Isolation and characterization of Vagococcus carniphilus from diseased crucian carp

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ABSTRACT
The bacterial genus Vagococcus has been described to include eight recognized species: Vagococcus fluvialis, Vagococcus fessus, Vagococcus salmoninarum, Vagococcus lutrae, Vagococcus elongates, Vagococcus penaei, Vagococcus acidifermentans and Vagococcus carniphilus. In this study, V. carniphilus was isolated from the myocardium, liver, spleen and kidney tissues of diseased crucian carp. After isolation, biochemical, 16S rDNA gene sequence and phylogenetic analysis was performed. Next, the phenotypic and genomic characteristics were analyzed using different biochemical tests and antibiotic susceptibility testing. The bacterial isolate was gram-positive. The biochemical testing results indicated that a positive reaction occurred in the tubes containing glycerol, galactose, mannitol, sucrose sorbitol and D-ribose, and a negative reaction with α-galactosidase, β-galactosidase and hydrogen sulphide. A DNA product (~1500 base pairs) was amplified from 16S rDNA. BLAST analysis revealed that V. carniphilus from crucian carp was highly similar to V. carniphilus isolated from other sources and showed the highest similarity (95%) with reference strains. Additionally, pathological analysis revealed that infected crucian carp tissues (myocardium, liver, spleen and kidney) and mouse tissues (myocardium, lung, liver, spleen and kidney) were severely damaged. In summary, we isolated V. carniphilus from diseased crucian carp, and this isolate induced pathological changes in crucian carp and mice.

Introduction
The bacterial genus Vagococcus was proposed and classified as a member of the family Enterococcaceae [1]. There are eight currently recognized species in the genus Vagococcus. Different species have been isolated from various sources, including Vagococcus fluvialis from pigs, cats, cattle and Culex quinquefasciatus mosquito [2,3]; human clinical samples such as blood, peritoneal fluid and wounds [4] and a root-filled tooth with periradicular lesions [5]; and V. fessus from a seal and harbour porpoise [6]; V. salmoninarum from diseased fish [7,8]; V. lutrae from the common otter (Lutra lutra) [9]; V. elongates from a swine-manure storage pit [10]; V. penaei from shrimp [11]; V. acidifermentans from an acidogenic fermentation bioreactor [11] and V. carniphilus from ground beef [12] and oil palm [13]. However, the pathogen characteristics of V. carniphilus have not been widely examined.

In the present study, we report the first systemic examination of V. carniphilus isolated from crucian carp (Carassius auratus). V. carniphilus was isolated from crucian carp and the morphological and biochemical characteristics were identified. After V. carniphilus was isolated, the 16S rDNA was amplified and phylogenetic analysis was carried out. Then, histopathological examinations of the crucian carp and infected mice were performed. The findings of this study may provide a basis for future studies on the treatment of infections caused by V. carniphilus.

Materials and methods
Sampling procedure and isolation
Samples were collected from the myocardium, liver, spleen and kidney tissues of diseased crucian carps from the aquaculture farm in Yunnan province. The samples were cut into 0.6 cm × 0.6 cm sections, and immersed in sterilized saline water. The samples were inoculated into nutrient broth and incubated at 37 °C for 15 h. After incubation, the bacterial liquid was plated on blood agar, chocolate agar, BBL agar, TPY agar, MRS agar, tryptic soy agar, eosin methylene blue agar, MacConkey agar,
mannitol agar and sodium chloride agar using streak cultivation. The plates were then incubated at 37 °C for 15 h.

**rDNA polymerase chain reaction (PCR) amplification and phylogenetic analysis**

Genomic DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). Next, the 16S rDNA gene was amplified by PCR using universal eubacteria-specific primers, F: AGAGTTTGATCATGGCTCA and R: ACGGTTACCTTGTTACGACT, according to a previous study [14]. The purified PCR products were then sequenced by Sangon Biotechnology Co. Ltd. (Shanghai, China). Finally, the 16S rDNA sequence was edited and compared to currently available microbiological sequences in GenBank. A phylogenetic tree was constructed using the neighbour-joining method of the MEGA program as reported previously [3].

**Histopathological examination**

Infected tissues (myocardium, spleen, liver, kidney) from crucian carp were fixed in 4% paraformaldehyde and embedded in paraffin for histopathological examination. To assess their pathogenicity, the bacteria were evaluated using small animal inoculation and bacterial plate counting. The murine model was challenged with the bacteria. Ten Balb/c mice from the Animal Lab Center of Kunming Medical University in Yunnan province were inoculated intraperitoneally with 0.5 mL bacterial solution containing $10^8$ CFU/mL. All sampling was accomplished according to the Animal Ethics Procedures and Guidelines of the People's Republic of China and Animal Care Guidelines. 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 [15]. The sections were stained with haematoxylin and eosin (HE) stain at room temperature, and examined under a microscope (Motic BA310 Digital).

**Results and discussion**

**Isolate identification**

The bacterial isolate was incubated on plates containing different media. White colonies were observed on the

![Figure 1](image-url). Growth of bacteria on different media and colony morphology following incubation for 15 h: (A) blood agar plate (white colonies); (B) chocolate agar plate (white colonies); (C) BBL agar plate (orange colonies); (D) TPY agar plate (light yellow colonies); (E) MRS agar plate (orange colonies); (F) tryptic soy agar plate (ivory white colonies); (G) eosin methylene blue agar plate (white colonies); (H) MacConkey agar plate (no crystal violet, pink colonies); (I) MacConkey agar plate (crystal violet, pink colonies).
blood agar plate (Figure 1(A)), chocolate agar plate (Figure 1(B)) and eosin methylene blue agar plate (Figure 1(G)). There were orange colonies on the BBL agar plate (Figure 1(C)) and MRS agar plate (Figure 1(E)). On the TPY agar plate, there were light yellow colonies (Figure 1(D)), and on the tryptic soy agar plate, the colonies were ivory white (Figure 1(F)). Pink colonies were observed on the MacConkey agar plate without crystal violet (Figure 1(H)) and with crystal violet (Figure 1(I)). However, no colonies were observed on the mannitol or sodium chloride agar plates.

Standard biochemical testing indicated that the bacterial isolate was gram-positive. A positive reaction was observed with glycerol, galactose, mannitol, sucrose, sorbitol and D-ribose, while a negative reaction was observed in α-galactosidase, β-galactosidase and hydrogen sulphide. PCR amplification yielded an amplicon of approximately 1500 base pairs (Figure 2). BLAST (Basic Local Alignment Search Tool) analysis showed that the sequence had maximum similarity (95%) with the reference strains (V. carniphilus, GenBank: KF767902.1, and V. teuberi, GenBank: FJ526386.1), indicating that our V. carniphilus isolate was closely related to the species V. carniphilus. This close relationship was further confirmed by phylogenetic clustering. Consistent with previous reports [12,19], we also found that V. carniphilus was gram-positive. Our isolate was positive for glycerol, galactose, mannitol, sucrose, sorbitol and D-ribose and negative for α-galactosidase, β-galactosidase and hydrogen sulphide, corresponding to the species description of V. carniphilus in Bergey’s Manual of Systematic Bacteriology [20].

**Histopathological analysis**

Histopathological examination of the infected crucian carps revealed obvious degeneration and necrosis of hepatocytes and hepatic cord destruction in the liver, as well as hepatic sinusoidal congestion (Figure 4(A,B)). The renal capsule was not present in the kidney; in addition, there was granular degeneration in the renal tubular epithelia, tumefaction and disappearance of the lumen, and presence of renal interstitial haemorrhaging with neutrophil infiltration (Figure 4(C,D)). Haemorrhaging in the
myocardium and necrosis of cardiac myocytes with inflammatory cell infiltration were observed (Figure 4(E, F)). Finally, there was serious haemorrhaging in the spleen and necrosis of lymphocytes (Figure 4(G,H)).

Histopathological observations of infected crucian carp revealed that the isolated strain had severely damaged the myocardium, liver, spleen, kidney and lung tissues. In a previous study, Enterococci were found to be involved in pathogenic infections and the transmission of food contamination [21]. The bacterial community may play essential roles in the developmental stages of pathogens. For example, the malaria transmission cycle may be affected by the presence of gram-negative bacteria, which promote the formation of malaria parasites.
However, although the specific clinical manifestations of *V. carniphilus* in humans have not yet been verified, this species may be transmitted from food-producing animals to humans through the food supply chain [23]. *V. carniphilus* should be considered a dangerous pathogenic microorganism in veterinary public health.

Therefore, we choose an animal model to study the virulence and pathogenicity of *V. carniphilus*. At present, in the study of human diseases, mice are the most commonly used animal models. In the small animal inoculation test, infected mice exhibited lethargy, tussled fur and poor appetite. Four mice died at 6 h, while the others died at 48 h. After the mice were dissected, bleeding sites were found on the surface of the liver and lungs. The infected mice exhibited obvious congestion of the alveolar wall, epithelial hyperplasia and inflammatory exudation in the alveolar space of the lung (Figure 5A); hepatic sinusoidal congestion, obvious degeneration and necrosis of hepatocytes, and hepatic cord destruction in the liver (Figure 5B); serious haemorrhaging in the spleen, necrosis of lymphocytes and splenic infarction (Figure 5C); fracturing of myocardial fibres and haemorrhaging in the myocardium (Figure 5D); and renal interstitial haemorrhaging, granular degeneration and necrosis in the renal tubular epithelia and tumefaction and disappearance of the lumen (Figure 5E).

These results of infected mice revealed that there were obvious degeneration and necrosis of the hepatocytes and destruction of the hepatic cord in the liver tissues, serious haemorrhaging in the spleen tissues, granular degeneration in the renal tubular epithelia, haemorrhaging in the kidney tissues and congestion of the alveolar wall in the lung tissues. Our findings provide a basis for additional studies of the pathogenesis of *V. carniphilus*. The results of the murine model showed that
the isolated bacteria may infect mammal animals. *V. car- niphilus* should be considered a dangerous pathogenic microorganism in veterinary public health. Thus, the transmission through the oral/food-borne or other route needs to be explored in future studies.

**Conclusions**

In this study, *V. carniphilus* was isolated from diseased crucian carp. The bacterial isolate was gram-positive and had some degree of pathogenicity to crucian carp and mice. Further studies are required to determine the roles of this bacterial species in the environment, and understanding these roles may provide insight into the development of preventive measures against this species.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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