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ORIGINAL RESEARCH

Characterization of Silver Resistance and Coexistence of *sil* Operon with Antibiotic Resistance Genes Among Gram-Negative Pathogens Isolated from Wound Samples by Using Whole-Genome Sequencing

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Purpose: Due to the extensive consumption of silver-containing compound, silver resistance spreads among gram-negative pathogens and is regarded as a great public problem. In this study, we investigated silver resistance mechanisms and antibiotic resistance genes co-harbored with *sil* operon among gram-negative pathogens isolated from wound samples.

Methods: A total of 193 strains of gram-negative pathogens were collected from wound samples between 2018 and 2020 in Xiangya hospital. Silver resistance was obtained by broth microdilution method. The silver resistance mechanisms and the prevalence, genetic environments, and coexistence with antibiotic resistance genes of *sil* operon were investigated by polymerase chain reaction (PCR) and whole genome sequencing (WGS).

Results: Among 193 strains, nine strains (4.7%) were resistant to Ag^+ and assigned to the following species: *Klebsiella pneumoniae* (n = 5) and *Enterobacter hormaechei* (n = 4). WGS confirmed that 24 strains carried the entire *sil* operon, including the four Ag^+-resistant *E. hormaechei* and 20 Ag^+-susceptible strains, while PCR failed to detect some *sil* genes, especially *silE*, due to sequence variations. In seven strains, Tn7 transposon was identified in the upstream of *sil* operon. Spontaneous mutants resistant to Ag^+ were induced in 15 out of 20 Ag^+-susceptible strains, including *K. pneumoniae* strains belonged to high-risk groups (ST11 and ST15). The *sil*-positive strains harbored various antibiotic resistance genes, including bla_{ESBL} and bla_{AmpC}. WGS revealed that a single mutation in *cusS* gene and loss of major porins conferred silver resistance in the five *K. pneumoniae* strains.

Conclusion: Our findings emphasize the cryptic silver resistance is prevalent among Enterobacteriaceae with *sil* operon or with the combination of *cus* operon and major porin loss and increase the understanding of the prevalence of *sil* operon with antibiotic resistance genes, especially bla_{ESBL} and bla_{AmpC}.

Keywords: silver resistance, gram negative pathogen, *sil* operon, antibiotic resistance gene, whole-genome sequencing

Introduction

The silver compounds exhibit excellent bactericidal effect on various pathogens and minimal toxicity towards human cells.[1] Silver targets a variety of bacterial components, ranging from cell wall to nucleic acid. Silver ions damage the integrality of cell wall, increasing the membrane permeability, and bind to the protein and enzymes through thiol groups, inhibiting the

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respiratory chain reaction. Furthermore, the silver ions generate reactive oxygen species and interfere with the replication of DNA.[2]

Silver has been employed as antibacterial material for thousands of years.[3] Nowadays, it has been intensively used in hospital and in daily life. However, the large and uncontrolled consumption of silver-containing materials raises the concern about the widely spread of silver resistance.

Both endogenous and exogenous mechanisms confer silver resistance. Endogenous mechanism involves a single mutation in *cusS*, which increases the expression of *cus* operon, and the loss of outer membrane porins. So far endogenous mechanism is only successfully induced in vitro.[4] The *sil* operon is firstly identified in a plasmid pMG101 from a *Salmonella enterica* strain in 1975 and plays an important role in exogenous silver resistance due to horizontal gene transfer.[5] The *sil* operon consists of nine genes, including *silCFBA (ORF105aa) PRSE*, and encodes efflux pumps (SilCBA and SilP), Ag^+ chaperone or binding proteins (SilF and SilE) and is regulated by a two-component regulatory system (genes *silRS*).[6,7] The *sil* operon and *pco* operon often locate on plasmid and compose the copper hemostasis and silver resistance island (CHASRI).[8]

Earlier studies indicate that silver resistance mainly distributes in Enterobacteriaceae. A research involved 752 strains isolated from bloodstream and demonstrated that 13% of *Enterobacter* spp. were phenotypic resistant to Ag^+.[9] Another study reported that 1006 strains of *Staphylococcus aureus* were susceptible to Ag^+ with the minimum inhibition concentration (MIC) values all below or equal to 16 μg/mL and no resistant mutants were selected during a 42-day exposure experiment.[10] But a recent study in 2019 at Egypt reported that both silver resistance and *sil* genes were firstly identified in *S. aureus, Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[11] Unfortunately, data on the occurrence of silver resistance and the prevalence of *sil* operon in China is lacking. In addition, previous studies usually employed polymerase chain reaction (PCR) method for the screening of *sil* and antibiotic resistance genes, whole-genome sequencing (WGS) analysis of the *sil*-positive strains is scarce.

In this study, we screened 193 strains of gram-negative pathogens isolated from wound samples and performed WGS on 26 strains with *sil* operon and/or silver resistance, in order to investigate silver resistant mechanisms and antibiotic resistance genes co-harbored with *sil* operon in Hunan province, China.

Materials and Methods

Bacteria Source

Non-duplicate gram-negative pathogens from wound samples were collected between 2018 and 2020 in Xiangya hospital, a university-affiliated tertiary teaching hospital with a 3000-bed capacity in Changsha, Hunan province, China. All samples were routinely tested in the microbiology laboratory. The strains were identified by Microflex™ MALDI-TOF MS system (Bruker Daltonik, Bremen, Germany). All the strains were stored at −80 °C for further analysis.

Antimicrobial Susceptibility Tests to Silver Nitrate and Antibiotics

MIC to silver nitrate of all strains in our study was determined by broth microdilution method with Mueller-Hinton broth (MHB, Oxoid, unipath, UK) according to the Clinical and Laboratory Standards institute (CLSI) guidelines.[12] Concentrations range between 2 and 512 μg/mL were tested in our study. Bacteria with MIC above or equal to 512 μg/mL were considered as strains resistant to Ag^+.[9,11]

Antibiotic susceptibility data were extracted from the laboratory database. The routine antibiotic susceptibility test was performed by VITEK-2 Compact system (bioMérieux, Marcy L'Etoile, France), followed by the manufacturers' instructions. *Escherichia coli* ATCC25922 and *P. aeruginosa* ATCC27853 were used as quality controls.

Detection of Silver Resistance Genes

Further detection experiments on silver resistance genes were completed by means of PCR. The genomic DNA were extracted by boiling method. The silver resistance genes, including *silS, silR, silE, silA, silB, silCBA, silP* and *silF* were analyzed in our study. The primers used in our study were listed in Supplemental Table S1. PCR products were electrophoresed with 1.2% agarose gel and visualized under a UV transilluminator.

In vitro Selection of Ag^+ Resistance Mutants

All the *silS*-positive strains with MIC value below 512 μg/mL were subjected to in vitro select spontaneous mutants resistant to Ag^+, according to previous work with modification.[13] Briefly, 100 μL of an overnight culture (~ 10^8 cfu/mL) was placed on MH agar supplemented with 128 μg/mL silver nitrate. Each strain was tested on ten agars to detect the mutation at frequencies of one in 10^9 bacteria. The experiments were repeated twice. The full length of *silS* and *silR* genes of the Ag^+ susceptible parent strains and corresponding resistant mutants were amplified, sequenced and compared with using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Relative change in the fitness cost of Ag^+ resistance was determined by growth curves between the susceptible parent strains and the corresponding mutants.[14]

Whole-Genome Sequencing of Strains with *sil* Operon and/or Silver Resistance

A chosen subcollection of strains (n = 26) with *sil* operon and/or silver resistance were analyzed by whole-genome sequencing (WGS). One colony of each strain was cultured in 3 mL LB broth (Oxoid, unipath, UK) at 37 °C for 12 h. The bacterial genomic DNA were extracted by Ezup Column Bacteria Genomic DNA purification Kit (Sangon Biotech, Shanghai, China), according to the manufacturer's recommendation. The quality and quantity of DNA were examined by agarose gel electrophoresis and Qubit fluorometric instrument (Invitrogen, USA). Fragmented DNAs were end repaired, A-tailed, adapter ligated and amplified using a NEB Next® Ultra™ DNA Library Prep Kit for Illumina® (NEB, USA). The libraries were sequenced with an Illumina HiSeq platform. The read quality was assessed using FastQC software (v0.11.2; http://www.bioinformatics.babraham.ac.uk) according to the developers' recommendations. Genome assembly was conducted by SPAdes (v3.5.0) (Bankevich et al, 2012). Draft genomes were annotated by use of the annotation software Prokka (v1.10) and RAST.[15,16] The antibiotics resistance genes and types of plasmids were identified by ResFinder and PlasmidFinder from the Centre for Genomic Epidemiology, respectively.[17,18] In *silico* analyses of ST type for *Klebsiella pneumoniae* strains was performed by MLST 2.0 (Multi-Locus Sequence Typing).[19] Pan-genome dendrograms describing single nucleotide polymorphisms of *K. pneumoniae* and *Enterobacter hormaechei* strains were constructed by Roary: the Pan Genome Pipeline.[20]

The WGS of the strains was deposited at GenBank and the accession numbers were listed in Supplemental Table S2.

Statistical Analysis

Numbers were presented for categorical variables. Pearson chi-square test or Fisher's exact test were employed to compare categorical variables, when appropriate. P values < 0.05 was considered as statistically significant. SPSS (version 22, IBM Corporation, USA) was used for all analysis.

Results

Bacterial Source

A total of 193 strains of gram-negative pathogens were collected during the study period. The strains belonged to *Acinetobacter* (n = 33), *Klebsiella* (n = 33), *Escherichia* (n = 30), *Pseudomonas* (n = 29), *Enterobacter* (n = 29), *Proteus* (n = 18), *Serratia* (n = 6), *Morganella* (n = 6), *Stenotrophomonas* (n = 4), *Citrobacter* (n = 3), *Myroides* (n = 1) and *Alcaligenes* (n = 1) genera.

Susceptibility Profile to Silver Nitrate

Among the 193 tested strains, the MIC range of silver nitrate for all strains was between 4 μg/mL and > 512 μg/mL. The MIC at which 50% and 90% strains were inhibited (MIC_{50} and MIC_{90}) were 16 μg/mL and 32 μg/mL, respectively. In total, nine (4.7%) strains were resistant to silver nitrate with MIC values above 512 μg/mL and were classified to the following species: *K. pneumoniae* (n = 5) and *E. hormaechei* (n = 4).

Screening for Silver Resistance Genes

PCR was employed to screen the prevalences of *sil* genes among 193 strains. The 68 strains of Nonfermenters were negative for all the silver resistance genes tested in our study.

Among the 125 strains of Enterobacteriaceae, PCR revealed that 26 strains carried one or more *sil* genes. Two *Enterobacter* spp. were positive for one (*silF*) or two *sil* genes (*silF* and *silA*). In the rest 24 strains, only ten strains were positive for the eight primers while the other strains mostly lacked *silE* gene. The MIC values of the 24 *sil* positive strains ranged from 8 to > 512 μg/mL. The distribution of *sil* genes and MIC values for the 24 strains were listed in Supplemental Table S2. Notably, none of the five Ag^+-resistant *K. pneumoniae* strains harbored any *sil* genes.

Whole-Genome Sequencing

In our study, a subcollection of strains (n = 26) were subjected to WGS, including two *sil*-negative Ag^+-resistant *K. pneumoniae* strains to reveal the resistant mechanism, and 24 *sil*-positive strains.

Molecular Mechanism of Ag^+ Resistance *K. pneumoniae*

Due to the same ERIC-PCR fingerprint (Supplemental Result Figure S1), two strains (WHC1 and WHC2) were analyzed by WGS to reveal the molecular mechanism of Ag^+ resistance. After assembled by SPAdes (v3.5.0), the sum of the contig length were 5,375,743 and 5,377,294 bp, respectively, and the G + C content were 57.31% for both strains. The Prokka software revealed the genome of WHC1 and WHC2 harbored 5195 and 5200 protein-encoding sequences, respectively. The average gene lengths were 914 bp for both strains.

According to the genes annotated by Prokka software, several heavy metal transport-related genes were identified in *K. pneumoniae* strains WHC1 and WHC2, consisting of cation efflux systems. The *cus* operon, including *cusRSCFBA* genes, was identified in both strains, while the *sil* operon was confirmed absent in both strains. The sequences of *cus* operon were identical to the corresponding region of *K. pneumoniae* strains SMKP03 (GenBank no. AP023148.1), except for gene *cusS*, in which a single mutation was found, resulting in an amino acid change (Pro209Ser). The sequences of *ompK35* and *ompK36* genes were analyzed by WGS and further verified with sanger sequencing and compared with those of *K. pneumoniae* KCTC 2242 (GenBank no. CP002910) and *K. pneumoniae* NTUH-K 2044 (GenBank no. AP006725). For gene *ompK36*, a deletion of a 1 bp (at nucleotide position 46) created a premature stop codon at amino acid position 32, resulting in early termination of translation. For gene *ompK35*, the sequences of WHC1 and WHC2 were the same as those in *K. pneumoniae* KCTC 2242. However, three-point mutations were identified in the promoter region at nucleotide 121, 163 and 453 upstream of the start codon.

The antibiotics resistance genes for two strains included *oqxAB, qnrS1, aac(6')*-Ib-cr, *fosA, tet(A), aadA16, dfrA27, sul1, sul2, mph(A)*, ARR-3, *aph(3')*-Ia, *aph(6)*-Id, *aac(3)*-IId, *aph(3")*-Ib, *floR,* bla_{SHV-27}, $bla_{CTX-M-3}$, bla_{TEM-1B} and bla_{NDM-1}. The strains belonged to ST967 and harbored IncFIB, IncFII, IncQ1 and IncX3 type plasmids.

WGS Results for *sil*-Positive Strains

WGS was performed on 24 strains with at least five *sil* genes according to PCR results, including four Ag^+-resistant strains and 20 Ag^+-susceptible strains. The strains belonged to *Klebsiella* spp. (n = 13), *Enterobacter* spp. (n = 9), *Escherichia* spp. (n = 1) and *Citrobacter* spp. (n =1). WGS revealed that the 24 strains carried the entire *sil* operon, ie, *silESRCFBAP*. Furthermore, 17 strains carried the *cus* operon, while the *pco* operon was detected alongside with *sil* operon in 23 strains and together formed a copper homeostasis and silver resistance island (CHASRI). In strain WHC182, only *pcoE* was detected with WGS and PCR also failed to detect *pcoD* and *pcoR* (data not shown). The sequence of *silS, silR* and *silE* were compared with the corresponding region of pMG101 (GenBank no. AF067954) (Table 1). The *silR* genes showed sequence variations between 7.42% and 1.31% at the nucleotide level. For *silE* gene, six strains showed 100% overall identities (Ident) to the *silE* gene in pMG101 while the rest strains showed a variation up to 9.49% at the nucleotide level. For *silS* gene, in strain WHC21, an IS5-like element was inserted into the *silS* gene and the full length for of *silS* gene amplified by PCR was 2548 bp, with a query coverage (QC) of 58% with *silS* gene in plasmid pMG101. The *silS* genes in other strains also show a variation between 5.89% and 1.10%.

As for the antibiotic resistance genes, the most prevalent gene was *fosA* (n =22). Various β-lactamase encoding genes were detected, including bla_{SHV} (n = 15), bla_{TEM-1B} (n = 13), bla_{CTX-M} (n = 10), bla_{ACT} (n = 7), bla_{OXA} (n = 6), bla_{DHA} (n =6), bla_{LAP} (n = 4), bla_{SFO} (n = 2), bla_{MIR} (n = 1), bla_{CMY} (n = 1), bla_{LEN} (n = 1) and bla_{OKP} (n = 1). Ten bla_{CTX-M} genes were classified as $bla_{CTX-M-15}$ (n = 5), $bla_{CTX-M-3}$ (n = 2), $bla_{CTX-M-65}$ (n = 1), $bla_{CTX-M-27}$ (n = 1) and $bla_{CTX-M-14}$

Table 1 Overall Identities of *sil* Genes Compared with pMG101

Strains	*silS*	*silR*	*silE*
WHC9	95.05%	98.69%	91.45%
WHC10	98.90%	98.40%	100.00%
WHC12	95.05%	98.69%	91.45%
WHC16	95.05%	98.69%	91.45%
WHC21	94.72%	93.01%	91.92%
WHC31	94.48%	94.03%	96.06%
WHC41	95.05%	98.69%	91.52%
WHC49	94.11%	92.58%	90.51%
WHC57	95.09%	98.54%	91.45%
WHC61	97.72%	98.54%	100.00%
WHC65	94.91%	92.87%	99.77%
WHC135	95.76%	92.72%	100.00%
WHC151	94.11%	92.58%	90.51%
WHC152	94.48%	94.03%	96.06%
WHC154	95.83%	98.11%	99.07%
WHC160	94.98%	94.03%	96.06%
WHC161	94.11%	92.58%	90.51%
WHC167	94.85%	92.87%	100.00%
WHC171	94.91%	92.87%	100.00%
WHC179	95.05%	98.69%	91.45%
WHC181	95.85%	98.11%	99.07%
WHC182	94.18%	93.16%	91.92%
WHC190	95.86%	92.87%	100.00%
WHC192	94.11%	92.58%	90.51%

(n = 1). Two carbapenem-resistance genes were identified as bla_{KPC} (n = 1) and bla_{NDM} (n = 2). The rates for *qnr-, sul-, dfr-, tet-, oqx-, aad-, mph-*, ARR- and *flo*-genes were 66.7% (n = 16), 62.5% (n = 15), 58.3% (n = 14), 58.3% (n = 14), 50.0% (n = 12), 45.8% (n = 11), 37.5% (n = 9), 33.3% (n = 8) and 29.2% (n = 7) (Table 2).

In four strains, no plasmid hit was found with PlasmidFinder. For other strains, various plasmid types were found, including IncFIB(K), IncFII(K), IncM2, IncQ1, IncHI2A, IncX3, IncR, IncI1-I, IncN, IncU, repB and Col. Only four strains carried IncHI type plasmids (Table 2).

According to the assembled sequences and annotations, the genetic environments of *sil* operon were also analyzed. Tn7 transposon was identified in the upstream of *sil* operon in seven strains, including the four strains in which no plasmid hit was found (ie, WHC61, 65, 167 and 171, Table 2). In five strains (ie, WHC9, 12, 16, 57 and 179), two mobile elements were identified in the upstream of *sil* operon and the assembled contigs showed an Iden of 100% with a QC of 100% with the corresponding region in *K. pneumoniae* strain C2972 plasmid pC2972-2 (GenBank no. CP039803), except for WHC179 (Iden 92% and QC 99.82%). In strain WHC181, a contig was assembled and carried *tra* and *sil*

Table 2 Antibiotic Resistance Genes and Plasmid Inc Type Among Gram-Negative Pathogens Harboring *sil* Operon

Isolate	Antibiotic Resistance Genes	Plasmid Inc Types
WHC9	*oqxA, oqxB, qnrS1, aac(6')*-Ib-cr, *fosA, tet(A), aph(3')*-Ia, *aadA2, aadA16, aac(3)*-IId, *mph(A), sul1, sul2, drfA12, drfA27*, ARR-3, *catB3, floR, bla*$_{SHV-28}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$	IncFIB(K), IncFII(K), IncM2, IncQ1
WHC10	*qnrB4, aph(3'')*-Ib, *aac(6')*-Ic, *fosA, dfrA19, sul1, bla*$_{DHA-1}$, *bla*$_{CTX-M-14}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1B}$, *bla*$_{ACT-16}$	IncHI2A
WHC12	*oqxA, oqxB, qnrS1, aac(6')*-Ib-cr, *aph(6)*-Id, *aac(3)*-IId, *aph(3')*-Ia, *aadA2, aadA16, tet(A), mph(A), dfrA12, dfrA27, sul1, sul2, catB3, floR*, ARR-3, *bla*$_{SHV-28}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *fosA*	IncFIB(K), IncFII(K), IncM2, IncQ1
WHC16	*oqxA, oqxB, qnrS1, aac(6')*-Ib-cr, *aph(6)*-Id, *aac(3)*-IId, *aadA16, aph(3')*-Ia, *aadA2, tet(A), mph(A), dfrA12, sul1, sul2, catB3, floR*, ARR-3, *bla*$_{SHV-28}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *fosA*	IncFIB(K), IncFII(K), IncM2, IncQ1
WHC21	*oqxA, oqxB, fosA, bla*$_{SHV-33}$	IncFIB(K)
WHC31	*oqxA, oqxB, qnrS1, aac(6')*-Ib-cr, *aph(3'')*-Ia, *aadA1, aadA2, dfrA12, dfrA27, sul1, sul3, tet(A), mef(B), mph(A), cmlA1, floR*, ARR-3, *bla*$_{SHV-1}$, *bla*$_{CTX-M-3}$, *bla*$_{TEM-1B}$, *fosA*	IncFIB(K), IncFII(K)
WHC41	*oqxA, oqxB, qnrS1, fosA, sul1, dfrA1, tet(A), bla*$_{OKP-B-45}$, *bla*$_{DHA-1}$	IncFIB(K)
WHC49	*oqxA, oqxB, fosA, bla*$_{SHV-11}$	repB
WHC57	*oqxA, oqxB, aac(6')*-Ib-cr, *fosA, tet(A), aph(3')*-Ia, *aac(3)*-IId, *aadA2, sul1, dfrA12*, *bla*$_{SHV-28}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *mph(A)*	IncFIB, IncFII
WHC61	*formA, fosA, bla*$_{MIR-9}$	No plasmid found
WHC65	*qnrB75, bla*$_{CMY-129}$	No plasmid found
WHC135	*qnrB4, aac(3)*-IId, *aadA2, aac(6')*-Ic, *aph(3')*-Ia, *aac(6')*-Ib-cr, *fosA, mph(A), dfrA12, dfrA19, sul1, catA2*, *bla*$_{NDM-1}$, *bla*$_{ACT-16}$, *bla*$_{SFO-1}$, *bla*$_{DHA-1}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-12}$	IncHI2A, IncX3
WHC151	*qnrS1, fosA, rmtB, aadA2, sul2, dfrA14, tet(A), bla*$_{LAP-2}$, *bla*$_{CTX-M-65}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1B}$, *bla*$_{KPC-2}$	IncFII, IncHI1B, IncR, repB, ColRNAI
WHC152	*qnrB52, aac(6')*-Ib-cr, *aph(3'')*-Ia, *aadA5, aac(3)*-IIa, *aadA16, mph(A), sul1, dfrA17, dfrA14, mdf(A), tet(A), sitABCD*, ARR-3, *bla*$_{TEM-1B}$	IncFIB, IncFII(K), IncI1-I, IncN, Col156
WHC154	*qnrB4, fosA, tet(A), mph(A), sul1, dfrA14, sul2, aph(3'')*-Ib, *aac(6')*-Ic, *aac(6')*-Ib-cr, *aac(3)*-IId, *catA2*, *bla*$_{TEM-1B}$, *bla*$_{ACT-16}$, *bla*$_{SFO-1}$, *bla*$_{DHA-1}$, *bla*$_{NDM-1}$, *bla*$_{SHV-12}$	IncFIB, IncHI2A, IncX3
WHC160	*qnrB4, oqxA, oqxB, qnrS1, fosA*, ARR-2, *aadA1, dfrA12, dfrA14, aph(6)*-Id, *aac(3)*-IId, *tet(A), floR, cmlA1*, *bla*$_{TEM-1B}$, *bla*$_{SHV-27}$, *bla*$_{DHA-1}$, *bla*$_{OXA-10}$, *bla*$_{LAP-2}$	IncFIB(K), IncFII(K)
WHC161	*oqxA, oqxB, fosA, bla*$_{SHV-11}$	IncHI1B, repB
WHC167	*qnrS1, fosA, aph(6)*-Id, *aph(3'')*-Ib, *sul2, tet(A), floR, bla*$_{CMH-3}$, *bla*$_{LAP-2}$	No plasmid found
WHC171	*fosA, bla*$_{ACT-56}$	No plasmid found
WHC179	*qnrS1, oqxA, oqxB, qnrB1, aac(6')*-Ib-cr, *fosA, sul1, dfrA27, dfrA14, tet(A), mph(A), aadA16, aph(3'')*-Ib, *aac(3)*-IId, *aph(6)*-Id, *aph(3')*-Ia, ARR-3, *bla*$_{LEN12}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	IncFIB, IncFII(K), IncQ1
WHC181	*qnrS1, fosA, aph(3'')*-Ib, *aph(6)*-Id, *dfrA14, sul2, tet(A), floR, bla*$_{ACT-16}$, *bla*$_{LAP-2}$	IncFIB
WHC182	*fosA, bla*$_{ACT-16}$	IncR, Col
WHC190	*qnrB4, aac(6')*-Ib-cr, *qnrS1, fosA, tet(A), dfrA19, sul1, aph(3'')*-Ib, *aadA2b, aac(6')*-Ic, *aph(6)*-Id, ARR-3, *bla*$_{CTX-M-3}$, *bla*$_{DHA-1}$, *bla*$_{ACT-16}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-12}$	IncHI2A, IncU, Col
WHC192	*oqxA, oqxB, fosA, bla*$_{SHV-1}$, *bla*$_{CTX-M-27}$	IncFIB, repB

operon. The only similar region was found in *E. hormaechei* strain SH19PTE2 plasmid pYUSHP2-2 (GenBank no. CP073773, BLASTn, NR database, Iden 100% and QC 100%).

The MLST type of *K. pneumoniae* were also analyzed. The 11 strains were assigned to the following ST type: ST15 (n = 4), ST35 (n = 1), ST45 (n = 1), ST39 (n = 1), ST11 (n = 1), ST65 (n = 1), ST3393 (n = 1) and ST412 (n = 1) (Table S2).

Pan-Genome Dendrogram Based on WGS Results

Pan-genome dendrograms of *K. pneumoniae* and *E. hormaechei* strains were constructed by Roary according to the prevalence of different genes categories, including core (genes shared by 99–100% of the strains), soft core (genes shared by 95–99% of the strains), shell (genes shared by 15–95% of the strains) and cloud genes (genes shared by 0–15% of the strains).

In *K. pneumoniae*, 3763 core, 3201 shell and 45 cloud genes were identified. The pan-genome dendrogram revealed that the 13 strains of *K. pneumoniae* were clustered into four groups (Figure 1). Notably, the *sil*-negative Ag^+-resistant strains (WHC1 and 2) were identified in one group with minor genetic variations. Strains WHC9, 12 and 16 also showed high genetic similarity.

In *E. hormaechei*, 2928 core, 2075 shell and 51 cloud genes were identified. The pan-genome dendrogram revealed that the 7 strains of *E. hormaechei* were clustered into three groups (Figure 2). The three Ag^+-susceptible strains (WHC181, 171 and 182) were identified into one group, while four resistant strains were clustered into two groups with different genetic similarities.

Co-Resistance Between Silver and Antibiotics

The antibiotic susceptibility profiles, silver resistance and the prevalence of *silS* gene among the Enterobacteriaceae were also analyzed. The results were listed in Table 3. For the nine Ag^+ resistant Enterobacteriaceae, eight strains were carbapenem resistant and were highly resistant to the tested antibiotics compared with Ag^+ susceptible strains, except for amikacin (0.0% vs 4.3%, P = 1.000). Considering the relationship between *silS* gene and antibiotics resistance, strains which harbored *silS* gene were more resistant to ceftriaxone (75.0% vs 45.5%, P = 0.012), ceftazidime (58.3% vs 25.7%, P = 0.003) and aztreonam (58.3% vs 28.7%, P = 0.009) than the *silS*-negative strains. No connection between the resistance of other antibiotics and the prevalence of *silS* was revealed in our study.

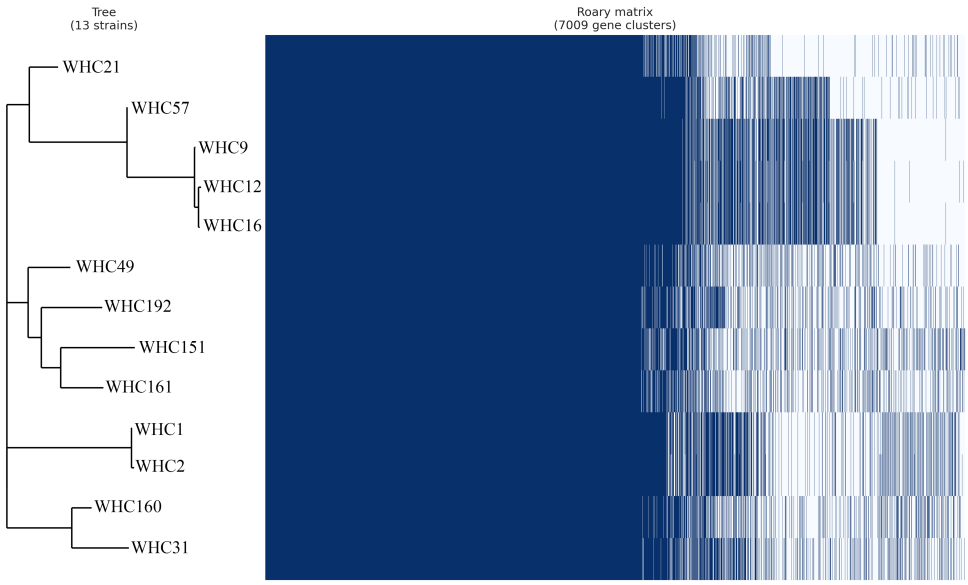

Figure 1 Phylogeny of *K. pneumoniae* isolated based on differences in SNPs.

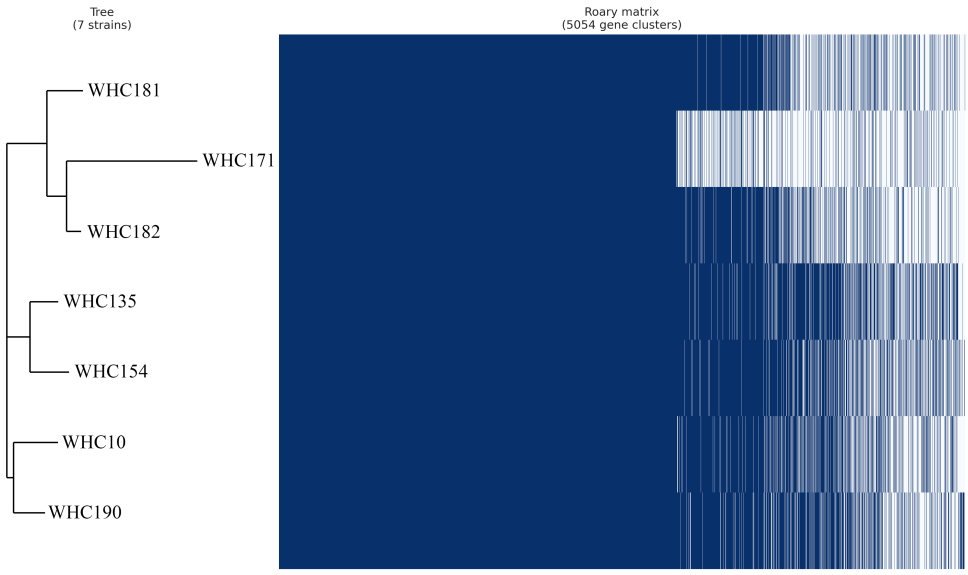

Figure 2 Phylogeny of *E. hormaechei* isolated based on differences in SNPs.

Spontaneous Mutation Resistance to Silver Nitrate

The spontaneous mutation frequency was also determined among the 20 *silS* positive strains. Fifteen strains showed selected mutants which exhibited silver resistance (MIC > 512μg/mL) and belonged to *Klebsiella* spp. (n = 10), *Enterobacter* spp. (n = 4) and *Escherichia* spp. (n = 1). The frequencies were between 2.2×10^{-7} and 9.8×10^{-9}. The silver resistance was induced in *K. pneumoniae* strains belonged to ST11 and ST15. The complete sequences of *silS* and *silR* genes were sequenced to reveal the amino acid substitution responsible for silver resistance. The results were listed in Table 4. Fourteen strains with selected mutants showed point mutations in different positions in *silS* gene. Only one strain showed a point mutation (Asp187Tyr) in *silR* gene. Growth curves were measured to determine the fitness cost of Ag^+ resistance. The parent strains and the derivative mutants showed similar growth curves, except for strain WHC182 (Figure S2).

Table 3 Antimicrobial Susceptibility Profiles in Enterobacteriaceae (n=125)

	Ag^+-R	**Ag^+-S**	**P**	***silS*-P**	***silS*-N**	**P**
	n=9 (%)	**n=116 (%)**		**n=24 (%)**	**n=101 (%)**	
Ceftriaxone	9 (100)	55 (47.4)	**0.003**	18 (75)	46 (45.5)	**0.012**
Ceftazidime	9 (100)	31 (26.7)	**<0.001**	14 (58.3)	26 (25.7)	**0.003**
Cefepime	9 (100)	19 (16.4)	**<0.001**	9 (37.5)	19 (18.8)	0.059
Aztreonam	9 (100)	34 (29.3)	**<0.001**	14 (58.3)	29 (28.7)	**0.009**
Imipenem	8 (88.9)	9 (7.8)	**<0.001**	4 (16.7)	13 (12.9)	0.740
Meropenem	8 (88.9)	8 (6.9)	**<0.001**	4 (16.7)	12 (11.9)	0.508
Amikacin	0 (0)	5 (4.3)	1.000	1 (4.2)	4 (4)	1.000
Gentamicin	9 (100)	32 (27.6)	**<0.001**	12 (50)	29 (28.7)	0.055
Levofloxacin	7 (77.8)	34 (29.3)	**0.006**	8 (33.3)	33 (32.7)	1.000
Ciprofloxacin	8 (88.9)	45 (38.8)	**0.004**	12 (50)	41 (40.6)	0.492
Trimethoprim/sulfamethoxazole	9 (100)	54 (46.6)	**0.003**	15 (62.5)	48 (47.5)	0.256

Note: Significant differences are indicated in bold font.

Table 4 In vitro Selection of Ag^+ Resistance Mutants of the *silS*-Positive Strains

Isolate	Ag+ MIC Parent Strain (μg/mL)	Ag^+ MIC Mutant (μg/mL)	Mutation Frequency	Amino Acid Substitution in *silS*	Amino Acid Substitution in *silR*
WHC9	32	>512	2.2×10^{-7}	Trp353Arg	No mutation
WHC12	32	>512	6.06×10^{-8}	Ala13Thr	No mutation
WHC16	32	>512	2.86×10^{-8}	Ala13Thr	No mutation
WHC21	32	>512	Failure	/	/
WHC31	16	>512	2.08×10^{-8}	Thr32Ile	No mutation
WHC41	32	>512	8.39×10^{-8}	Asp436Val	No mutation
WHC49	16	>512	1.91×10^{-8}	Phe249Leu	No mutation
WHC57	16	>512	3.02×10^{-8}	Leu322Gln	No mutation
WHC61	16	>512	1.18×10^{-8}	Ser196Ile	No mutation
WHC65	8	>512	Failure	/	/
WHC151	32	>512	3.40×10^{-8}	Gln351Lys	No mutation
WHC152	16	>512	3.00×10^{-8}	Gly210Gln	No mutation
WHC160	32	>512	Failure	/	/
WHC161	32	>512	3.50×10^{-8}	Gly210Gln	No mutation
WHC167	32	>512	Failure	/	/
WHC171	32	>512	7.30×10^{-8}	No mutation	Asp187Tyr
WHC179	32	>512	9.8×10^{-9}	Leu242Gln	No mutation
WHC181	32	>512	3.75×10^{-8}	Ser196Ile	No mutation
WHC182	64	>512	9.28×10^{-8}	Leu212Pro	No mutation
WHC192	32	>512	Failure	/	/

Discussion

In the current study, 193 strains of gram-negative pathogens isolated from wound samples were collected in Xiangya hospital in Hunan province, China. Among the pathogens, *Acinetobacter* spp. were predominant, followed by *Klebsiella* spp., *Escherichia* spp. and *Pseudomonas* spp. The distribution of species was in accordance with studies conducted in St. Louis and Mashhad, which both reported *P. aeruginosa, Acinetobacter* and *Klebsiella* were the leading causes among gram-negative pathogens from burn samples.[21,22] However, another study demonstrated that *K. pneumoniae, P. aeruginosa* and *E. coli* were the leading causes for skin and skin structure infection among gram-negative pathogens in Greece.[23] These differences could be explained by the antibiotics commonly used in the areas where the studies were conducted.

It is not surprising that only nine strains were resistant to Ag^+, while all strains of Nonfermenters were susceptible to Ag^+. Previous studies confirmed that the silver resistant strains were not prevalent, and all belonged to Enterobacteriaceae.[24,25] However, the MIC results from ours and previous studies were not comparable because the methods chosen to detect silver resistance were variable. The differences include the culture medium (MHB, Luria-Bertani broth without salt or IsoSensitent broth) and the MIC cut-off value chosen.[24,26,27] The culture medium significantly influences the bactericidal potency of Ag^+ due to NaCl or thiol-containing components, which can precipitate or bind to

Ag^+.[28] Moreover, no widely accepted cut-off value for Ag^+ is available to date. It is urgent to standardize the procedure of antimicrobial susceptibility test for Ag^+.

In our study, WGS confirmed that the 24 strains carried the entire *sil* operons. To the best of our knowledge, this is the first time to verify PCR results for *sil* genes with WGS. In earlier studies, the *sil* genes were only detected by PCR and the authors claimed in some strains one or more *sil* genes were missing, mostly *silE* and *silRS* genes.[11,24] Our PCR results also showed the absent of several *sil* genes, mainly *silE*. We further verified primer *silRS* used in Finley et al and only ten *silRS* genes were detectable out of 24 strains (Supplemental Table S2).[24] The low detection rates for PCR method is due to the variation of *sil* genes. The sequences of *silE, silR* and *silS* genes showed variations (0–10%) when compared with that of the corresponding regions in pMG101, which is in consistence with minor variation (up to 4%) found in previous work.[29] Our results emphasize that the primers for *sil* genes should be designed specifically for the highly conserved regions.

Among the 24 stains with *sil* operon, only four *E. hormaechei* strains were phenotypically resistant to Ag^+. Ag^+ susceptible strains with *sil* operon were reported in earlier studies and it is presumed that the *sil* operon is not constitutively expressed due to the regulation mediated by *silRS*. But importantly, the phenotypic resistance was easily induced, as indicated in our study that 15 out of 24 *sil* positive strains showed spontaneous mutants resistant to Ag^+. Previous study demonstrated that spontaneous mutants resistant to Ag^+ is prevalent among Enterobacteriaceae and the phenotypic change is due to the single missense mutations in *silS*, while no differences were found in other *sil* genes.[9,26] In our study, only a single mutation in *silR* gene of strain WHC171 was identified before and after the exposure of Ag^+, indicating that *silR* also involved in the spontaneous development of silver resistance. MLST analysis showed that silver resistance can be induced within *K. pneumoniae* strains belonged to both high-risk group (ST11 and ST15) and minor clone groups, which is in line with previous study.[14]

The most important factor contributing to the dissemination of *sil* operon is Tn7 transposon, which presents in the upstream region of *sil* operon. Tn7 element, encoding *tnsABCDE*, recognizes the attachment site (*attTn7*) though *tnsD*, which locates downstream of *glmS* gene, and can be inserted into chromosomes of all bacteria.[30] In four strains, no plasmid hit was found by PlasmidFinder and their *sil* operon were all flanking with Tn7 element, so the *sil* operon may be transposed into the chromosome.[31] Another important pathway for Tn7 is mediated by *tnsE* and targets mobile plasmids and filamentous bacteriophage, which facilitates the spread of *sil* operon into new host.[30] The *sil* operon is first discovered in plasmid belonged to the Incompatibility group H. The IncH plasmids are large plasmid (> 150 Kb) with various antibiotic resistance genes and are transmissible under the temperature between 22 to 30 °C, contributing to the dissemination of resistance genes in soil or water.[32] In our study, WGS confirmed the plasmid types in isolates with *sil* operon were variable, including IncFIB, IncFII, IncM2, IncQ1, IncHI2A, IncX3, IncR, IncI1-I, IncN, IncU, repB and Col, which is in line with previous study.[11] The transmission of *sil* cassette into different plasmids may be mediated by Tn7 transposon.

Another important route for the spread of *sil* operon is co-selection under the pressure of antibiotics, especially β-lactams.[33,34] Previous works have reported the relationship of *silE* and CTX-M-15 in *E. coli* strains from human and avian.[35] Another study showed higher prevalence of *silC* gene in $bla_{\text{NDM-1}}$-positive Enterobacteriaceae than that in $bla_{\text{CTM-M-15}}$-producing strains and in susceptible strains.[36] In current study, we also disclosed the relationship between *silS* and resistance to ceftriaxone, ceftazidime and aztreonam and detected various β-lactamase encoding genes, including bla_{ESBL} and bla_{ApmC}, in *sil*-positive strains. Furthermore, various plasmids with *sil*-operon were reported. A conjugative plasmid pSTM6-275 was reported to harbor *sil* operon and various antibiotics resistance genes, including bla_{TEM}, *strA, strB, sul3, aadA2, cmlA, aphA2* and *tet(A)*.[37] Similarly, an IncHI2 plasmid with *sil* operon, $bla_{\text{CTX-M-14}}$ and *aac(6')*-Ib-cr was identified from a *E. coli* strain isolated among a pig in Guangzhou province, China.[38] In our study, a contig carrying *tra* operon, a well-known conjugation component, and *sil* operon was assembled in strain WHC181, indicating a putative conjugative plasmid.[39] In BLAST NR database, this contig in WHC181 showed identical to the corresponding region in *E. hormaechei* strain SH19PTE2 plasmid pYUSHP2-2, isolated from pig feces in Shanghai, China in 2021. The geographic distance and the different sources indicated the possible dissemination of *sil* operon through a conjugative plasmid.

As for *sil*-negative *K. pneumoniae* strains, WGS revealed that a single mutation of *cusS* in *cus* operon with loss of outer membrane porins conferred high-level resistance, which is identical to endogenous resistance mechanism elaborated in *E. coli* BW25113 by Randall et al.[4] Silver resistance was also induced in vitro in *K. pneumoniae* K5024 strain, due to a single mutation in *cusS* and a nonsense mutation in *ompK36* gene.[14] However, the authors failed to investigate the sequence of *ompK35*. In our study, we identified three-point mutations in the promoter region of *ompK35*, which was linked to loss of OmpK35.[40] Roary identified the genetic variations between two strains were negligible, which were consistent to ERIC-PCR result, indicating that clone dissemination was possible. To the best of our knowledge, this is the first time to identify silver resistance strains from clinical samples with endogenous resistance mechanism. This finding emphasized that endogenous resistance mechanism also contribute to cryptic silver resistance. Because *cus* operon distributes widely in Enterobacteriaceae, including *E. coli, C. freundii, Shigella sonnei* and *K. pneumoniae*.[41] Major porin loss is also prevalent, especially in multi-drug resistant *K. pneumoniae*. Recently, a global analysis based on WGS results of 2076 *K. pneumoniae* strains reported 29% strains lacked *ompK35* and mainly distributed in *K. pneumoniae* CC258 while 3.7% strains showed mutations in *ompK36*.[42] Another global research among 487 strains of ertapenem-non-susceptible *K. pneumoniae* reported 83.0% strains showed mutations in either or both of *ompK35* and *ompK36* gene.[43] A study in Taiwan revealed 46.4% strains lost OmpK35 and OmpK36 among 347 carbapenem non-susceptible *K. pneumoniae* strains.[44] It is reasonable to believe that silver resistant strains with endogenous mechanism could be induced during Ag^+-based therapeutics.

Taken together, although phenotypic silver resistance is not prevalent in current study, our findings indicate that gram-negative pathogens can develop silver resistance via two different routes: a single mutation in *silS* or *silR* in strains with *sil* operon or a single mutation in *cusS* in strains with *cus* operon and major porins loss. Due to cryptic silver resistance, it is not sufficient to detect phenotypic silver resistance only. The screening at genetic level is essential but the researchers must be aware of the variation of *sil* genes. Moreover, silver or silver-containing materials should be used more discreetly, especially against multi-drug resistant pathogens, to eliminate the possibility to develop silver resistance and to prevent further spread of silver resistance genes.

Conclusion

The silver resistant rate for gram negative pathogen isolated from wound samples in our hospital is low. However, our findings indicate that silver resistance is easily induced in pathogens with two genetic backgrounds: strains with *sil* operon or with the combination of *cus* operon and major porin loss, especially in *Klebsiella* spp., so the screening of silver resistance at genetic level is essential. Furthermore, strains with *sil* operon often harbor various antibiotic resistance genes, including bla_{ESBL} and bla_{ApmC}. Therefore, it is of great significance to restrict the uncontrolled use of silver to prevent the further spread of silver resistance.

Abbreviations

BLAST, the Basic Local Alignment Search Tool; CHASRI, a copper homeostasis and silver resistance island; CLSI, Clinical and Laboratory Standards institute; Iden, overall identity; MIC, minimum inhibition concentration; MLST, multilocus sequence typing; PCR, polymerase chain reaction; QC, query coverage; WGS, whole genome sequencing.

Ethics Statement and Informed Consent

This research was conducted according to the recommendations of the Ethics Committee of Central South University (Changsha, Hunan Province, China). Ethical review and approval were waived for this study since the human samples were routinely collected and patients' data remained anonymous.

Consent for Publication

All authors approved the manuscript and gave their consent for submission and publication.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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