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RESEARCH ARTICLE

Pathogenicity Analysis of *Klebsiella oxytoca* Isolated from *Larus ridibundus* Migratory Birds

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ABSTRACT

Klebsiella oxytoca is one of the most common pathogens causing septicemia, pneumonia, and urinary tract infections in humans and animals and is a major concern for public health. In the present study, we aimed to elucidate the pathogenicity of *K.oxytoca* isolates obtained from *Larus ridibundus* using an in vivo system. The results revealed that the bacteria grew on EMB, lysogeny broth agar, MacConkey medium, nutrient broth, sheep blood agar, and *Salmonella Shigella* agar. *K. oxytoca* from *L. ridibundus* showed highest similarity (96.6%) to the reference strain (AB476819.1). Pathological biopsy revealed that *K. oxytoca* severely damaged hepatocytes, renal tubular epithelial cells, lymphocytes, and alveolar bronchioles in infected mouse tissues. The presence of this pathogen in *L. ridibundus* suggested that infectious diseases may be transmitted via pollution of water, allowing human infection to occur during the migration process. The findings will provide the foundation for the studies on the treatment of *K.oxytoca*.

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INTRODUCTION

Klebsiella bacteria belong to the family Enterobacteriaceae, which is divided into five species, including *K. pneumoniae*, *K. oxytoca*, native *K. terrigena*, *K. planticola*, and deformation *K. preteus.* Of these species, only *K. pneumoniae* and *K. oxytoca* cause infectious diseases in animals. Although *K. pneumoniae* has been well studied (Seyedpour *et al.,* 2014), few reports have described *K. oxytoca*. *K. oxytoca* is a well-known opportunistic pathogen capable of causing several infectious diseases (Högenauer *et al.,* 2006. *K. oxytoca* has been isolated from humans, peacocks, horses, and aquatic environments (Ai *et al.,* 2015; Pereira *et al.,* 2015). However, no reports have described isolation of *K. oxytoca* from *Larus ridibundus*.

L. ridibundus is a species of migratory birds distributed in Eurasia and along the east coast of North America. They migrate to North Africa, India, the Philippines, Japan, and China in the winter. Since November 1985, *L.ridibundus* has been found to migrate from mid-October to mid-April of the following year, following a route along Dianchi, Panlong River, and Green Lake in Kunming, China. Kunming is located in the middle of the Yungui Plateau in southwest China and has a monsoon climate in the plateau mountains of the low latitude subtropics of northern latitudes; the annual temperature difference is small, and the average temperature is 14.6°C. If *L. ridibundus* carries these pathogens, infectious diseases maybe transmitted through water, resulting in infection in humans during the migration process.

Because of our limited knowledge on the pathogenicity of *K. oxytoca* strains from wild birds, in this study, we aimed to characterize *K. oxytoca* strains isolated from the feces of *L. ridibundus*. Additionally, we assessed the pathogenicity of *K. oxytoca.*

MATERIALS AND METHODS

Samples and bacterial isolation: During October and November 2017, we collected yellow feces from *L. ridibundus* in Dianchi, Yunnan province, China. The feces samples were collected from anususing aseptic cotton swabs and placed in sterile EP tubes containing 0.5 mL sterile phosphate-buffered saline. Samples were then transferred to our microbiological laboratory for bacterial analysis. To obtain bacteria, the samples were inoculated

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into nutrient broth at 37°C for 36h, and morphologically different bacterial colonies were then aseptically picked and purified by repeatedly streaking onto fresh agar plates (eosinmethylenebluemedium, EMB), lysogenybroth (LB) agar, MacConkey medium, SS agar medium, and blood agar medium (Sigma Chemical Co., Shanghai, China). The strain was characterized and identified using standard morphological and biochemical tests. To characterize the strain at the molecular level, 16S rDNA sequencing and phylogenetic analyses were also performed. Nucleotide sequence data were deposited in GenBank Nucleotide Sequence Data Libraries. The computer program BLASTN was used to identify the species closest to the sequence of strain 20. Phylogenetic trees were constructed using the neighbor-joining method of MEGA (version 4.0) based on 16S rDNA sequences.

Histopathological examination of the bacteria in infected Balb/c mice: To assess their pathogenicity, the bacteria were evaluated using small animal inoculation and bacterial plate counting. The murine model was challenged with the bacteria. Five-week-old mice weighing between 16 and 20g were obtained from Kunming Medical University. Ten Balb/c mice were inoculated intraperitoneally with 0.5 mL bacteria solution containing 10^8 CFU/mL. Mice were kept under conventional conditions and fed on *K. oxytoca*-free commercial diet. Food and water were supplied ad libitum until the end of the experiment. All mice were examined for gross lesions on the day of death. After gross examination, the tissues (myocardium, spleen, liver, and kidney) were aseptically removed and processed routinely using established methods. The samples were then cultured in MRS medium for isolation, after which they were fixed in 4% paraformaldehyde and embedded in paraffin for histopathological examination. The samples were sectioned at 5-µm thickness and stained with hematoxylin and eosin (HE) for examination under a light microscope.

RESULTS AND DISCUSSION

In this study, we identified *K. oxytoca* isolate from wild *L. ridibundus* in China for the first time. The bacteria isolated on EMB agar plates appeared as small, smooth, blue colonies after 32h; however, all colonies became pink in color after 48h (Fig. 1A) and gave an off odor. The bacteria also grew on LB agar, MacConkey medium, nutrient broth, sheep blood agar medium, and SS agar medium (Fig. 1B-F). Standard biochemical analyses showed that the bacteria were negative for Gran staining; positive for lactose and amylomaltose; positive for V-P, indole, urease, simmons citrate; negative for H_2S, MR, semisolid agar; and positive for amylomaltose, mannitol, sucrose. According to Bergey's Manual of Systematic Bacteriology, these descriptions fitted the characteristics of *K. oxytoca*.

To characterize the bacteria further, the sequencing analysis of 16S rDNA PCR and phylogenetic trees were confirmed. An amplicon of approximately 1500 bp were yielded by PCR amplification (Fig. 2A). The amplification product were sequenced, and the sequence results were analyzed by the BLASTN program. The results showed

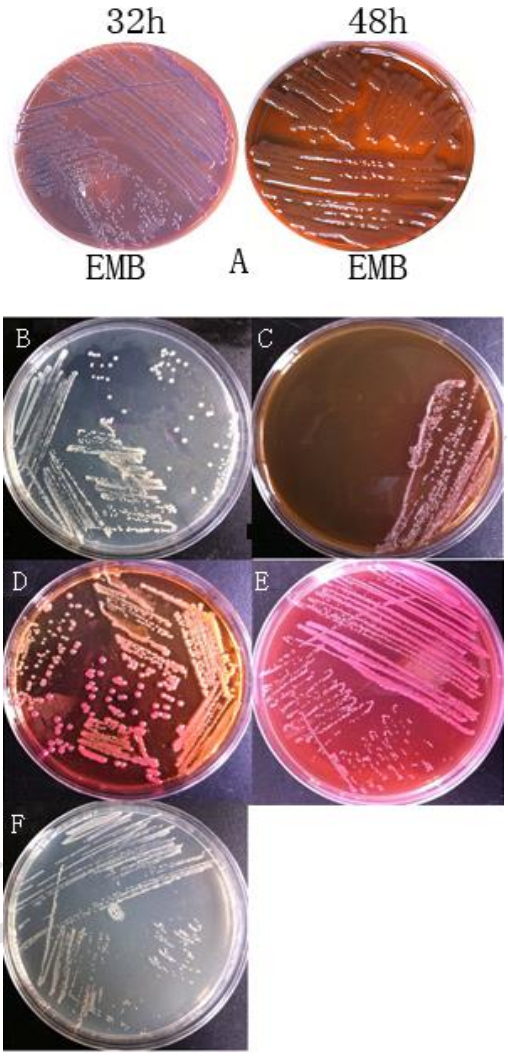

Fig. 1: Culture characteristics of *K. oxytoca* under different conditions. A) Colony morphologies of *K. oxytoca* on EMB medium after incubation for 32 h and on EMB medium after incubation for 48h. B) Nutrient broth agar plates; C) sheep blood agar medium; D) MacConkey medium; E) SS agar medium; F) LB agar.

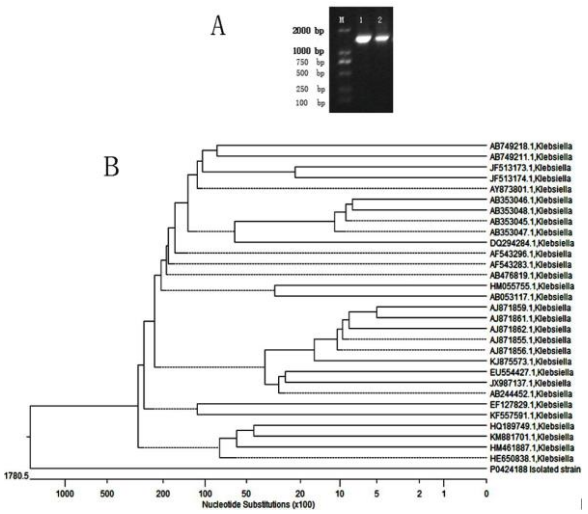

Fig. 2: PCR amplification and phylogenetic tree analysis of *K. oxytoca* 16S rDNA. A) PCR amplification of *K. oxytoca* 16S rDNA. Marker: DNA DL2000; 1–2: amplified product of 16S rDNA of *K. oxytoca* from *L. ridibundus* (about 1500bp). B) Phylogenetic tree of *K. oxytoca* isolated from *L. ridibundus* based on 30 references of the *K. oxytoca* 16S rDNA sequences from different species using the MEGA program.

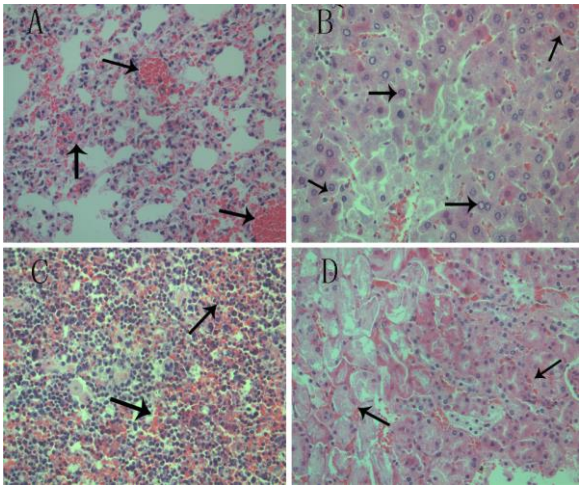

Fig. 3: Histopathological examination in the lungs, liver, spleen, and kidneys of infected mice. A) Congestion of the alveolar wall, epithelial hyperplasia, and interstitial thickening in the lungs. B) Obvious degeneration and necrosis of hepatocytes and destructive hepatic cords in the liver, and congestionof the hepatic sinusoid. C)Serious hemorrhage in the spleen. D) Granular degeneration in renal tubular epithelia, tumefaction and disappearance of lumen, and hemorrhage in the kidneys (The positions of the lesion are indicated by arrows).

that the bacteria were closely related to *K. oxytoca* (96.6% similarity with the reference strain, AB476819.1 and W-2). These results further confirmed the strain as *K. oxytoca*. Phylogenetic results were constructed by the MEGA program together with the reference strain, and the results revealed that *K. oxytoca* isolated from *L. ridibundus* formed an independent cluster (Fig. 2B). Moreover, *K. oxytoca* isolated from different species may have undergone genetic variations, gene mutations, or gene deletions because of changes in the environment; however, further studies are needed to confirm this hypothesis.

L. ridibundus migrate from mid-October to mid-April of the following year, following a route along Dianchi, Panlong River, and Green Lake in Kunming, China. Importantly, the presence of this pathogen in *L. ridibundus* suggested that infectious diseases maybe transmitted via pollution of water, allowing human infection to occur during the migration process. *K. oxytoca* is an opportunistic pathogen in humans, particularly in hospitalized and immunocompromised patients (Sohn *et al.*, 2012; MigliaVacca *et al.*, 2013; Herzog *et al.*, 2014; Leila *et al.*, 2015; Herruzo *et al.*, 2017; Tavakoly *et al.*, 2018). *K. oxytoca* is harmful for human health, and can lead to severe diseases, such as urinary tract infections, pneumonia, and septicemia. *K. oxytoca* should be considered a dangerous pathogenic microorganism. So, in order to analyze the pathogenicity of *K. oxytoca*, the appropriate animal model was established. But *L.ridibundus* had been listed in the preserved animals in China, so we set up the animal model, such as mice.

Histopathology revealed some lesions in the lungs, liver, spleen, and kidneys. Alveolar wall congestion, epithelial hyperplasia, and pulmonary interstitial thickening were observed (Fig. 3A). Significant degeneration and necrosis of hepatocytes, hepatic destructive hepatic cords and hepatic sinusoids are also noted (Fig. 3B). Severe hemorrhage was observed in the spleen (Fig. 3C), and degeneration of tubular epithelial cells, swelling and disappearance of lumen, and hemorrhage were observed in the kidney (Fig. 3D).

At present, vivo analyses revealed severe damage to the lungs, liver, spleen, and kidneys, consistent with the ability of the bacteria to invade the blood system and infect organs, thereby causing major damage throughout body. Accordingly, our findings indicated that *K. oxytoca* should be considered an important organism in veterinary public health. Infectious diseases maybe transmitted via pollution of water, allowing human infection to occur. Our findings will provide the foundation for further analysis of the pathogenesis of *K. oxytoca.* This information will be valuable for controlling this pathogen. Further studies are needed to determine the prevalence of *K. oxytoca* in *L. ridibundus* in order to prevent disease outbreaks in China.

Conclusions: In conclusion, *K. oxytoca* was isolated from *L. ridibundus*. Bacterial isolates have a certain degree of pathogenicity in mice. Further research will confirm the role of the bacteria in the environment, which may provide insights into developing preventive measures for the bacteria.

Authors contribution: CH and DG participated in the design of both studies, performed clinical evaluations and helped to draft the manuscript. DFY, ZSY, LHL, XX helped with study design, and drafted the manuscript. All authors read and approved the final manuscript.

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