associated with aminoglycoside resistance (i.e., *aadA1*, *aadA2, aadA16*, *aph(6)-Id*, *aph(4)-Ia*, *aph(3″)-Ib*, *aph(3′)-Ia*, *aph(3′)-IIa*, *ant(2″)-Ia*, *aac(6′)-Ib-cr*, *aac(3)-IId*, *aac(3)-Iva*, and *armA*) were detected in 15 *K. pneumoniae* isolates, which could be responsible for resistance phenotypes of strains resistant to gentamicin and amikacin. The 15 isolates harbored at least one genes associated with β-lactam resistance (e.g., bla_{SHV-26}, bla_{SHV-28}, bla_{SHV-13}, $bla_{SHV-182}$, bla_{SHV-67}, bla_{SHV-2}, bla_{DHA-1}, bla_{TEM-1A}, bla_{TEM-1B}, $bla_{TEM-116}$, $bla_{CTX-M-3}$, $bla_{CTX-M-14}$, $bla_{CTX-M55}$, or bla_{OXA-1}). These genes might be the reason that fourteen strains, twelve strains, and eight strains were resistant to amoxicillin/clavulanic acid, ceftizoxime, and aztreonam, respectively. The *fosA* and *tet* genes are associated with fosfomycin/tetracycline resistance and were detected in 15 *K. pneumoniae* isolates. Specifically, the SCsl1 strain contained 16 resistance genes, including *mcr-1.1*, *aadA1*, *aph(6)-Id*, *aph(4)-Ia*, *aadA2*, bla_{SHV-26}, *qnrB4*, *oqxA*, *oqxB*, *fosA*, *cmlA1*, *sul1*, *sul3*, *sul2*, *tet*(A) and *dfrA12*, but no carbapenemase-encoding genes were identified.

Virulence-associated genes were identified in the 15 strains of *K. pneumoniae* (Table 1). Gene clusters (*mrk*A, *mrk*B, *mrk*C and *mrk*D) encoding type 3 fimbriae were identified in all of the *K. pneumoniae* isolates. Seven *K. pneumoniae* isolates had iron ABC transporter genes (*kfu*A, *kfu*B and *kfu*C). The SCsl1 strain carried 16 virulence genes, including aerobactin-related genes (*iutA*, *iuc*A, *iuc*B, *iuc*C, *iuc*D), *kfu*A, *kfu*B, *kfu*C, *mrk*A, *mrk*B, *mrk*C, *mrk*D, *mrk*F, *mrk*H, *mrk*I and *mrk*J.

3.4. Integrative and conjugative element in K. pneumoniae SCsl1

A 70.2-kb DNA fragment was identified in the genome of the *K. pneumoniae* strain SCsl1 (Fig. 3). This fragment can be divided into four functional regions: integration and excision, conjugation, regulation modules and the yersiniabactin core region. Since it contains a typical ICE structure and thus this element was designated as ICE*KpSL1*. The ICE*KpSL1* was inserted at the 3′ -end of a tRNA gene (asn-tRNA) and was flanked by a 17-bp direct repeats (CCAGTCAGAGGAGCCAA) (Fig. 2). Of note, the ICE*KpSL1* contains a *Yersinia* high-pathogenicity island but has no other virulence genes and resistance genes (Fig. 2).

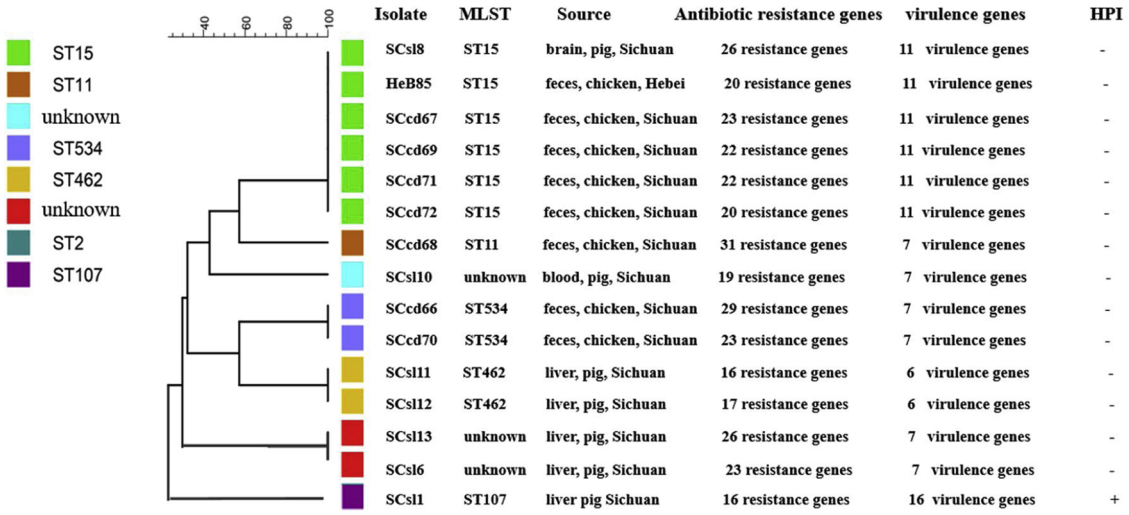
ICE*KpSL1* has an average GC content of 53% with 53 predicted ORFs. A prophage CP4-57 integrase-encoding gene *int*A was identified in ICE*KpSL1*, and a putative helicase gene (ORF17) *traC* was found in this segment. ORF19 to ORF29 (*virB1-virB11*) were all oriented in the same direction, and encode a conjugation transfer associated type IV secretion system. MobB (ORF33) is associated with mobilization of conjugative plasmids. ORF49 encodes PmrD, which is responsible for activation of PhoP and PmrA. ORF52 encodes *MdtK*, which belongs to the Multi Antimicrobial Extrusion family of efflux pumps. This pump is responsible for resistance to quinolones, tetracyclines, and chloramphenicol in various MDR isolates (Ferreira et al., 2018).

Importantly, 11 ORFs larger than 150 nucleotides were identified in the ICE*KpSL1* (ORF2-ORF12), with 99% identity to the HPI of ICE*EcoUMN026-1* (Fig. 3). The genes *ybtS*, *ybtT*, *ybtE*, *ybtU*, *irp1*, and *irp2* are responsible for yersinabactin synthesis, and *ybtP*, *ybtQ* are involved in iron transport. Expression of the cluster is transcriptionally regulated by the product of *ybtA*. The function of *ybtX* has yet to be elucidated (Petermann, 2008).

A 17-bp direct repeat sequence (CCAGTCAGAGGAGCCAA) was identified at both ends of ICE*KpSL1*. Inverse PCR and sequencing confirmed that ICE*KpSL1* could be precisely excised and circularized from the chromosome of *K. pneumoniae,* which may help the transmission of the *Yersinia* pathogenicity island.

4. Discussion

In this study, we isolated 15 multidrug-resistant *K. pneumoniae* from pigs and chickens. Most multidrug-resistant *K. pneumoniae* isolates are resistant to β-lactam, quinolone and aminoglycoside. The findings here are consistent with previous reports (Bialek-Davenet et al., 2014; Woodford et al., 2011). Seven strains were found to be resistant to colistin, which might be related to the widespread use of this antibiotic in China (Apostolakos and Piccirillo, 2018). Furthermore, one *K. pneumoniae* strain (SCsl1) isolated from the liver of a sick pig not only showed resistance to multiple clinical important antibiotics including the last-resort antibiotic colistin, but was also hypervirulent. The



Isolate	MLST	Source	Antibiotic resistance genes	virulence genes	HPI
SCsl8	ST15	brain, pig, Sichuan	26 resistance genes	11 virulence genes	-
HeB85	ST15	feces, chicken, Hebei	20 resistance genes	11 virulence genes	-
SCcd67	ST15	feces, chicken, Sichuan	23 resistance genes	11 virulence genes	-
SCcd69	ST15	feces, chicken, Sichuan	22 resistance genes	11 virulence genes	-
SCcd71	ST15	feces, chicken, Sichuan	22 resistance genes	11 virulence genes	-
SCcd72	ST15	feces, chicken, Sichuan	20 resistance genes	11 virulence genes	-
SCcd68	ST11	feces, chicken, Sichuan	31 resistance genes	7 virulence genes	-
SCsl10	unknown	blood, pig, Sichuan	19 resistance genes	7 virulence genes	-
SCcd66	ST534	feces, chicken, Sichuan	29 resistance genes	7 virulence genes	-
SCcd70	ST534	feces, chicken, Sichuan	23 resistance genes	7 virulence genes	-
SCsl11	ST462	liver, pig, Sichuan	16 resistance genes	6 virulence genes	-
SCsl12	ST462	liver, pig, Sichuan	17 resistance genes	6 virulence genes	-
SCsl13	unknown	liver, pig, Sichuan	26 resistance genes	7 virulence genes	-
SCsl6	unknown	liver, pig, Sichuan	23 resistance genes	7 virulence genes	-
SCsl1	ST107	liver pig Sichuan	16 resistance genes	16 virulence genes	+

Fig. 1. Dendrogram tree showing relationship of 15 *K. pneumoniae* isolates from china. The columns from left to right include isolate number, MLST-based housekeeping genes, source of isolates, data of antibiotic resistance genes and virulence genes, detection results of HPI.

emergence of multidrug-resistant and hypervirulent *K. pneumoniae* isolates of animal origin is concerning.

K. pneumoniae ST107 that harbors a carbapenem resistance genes (bla_{NDM-9}) was recovered from a urine culture of a child with acute lymphocytic leukaemia in April 2013 in Beijing (China) (Wang et al., 2014). And a high biofilm- and bla_{KPC-2}-producing *Klebsiella pneumoniae* ST107 strain from hospital was also reported in March 2018 in Sichuan (China) (Fu et al., 2018;). Recent studies also found that multidrug-resistant and hypervirulent *K. pneumoniae* was associated with a few STs, such as ST660, ST412 and ST700 (Lu et al., 2018). In our study, the multidrug-resistant and hypervirulent ST107 *K. pneumoniae* SCsl1 strain SCsl1 isolated from the liver of a diseased pig was found to harbor 16 antibiotic resistance genes, 16 virulence genes (including aerobactin) and HPI. Importantly, the toxicity of SCsl1 was tested and confirmed with our mouse lethality assay. As far as we know, this is the first report about ST107 multidrug-resistant hvKP from animals, which expands the spectrum of resistant hvKP.

Recent studies also documented that the occurrence of antibiotic resistance genes in hvKP posing a direct threat to the public health. In one case, bla_{KPC-2} was detected in a carbapenem-resistant clinical hvKP strain (Wei et al., 2016). In another case, three aminoglycoside resistance determinants (ARDs) and four common quinolone resistance determinants (QRDs) were found in a novel clinical *K. pneumoniae* ST1137 (Liu et al., 2014). While these previous studies focused on investigating clinical hvKP strains of human sources, our work was designed to characterize the multidrug-resistant hvKP of pig origin. Surprisingly, the hvKP isolates were resistant to β-lactams and colistin. Considering that colistin is a last-resort antibiotic for the treatment of infections caused by Gram-negative bacteria (Biswas et al., 2012), the emergence of *mcr-1* in hvKP isolates will limit available therapeutic options.

Whole genome sequencing and analysis revealed that the 15 strains of *K. pneumoniae* not only encode multiple resistance genes, but also encode different types and numbers of virulence genes. The main virulence factors included type 3 fimbrial gene cluster (*mrkABCDFHIJ*) (Korhonen, 1998). *KfuABC* was found to be involved in iron acquisition and associated with invasive infection and increased virulence in mice (Ma et al., 2005). The identified virulence factors included a major siderophore aerobactin (*iucABCD*, *iutA*), which is expressed by hvKP strains andcan potentiate invasive infection. *Yersinia* HPI mediates iron acquisition and is tightly linked to the ability of bacteria to cause invasive disease(Holden and Bachman, 2015).

In this study, the *Yersinia* HPI was encoded in a new ICE variant termed ICE*KpSL1*, belonging to the ICE*Kp1* family. A prophage encoding-integrase mediated its integration into Asn-tRNA, a known "hotspot" for the integration of ICE*Kp1* family. Inverse PCR confirmed the circular form of ICE*KpSL1*. Other ICE in the ICE*Kp1* family can form cyclization structures, such as ICE*Ec1* and ICE*Kp1* (Lin et al., 2008; Putze et al., 2009). ICE*KpSL1* also has *VirB1-11* coding genes, indicating potential conjugal transfer ability. Further work is needed to identify the reservoirs of high pathogenicity elements, and decipher the mechanisms of transferring that contribute to the emergence of highly virulent *Klebsiella* strains.

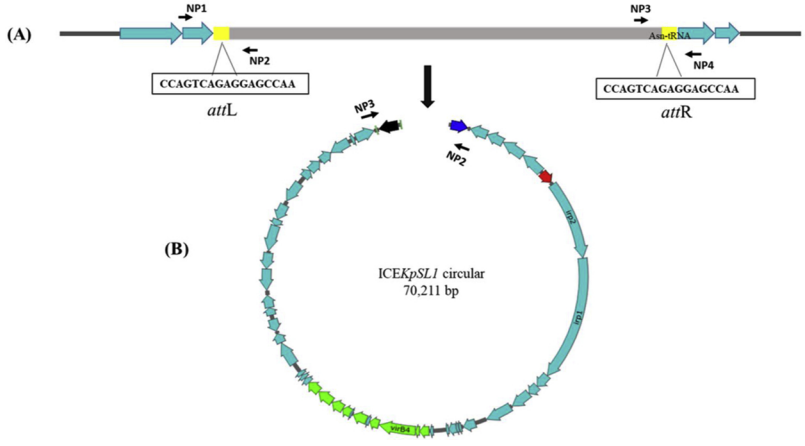


Fig. 2. (A). The integration site and cyclization structure of the ICE*KpSL1*. Gray and orange fragments represent ICE*KpSL1* and the asn-tRNA gene, respectively. The surrounding genes are colored blue. The site-specific integration of ICE*KpSL1* into the asn-tRNA gene is clearly visible. The 17-bp *att*L and *att*R sequences of ICE*KpSL1* and primers NP1, NP2, NP3, NP4, are also shown. (B). Schematic diagram of the circular form of ICE*KpSL1* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

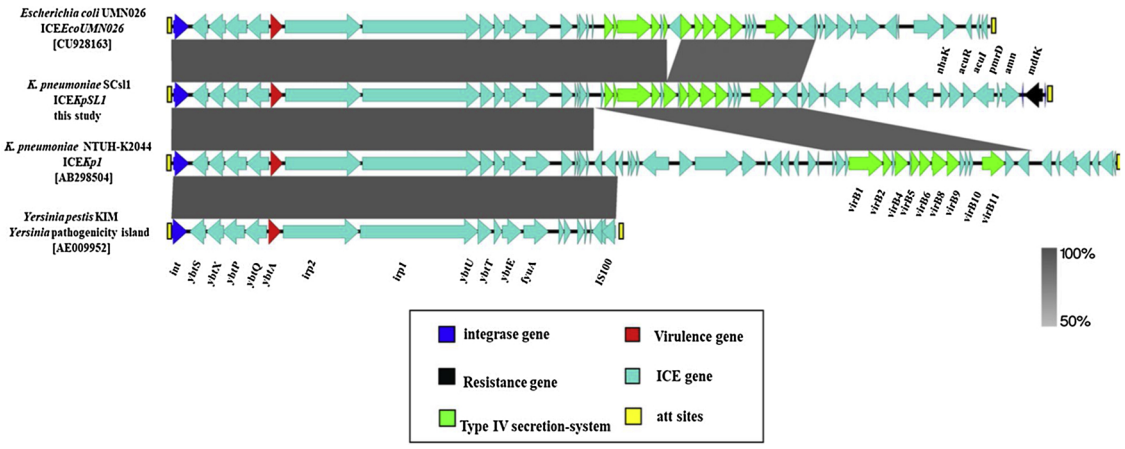


Fig. 3. Structural comparison of the ICE*KpSL1* with other ICEs and *Yersinia* pathogenicity island. The 70,211-bp ICE*KpSL1* element from *Klebsiella pneumoniae* SLZN1. The 65732-bp ICE*EcoUMN026-1* element from *Escherichia coli* UMN026, 35667-bp *Yersinia* pathogenicity island from *Yersinia pestis* KIM and 75935-bp ICE*Kp1* element from *Klebsiella pneumoniae* NTUH-K2044. Homologous regions are shaded in gray.

5. Conclusion

We characterized the antibiotic resistance and virulence profiles of 15 multidrug-resistant *K. pneumoniae* isolated from pigs and chickens in China. Seven *K. pneumoniae* isolates were found carrying the *mcr-1.1* gene. Of note, a multidrug-resistant and hypervirulent *K. pneumoniae* strain SCsl1 was found, which contains 16 resistant genes (including *mcr-1.1*), 16 virulence genes and the *Yersinia* HPI. This is the first report of the presence of ST107 multidrug-resistant hvKP strain from animals. Moreover, a novel ICE variant termed ICE*KpSL1* carrying the *Yersinia* HPI was also detected, which likely play an important role in the transmission of *Yersinia* HPI. More attention should be paid to the emergence of multidrug-resistant and hypervirulent *K. pneumoniae* isolates in livestock.

Nucleotide sequence accession numbers

The nucleotide sequence of ICE*KpSL1* has been deposited in the GenBank sequence database under the accession number MK257718.

Declaration of Competing Interest

None.

Acknowledgments

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