Klebsiella pneumoniae:A pathogenic bacteria that may be transmitted through Hirudo nipponia and cause death in humans

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June 1, 2020

Abstract

Hirudo nipponia is not only an important economic pillar for farmers, but is also a precious raw material for medicinal materials. However, in recent years, H. nipponia suffered from diseases with symptoms including systemic edema and hyperemia. It has not yet been demonstrated which pathogen causes this disease and whether this could be transmitted to humans. In this study, Klebsiella pneumoniae was isolated and identified from diseased H. nipponia and the pathogenicity of the isolated strain was confirmed. Furthermore, by comparing the sequence of the pathogen isolated from leeches to the same pathogen infecting humans, we identified that the isolated strain is a threat to human health. This work emphasizes the importance of the first discovery of pathogenic bacteria from leeches similar to human pathogens, as well as the need for identifying comorbidities for both humans and aquatic animals.

Full title:*Klebsiella pneumoniae*:A pathogenic bacteria that may be transmitted through *Hirudo nipponia* and cause death in humans

Short running title:New ways of transmission of pathogenic bacteria

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Abstract: *Hirudo nipponia* is not only an important economic pillar for farmers, but is also a precious raw material for medicinal materials. However, in recent years, *H. nipponia*suffered from diseases with symptoms including systemic edema and hyperemia. It has not yet been demonstrated which pathogen causes this disease and whether this could be transmitted to humans. In this study,*Klebsiella pneumoniae* was isolated and identified from diseased*H. nipponia* and the pathogenicity of the isolated strain was confirmed. Furthermore, by comparing the sequence of the pathogen isolated from leeches to the same pathogen infecting humans, we identified that the isolated strain is a threat to human health. This work emphasizes the importance of the first discovery of pathogenic bacteria from leeches similar to human pathogens, as well as the need for identifying comorbidities for both humans and aquatic animals.

Keywords: *Hirudo nipponia* ;*Klebsiella pneumoniae* ;human disease;pathogenic bacteria

1. Introduction

Hirudo nipponia, commonly known as the leech, belongs to the Hirudinidae and Hirudo families, is widely distributed in Northeast, North and Southwest China (Michalak et al., 2009) and is the main representative species of leeches. Leeches were used to promote blood circulation and eliminate blood stasis. In addition to being an anticoagulant, leeches also showed anti-inflammatory, anti-tumor and anti-fibrosis effects, playing an important role in the prevention and treatment of a variety of diseases(Ahmad, 2006; Rowghani et al., 2007). For example, Hirudin extracted from leech saliva can inhibit blood coagulation and was known as the strongest natural anticoagulant in the world. The effects of Hirudin were stronger than heparin and showed a significant effect on cardiovascular and cerebrovascular diseases(El-Husseiny et al., 2008). There are 10 types of Chinese patent medicines where leeches as the main raw material, that are used to treat many diseases(Mo et al., 2003). In recent years, the living environment of leeches was severely damaged and the number of wild leech population was sharply reduced due to an increase in overhunting(Yu et al., 2020).

To protect wild resources and meet the needs of clinical and scientific research, leeches were artificially bred and cultured. Presently, Russia, the United Kingdom, France and Turkey have large leech breeding industries(Zhang et al., 2010). Leech breeding technology research in China is less prominent, resulting in only a few large-scale breeding enterprises. At the same time, there was low leech reproduction technology, wild leech sources were reduced and the quality of leech seedlings was declining, leading to leech diseases. However, sick leeches may reduce clinical treatment effects and may even bring disease to patients. Yet, there have been few reports on leech disease research in China and abroad. Only some experiments reported diseases related pathogens and conducted drug screenings (Gouda et al., 2006; Hamilton et al., 2005; Zhang et al., 2009; Zhang et al., 2006), but have not evaluated the possibility that leeches may be used as a vector to spread certain pathogens to cause further harm to humans.

From 2016 to 2018, *Hirudo nipponia* died on farms in the Hubei Province in China. These leeches showed reduced food intake, poor swimming ability, edematous bodies and hyperemia. Dissections revealed that *H. nipponia* bodies were full of water and eroded, but a parasite infection was not found. Based on a bacteriological study, a number of bacteria was isolated from diseased*H. nipponia* , which were collected in different batches and times. Many of the dominant bacteria were identified as the same based on their morphology and dominance. Here, the pathogenicity of the bacteria isolated from *H. nipponia* was studied. Our results confirmed that pathogenic bacteria caused the disease in *H. nipponia* and also caused diseases in humans. This provided important methods for the diagnosis and control of this emerging disease from leeches and suggested there may be a risk in Traditional Chinese medicine such as Hirudin.

2. Material and methods

2.1. Sampling

Diseased *H. nipponia* were collected in the Jingzhou area of Hubei Province in China. These leeches contained a body length of 3-8 cm and showed typical pathological symptoms. Dying leeches were stored in oxygen containing bags and immediately transported to the laboratory for diagnosis and pathogen isolation. Meanwhile, normal*H. nipponia* purchased from the farm were 3-5 cm in length and cultured in an inflated

water tank at 28 for one week for the pathogenicity test.

2.2. Pathogen isolation

Typical symptoms of skin mucus and visceral tissues in *H. nipponia* were examined for parasites. Bacteria were isolated in a secondary biosafety cabinet (ESCO Singapore). Dying, diseased leeches were placed on ice and sterilized using 75% ethanol. Visceral tissue of each *H. nipponia* was added to an inoculation ring and placed on brain heart infusion agar (BHIA; Difco, USA) for 24 hours at 28 . The dominant strain was selected and purified with the purified strain temporarily named SZ01. Next, 15% glycerin was added into the purified strain, mixed and frozen at - 80 .

2.3. Biochemical characterization of bacterial isolates

The SZ01 strain was inoculated on brain heart infusion agar and kept at a constant temperature of 28 for 24 hours before being stained by gram. The physical and chemical indexes of the strain were determined using the micro biochemical identification tube of bacteria based on the manual of common bacterial system identification (Dong and Cai, 2001).

2.4. 16S ribosomal DNA sequencing analysis

The isolated strain SZ01 was inoculated on brain heart infusion agar, cultured at 28 for 24 hours and a single colony was dissolved in 10 μL sterile water, which was used as a PCR template.

The primer used to detect the 16S rRNA sequence was 27F: 5'-AGAGTTTGATC(C/A)TGGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3'(Polz and Cavanaugh, 1998). The PCR reaction system included 2×Taq PCR mix, 50 μL; ddH2O, 47 μL; 1 μL for each primer and 1 μL for each template. Reaction conditions included 95 , 1 min; 98 , 15s, 55 , 30s; 72 , 2min; 35 cycles in this stage, 72 , 15min. The amplified product was verified using 1% agarose gel electrophoresis as the target fragment size and then sent to Shanghai bioengineering for purification and sequencing. The 16S rRNA gene sequence of the SZ01 strain was put into the NCBI for comparison. The 16S rRNA sequences of *Klebsiella* and important aquatic pathogens were selected and analyzed by cluster x software. The phylogenetic tree was constructed by the Neighbor-Joining method using the mega 6.0 software and the confidence of the bootstrap test was 10000 times.

2.5. Pathogenicity

Based on Koch's rule, challenge tests were performed to test the pathogenicity of the isolated strains. The isolated bacteria SZ01 was cultured in liquid medium at 28 °C for 18h and then collected at 4 ° C for 5 minutes at 4000 rpm. Contents were resuspended in sterile PBS buffer. The concentration of the bacterial suspension was adjusted to 10^6,10^7,10^8 and 10^9 CFU/mL using sterile PBS. A total of 150 normal*H. nipponia* were randomly divided into 5 groups termed ABCDE, where ABCD were the experimental groups and group E was the control group. Each group contained 30 leeches. In groups A-D, 0.1mL of bacterial solution was intraperitoneally injected into each *H. nipponia* . The bacterial concentrations were 10^9, 10^8, 10^7 and 10^6CFU/mL respectively, which was equivalent to 10^8, 10^7, 10^6 and 10^5CFU for each *H. nipponia* . The same volume of PBS was injected into the same area of the *H. nipponia* for the control group. During the experiment, dissolved oxygen was kept at 7.5-8.5, water temperature was 24-26 and fully aerated tap water was used for breeding until death. Clinical symptoms and mortality were both recorded daily. At the same time, dead *H. nipponia* were dissected and bacteria were isolated and purified. Purified bacteria were identified using 16S rRNA sequencing.

2.6. Analysis of drug sensitivity of the SZ01 strain

The paper diffusion method was used to analyze drug sensitivity of SZ01 based on the NCCLS standard (Wikler, 2009). The SZ01 strain was inoculated onto brain heart infusion agar and cultured for 24 hours with constant shaking (200r/min) at 28 , then diluted to a 10^7 CFU/mL bacterial suspension using sterile PBS. A total of 100 μL of bacterial solution was added to MH agar, selected drug sensitive paper was placed on the plate (see the table 2 for paper drug content), cultured bacteria at 28 for 24 hours and measured the diameter of the bacteriostatic circle.

3. Results

3.1. The epidemic time and clinical symptoms of diseased *H. nipponia*

Cultivation of *H. nipponia* is shown in Figure 1. The epidemic time of *H. nipponia* was from May to October each year, when temperature is between 25-35 . Diseased *H. nipponia* obviously showed reduced food intake, swam slowly and floated on the water. The incidence and mortality rates of *H. nipponia* were very high, causing serious economic damage to farmers. Only a small part of the body surface of diseased *H. nipponia* was congested and most of the body surface was normal without debonding (Figure 2). Internal organs were found to be edematous and erosive.

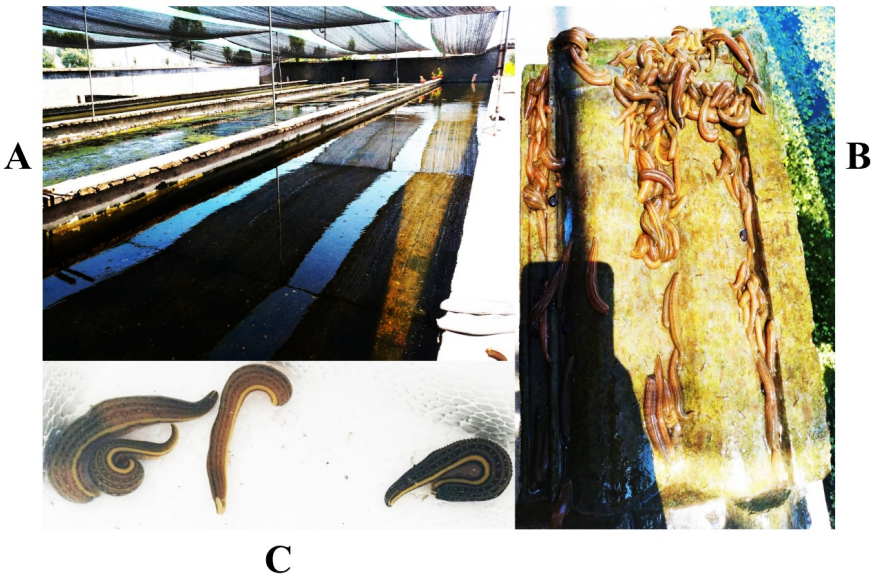

Figure 1. Cultivation of *H. nipponia*

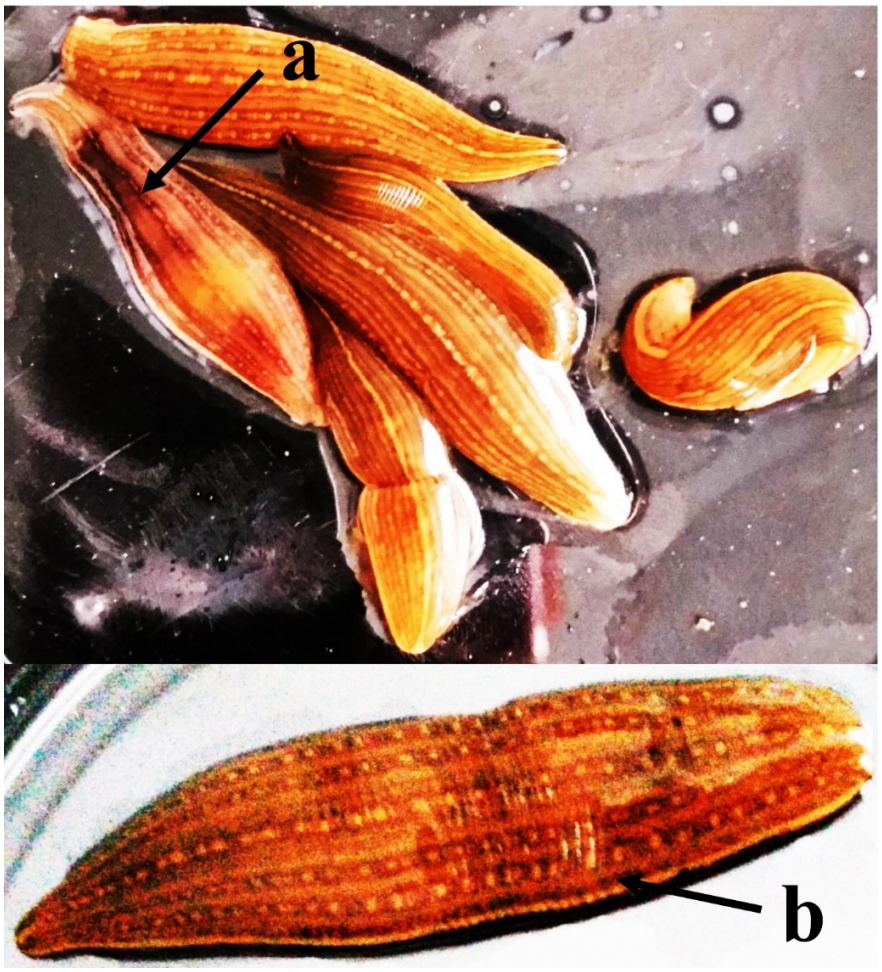

Figure 2. Diseased *H. nipponia* : a: hyperemia; b: edematous

3.2. Biochemical characterization and molecular identification of bacteria

Gram staining revealed that the SZ01 strain was red, indicating that it was a gram-negative bacterium. The specific physical and chemical characteristics of this bacterium are shown in Table 1. The physical and chemical characteristics of the SZ01 strain suggest that this bacterium was *Klebsiella pneumoniae* . After gene amplification, the size of the 16S rRNA fragment of the SZ01 strain was about 1500 bp, which was consistent with the expected size for 16S rRNA. The 16S rRNA gene sequence(GenBank login No.: MT192711)of the SZ01 strain was incorporated into the gene library and analyzed using the NCBI-blast program. The results showed that isolates showed the highest homology with *K. pneumoniae* . The 16S rRNA gene sequences of several*Klebsiella* species and important aquatic pathogenic bacteria were selected to construct a development tree based on the 16S rRNA gene sequence (Figure 3). Results showed that the isolated bacteria and*K. pneumoniae* (GenBank login No. is KF733734.1) were clustered into the same branch(Figure 3),and had the highest homology. However, *K. pneumoniae*(GenBank login No. was KF733734.1) was isolated from the human oral cavity. Therefore, physical and chemical characteristics as well as gene analysis of the SZ01 strain all comprehensively suggest that the isolated SZ01 strain was *K. pneumoniae.*

Table1. Physiological and biochemical characteristics of the SZ01 strain

Test items	SZ01	*K. pneumoniaea*
Catalase	+	+
Oxidase	-	-
H_2S	-	-
Indole	-	-
Methyl red	-	-
Indigo	-	-
Vp	+	+
Denitrification	+	+
Urea	+	+
Malonate	+	+
Nitrate reduction	+	+
Gelatin liquefaction	+	+
Glucose fermentation	+	+
Sucrose	+	+
Mannitol	+	+
Maltose	+	+
Ornithine decarboxylase	-	-
Arginine	-	-
Lysine decarboxylase	+	+

Note: +, positive; –, negative.

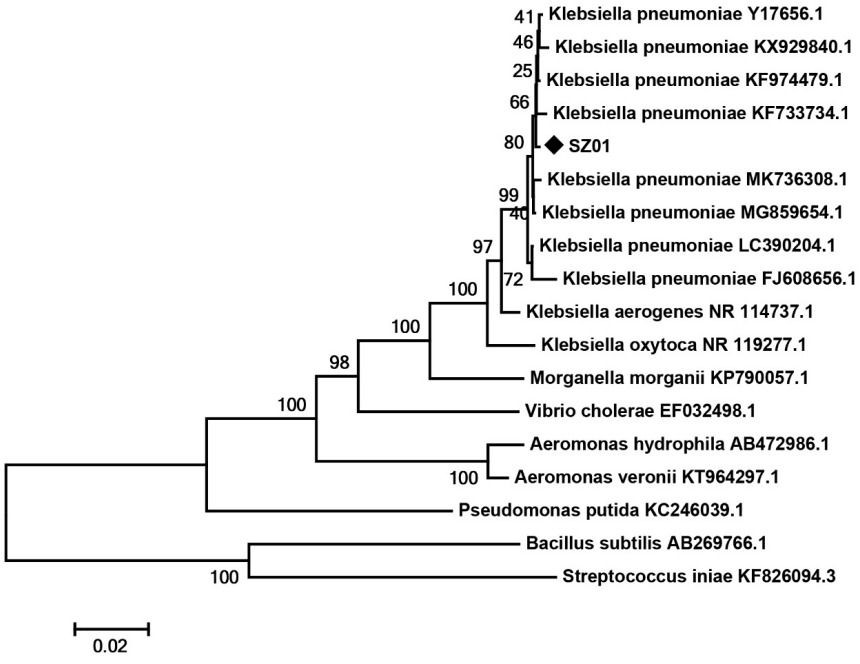

Figure 3. The phylogenetic tree for the 16s rRNA sequence of SZ01

3.3. Pathogenicity

In the pathogenicity study, the different experimental groups of*H. nipponia* showed different degrees of death (Figure 4). In groups A and B, the mortality rate of *H. nipponia* was 100% and the dead leeches showed

similar symptoms in accordance with natural diseases. However, there were no diseases or death observed in the control group. *K. pneumoniae* was isolated from the body of dying*H. nipponia* again. Therefore, this experiment abided by Koch's rule, which showed that *K. pneumoniae* was the pathogen of this disease in *H. nipponia* .

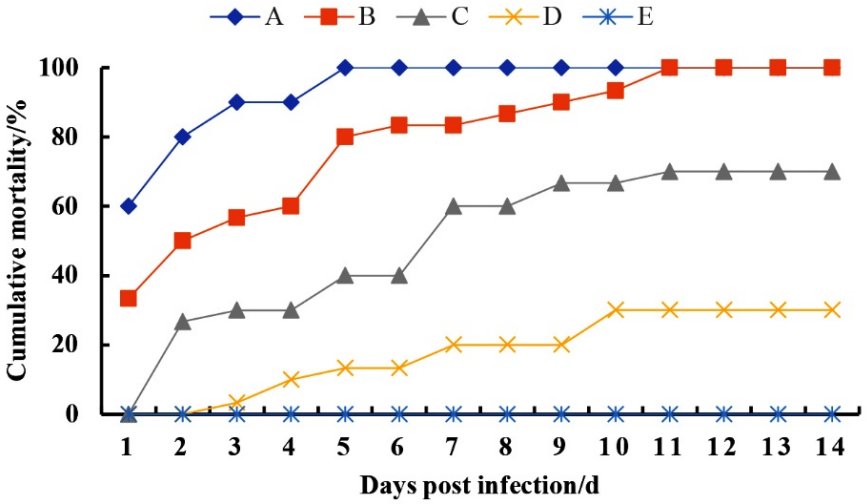

Figure 4. The pathogenicity of isolated the SZ01 strain in healthy*H. nipponia* experimentally infected with 10^8(A), 10^7 (B), 10^6 (C), 10^5 (D) cfu/leech doses or 0.1 ml PBS (E).

3.4. Drug sensitivity tests for the SZ01 strain

Next, the sensitivity of the SZ01 strain to 17 different antibiotics was investigated. Results showed that SZ01 was only sensitive to ciprofloxacin and sulfisoxazole and moderately sensitive or resistant to the other drugs. Among these drugs, sulfisoxazole, florfenicol and neomycin are allowed to be used in aquaculture (Table 2).

Table 2. Susceptibility of SZ01 to antibiotics.

Antibiotics	Content (μg/disc)	Inhibition zone diameter (mm)
Chloramphenicol	300	8.32 ± 0.25^{R}
Cefotaxime	30	15.76 ± 0.33^{R}
Cefradine	30	10.22 ± 0.12^{R}
Ciprofloxacin	5	20.55 ± 0.11^{S}
Clindamycin	2	0 ± 0^{R}
Doxycycline	30	18.14 ± 0.15^{R}
Erythrocin	15	12.06 ± 0.14^{I}
Florfenicol*	75	12.01 ± 0.21^{R}
Furazolidone	300	8.11 ± 0.24^{R}
Levofloxacin	5	9.48 ± 0.16^{R}
Norfloxacin	10	12.13 ± 0.19^{R}
Neomycin*	30	13.95 ± 0.13^{R}
Oxacillin	1	0 ± 0^{R}
Penicillin	10	0 ± 0^{R}
Rifampicin	5	10.69 ± 0.24^{R}
Streptomycin	10	12.35 ± 0.17^{R}
Sulfisoxazole*	300	22.06 ± 0.14^{S}

Note: Data are presented as the mean ± standard deviation; S: Sensitive; I: Intermediately sensitive; R: Resistant. *: Veterinary antibiotics used in aquaculture.

4. Discussion

In recent years, cultivation of leeches has been growing China. Among the leeches being cultured, *H. nipponia* is the important representative species(Zhang., 2009). As Hirudin harbors anticoagulation, antithrombotic, anti-inflammatory, anti-tumor and anti-fibrosis activities, it plays an important role in the prevention and treatment of disease(Ahmad, 2006; Rowghani et al., 2007). Therefore, the market demand of Hirudin continues to expand and lead to the rapid development of the leech breeding industry. However, with the continuous expansion of the leech breeding industry, disease rates have also increased. Although, a small number of leech parasitic diseases were identified (Gouda, 2006; Hamilton et al., 2005), there are only a few reports discussing bacterial pathogens of leeches, such as*Escherichia coli* , *Proteus mirabilis* , *Salmonella* and*Aeromonas hydrophila* (Mo et al, 2003; Zhang et al., 2009; Zhang et al., 2006). In this study, we first isolated a dominant SZ01 bacteria strain from *H. nipponia* and identified it as *K. pneumoniae*using biochemical identification, 16S rRNA sequence analysis and a phylogenetic tree. According to the phylogenetic tree analysis, the isolated bacteria and *K. pneumoniae* (GenBank login No. KF733734.1) were clustered into the same branch(Figure 3)and showed the highest homology. However, *K. pneumoniae* (GenBank login No. KF733734.1) was isolated from the human oral cavity. Results of the pathogenicity experiment revealed that *K. pneumoniae* was the main pathogen of this disease in *H. nipponia* . *K. pneumoniae* was found to infect *H. nipponia* and lead to a large number of deaths.

K. pneumoniae includes many virulence genes such as *uge*(encodes uridine diphosphate galacturonate4-epimerase), *wab* G (is involved in the biosynthesis of the outer core lipopolysaccharide),*urea* (related to the urease operon), *mag* A (mucoviscosity-associated gene A), *mrk* D (type 3 fimbriae adhesion), *all* S (activator of the allantoin regulon),*kfu* BC (iron-uptake system), *rpm* A (regulator of mucoid phenotype) and *fim* H (fimbrial gene encoding type 1 fimbrial adhesion), all which play prominent roles in bacterial pathogenesis (Gao et al., 2014; Lascols et al., 2013). *K. pneumoniae* widely exists in water, soil and grain, and is a parasitic pathogen in the respiratory and intestinal tracts of terrestrial animals. *K. pneumoniae* is ubiquitous in nature and has the ability to infect a wide range of mammalian organisms, including *Homo sapiens* and the California black langur (Holden et al., 2016; Jang et al., 2010; Magill et al., 2014). In humans, *K. pneumoniae* infects the human respiratory tract and is associated with pneumonia, liver abscesses and wounds (Bubeck et al., 2007; Clegg et al., 2016). *K. pneumoniae* had also been reported in aquatic animals such as fish, shrimp and crabs (Singh et al., 1992) and can cause bacterial diseases in soft shelled turtles, Eel, *Nemipterus japonicus* , *Cyprinus carpio* and *Labeo rohita* (Das et al., 2018; Xu et al., 2002; Deng et al., 2009; Diana et al., 2012; Oliveira et al., 2014). In this study, *K. pneumoniae*was isolated from the body of *H. nipponia* and caused symptoms of "edema and congestion" in *H. nipponia* , with a high mortality and high pathogenicity. The SZ01 strain that was isolated showed the highest homology with *K. pneumoniae* from humans, indicating that it is likely to cause human disease. Based on drug sensitivity studies, SZ01 appeared to only be sensitive to ciprofloxacin and sulfisoxazole. Otherwise, due to a large number of antibiotics and other drugs used as treatment, *K. pneumoniae* isolated from *H. nipponia* was a strong resistant strain, which makes it difficult to prevent and control of this disease. When cultivating *H. nipponia* , to treat noticeable disease as quickly as possible, a large number of antibiotics and other drugs are used, which may lead to resistance.

Modern biology supports the identification, isolation and synthesis of anticoagulant substances from leeches, among which Hirudin is the most representative. Hirudin is a medicinal substance extracted from leeches, with a peptide chain containing 65-67 amino acid residues. It is slightly acidic and has a molecular weight of 7 kDA. It can also exist stably in a dry state. Compared to other anticoagulants, Hirudin is the most efficient and does not lead to allergic reactions, making it ideal as an anticoagulant(Pan et al., 2006). Hirudin is now widely used as an antithrombotic, anti-congestion, anti-edema, anti-inflammatory and analgesic. Some studies show that Hirudin can be used to inhibit tumorigenesis(Lv et al., 2003). Extraction methods of Hirudin mainly include sun drying, low-temperature drying and freeze-drying(Elmadhun et al., 2013).

During the Hirudin extraction process, the leech may contain *K. pneumoniae* but extraction conditions are not able to destroy bacterial toxins. Therefore, toxins from *K. pneumoniae*will exist in extracted Hirudin. Furthermore, this will affect therapy and may cause a threat to humans.

To solve this issue, close attention should be paid to the prevention of leech diseases, which may occur through improved feeding conditions and methods. We have identified many issues in the cultivation of *H. nipponia* in Hubei Province. The cultivation of leeches has shown a state of disordered development and lacked government management and guidance, with a supply exceeding demand. Due to disordered development of leech breeding, the number of wild leech seedlings decreased resulting in a decline in the quality of the whole seedling. On the other hand, human pathogenic bacteria, such as *K. pneumoniae,* may also cause leeches to get sick. Human pathogens might release toxins and remain in leeches. Thus, the toxin would further pollute Hirudin and other pharmaceutical products, causing major medical issues. In addition, the direct consequence of frequent occurrence of disease is drug abuse. The abuse of drugs, especially antibiotics, leads to the emergence of drug-resistant strains such as highly resistant *K. pneumoniae* . At last, leeches in the Hubei province, especially *H. nipponia* , are mainly fed snails that are important hosts for*Schistosoma* (Lv et al., 2019). Therefore, *Schistosoma*eggs may be mixed in leeches, which endanger the safety of farmers and also contaminate products related to Hirudin, and then endanger patient safety.

To this end, we suggest: 1. To designate the government regulatory department for leech breeding, strengthen the guidance of its breeding industry and perform orderly breeding of leeches; 2. Measures should be taken to reduce the density and incidence of disease in leech culture to avoid contamination by bacterial toxins; 3. To reduce the use of drugs in the process of leech breeding, perform green scientific prevention and control of leech diseases to reduce the use and residue of drugs; 4. Strengthen the inspection and quarantine of leech related products, resolutely stop the sale of problematic products and destroy them. In conclusion, we propose that there are some factors threatening human safety in products that are related leeches. *K. pneumoniae* is an important representative pathogenic bacterium that deserves attention.

Acknowledgments

This work was supported by the China Agriculture Research System CARS-46 and Modern Agricultural Talent Support Plan (2016-139). Thanks are also due to the anonymous reviewers who provided detailed comments that helped to improve the manuscript.

Data Availability Statement

All data generated or used during the study appear in the submitted article.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of the work described in this manuscript.

Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as human or animal subjects were not involved in this study.

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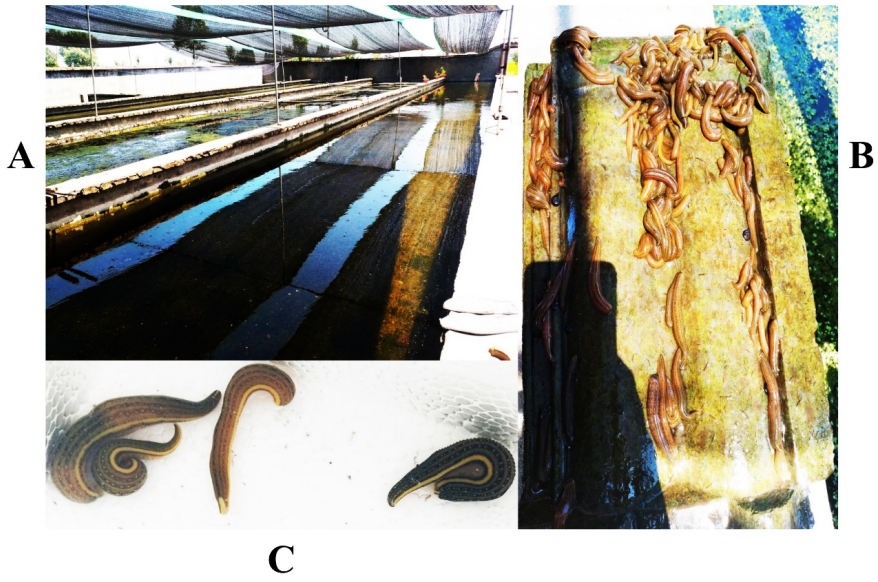

Figure 1. Cultivation of *H. nipponia*

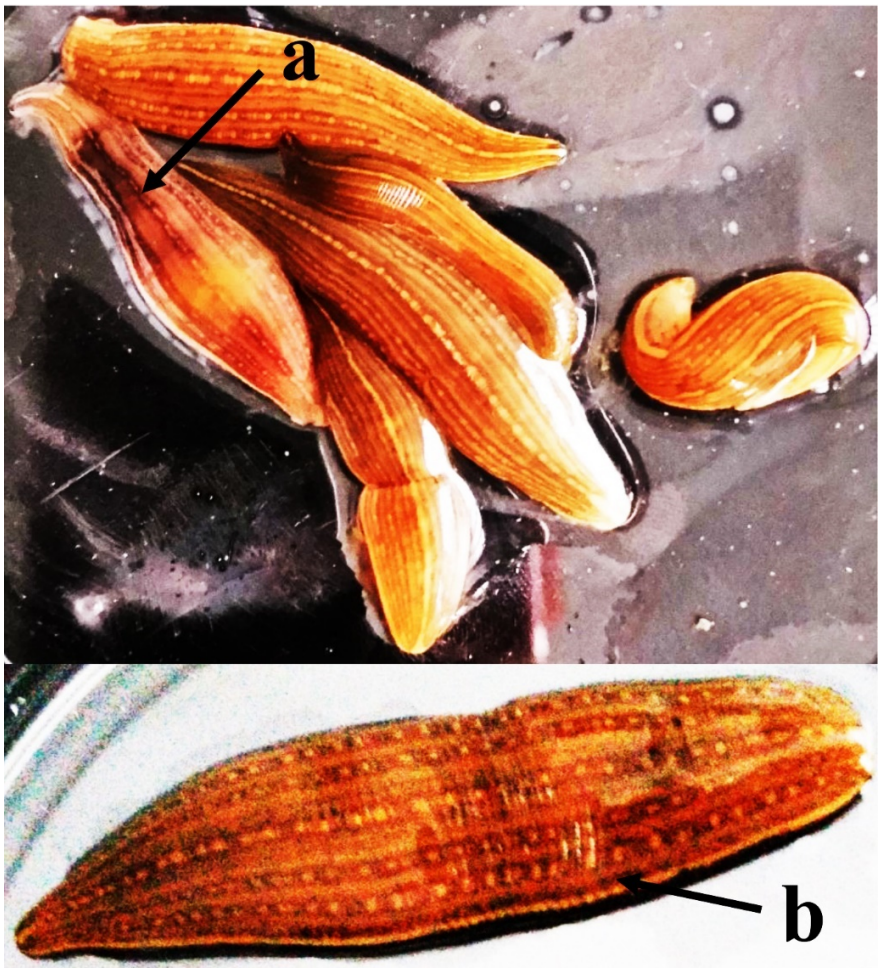

Figure 2. Diseased *H. nipponia* : a: hyperemia; b: edematous

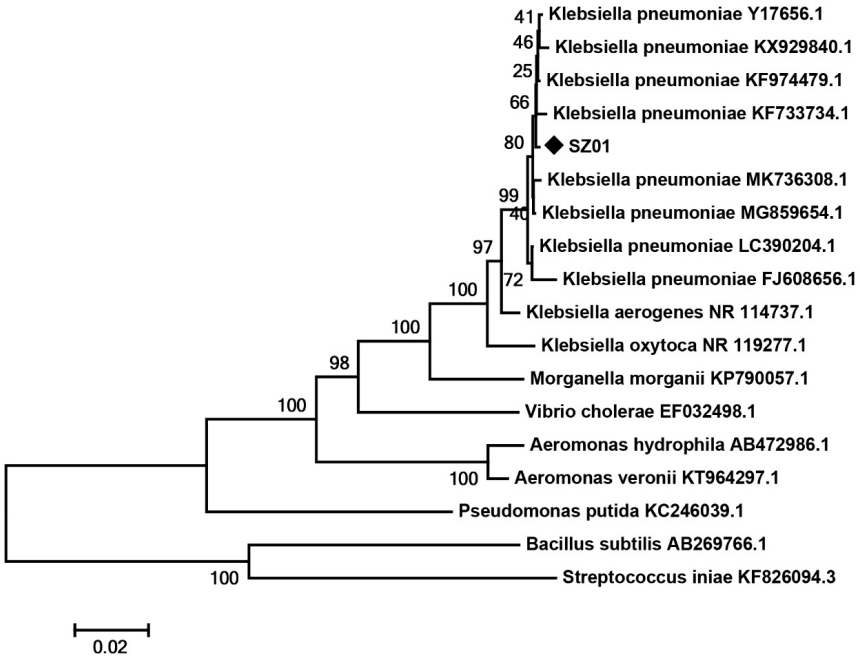

Figure 3. The phylogenetic tree for the 16s rRNA sequence of SZ01

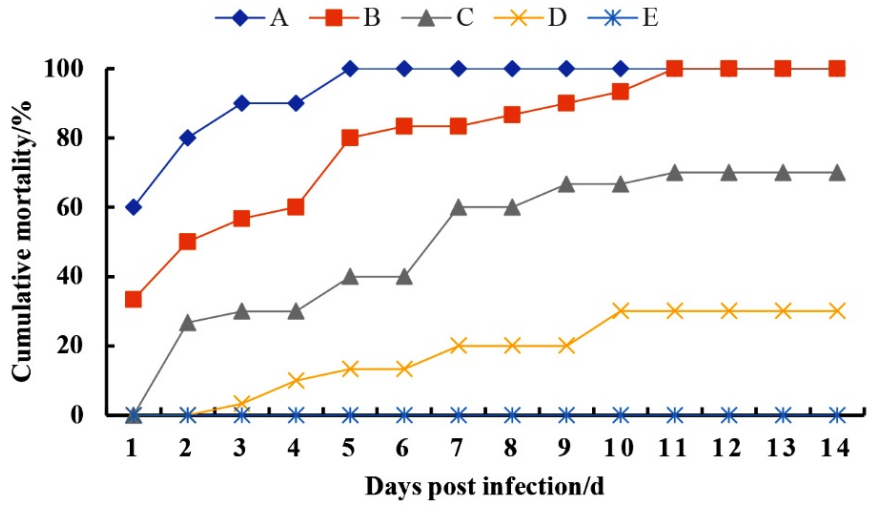

Figure 4. The pathogenicity of isolated the SZ01 strain in healthy *H. nipponia* experimentally infected with 10^8(A), 10^7 (B), 10^6 (C), 10^5 (D) cfu/leech doses or 0.1 ml PBS (E).

Table1. Physiological and biochemical characteristics of the SZ01 strain

Test items	SZ01	*K. pneumoniaea*
Catalase	+	+
Oxidase	-	-
H_2S	-	-
Indole	-	-

Test items	SZ01	*K. pneumoniaea*
Methyl red	-	-
Indigo	-	-
Vp	+	+
Denitrification	+	+
Urea	+	+
Malonate	+	+
Nitrate reduction	+	+
Gelatin liquefaction	+	+
Glucose fermentation	+	+
Sucrose	+	+
Mannitol	+	+
Maltose	+	+
Ornithine decarboxylase	-	-
Arginine	-	-
Lysine decarboxylase	+	+

Note: +, positive; –, negative.

Table 2. Susceptibility of SZ01 to antibiotics.

Antibiotics	Content (μg/disc)	Inhibition zone diameter (mm)
Chloramphenicol	300	8.32 ± 0.25^{R}
Cefotaxime	30	15.76 ± 0.33^{R}
Cefradine	30	10.22 ± 0.12^{R}
Ciprofloxacin	5	20.55 ± 0.11^{S}
Clindamycin	2	0 ± 0^{R}
Doxycycline	30	18.14 ± 0.15^{R}
Erythrocin	15	12.06 ± 0.14^{I}
Florfenicol*	75	12.01 ± 0.21^{R}
Furazolidone	300	8.11 ± 0.24^{R}
Levofloxacin	5	9.48 ± 0.16^{R}
Norfloxacin	10	12.13 ± 0.19^{R}
Neomycin*	30	13.95 ± 0.13^{R}
Oxacillin	1	0 ± 0^{R}
Penicillin	10	0 ± 0^{R}
Rifampicin	5	10.69 ± 0.24^{R}
Streptomycin	10	12.35 ± 0.17^{R}
Sulfisoxazole*	300	22.06 ± 0.14^{S}

Note: Data are presented as the mean ± standard deviation; S: Sensitive; I: Intermediately sensitive; R: Resistant. *: Veterinary antibiotics used in aquaculture.