# Isolation and Characterization of Uropathogenic *Escherichia coli* (UPEC) from Red Panda (*Ailurus fulgens*)

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# Abstract

Background Disease prevention and control is a significant part of the ex-situ conservation of the endangered red panda (Ailurus fulgens), with bacterial infection being one important threat to the health of the captive population. To date, there are no systematic and detailed publications about E. coli-associated disease in red pandas. This study was conducted to determine the cause of death, etiology and pathogenesis in a captive 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 Escherichia coli ( E. coli ) was isolated from one captive red panda post mortem. This strain is resistant to most of the β-lactam antibiotics and a small number of aminoglycoside medications. The mouse pathogenicity test results showed that the strains isolated post mortem from mice were identical to that in the red panda, and the pathological findings were similar to those seen in the red panda. Whole-genome sequencing of the E. coli isolated from the red panda showed the complete sequences of the chromosome was 4.99 Mbp. PapA , PapG , OmpA , OmpU and other virulence factors were specific to Uropathogenic Escherichia coli (UPEC). Among the virulence factors, P pili, type I pili and iron uptake system related factors were associated with nephrotoxicity.

Conclusion The red panda died of septicemic 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* is a common species of bacteria distributed in the digestive tract of animals. Most *E. coli* strains are not pathogenic, but certain strains with virulence factors will cause serious 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]. Uropathogenic *Escherichia coli* (UPEC) can induce urinary tract infection [6] through entering the urethra, then causing 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 International Union for Conservation of Nature(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 of the *ex-situ* conservation of the red panda, with bacterial infection being one of the most important threats to the health of the captive population. To date, there are no systematic and detailed publications about *E. coli*-associated disease in red pandas. 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 to determine the cause of death, etiology and pathogenesis in a red panda. The cause of death was identified through clinical symptoms, complete blood count, biochemical analysis, gross and histopathological diagnosis and animal experiments. A strain of *E. coli* was isolated and identified, and the antimicrobial susceptibility test, mouse pathogenicity test, and bacterial whole genome sequencing were used to explore the pathogenic mechanism of the bacteria. The results 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 table1. 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 higher while the other parameters were normal[11].

Table 1 Complete blood count and biochemical analysis for the red panda

| Projects | Units | Value |
|---|---|---|
| WBC | $\times 10^9$/L | 9.66 |
| Neu | $\times 10^9$/L | 6.97 |
| Lym | $\times 10^9$/L | 1.69 |
| Mon | $\times 10^9$/L | 0.81 |
| Eos | $\times 10^9$/L | 0.16 |
| Bas | $\times 10^9$/L | 0.03 |
| Neu% | - | 72.2 |
| Lym% | - | 17.5 |
| Mon% | - | 8.4 |
| Eos% | - | 1.6 |
| Bas% | - | 0.3 |
| RBC | $\times 10^9$//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 findings showed significant glomerular congestion in the renal cortex, necrosis of renal tubular epithelial cells, collapse of renal tubular lumens and interstitial infiltration of neutrophils, lymphocytes, plasma cells and macrophages (fig.1 A). Cord-like interstitial inflammatory lesions were noted in renal medullary regions, as normal collecting duct structures were replaced by extensive inflammation (fig.1 B). In the liver, there was vesicular degeneration or steatosis of hepatocytes, mild congestion, and perivascular inflammatory cell (neutrophils, macrophages and lymphocytes) infiltration (fig.1 C). In the lung, alveolar walls were thickened by lymphoplasmacytic infiltration and congestion. Some alveolar spaces collapsed, while others were dilated due to compensatory

emphysema (fig.1 D). Expanded lymphoid follicular germinal centers increased splenic white pulp, while red pulp showed congestion, hemorrhage and inflammation with nuclear debris (fig.1 E). Mesenteric lymph node sinuses were dilated with increased macrophages and lymphocytes. (fig.1 F). Other tissues (heart, pancreas, stomach, digestive tract) did not show obvious pathological damage.

### Bacterial isolation and identification

Strains were isolated from the kidney, liver and lung samples of the red panda. The amplified 1500 bp 16S rRNA genes of the isolated strains shared the highest identity (99.45%) with that of *E. coli*（GenBank: LC050175.1）, suggesting that these isolates were *E. coli* strains.

### Antimicrobial susceptibility test

The results of antimicrobial susceptibility test showed that *E. coli* isolates were sensitive to penicillin (piperacillin), cephalosporins (cefoxitin, ceftriaxone, cefepime) and non-classical β-lactam antibiotics (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).

Table 2 Antimicrobial susceptibility test results for the *E. coli* isolated from red panda

| Group | Antibiotics | Dose (μg/disc) | Diameter of inhibition zone (mm) | Sensitivity |
|---|---|---|---|---|
| β-lactams | Piperacillin | 100 | 23 | S |
| | Ampicillin | 10 | - | R |
| | Carbenicillin | 100 | - | R |
| | Cephalexin | 30 | - | R |
| | Cefradine | 30 | - | R |
| | Cefazolin | 30 | - | R |
| | Cefoxitin | 30 | 26 | S |
| | Cefuroxime | 30 | - | R |
| | Cefoperazone | 75 | 10 | R |
| | Ceftazidime | 30 | - | R |
| | Ceftriaxone | 30 | 31 | S |
| | Cefepime | 30 | 33 | S |
| Nonclassical-β-lactams | Meropenem | 10 | 33 | S |
| | Piperacilin-Tazobactam | 100/10 | 26 | S |
| | Ampicillin-Sulbactam | 10/10 | 12 | S |
| Aminoglycosides | Kanamycin | 30 | 23 | S |
| | Amikacin | 30 | 24 | S |
| | Gentamicin | 10 | 12 | R |
| | Neomycin | 30 | 18 | S |
| Tetracyclines | Acheomycin | 30 | - | R |
| | Minocycline | 30 | 24 | S |
| | Doxycycline | 30 | - | R |
| Chloramphenicols | Chloramphenicol | 30 | - | R |
| Quinolones | Ciprofloxacin | 5 | - | R |
| | Norfloxacin | 10 | - | R |
| | Levofloxacin | 5 | 33 | S |
| | Ofloxacin | 5 | - | R |
| Sulfonamides | Cotrimoxazole | 23.75/1.25 | - | R |
| Nitrofurans | Furazolidone | 300IU | 19 | S |
| Polypeptide | Polymyxin B | 300IU | 14 | S |

**Note:** “S” means sensitive; “R” means resistant; “-” means no inhibition zone. The results were based on CLSI M100 2018.

## Pathogenicity test

Six hours after injection, the mice in the high-dose group ($1.2\times10^9$cfu/mL) showed clinical symptoms such as tremors and lethargy and died12 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

$8.42\times10^7$cfu/mL. The bacterial isolates recovered from the heart, liver, spleen, lung and kidney tissues of the test mice showed the same morphological characteristics as the isolates recovered from the red panda. The isolates recovered were identified by 16S rRNA sequencing. Similarity between the isolated bacteria and *E. coli* sequences in different tissues was highly consistent (99.66%). Histopathological findings in the injected mice showed bacterial colonies (sepsis) in the heart, liver, and kidney (fig.2). Lesions of edema, congestion, cell degeneration andnecrosis, and inflammatory cell infiltration were found in many tissues including the heart, liver, kidney, and pancreas (fig.2).

### Whole genome sequencing results

The size of *E. coli* chromosome isolated from the red panda was 4,990,420 bp.  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*, and *mdtf*. These genes can mediate resistance to aminoglycosides, β-lactams, macrolides and other antibiotics. 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 were identified. Among these virulence factors, *PapA*, *PapG*, *OmpA*, *OmpU* and other virulence factors were specific to Uropathogenic *Escherichia coli* (UPEC)[8], thus confirming its identity.

## Discussion

Understanding and addressing disease threats in the *ex-situ* population of the red panda is crucial to the conservation of *in situ* populations of red pandas in China. In this study, blood examination revealed leukocytosis with increased ALT, AST and BUN, suggesting that bacterial infection caused liver and kidney damage in this case, and then fatality [12, 13, 14]. Histopathological findings revealed major pathological damage in kidney, liver and lung, characterized by hyperemia, parenchymal cell degeneration and necrosis, inflammatory cell infiltration which was predominantly neutrophilic. These findings are consistent with the results of the complete blood count and biochemical analysis. Researchers have reported acute pyelonephritis caused by UPEC in the mouse model, with renal histopathological lesions including  infiltration of inflammatory cells (predominantly neutrophils) in renal pelvis submucosa, 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. We postulate that UPEC induced pyelonephritis in this red panda, through retrograde infection from the urethra, then leading to septicemia through the blood.

In the antimicrobial susceptibility test, the strain was resistant to some β-lactam antibiotics and a small number of aminoglycoside antibiotics; this pattern is consistent with the sequencing results of antibiotic resistance gene. Although copy numbers of *acra, tolc* and *emr* genes (included in multi-drug efflux systems which mediate the aminoglycoside□polymyxin and beta_lactamantibiotic resistance) were detected, this *E. coli* isolate was sensitive to gentamycin, polymixin B, and cefoperazone. A 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 result is consistent with the results of antimicrobial susceptibility test and antibiotic resistance, showing resistance to gentamycin and cefoperazone. Current increasing usage of clinical antibacterial drugs forces antibiotic resistance to occur in both pathogenic and zoonotic bacteria in animals [19]. Previous research showed that the appearance of antibiotics 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 bacterial infections in clinical practice.

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 epithelia in 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. One, they belong to different species and have different sensitivities to the same strain, and the other reason, 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 septicemic 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 report of UPEC strain isolation in the red panda, further research is needed for a better understanding of the epidemiology, susceptibility and antibiotic resistance of this 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 beginning the morning of November 17, 2017. Oral and nasal swab specimens were collected for Canine Distemper Virus (CDV) and Canine Parvovirus (CPV) tests using Asan Esay Test (ASAN PHARM. Co., LTD); the results were negative. The red panda was anaesthetized with 21mg Zoletil (intramuscular injection) at a dose of 3 mg/kg body weight, and intravenous injection of 350 mg Cefoperazone Sodium and Sulbactam Sodium in 150 mL 0.9% NaCl was given. Blood samples were collected for complete blood count and biochemical analysis before the treatments. The red panda was moved to the quarantine area of the Chengdu Research Base of Giant Panda Breeding for daily treatment of oral cefpodoxime proxetil 50mg. During this period, she did not eat or defecate, and died at 15:00 on November 21.

During gross necropsy, the red panda's skin and fur was intact, no traumatic injuries were observed. Bilaterally, the kidneys were swollen and there were several white pinpoint spots on cutsurface, with white cord-like streaks between the medullary rays. The liver was icteric, with soft and brittle texture, and few dark red ecchymoses on the cut surface. The lungs were dark red with multifocal plaques of black-red hemorrhage. The spleen was swollen and dark red with a small number of black-red plaques. The heart and intestines were intact, with 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 evaluated

for histopathological changes under the microscope (Leica DM4B optics). The use of the animal was approved by the Chengdu Research Base of Giant Panda Breeding Institutional Animal Care and Use Committee (IACUC). After sampling, the body was processed non-hazardously by the company (Chengdu Yongxin Harmless Disposal Co, LTD).

### Isolation of the *E. coli*

Each organ (heart, liver, spleen, lung and kidney) of the red panda was sterilized on the surface with hot scissors, then incised with a sterilized scalpel and the inoculation loop sampled the deep tissue then inoculated Brain Heart Infusion (BHI) Agar through standard streaking technique. After 12-24 hours incubation at 37℃, bacterial colonies were transplanted to MacConkey Agar and finally stored at − 80 ℃ in 20% glycerol-Brain Heart Infusion (BHI) Broth for further characterization.

### DNA extraction and identification

Genomic DNA was individually extracted from the isolates using the TIANamp Bacteria DNA kit according to the manufacturer's instructions (Tiangen Biotech Co., Ltd., Beijing, China). The 16S rRNA gene was amplified by PCR using the universal primer, forward primer (27F): 5′AGAGTTTGATCCTGGCTCAG3′ and reverse primer (1492R): 5′TACGGCTACCTTGTTACGACTT3′ which produced a 1500-bp fragment [24]. After the completion of the amplification, the PCR products were subjected to DNA sequencing (completed by Sangon Biotech (Shanghai) Co, LTD.). The obtained sequences were aligned using NCBI BLAST in Gen Bank to find similar strains and define their species status.

### Antimicrobial susceptibility test

The K-B disk diffusion method was performed, and the antimicrobial susceptibility test was carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI M100 2018 standard). The 30 categories of antimicrobials (Hangzhou Microbial Reagent., Ltd.) were used in this study: piperacillin (100 μg), ampicillin (10 μg), carbenicillin (100 μg), cephalexin (30 μg), cefradine (30 μg), cefazolin (30 μg), cefoxitin (30 μg), cefuroxime (30 μg), cefoperazone (75 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), meropenem (10 μg), piperacilin-tazobactam (100/10 μg), ampicillin-sulbactam (10/10 μg), kanamycin (30 μg), amikacin (30 μg), gentamicin (10 μg), neomycin (30 μg), acheomycin (30 μg), minocycline (30 μg), doxycycline (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), levofloxacin (5 μg), ofloxacin (5 μg), cotrimoxazole (23.75/1.25 μg), furazolidone (300 IU), polymyxin B (300 IU). The *Escherichia coli* ATCC 25922 was invoked as the control organism.

### Pathogenicity test

The mouse test is used as a pathogenicity confirmatory test on the Escherichia coli (E. coli) isolated from the red panda post mortem. 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 IACUC.) were randomly divided into 10 groups. Groups of 10 mice were intraperitoneally

inoculated (0.5 mL) with different bacterial concentration ranging from $1.2\times10^9$ to $2.0\times10^7$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 were immediately necropsied and tissues from the heart, liver, spleen, lung and kidney were taken for bacterial isolation and histopathological 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* isolated from the kidney of red panda was reactivated, and then the culture was enriched in Brain Heart Infusion (BHI) Broth. Genomic DNA was extracted using the TIANamp Bacteria DNA kit according to the manufacturer's instructions (Tiangen Biotech Co., Ltd., Beijing, China).A sample of high-quality, high-molecular-weight DNA was sent to an external laboratory for whole genome 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

**BHI:** Brain Heart Infusion

**IACUC:** Institutional Animal Care and Use Committee

# 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 Institutional Animal Care and Use Committee protocol #2019013.

### 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 been published or submitted anywhere.


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### Authors' contributions

SL and YL designed the work and performed main experimental operation, and were major contributors in writing the manuscript; CY, DZ, XS, XY, KY, XC and GZ performed the necropsy work and acquired the samples. TC and JL participated in the experimental operation; RH and XP verified all the data, figures and materials (including reagents), and proofreading. All authors read and approved the final manuscript.


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# Figures

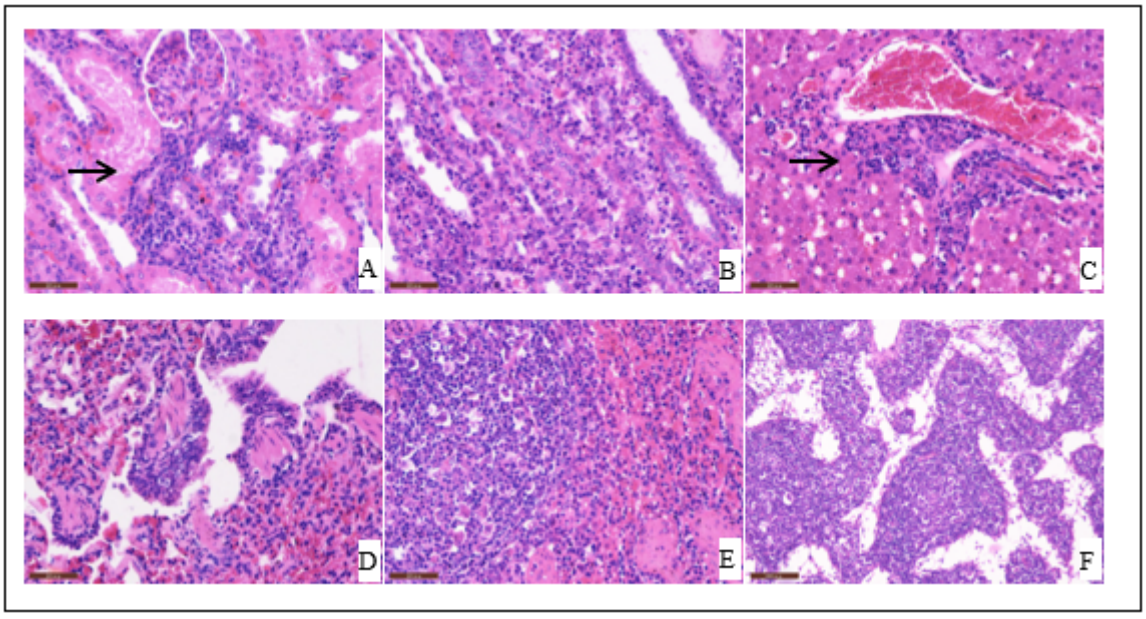


**Figure 1**

Histopathological finding in red panda with UPEC infection. A: Glomerular congestion, epithelial cell necrosis in small renal tubules, and focal infiltration of inflammatory neutrophils and fewer lymphocytes. B: Cord-like lymphoplasmacytic infiltration in the renal medullary interstitium. C: Mild hepatic congestion, perivascular lymphoplasmacytic infiltration, and hepatocellular vacuolar degeneration. D: Thickening, hyperemia, and lymphoplasmacytic expansion of the pulmonary alveolar wall. E: Congestion and hemorrhage in splenic red pulp. F: Eexpansion of the mesenteric lymph node sinuses by lymphoplasmacytic infiltration. H&E, bar = 50μm.

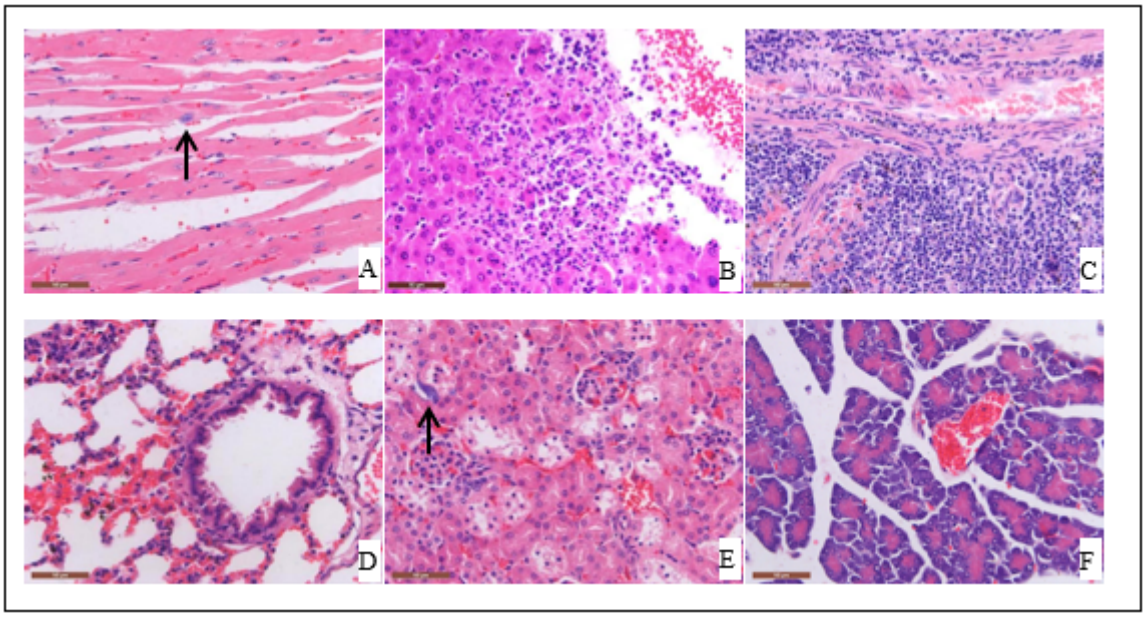


**Figure 2**

Histopathological findings in mice in the pathogenicity study. A: Myocardial interstitial edema with discrete intramyofiber bacterial colony (arrow). B: Focal hepatocellular necrosis, with infiltration of neutrophils, macrophages, and lymphocytes. C: Increased nuclear debris in splenic white pulp. D: Alveolar telangiectasia and hyperemia, with inflammatory cell infiltration of the alveolar wall. E: Renal glomerular congestion, partial tubular epithelial cell necrosis and interstitial bacterial embolus (arrow). F: Pancreatic interstitial edema with mild congestion. H&E, bar = 50μm.

## Supplementary Files

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- Highlights.docx
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