Isolation and Identification of Kidney-derived Pathogenic *Escherichia coli* from Red Panda (*Ailurus fulgens*)

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Abstract

Background Disease prevention and control is a significant part during the ex-situ conservation of the red panda (Ailurus fulgens) with bacterial infection being one of the important threats to the health of the captive population. So far, there was no systematic and detailed publications about the red panda-related E. coli disease. This study was conducted for the purpose of determining the cause of death, etiology and pathogenesis on a red panda through clinical symptoms, complete blood count, biochemical analysis, pathological diagnosis, antimicrobial susceptibility test, mouse pathogenicity test, and bacterial whole genome sequencing.

Results A bacterial strain confirmed as Uropathogenic Escherichia coli (UPEC) was isolated from one captive dead red panda, which is resistant to most of the β-lactam drugs and a small number of aminoglycoside medications. The mouse pathogenicity test results showed the strains isolated postmortem from mice were the same as from the dead red panda, and the pathological findings were similar to the red panda while they were not completely the same. These pathological differences between red panda and mice may be related to the routes of infection and perhaps species differences and tolerance. The whole genome sequencing results showed that the isolated strain contained P pili, type I pili and iron uptake system related factors, which were closely related to its nephrotoxicity.

Conclusion The red panda died of bacterial infection which was identified as Uropathogenic Escherichia coli. The pathogenic mechanisms of the strain are closely related to the expression of specific virulence genes.

Background

Escherichia coli are a common strain of bacterium distributed in the digestive tract of
animals. Most of the strains are not pathogenic, but once they obtain the virulence factors of pathogenic, they will turn into the pathogenic bacterium and can cause infections in animals and humans [1]. For example, E. coli infection occurs in poultry and livestock as well as in wild animals such as snub-nosed monkeys and giant pandas [2, 3, 4, 5]. The Uropathogenic Escherichia coli (UPEC) are the main bacterium strain which can induce urinary tract infection [6]. Once E. coli enters the urethra, it can cause pyelonephritis, cystitis and urethritis [7, 8]. If the bacterial infections cannot be controlled in time, renal function damage may occur and pose a serious threat to health and life.

The red panda (Ailurus fulgens) has been classified as endangered by the IUCN as its wild population is estimated at less than 10,000 mature individuals, and its survival in the wild is threatened by deforestation, loss of habitat and fragmentation of the existing wild populations [9]. Disease prevention and control is a significant part during the ex-situ conservation of the red panda with bacterial infection being one of the important threats to the health of the captive population. So far, there are no systematic and detailed publications about the red panda-related E. coli disease. Analysis of post-mortem reports is an important tool in increasing our understanding of the red panda in captivity and improving our husbandry and management procedures for this species [10]. Due to its special ecological status, the prevention and treatment of E. coli in the red panda needs high attention.

This study was conducted for the purpose of determining the cause of death, etiology and pathogenesis on a red panda. The cause of death was identified through clinical symptoms, complete blood count, biochemical analysis, pathological diagnosis and animal experiments. A strain of E. coli was isolated and identified, the antimicrobial susceptibility test, mouse pathogenicity test, and bacterial whole genome sequencing were used to explore the pathogenic mechanism of the bacteria. The results could provide a scientific
basis for the diagnosis and clinical treatments of the bacterial infection of the red panda.

Results

**Complete Blood Count (CBC) and biochemical analysis results**

The complete blood count and biochemical analysis results of the red panda are shown in table 1. Compared with a report which studied the range of blood physiological and biochemical parameters of 28 healthy red pandas, the values of neutrophils, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Blood urea nitrogen (BUN) in this red panda were significantly increased while the other parameters were normal [11].

| Projects | Units | Value |
|----------|-------|-------|
| WBC      | ×10^9/L | 9.66  |
| Neu      | ×10^9/L | 6.97  |
| Lym      | ×10^9/L | 1.69  |
| Mon      | ×10^9/L | 0.81  |
| Eos      | ×10^9/L | 0.16  |
| Bas      | ×10^9/L | 0.03  |
| Neu%     | -       | 72.2  |
| Lym%     | -       | 17.5  |
| Mon%     | -       | 8.4   |
| Eos%     | -       | 1.6   |
| Bas%     | -       | 0.3   |
| RBC      | ×10^9/L | 6.48  |
| HGB      | g/L     | 91    |
| MCH      | pg      | 14.1  |
| MCHC     | g/L     | 342   |
| PLT      | -       | 571   |
| TP       | g/L     | 83.1  |
| TB       | μmol/L  | 2.37  |
| TBA      | μmol/L  | 7.6   |
| ALT      | U/L     | 1127  |
| AST      | U/L     | 482   |
| ALP      | U/L     | 90    |
| CK       | U/L     | 485   |
| TC       | mmol/L  | 6.22  |
| TG       | mmol/L  | 0.44  |
| BUN      | mmol/L  | 13.64 |
| Cr       | μmol/L  | 110   |
| UA       | μmol/L  | 42.3  |
| GLU      | mmol/L  | 5.01  |

**Histopathological observations of the red panda**

Histopathological observation showed that glomerular congestion in the renal cortex, necrosis of the renal tubule, disappearance of the luminal structure, and infiltration of neutrophils, lymphocytes, plasma cells and macrophages were seen in the slides(fig.1 A).
The cord-like lesions can be seen in the medulla area, shown as part of the normal collecting duct structure were replaced by a large amount of inflammatory cell infiltration (fig.1 B). In the liver, there was vesicular degeneration or steatosis of hepatocytes, mild congestion, and inflammatory cell (neutrophils, macrophages and lymphocytes) infiltration around the blood vessels (fig.1 C). In the lung, significant thickening of the alveolar walls was due to inflammatory cells infiltration and congestion; some of the alveolar space turned narrow, and a few alveolar spaces are dilated showing compensatory emphysema (fig.1 D). The increase in size of the white pulps of the spleen were a result from the expanded germinal center of lymphoid follicles, and the red pulps were congested and hemorrhaged accompanied with the infiltration of inflammatory cells and the increase of nuclear debris (fig.1 E). The lymphoid sinus of the mesenteric lymph nodes was dilated and filled with lymph, in which macrophages and lymphocytes were increased (fig.1 F). The rest of the tissues did not show obvious pathological damage.

**Bacterial isolation**

Bacteria were isolated from the kidney, liver and lung. On the MacConkey agar, the colonies isolated from these three tissues were pink and round, with a diameter of about 2 mm, and the surface was smooth and moist. After Gram staining, the morphology of the bacteria was short rod-shaped under the oil microscope, indicating Gram-negative bacteria.

**16S rRNA**

The results of the electrophoresis test illustrated that there was no banding in the negative control, and the isolated strain had a clear band at about 1500 bp, which was consistent with the expected size of the 16S rRNA gene (fig.2). Three strains from different tissues were sequenced. The sequencing results revealed that the three strains were highly consistent. The similarity among three isolated bacteria was 100%, and
similarity between the isolated strains and *Escherichia coli* (*E. coli*) was 99.45%.

**Antimicrobial susceptibility test**

The results of antimicrobial susceptibility test showed that the isolated *E. coli* were sensitive to penicillin drugs (piperacillin), cephalosporins (cefoxitin, ceftriaxone, cefepime) and non-classical β-lactam drugs (Meropenem, piperacillin-tazobactam, ampicillin-sulbactam), aminoglycosides (kanamycin, amikacin, neomycin), tetracycline (minocycline), quinolone (levofloxacin), nitrofuran (furazolidone), peptide (polymyxin B); but resistant to other antibiotics (table 2).

| Type                  | Drug                        | Dose (μg/disc) | Diameter of inhibition zone (mm) | Sensitivity |
|-----------------------|-----------------------------|----------------|----------------------------------|-------------|
| Penicillin            | Piperacillin                | 100            | 23                               | S           |
|                       | Ampicillin                  | 10             | -                                | R           |
|                       | carbenicillin               | 100            | -                                | R           |
| Cephalosporins        | Cephalaxin                  | 30             | -                                | R           |
|                       | Cefradine                   | 30             | -                                | R           |
|                       | Cefazolin                   | 31             | 26                               | S           |
|                       | Cefoxitin                   | 30             | 26                               | S           |
|                       | Cefuroxime                  | 30             | R                                |             |
|                       | cefoperazone                | 75             | 10                               | R           |
|                       | Ceftadizime                 | 30             | R                                |             |
|                       | Ceftriaxone                 | 30             | 31                               | S           |
|                       | Cefepime                    | 30             | 33                               | S           |
| Nonclassical-β-lactams| Meropenem                  | 10             | 33                               | S           |
|                       | piperacillin-tazobactam     | 100/10         | 26                               | S           |
| aminoglycosides       | Kanamycin                   | 30             | 23                               | S           |
|                       | Amikacin                    | 30             | 24                               | S           |
|                       | Gentamicin                  | 10             | 12                               | R           |
|                       | Neomycin                    | 30             | 18                               | S           |
| tetracyclines         | acoheomycin                 | 30             | R                                |             |
|                       | Minocycline                 | 30             | 24                               | S           |
|                       | doxycycline                 | 30             | -                                | R           |
| Chloramphenicol        | chloramphenicol             | 30             | R                                |             |
| quinolones            | Ciprofloxacin               | 5              | -                                | R           |
|                       | Norfloxacin                 | 10             | -                                | R           |
|                       | levofloxacin                | 5              | 33                               | S           |
|                       | Ofloxacin                   | 5              | -                                | R           |
| sulfonamides          | cotrimoxazole               | 23.75/1.25     | -                                | R           |
| polypeptide           | furazolidone                | 300IU          | 19                               | S           |
| polypeptide           | Polymyxin B                 | 300IU          | 14                               | S           |

**Note:** “S” means sensitive; “R” means resistant.

**Pathogenicity test**

Mice were injected with different doses of bacterial fluid, while the control group was given the same amount of sterile physiological saline. Six hours after injection, the mice in the high-dose group showed clinical symptoms such as tremors and slowness of
movements and died 12 hours post injection. The other test groups of mice died within 72 hours, while no death was noted in the control group. According to the modified Karber method, the LD$_{50}$ of the median lethal dose was $8.42 \times 10^7$ cfu/mL. The isolated strains of bacteria collected from the heart, liver, spleen, lung and kidney tissues the test mice showed the same morphological characteristics as the isolated strains from the red panda. The isolated strains were identified by 16S rRNA sequencing. The results showed that the similarity between the isolated bacteria and *E. coli* sequences in different tissues was highly consistent, the similarity was 99.55%. Histopathological observations from the mice showed blue-stained bacterial masses in the heart, liver, and kidney. The edema, congestion, cell degeneration even necrosis, inflammatory cell infiltration and other phenomena were found in many tissues including the heart, liver, kidney, and pancreas.

**Whole genome sequencing results**

By genomic assembly of strain sequence isolated from the red panda, it was found that the isolated strain had a genome size of 4,990,420 bp. The BLAST alignment of the predicted protein sequence with the Antibiotic Resistance Gene Database (ARDB) showed that the strain contained 20 resistance genes including *acra, acrb, mdte, mdtf*. These genes can mediate resistance to aminoglycosides, β-lactams, macrolides and other drugs. BLAST alignment with the Virulence Factor Database (VFDB) showed that the measured *E. coli* contained 713 virulence factors, including outer membrane protein, flagella, P pili, S pili, type I pili, cytotoxic necrosis factor, and hemolysin. In addition, factors related to the iron uptake system and other types of systems are also included. Among these virulence factors, *PapA, PapG, OmpA, OmpU* and other virulence factors were specific to Uropathogenic *Escherichia coli* (UPEC) [8], which helped confirm that the bacteria is UPEC.

**Discussion**
Understanding and addressing disease threats in the ex-situ population of the red panda is crucial to the conservation of red panda in China. In this study, blood examination revealed leukocytosis with increased ALT, AST and BUN, suggesting that bacterial infection may cause the liver and kidney damage then cause the death [12, 13, 14]. Histopathological findings revealed that the main pathological damage were kidney, liver and lung, which were characterized by hyperemia, degeneration and necrosis of parenchymal cells and inflammatory cell infiltration. In addition, the infiltrating inflammatory cells in each tissue were mainly neutrophils, which were consistent with the results of complete blood count and biochemical analysis. Bacterium was isolated from the kidney, liver, and lung. The results of whole genome sequencing of the bacterium showed that the strain contained PapA, PapG, OmpA, OmpU and other virulence factors specific to UPEC [8], confirming that the strain infecting the red panda was UPEC. Researchers have reported in the mouse model of acute pyelonephritis caused by UPEC, the pathological manifestations of the kidney were infiltration of inflammatory cells mainly by neutrophils under the renal pelvis mucosa, renal interstitial dilatation, partial renal tubular epithelial cell necrosis and shedding [15, 16, 17]. These were similar to the pathological manifestations of the kidney in this red panda. Therefore, the pathogen of the red panda was diagnosed as UPEC, which induced pyelonephritis through retrograde infection from the urethra, and then leading to septicemia through the blood.

In the antimicrobial susceptibility test, the isolated strain was only sensitive to a small number of β-lactam drugs, tetracycline drugs, quinolones, and polypeptide drugs and some non-classical β-lactam drugs and aminoglycosides. The strain was resistant to some β-lactam drugs and a small number of aminoglycoside drugs, which was basically consistent with the sequencing results of antibiotic resistance gene. However, some results from antimicrobial susceptibility were not in accord with the gene sequencing
results. Namely, although the copy numbers of acraTolc and emregene which mediated
the aminoglycoside-polymyxin and beta-lactam drug resistance were detected, the E. coli
was sensitive to gentamycin, polymixin B, cefoperazone. The possible reason is related to
“Gene Silencing”, which is due to the immature expression of these genes [18]. As far as
the clinical treatment of the red panda, when symptoms were first noted, sulperazon
(cefoperazone sodium and sulbactam sodium) and gentamicin were used, but there was no
obvious curative effect. This condition is compatible with the results of antimicrobial
susceptibility test and antibiotic resistance, which showed that the E. coli was resistant to
gentamycin and cefoperazone. The increasing usage of the clinical antibacterial drug
forces the resistance to occur in both pathogenic and zoonotic bacteria in animals [19].
Previous research showed that the appearance of drug resistant strains can make
treatment of bacterial infections more difficult, which may lead to an overall increase in
transmission, morbidity and mortality [20, 21]. These results suggest that effective
treatment should be based on the results of antimicrobial susceptibility tests and clinical
efficacy in the control of bacteriosis in clinical practice.
The mouse pathogenicity test showed that the isolated UPEC strain had pathogenicity to
mice, and the median lethal dose was calculated to be $8.42 \times 10^7$ cfu/mL. Bacteria were
isolated from the heart, liver, spleen, lung, and kidney of the infected mice, and the
strains are consistent with the strains isolated from the red panda, according to the
results by 16S rRNA sequencing. The Blast alignment showed that the sequence similarity
of the isolated strains from different tissues was 100%, and there were 99.55% sequence
similarity compared with the sequence of UPEC isolated from the red panda.
Histopathologically, in the mice, blue-brown bacterial mass could be observed in the
heart, liver and kidney, and the edema, hyperemia, cell degeneration and necrosis,
inflammatory cell infiltration could be found in the heart, liver, kidney and pancreas.
These lesions are characteristic of bacterial sepsis [12] and are similar to the pathological manifestations characterized as widespread degeneration, necrosis and inflammatory cells infiltrate in the parenchymal organ of the dead red panda. In the red panda, the suppurative nephropyelitis suggested the specific nephrophilic toxicity of this UPEC. In accordance with this lesion, extensive cell swelling and severe necrosis of the renal tubular epithelial mice revealed that the strain was more toxic to the kidney than other organs, which was the confirmation of the kidney toxicity of the strain. However, unlike the pathological manifestations of the red panda, lesions of the intestinal villus epithelial detachment, lamina propagating telangiectasia, and congestion were observed in each intestinal segment of the mouse. We speculate that there are two possible reasons for the pathological difference between the mouse and the red panda: 1) they belong to different species and have different sensitivities to the same strain; 2) the routes of infection are different. Intraperitoneal injection was used in mice, but natural infection through the urethra appeared in the red panda.

Previous researchers found that 80% of E. coli containing P pili may cause pyelonephritis [22]. In our study, the whole genome sequencing results found high express of P pili and type I pili genes. The P pili receptors exist on renal epithelia, while type I pili mediates the biofilm formation and colonization of the bacteria in the renal epithelia [8], which makes the strain more nephrotoxic. In addition, the virulence factors associated with the iron uptake system are also high in this case. The presence of the iron uptake system enhances the pathogenicity of the bacteria, for it contributes to bacteria survival through the host’s heme and ferritin [23].

Conclusions

Based on the results of blood physiology and biochemistry, histopathological damage, bacterial isolation and identification, mouse pathogenicity test, and whole genome
sequencing, the red panda died of bacterial infection and the isolated bacterial strain was identified as Uropathogenic Escherichia coli. The pathogenic mechanisms of the strain are closely related to the expression of specific virulence genes. Since this is the first time to isolate the UPEC strain in the red panda, further research is needed for a better understanding of the epidemic, susceptibility and antibiotic resistance of the strain in the red panda.

Methods

Animal and pathology

One 1.5 year old captive female red panda (*Ailurus fulgens styani*) was found sick in Panda Valley, Chengdu Field Research Center for Giant Pandas in Dujiangyan, Chengdu, China. She was lethargic and had low appetite start from the morning of November 17, 2017. Oral and nasal swab specimens were collected for Canine Distemper Virus (CDV) and Canine Parvovirus (CPV) tests and the results were all negative. The veterinary staff gave antibacterial treatment to the red panda. The red panda was anaesthesia with 21 mg Zoletil (intramuscular injection) at a dose of 3 mg/kg body weight. Then intravenous injection of 350 mg Cefoperazone Sodium and Sulbactam Sodium in 150 mL 0.9% NaCl was performed. They also collected blood samples for complete blood count and biochemical analysis. Then the red panda was moved to the quarantine area of the Chengdu Research Base of Giant Panda Breeding for treatment. A daily dose of cefpodoxime proxetil 50 mg was prescribed by oral administration. During this period, she did not eat or defecate and natural died at 15:00 on November 21.

The red panda's skin and fur was intact, no traumatic injuries such as fractures or skin ulcerations were observed. The necropsy revealed that the two kidneys were swollen and there were several white spots of millet size on the incision surface, and white cord-like streaks were also seen between the medullary rays. The liver was yellowish while the
texture was soft and brittle with a few dark red ecchymoses on the surface. The lungs
were dark red with plaques of black-red hemorrhage locally. The spleen was swollen and
dark red with a small amount of black-red plaques. The heart and intestines were intact
and there were no obvious lesions.

Specimens, including the heart, liver, spleen, lung, kidney, gastrointestinal tract and other
tissues were collected and fixed in 4% paraformaldehyde for more than 24 hours. These
tissue samples were routinely processed in paraffin. Sections (5μm) were stained with
haematoxylin and eosin Y (H&E) and were observed for recording characteristic
histopathological changes under the microscope (Leica DM4B optics). The use of the
animal was approved by the Chengdu Research Base of Giant Panda Breeding Animal Care
and Use Committee. After sampling, the body was processed non-hazardously by the
company (Chengdu Yongxin Harmless Disposal Co, LTD).

**Bacterial separation**

The heart, liver, spleen, lung and kidney of the red panda were collected and streaked on
Brain Heart Infusion Agar. After incubation at 37 °C for 12-24 hours, the dominant colonies
were selected for further purification. Gram staining was carried out to observe the
morphology and identify the type of different colonies. The isolated colonies were stored
in the brain heart broth with 20% glycerin for further experiment.

**DNA extraction and identification**

The overnight culture strain solution was taken to extract DNA using the bacterial genomic
DNA rapid extraction kit. The 16S rRNA gene was amplified by PCR using the universal
primer, forward primer (27F): 5’AGAGTTTGATCCTGGCTCAG 3’, reverse primer (1492R): 5
‘TACGGCTACCTGTAGACAGTT3’. After the completion of the amplification, the PCR
products were sent for sequence detection (completed by Sangon Biotech (Shanghai) Co,
LTD.). The sequencing results were aligned using Blast alignment function in Gen Bank to
find similar strains and define their species status.

**Antimicrobial susceptibility test**

The K-B disc diffusion method was performed in this study, and the test was carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI M100 2018 standard). The bacterial solution after resuscitation was diluted, applied to MH plate, and the susceptibility paper was attached at a certain interval, and cultured at 37℃ for a certain period of time to measure the diameter of the inhibition zone and determine microbial sensitivity.

**Pathogenicity test**

One hundred SPF mice (Provided by the Experimental Animal Center of North Sichuan Medical College, the use of animals was approved by the Chengdu Research Base of Giant Panda Breeding Animal Care and Use Committee.) were randomly divided into 10 groups. Groups of 10 mice were intraperitoneally inoculated (0.5 mL) with different bacterial doses ranging from $1.2 \times 10^9$ to $2.0 \times 10^7$ cfu/mL each group with the dilution ratio 1:0.6. One group was the control group which was injected with sterile physiological saline. After the injection, each group was kept for 7 days consecutively and all of the mice in different groups were housed in individual cages. The morbidity and mortality of each group was recorded. The dead mice caused by the infection were immediately necropsied and tissues from the heart, liver, spleen, lung and kidney were taken for bacterial isolation and pathological observation. The remaining animals in control group and low dose groups at the end of this study were euthanized by carbon dioxide ($CO_2$) inhalation (concentration: 30%, 240s) and the bodies were processed non-hazardously by the company (Chengdu Yongxin Harmless Disposal Co, LTD).

**Whole genome sequencing**
The preserved *E. coli* was resuscitated, then the culture was enriched and bacterial genomic DNA was extracted, its purity detected and sent to an external laboratory for sequencing (completed by Biomarker Technologies Co, LTD.).

**abbreviations**

**UPEC:** Uropathogenic *Escherichia coli*

**IUCN:** International Union for Conservation of Nature

**CBC:** Complete Blood Count

**ARDB:** Antibiotic Resistance Gene Database

**VFDB:** Virulence Factor Database

**CDV:** Canine Distemper Virus

**CPV:** Canine Parvovirus

**WBC:** White Blood Cells

**Neu:** Neutrophils

**Lym:** Lymphocytes

**Mon:** Monocytes

**Eos:** Eosinophils

**Bas:** Basophils

**RBC:** Red Blood Cells

**HGB:** Hemoglobin

**MCH:** Mean Corpuscular Hemoglobin

**MCHC:** Mean Corpuscular Hemoglobin Concentration

**PLT:** Platelets

**TP:** Total protein

**TB:** Total bilirubin

**TBA:** Total bile acid
**ALT**: Alanine aminotransferase  
**AST**: Aspartate aminotransferase  
**ALP**: Alkaline Phosphatase  
**CK**: Creatine Kinase  
**TC**: Total Cholesterol  
**TG**: Triglyceride  
**BUN**: Blood Urea Nitrogen  
**Cr**: Creatinine  
**UA**: Uric acid  
**GLU**: Glucose

declarations

**Ethics approval and consent to participate**

The use of materials and all experimental procedures involving animals were approved by the Chengdu Research Base of Giant Panda Breeding Animal Care and Use Committee.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

We are sure that there is no potential conflict of interest and no part of this paper has published or submitted anywhere.

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Authors' contributions

LS and LY designed the work and performed main experimental operation, and were major contributors in writing the manuscript; YC, ZD, SX, YX, YK, CX and ZG performed the necropsy work and acquired the samples. CT and LJ participated the experimental operation; HR and PX verified all the data, figures and materials (including reagents), and proofreading. All authors read and approved the final manuscript.

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Figures

![Histopathological observation images](image)

**Figure 1**

Histopathological observation. A: Glomerular congestion, epithelial cell necrosis on some small renal tubules, and focal infiltration of inflammatory cells. B: A cord-like inflammatory cell infiltration zone is seen in the medulla of the kidney. C: Mild hepatic congestion, inflammatory cell infiltration, hepatocyte vacuolar degeneration. D: Significant thickening, hyperemia, and inflammatory cell infiltration of the alveolar wall. E: Congestion and hemorrhage in red pulp of the spleen. F: Lymphatic sinus expansion of the mesenteric lymph nodes, filled with lymphocytes and varying numbers of inflammation cells. H&E, bar = 50μm.
Figure 2

Electrophoresis results of amplified products Note: 1: negative control; 2: isolated strain from the kidney; 3: isolated strain from the liver; 4: isolated strain from the lung.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Checklist.docx
Highlights.docx