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A fatal case of liver abscess caused by hypervirulent *Klebsiella pneumoniae* in a diabetic adolescent: A clinical and laboratory study

Yue Li[1*] | Zheng Li[2*] | Suyun Qian[2] | Fang Dong[3] | Qing Wang[1] | Pengfei Zhang[2] | Kaihu Yao[1]

[1]MOE Key Laboratory of Major Diseases in Children, National Key Discipline of Pediatrics (Capital Medical University), National Clinical Research Center for Respiratory Diseases, Beijing Key Laboratory of Pediatric Respiratory Infection Diseases, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, China
[2]Department of Pediatric Intensive Care Unit, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, China
[3]Department of Clinical Microbiology Laboratory, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, China.

Correspondence
Kaihu Yao, Beijing Children's Hospital, Capital Medical University, Beijing 100045, China
Email: jiuhu2655@sina.com
Suyun Qian, Beijing Children's Hospital, Capital Medical University, Beijing 100045, China
Email: syqian1211@163.com

*These authors contributed equally to this study.

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ABSTRACT

Importance: Hypervirulent variants of *Klebsiella pneumoniae* (hvKp) are capable of causing life-threatening pyogenic liver abscesses (PLAs), but hvKp caused PLAs was seldom reported in pediatric populations. Hence, there is an urgent need to raise our awareness of this phenomenon in pediatric populations.

Objective: This study aimed to report the clinical characteristics of hvKp that caused fatal PLA complicated by bacteremia in an adolescent and further identify the microbiological and genomic features of the causative strain.

Methods: A 14-year-old boy with diabetes mellitus was admitted to our hospital with a diagnosis of PLA complicated by bacteremia. A hypermucoviscous hvKp strain, KPN_19-106, was isolated from the drainage fluid present within the liver abscess cavity and blood. The hypermucoviscosity phenotype of the causative strain was determined by string test. Its virulence was measured using serum resistance assay and *Galleria mellonella* larvae-killing assay. Antimicrobial susceptibility was determined by broth microdilution method. Genetic information was obtained by whole-genome sequencing and bioinformatics analysis.

Results: KPN_19-106 belonged to sequence type 380 and serotype K2 and exhibited stronger serum resistance and higher *in vivo* lethality than the well-characterized hvKp NTUH-K2044 strain. Although KPN_19-106 is susceptible to most antibiotics, no sign of improvement was observed during treatment with such drugs. Whole-genome sequencing revealed that the isolate had integrated multiple mobile genetic elements related to virulence.

Interpretation: Antibiotic-susceptible hvKp can cause fatal PLA complicated by bacteremia in adolescents, with no improvement during antimicrobial therapy. The causative strain in this case had integrated multiple virulence genes and thus exhibited higher virulence both *in vitro* and *in vivo* when compared with NTUH-K2044.

KEYWORDS
Hypervirulent, *Klebsiella pneumoniae*, Pyogenic liver abscess, Pediatric populations, Diabetes mellitus

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INTRODUCTION

Within the past 30 years, a new hypervirulent variant of *Klebsiella pneumoniae* (hvKp), which emerged in the Asian Pacific Rim, has spread globally.[1,2] HvKp is capable of infecting healthy individuals and causing pyogenic liver abscesses (PLAs), endophthalmitis, pneumonia, central nervous system infection, and bacteremia.[3] However, only a limited number of cases have been reported in the pediatric populations. Herein, we present a fatal case of PLA complicated by bacteremia caused by an hvKp strain of the sequence type 380 and serotype K2 (ST380/K2) in a 14-year-old boy with type 2 diabetes mellitus (DM). No sign of improvement was observed during treatment with antibiotics to which the causative strain is susceptible. Clinical and microbiological studies revealed that the strain exhibited a strong hypervirulent phenotype, and whole-genome sequencing analysis indicated that the strain had integrated multiple mobile gene elements related to virulence.

To the best of our knowledge, this is the first reported fatal case of PLA caused by hvKp in an adolescent in mainland China. This study aimed to highlight the poor prognosis associated with evolving hvKp strains and raise awareness regarding the clinical identification, therapy, prevention, and surveillance of PLA caused by hvKp in pediatric populations.

METHODS

Ethical approval

This study is approved by the Ethical Committee of Beijing Children's Hospital and written consent were needed (2020-Z-129). Written formed consent was obtained from the patient's guardians.

Bacteria isolates

All the *K. pneumoniae* isolates used in this study were identified by both 16SrDNA sequencing and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Microflex, Bruker Daltonics, Bremen, Germany). NTUH-K2044 is a well-known ST23/K1 type clinical hvKp isolate generally used in hvKp studies.[4] KPN_18-01 is a ST11 type classic *K. pneumonia* (cKp) isolated from midstream urine of a four-month-old boy.

Multilocus sequence typing (MLST)

MLST were performed as the described on Pasteur Institute MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html), including sequencing analysis of seven housekeeping genes.

Wzi sequencing of K-serotype

K-serotype was determined by sequencing analysis of a cps locus conserved gene *wzi*.[5] PCR amplification of *wzi* (580-bp) was achieved using the primers wzi_for (5'-GTGCCGCGAGCGCTTTCTATCTTGGTATTCC-3') and wzi_rev (5'-GAGAGCCACTGGTTCCAGAACTTCACCGC-3'). Sequencing analysis was performed on Pasteur Institute MLST website.

String test

The hypermucoviscous phenotype of *K. pneumoniae* isolate was identified by a positive string test as previously described,[6] which is defined as the formation of a viscous string >5 mm in length when a single colony grown overnight on Columbia blood agar plate at 37°C was stretch by a standard bacteriology inoculation loop.

Antimicrobial susceptibility tests

Minimum inhibitory concentration (MIC) values were determined by broth microdilution method. Methodology and breakpoint interpretations were developed by reference to Clinical and Laboratory Standards Institute (CLSI) guidelines.[7] All the antimicrobial agents were serially diluted two-fold across the wells of 96-well plates (Corning 3370) with concentration ranging from 1024 μg/mL to 0.06 μg/mL. Bacteria were cultured in cation-adjusted Mueller-Hinton broth (CAMHB) to mid-log phase, and then diluted into sterilized saline to get the McFarland turbidity at 0.5, and then 20-fold dilution of 1×10^8 CFU/mL culture were added by 10 μL to each well of the 96-well plates giving a final concentration of 1×10^5 CFU/mL. All the plates were covered and incubated at 37°C for 16–20 h. MICs were determined visually as the lowest concentration showing no visible growth. *Escherichia coli* ATCC 25922 served as a control strain. All the MIC assays were repeated in triplex.

Infection of *G. mellonella* larvae

G. mellonella larvae assay was performed as previously described.[8] Larvea weighing approximately 300 mg (Tianjin Huiyude Biotech Company, Tianjin, China) were selected for experiments. Overnight cultures of *K. pneumoniae* isolates were washed by sterilized normal saline, and were adjusted to 1×10^8 CFU/mL subsequently. After surface disinfection with 75% ethanol, each group of ten larvae were injected with 10 μL bacterial suspension into the last right proleg, and larvae injected with the same volume of sterilized normal saline were used as control. Larvae were kept at 37°C in dark with food, and pigment and time of death were recorded continuously for 72 h. All experiments were performed in triplicate. The hvKp strain K2044 and clinical isolated classical cKp strain KPN_18-01 were used as comparators.

Serum resistance assay

Serum resistance assay was performed as previously described.[9] Pooled normal human serum from healthy

volunteer was stored at −70 °C before use. Bacteria grown to log phase in trypticase soy broth were collected by centrifuge and washed twice by sterilized normal saline. The bacterial counts were adjusted to 1×10^6 CFU/mL. A mixture of 250 μL of bacterial suspension and 750 μL of human serum were incubated at 37 °C for 15 min. The number of bacteria was determined by plate counting. Survival rate was calculated as the number of bacteria incubated with human serum compared to the number of bacteria pretreatment. All the serum resistance assays were repeated in triplex.

Genome sequencing and analysis

The whole genomes of KPN_19-106, NTUH-K2044 and KPN_18-01 were sequenced using Illumina NovaSeq PE150 (Illumina, USA) platforms at the Beijing Novogene Bioinformatics Technology Co., Ltd. All good quality paired reads were *de novo* assembled using the SOAP denovo, SPAdes and ABySS into a number of scaffolds. The whole genome of KPN_19-106, NTUH-K2044 and KPN_18-01 were bacteria draft, in which plasmid sequence was not completely assembled. We use bioinformatics database Virulence Factors of Pathogenic Bacteria (VFDB)[10] and Antibiotic Resistance Genes Database (ARDB)[11] to perform pathogenicity and drug resistance analyses.

RESULTS

Case presentation

A 14-year-old boy with a 6-year history of type 2 diabetes mellitus (DM) and poorly controlled blood sugar level was admitted to our hospital in June 2019 for fever and abdominal pain. He had been initially diagnosed with type 2 DM with low insulin level and insulin resistance in July 2013 at the age of 8-year-old with insulin and C-peptide (C-P) in the normal range (1.53 μIU/mL and 10.13 ng/mL), his diabetes autoantibodies were negative. During the period of hospitalization in June 2019, the patient's insulin and C-P decreased to 0.55 μIU/mL and 0.9 ng/mL, respectively, and his hemoglobin A1c was 11.9%. The patient was not morbidly obese. His mother had been diagnosed with type 2 DM at the age of 45 years, but his father and sister (aged 29 years) was healthy.

Prior to hospitalization, he had experienced a 5-day history of recurrent high fever (with a peak temperature of 39.5 °C) complicated by diarrhea, cough, and vomiting. He had also faced persistent pain throughout the abdomen continually for 4 days before admission. Upon admission, the boy was in a slight coma (Glasgow score of 11 points). His C-reactive protein level was 231 mg/L, and procalcitonin level was >50 ng/mL. His serum aspartate aminotransferase and alanine aminotransferase levels were 154.5 U/L and 160.1 U/L, respectively, and his total bilirubin was 92.22 μmol/L. Computed tomography scan of the abdomen revealed a large abscess in the right liver lobe. Collectively, the clinical findings suggested PLA complicated by bacteremia, which was subsequently confirmed by microbial culture. However, microbial culture of the cerebrospinal fluid was negative. We surmised that the spread of the infection through the circulatory system had led to the observed effects. The patient received empirical treatment of intravenous meropenem (2 g/d) upon arrival, which was increased to 3 g/d from the second day of hospitalization. To enhance the efficacy of antibacterial treatment, intravenous amikacin (3 g/d) was added on the fourth day of hospitalization. However, no sign of improvement was observed during the course of the treatment. On the third day of hospitalization, the patient fell into a deep coma (Glasgow Coma Scale score 3T). After 8 days of treatment, his guardians requested the boy's discharge, and he died soon after discharge.

Microbiological study

Microbial cultures of lumbar puncture, blood, and drainage fluid within the liver abscess were performed. The cerebrospinal fluid was clear, the physiological indexes were normal, and microbial cultures yielded negative results. Four days after admission, a strain of *K. pneumoniae* was identified from the drainage fluid within the liver abscess cavity and was labeled as KPN_19-106. A *K. pneumoniae* strain was also identified in two sets of blood cultures, which is identified as hypermucoviscosity and belonged to ST380/K2.

Analysis of MLST and serotype revealed that KPN_19-106 could be classified as ST380 and serotype/K2. Although the dominant type of hvKp is characterized by sequence type 23 and serotype K1 (ST23/K1),[12] ST380/K2-type hvKp strains have also been reported to cause severe infections in clinical settings.[13] In this case, a single bacterial colony of KPN_19-106 formed a viscous string of >5 mm, which was defined as hypermucoviscosity (Figure 1).

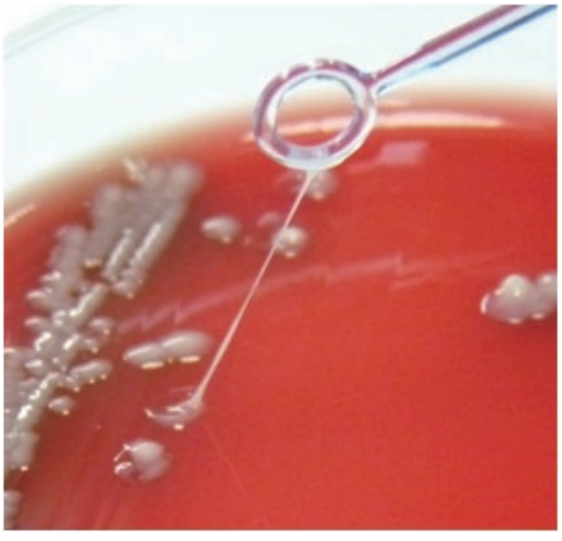

FIGURE 1 Hypermucoviscosity phenotype of KPN_19-106.

Antimicrobial susceptibility of the strain was tested against 11 antibiotics, and the MIC are listed in Table 1. KPN_19-106 was found to be susceptible to most of the tested antibiotics, including meropenem (MIC = 0.5 μg/mL) and amikacin (MIC = 2 μg/mL), which were used for therapy, while the strain appeared to be resistant to ampicillin, trimethoprim–sulfamethoxazole, and azithromycin.

TABLE 1 Antimicrobial susceptibility of the KPN_19-106 strain of hypervirulent variants of *Klebsiella pneumoniae*

Antimicrobial agent	MIC (μg/mL)	Susceptibility
Ampicillin	32	R
Cefuroxime	2	S
Ceftazidime	1	S
Amoxicillin/Clavulanic	2/1	S
Meropenem	0.5	S
Imipenem	0.25	S
Lexvofloxacin	1	S
Trimethoprim/Sulfamethoxazole	≥4/76	R
Gentamicin	2	S
Amikacin	2	S
Azithromycin	32	R

MIC, minimum inhibitory concentration; R, resistant; S, susceptible.

For virulence determination, a well-characterized hvKp strain, NTUH-K2044 (ST23/K1), and a cKp strain, KPN_18-01, served as comparators. As shown in Figure 2A, 82.81% of the cells remained viable after being treated with human serum and the survival rate of KPN_19-106 was significantly higher than that of both NTUH-K2044 (66.55%) and KPN_18-01 (29.16%). The strong serum resistance was considered to be associated with the high capability to evade the immune system and infect the blood. Next, we determined the dynamic process of KPN_19-106-induced lethality using a *Galleria mellonella* infection model. KPN_19-106 caused 100% mortality of the *G. mellonella* larvae within 48 hours (Figure 2B). Moreover, in contrast with NTUH-K2044 and KPN_18-01, KPN_19-106 was able to cause death more rapidly. Both serum resistance assay and *G. mellonella* larvae-killing assay identified the strong hypervirulent phenotype of ST380/K2-type KPN_19-106, and the strain also exhibited higher virulence than the comparator NTUH-K2044 and KPN_18-01.

Whole-genome analysis

KPN_19-106 was found to harbor a 5 501 604 bp genome and was assembled into 30 scaffolds with 5215 genes. The overall bacterial draft genomic information of KPN_19-106 could be located in GenBank (Accession No. SUB6521505). Virulence and antimicrobial-resistance genes were searched throughout the genome of KPN_19-106 in the VFDB and ARDB, respectively. Besides, the genomes of NTUH-K2044 and KPN_18-01 were also searched for in the Virulence Factor Database. Virulence genes found in the genome of KPN_19-106, NTUH-K2044, and KPN_18-01 are listed in Table S1.

A total of 422 virulence genes, both specific and nonspecific for hvKp, were identified in the genome of KPN_19-106. Moreover, at least four iron-acquisition systems were located in the KPN_19-106 genome, including *iutAiucABCD* (aerobactin), *iroBCDEN* (salmochelin), *fyuA-irp1-irp2-ybtSXQPAUET* (yersiniabactin), and *fepABCDG-entABCDFSE* (enterobactin). Aerobactin and salmochelin are known to be encoded by genes located on hvKp-specific, pLVPK-like virulence plasmids,[14,15] while enterobactin and yersiniabactin are produced by both hvKp and cKp strains. Another hvKp-specific virulence gene, *rmpA* (regulator

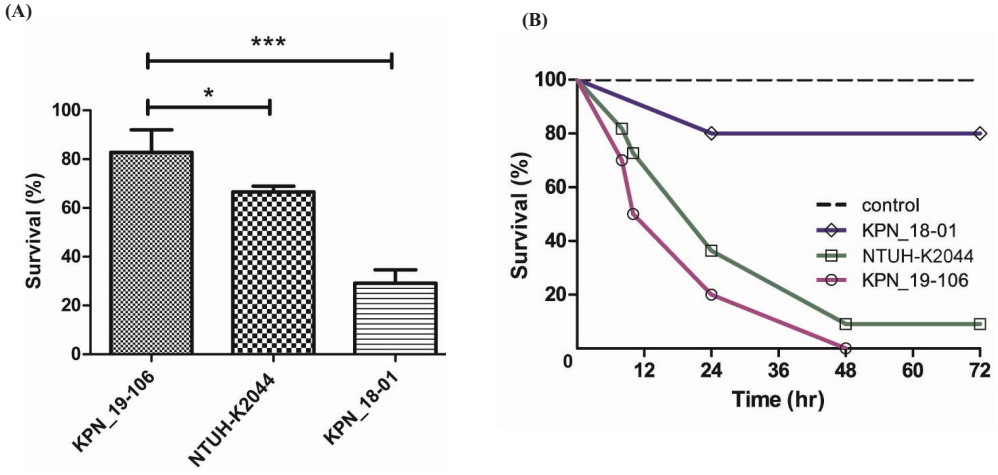

FIGURE 2 Virulence determination of KPN_19-106 as compared with that of both NTUH-K2044 and KPN_18-01. (A) The serum resistance of KPN_19-106, NTUH-K2044, and KPN_18-01 was measured by assessing the relative viability of bacteria after treatment with human serum. $^{*}P < 0.05$, $^{***}P < 0.01$. (B) Survival percentages of *Galleria mellonella* larvae following infection with KPN_19-106, NTUH-K2044, or KPN_18-01 within 72 hours. Larvae injected with sterilized saline served as the control group.

of mucoid phenotype A), which has been confirmed to enhance capsular polysaccharide (CPS) expression and confer hypermucoviscosity was also found.[16] Genes encoding colibactin, which is a genotoxic metabolite, was also identified as a putative virulence factor that accounted for the severity of the reported hvKp infections.[17] Other virulence genes nonspecific for hvKp were also noted. Filamentous appendages are important virulence factors for adhesion, biofilm formation, and conjugation. Additionally, a variety of fimbriae-related genes, such as *fimABCDEFGHIK* (type 1 fimbriae) and *mrkABCDF* (type 3 fimbriae), and pili-encoding genes, such as *pilRTDW* (type IV pili) and *ecpBCDER* (*Escherichia coli* common pilus), were observed in the KPN_19-106 genome. Additional virulence-related genes for the biosynthesis or transport of lipopolysaccharides, capsular polysaccharides, heme, and urease, together with genes related to secretion systems, were found. When compared with the virulence genes identified in the genomes of NTUH-K2044 and KPN_18-01, we found that colibactin is specific for KPN_19-106.

Plasmids harbored by the KPN_19-106 genome were determined based on the scaffolds in bacterial drafts using the PlasmidFinder software program (Center for Genomic Epidemiology, Lyngby, Denmark). PlasmidFinder was able to match the bacterial drafts of KPN_19-106 with plasmid sequences recorded in plasmid banks, as a result, plasmids pRJA116b (harbored by the ST23/K1-type hvKp strain RJA116[18]) and pK2044 (harbored by NTUH-K2044) were identified with 99% and 94% identity to bacterial drafts of KPN_19-10, respectively. Both pRJA116b and pK2044 are pLVPK-like plasmids, and the results highly suggested the existence of a similar virulence plasmid in the KPN_19-106 genome. Besides, another plasmid, pKPN_CZ, was identified with 99.7% identity. Notably, pKPN_CZ was harbored by a multidrug-resistant classic *K. pneumoniae* sequence type 416 strain and showed an increased number of putative virulence clusters.[19]

Genes attributed to Antibiotic ARDB revealed that KPN_19-106 carried the class A betalactamase encoding gene bla_{SHV} and the macrolide-specific efflux system encoding gene macab, which may confer resistance to ampicillin and azithromycin, respectively (Table 1).

DISCUSSION

In the present case, KPN_19-106 caused severe PLA with high fever, abdominal pain, cough, diarrhea, and vomiting in a 14-year-old boy who fell into a coma within 5 days of hospitalization and later died after his guardians opted for his discharge. Although the causative strain was susceptible to meropenem and amikacin, no sign of improvement was observed during antimicrobial treatment. When compared with the previously reported PLA cases,[20,21] the clinical findings and unfavorable outcome of this case suggest the strong hypervirulent phenotype of the causative hvKp strain. The results of the microbiological study correlated well with the clinical presentations, and both the degree of serum resistance and the lethality rate of the causative strain KPN_19-106 (ST380/K2) were higher than that of NTUH-K2044.

Whole-genome bioinformatics analysis identified multiple virulence genes and several antibiotic resistance genes, with colibactin being more specific for KPN_19-106 than for both NTUH-K2044 and KPN_18-01. Colibactin is a bacterial toxin that interferes with the cell cycle by inducing chromosomal instability and DNA damage in eukaryotes, leading to the senescence of epithelial cells and the apoptosis of immune cells. We surmise that KPN_19-106 may act by evading the killing ability of neutrophils and macrophages by producing colibactin and then disseminating through the bloodstream rapidly.

The putative virulence plasmid harbored by KPN_19-106 highly resembled several hvKp-specific pLVPK-like plasmids and also showed close similarity to a virulence plasmid in one classic *K. pneumoniae* strain, suggesting that the virulence plasmids of hvKp are highly variable. Other than plasmids, virulence genes, such as those encoding colibactin and yersiniabactin or linked to secretion system function, were also found located on mobile pathogenic islands.[17,22] We hypothesize that horizontal gene transfer and gene recombination mediated by mobile gene elements, such as plasmids and pathogenic islands, might increase the virulence of hvKp strains.

As it is a newly evolving pathogen, the current lack of detection methods exacerbates the difficulty in identifying hvKp. Molecular detection methods attempt to identify hvKp via the detection of known virulence genes using multiplex polymerase chain reaction assay.[23] However, it has been found that some hvKp strains identified by molecular detection do not exhibit hypervirulent pathogenicity in clinical settings, while some atypical hvKp strains found in clinical settings may not possess known hvKp virulence genes.[24] The complexity and diversity of hvKp strains suggest that molecular detection alone is insufficient for the clinical diagnosis and treatment of hvKp infections. In the present case, the results of serum resistance assay and *G. mellonella* larvae-killing assay might partly indicate the strain's capability to evade the immune system and spread through the circulatory system, and these methods could potentially highlight the variations in the virulence of these strains. These nonmolecular detection methods are more conducive to clinical diagnosis and treatment, and we suggest that they be considered as promising additional methods for hvKp identification. However, there is a long way to go before these laboratory methods become appropriate for large-scale clinical detections.

DM is a strong risk factor for PLA caused by hvKp

infection, and a poorly controlled blood sugar level is thought to increase the metastatic complications.[25] The rise in the prevalence of DM, together with that of obesity, in pediatric populations suggests the potential danger of hvKp infection leading to PLA in youngsters. As compared with adults, adolescent DM patients exhibit poor self-regulation and hence their blood sugar levels are more likely to be erratic. The present case showed that a strong hvKp strain could cause a fatal outcome in an adolescent with a persistent poorly controlled blood sugar level despite them receiving susceptible antibiotic treatment. To date, hvKp infections in pediatric populations have not received enough attention and both pediatricians and parents alike are seldom aware of the high risk and poor prognosis of PLA caused by hvKp infection in pediatric DM patients.

Given the lack of clinical therapeutic guidelines, there is an urgent need to focus on the control and prevention of hvKp infections. Although the initial entry route of these pathogens is unclear, colonic colonization is presumed to be a potentially important ingress method.[26] We hence propose early screening for the detection of hvKp pathogens in fecal samples because a proper prophylaxis may assist in the prevention of PLA caused by hvKp infections.

In conclusion, the present case of PLA caused by hvKp in an adolescent with type 2 DM in mainland China suggests that increased attention in this regard is warranted in clinical practice in the future. The clinical, microbiological, and genomic data of the hvKp strain KPN_19-106 provided herein will be beneficial for further research in this field.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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