Research Article

Secondary Bacterial Infections Associated with Koi Herpesvirus Disease Outbreak in Common Carp (*Cyprinus carpio* L.) in Al-Najaf Province, Iraq

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Abstract | Alongside Koi herpesvirus (KHV) infection, secondary bacterial diseases can significantly increase mortality rates in affected fish populations. This study aimed to investigate and identify secondary bacterial infections, particularly *Aeromonas* species and other gram-negative bacteria, associated with KHV outbreaks in common carp (*Cyprinus carpio* L.). A total of 40 infected common carp specimens were collected from five cage system farms located along the Euphrates riverbed in Al-Najaf Governorate, Iraq, with each farm providing eight fish. For bacterial isolation, tissue samples were first incubated in ampicillin-supplemented nutrient broth to suppress non-target bacterial growth, and then cultured on bacteriological media. Macroscopic examination of the infected fish revealed brown to crimson hemorrhagic lesions on the skin and widespread gill damage, characterized by pale white to gray discoloration and filament degradation. Microscopically, colonies of *Aeromonas hydrophila* and *Aeromonas sobria* displayed clear zones of β-hemolysis on sheep blood agar, while *Aeromonas caviae* formed non-hemolytic colonies. On MacConkey agar, *Aeromonas* species produced pale, lactose-negative colonies. Further identification using the VITEK® 2 systems confirmed the presence of three *Aeromonas* species (*A. sobria*, *A. hydrophila*, and *A. caviae*) along with two other gram-negative bacteria: *Raoultella ornithinolytica* and *Shewanella putrefaciens*. This study highlights the role of secondary bacterial infections, particularly *Aeromonas* species and other gram-negative bacteria, in exacerbating the mortality associated with Koi herpesvirus outbreaks in common carp, underscoring the need for integrated disease management and improved biosecurity in aquaculture systems.

Keywords | *Aeromonas* spp, Koi herpesvirus, VITEK-2 system, Isolation, Culture

Received | March 13, 2025; **Accepted** | May 25, 2025; **Published** | July 10, 2025
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Citation | Kane AM, Al-Muhana IR, AbdulAmeer AK, Al-Aqbi S (2025). Secondary bacterial infections associated with koi herpesvirus disease outbreak in common carp (*Cyprinus carpio* L.) in Al-Najaf Province, Iraq. J. Anim. Health Prod. 13(3): 660-665.
DOI | https://dx.doi.org/10.17582/journal.jahp/2025/13.3.660.665
ISSN (Online) | 2308-2801

INTRODUCTION

Cyprinid herpesvirus 3 (CyHV-3), as identified by Boutier *et al.* (2019), is the causative agent of Koi herpesvirus disease (KHVD), a severe illness affecting both wild and cultured common carp populations (*Cyprinus carpio* L.). With mortality rates reaching up to 80%, KHVD has prompted extensive research into its biology, epidemiology, and transmission due to the substantial economic impact on the aquaculture industry (Monaghan *et al.*,

2015). Infected fish are significantly more susceptible to secondary infections caused by bacteria, parasites, or fungi, which can further elevate mortality at the population level (Rakus *et al*., 2013).

Aeromonas species typically cause infections in common carp following host damage or stress responses triggered by viral illnesses (Chen *et al*., 2019). Widely distributed in aquatic environments and soils, *Aeromonas* spp. is gram-negative, non-spore-forming, rod-shaped, facultative anaerobic bacteria (Daskalov, 2006). The genus includes both motile and non-motile species; motile aeromonads belong to a mesophilic group primarily represented by *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii*, while *A. salmonicida* is classified as a psychrophilic, non-motile aeromonad (Fernández-Bravo and Figueras, 2020). According to Figueras and Beaz-Hidalgo (2015), these opportunistic pathogens can lead to significant epidemic outbreaks, often triggered by stressors such as poor water quality, overcrowding, or improper handling.

The primary causative agent of the ulcerative condition known as hemorrhagic septicemia is *A. hydrophila*, which manifests as red skin sores in infected fish (Khamees *et al*., 2013). *A. hydrophila* induces septicemia primarily through the production of two key virulence factors: extracellular hemolysin and aerolysin (Nordmann and Poirel, 2002). Recent studies have also identified *Shewanella putrefaciens* as a novel etiological agent responsible for major health issues in fish, a condition now recognized as Shewanellosis (Müller *et al*., 2023). Additionally, the ingestion of *Raoultella ornithinolytica*, an uncommon histamine-producing bacterium found in fish, has been associated with food poisoning in humans (Feng *et al*., 2016).

This study investigated the distribution of bacterial infections in common carp (*Cyprinus carpio* L.) in Al-Najaf Province, Iraq. Specifically, it aimed to isolate and identify *Aeromonas* species and other gram-negative bacteria associated with septicemia in fish affected by KHVD along the banks of the Euphrates River.

MATERIALS AND METHODS

Subject of Study

To investigate and identify secondary bacterial infections associated with KHV in Iraqi common carp (*Cyprinus carpio* L.), the Faculty of Veterinary Medicine at the University of Kufa, located in Najaf Province conducted a comprehensive survey of infected fish between October and November 2022. A total of 40 infected common carp specimens were collected from five cage system farms located along the Euphrates riverbed in Al-Najaf Governorate, Iraq, with each farm providing eight fish.

Viral Detection

Koi herpesvirus (KHV) was detected using reverse transcription polymerase chain reaction (RT-PCR), based on protocols developed during previous investigations of KHV outbreaks in Iraq.

Clinical Signs and Postmortem

Clinical manifestations in infected fish were evaluated through direct visual inspection, with a focus on external signs affecting the skin, gills, fins, and eyes. Post-mortem examinations were conducted to facilitate a more comprehensive assessment. During necropsy, tissue samples were systematically collected from multiple organs, including the skin, gills, fins, liver, kidneys, and spleen. These samples were then processed for bacterial isolation and identification.

Culture and Biochemical Tests

Fish infected with KHV were subjected to bacterial culture and biochemical identification. Samples were aseptically collected from the gills, kidneys, liver, spleen, and skin using sterile cotton swabs. These were inoculated into nutrient broth containing ampicillin to suppress contaminating flora and incubated at 37°C for 24 hours, following the method described by Mama *et al*. (2014). After incubation, samples were streaked onto blood agar and MacConkey agar plates. Suspected bacterial colonies were then selected and further cultured on triple sugar iron (TSI) agar.

For biochemical identification, isolates were analyzed using the VITEK® 2 system (BioMérieux, France) with a GN (Gram-negative) card. This automated system identified 135 taxa, representing the most clinically relevant fermenting and non-fermenting Gram-negative bacilli.

Data Analysis

Data were initially entered into Microsoft Excel spreadsheets. The percentage incidence of each bacterial species across the various farms was then calculated based on the number of isolates identified in the collected samples.

RESULTS

Pathological Findings

Macroscopic lesions were observed in the skin, gills, fins, liver, kidneys, and spleen of the affected fish (Figure 1). The skin exhibited widespread hemorrhaging, including petechial hemorrhages extending from the gills to the caudal fins. Upon necropsy, the liver, kidneys, and spleen appeared markedly swollen and congested, and muscle hemorrhages were also evident. In several fish, gill tissues showed signs of bleeding, while others exhibited necrosis characterized by the dissolution of gill filaments. Cutaneous lesions ranged in color from white to gray, indicative of skin layer

degradation. In some cases, necrotic areas along the lateral body surfaces were accompanied by scale loss.

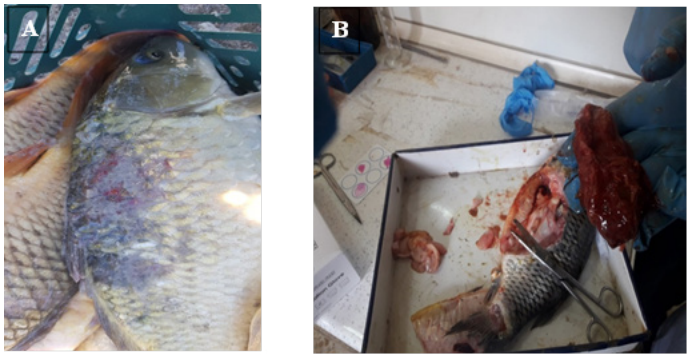

Figure 1: Koi herpesvirus-infected *Cyprinus carpio*. **A:** White lesions and sloughing of the skin observed on the head and lateral body surfaces; **B:** Marked enlargement and congestion of internal organs, accompanied by muscular hemorrhages.

Microbiological Results

To isolate bacteria, tissue samples from lesioned organs including the skin, gills, liver, kidneys, and spleen were cultured on blood agar and MacConkey agar. Biochemical analyses were then performed to identify bacterial colonies that developed on the media, enabling the differentiation and identification of pathogens associated with the lesions.

The bacteriological findings revealed five gram-negative bacteria, consisting of three Aeromonas species (*A. sobria, A. hydrophila,* and *A. caviae*), as well as *Raoultella ornithinolytica* and *Shewanella putrefaciens*. On sheep blood agar, *A. hydrophila* and *A. sobria* exhibited β-hemolytic activity, producing clear zones around the colonies with a coloration ranging from pale white to grey. In contrast, *A. caviae* demonstrated no hemolytic activity on sheep blood agar. On MacConkey agar, Aeromonas species formed pale colonies, indicating their inability to ferment lactose (Figure 2).

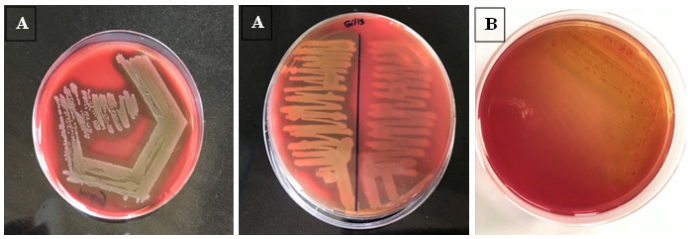

Figure 2: *Aeromonas hydrophila* growth on culture media. **A:** Distinct β-hemolytic colonies of *A. hydrophila* on sheep blood agar after 48 hours of incubation; **B:** Pale, non-lactose-fermenting colonies of *A. hydrophila* on MacConkey agar.

The study findings exhibited that among 40 samples *Aeromonas* spp. was found 52.5% samples. Similarly, the incidence of *R. ornithinolytica* and *S. putrefaciens* was recorded in 22.5% and 25% samples respectively (Table 1).

Table 1: Number and percentage incidence of bacterial species in fish samples collected from various farms infected with koi herpesvirus (KHV).

Bacterial Species	No (%) incidence of bacteria					
	Farm number*					
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Total
Aeromonas spp.	6 (75)	2 (25)	6 (75)	3 (37.5)	4 (50)	21 (52.5)
Raoultella ornithinolytica	2(25)	0 (0)	3 (37.5)	2 (25)	2 (25)	9 (22.5)
Shewanella putrefaciens	3 (37.5)	2 (25)	3 (37.5)	2 (25)	0 (0)	10 (25)

* n= 8 collected from each farm.

Biochemical Findings

The VITEK® 2 system identified five Gram-negative bacterial species among the isolates. These included three *Aeromonas* species: *Aeromonas sobria* (98% probability), *Aeromonas hydrophila* (98%), and *Aeromonas caviae* (98%), along with *Raoultella ornithinolytica* and *Shewanella putrefaciens*.

Biochemical characterization revealed that all *Aeromonas* isolates were hydrogen sulfide (H_2S) negative and capable of utilizing citrate and fermenting mannitol. In contrast, *R. ornithinolytica* and *S. putrefaciens* tested positive for H_2S production. All *Aeromonas* isolates fermented glucose, maltose, and sucrose, but lacked both gelatinase and ornithine decarboxylase activity. Conversely, *R. ornithinolytica* demonstrated positive ornithine decarboxylase activity (Table 2) and also fermented glucose, maltose, and sucrose.

DISCUSSION

Aeromonas septicemia is the primary disease impacting carp farms, as reported by Garabowicz *et al.* (2022). Figueras and Beaz-Hidalgo (2015) identified and isolated pathogenic bacteria from five floating cage farms along the Euphrates River bank. These bacteria pose a significant public health risk when consumed and can spread rapidly among fish populations, leading to severe illness and hemorrhagic septicemia.

In our investigation, affected fish exhibited pronounced skin hemorrhaging and petechial bleeding, particularly in the ventral region, extending from the gills to the caudal fins. Additional findings included white to gray patches on the skin, gill bleeding, and areas of necrosis, especially in the gills, characterized by the dissolution of gill filaments. Several factors were found to influence disease progression, including the virulence and concentration of the virus, environmental conditions, seasonal variations, host stress levels, and the viral replication sites within host tissues (Monaghan *et al.*, 2015).

Table 2: Biochemical characteristics of bacterial isolates of fish samples infected with koi herpesvirus using the VITEK 2 system.

Biochemical characteristics	Aeromonas Sobria	Aeromonas hydrophila	Aeromonas caviae	Raoultella ornithinolytica	Shewanella putrefaciens
APPA	+	-	-	-	+
H_2S	-	+	+	+	+
BGLU	-	+	+	-	-
ProA	+	+	+	-	+
SAC	+	-	-	+	-
GlyA	-	-	-	-	+
ADO	-	-	-	-	-
dMAL	+	+	+	+	+
LIP	-	+	+	-	-
dTAG	-	-	-	-	-
ODC	-	-	-	+	-
PyrA	-	-	-	+	+
AGLTp	-	-	-	-	+
dMAN	+	+	+	+	-
dMNE	+	+	+	+	-
TyrA	+	+	+	+	+
CIT	+	-	-	+	-
IHISa	-	-	-	-	-
URE	-	-	-	+	-
ILATa	-	-	-	-	-
dSOR	-	-	-	+	-
OFF	+	+	+	+	-

Abbreviations: APPA: Phe-Pro arylamidase; H_2S: hydrogen sulfide production; BGLU: β-glucosidase; ProA: L-proline arylamidase; SAC: saccharose (sucrose); GlyA: glycine arylamidase; ADO: adonitol; dMAL: D-maltose; LIP: lipase; dTAG: D-tagatose; ODC: ornithine decarboxylase; PyrA: L-pyrrolydonyl arylamidase; AGLTp: glutamyl arylamidase pNA; dMAN: D-mannitol; dMNE: D-mannose; TyrA: tyrosine arylamidase; CIT: citrate (sodium); IHISa: L-histidine assimilation; URE: urease; ILATa: L-lactate assimilation; dSOR: D-sorbitol; OFF: glucose fermentation. + positive; - negative.

These observations align with the findings of Bergmann *et al.* (2020), who reported extensive skin necrosis, petechial to severe hemorrhaging of the skin and fins, excessive mucus discharge, and a rough, sandpaper-like skin texture resulting from severe gill necrosis.

Necropsy findings in this study revealed marked congestion and enlargement of the kidneys, spleen, and liver. Additional external signs included white to gray patches, gill bleeding, and necrosis in both the skin and gill tissues, with notable dissolution of gill filaments. According to Haenen *et al.* (2004), Koi herpesvirus (KHV) is disseminated via white blood cells from the gills and intestines to other organs, with extensive viral replication occurring within the interstitial tissues of the kidney. The virus further spreads through the bloodstream and infected organs, facilitating systemic infection.

The role of *Aeromonas* spp. in co-infection is significant, as these bacteria enhance pathogenicity through the production of virulence factors such as hemolysins, aerolysins, leukocidins, cytotoxins, and enterotoxins (Yu *et al.*, 2015). These factors contribute to ulcerative skin lesions, fin rot, splenic tissue damage, renal tubular necrosis, liver congestion and enteritis, thereby increasing mortality in infected fish. Our findings are consistent with previous studies by Al-Salih *et al.* (2020) and Panicz *et al.* (2019), which documented similar pathological outcomes.

In line with observations from earlier KHV outbreaks, this study also demonstrated necrosis in the kidneys, liver, and gills, along with hemorrhaging in internal organs and skin, supporting the established understanding of KHV pathology and secondary bacterial complications.

Our bacterial culture analysis identified three *Aeromonas* species *A. sobria*, *A. hydrophila*, and *A. Caviae* among the Gram-negative bacteria isolated from fish samples. These results corroborate previous studies that recognize these species as pathogenic to fish and other animals (Aravenarromán *et al.*, 2014; Li *et al.*, 2019). Notably, while *A. caviae* exhibited no hemolytic activity, *A. hydrophila* and *A. sobria* demonstrated β-hemolysis by producing clear zones around colonies on sheep blood agar. These findings align with those of Sadique *et al.* (2021), who reported that 74% of *Aeromonas* strains, including *A. hydrophila* and *A. sobria*, were β-hemolytic, whereas 14%, including *A. caviae*, were α-hemolytic. The hemolytic activity of *Aeromonas* species is attributed to the virulence factor hemolysin (hlyA) (Mangoudehi *et al.*, 2020).

In addition to *Aeromonas* spp, our study identified two other Gram-negative bacteria: *Raoultella ornithinolytica* and *Shewanella putrefaciens*. *R. ornithinolytica* is commonly found in aquatic environments (Park *et al.*, 2011; Dang *et al.*, 2020) and is an uncommon histamine-producing bacterium associated with food poisoning upon fish consumption (Feng *et al.*, 2016; Mooraki and Sedaghati, 2019). Although *S. putrefaciens* is rarely considered a major fish pathogen, it can induce lesions in the digestive tract and skin (Kozińska and Pekala, 2004; Paździor, 2016). The first reports of fish infections caused by *S. putrefaciens* in marine life were documented by Korun *et al.* (2009). Disease outbreaks linked to *S. putrefaciens* in freshwater fish—including common carp (*Cyprinus carpio* L.), rainbow trout (*Oncorhynchus mykiss*) and silver carp (*Hypophthalmichthys*

molitrix) were first reported in Poland in 2004 (Kozińska and Pękala, 2004). Since then, significant health issues associated with *S. putrefaciens* infections have been reported in various freshwater fish species (Qin *et al*., 2012; Pękala *et al*., 2015; Rusev *et al*., 2016).

Previous studies have reported that *Aeromonas* spp. induce skin lesions characterized by hemorrhagic effects in both freshwater and farmed fish (Skwor *et al*., 2014; Gao *et al*., 2016; Al-Haider *et al*., 2019).

CONCLUSIONS AND RECOMMENDATIONS

This study focused on the significance of secondary bacterial infections in fish infected with Koi herpesvirus. Among these, *Aeromonas* spp. is considered some of the most critical bacterial pathogens affecting common carp, causing severe infections and hemorrhagic septicemia. Additionally, such infections pose a considerable public health risk. For example, the consumption of fish contaminated with *Raoultella ornithinolytica* can result in food poisoning. Moreover, fish have suffered major health issues linked to *Shewanella putrefaciens*, which causes a disease known as shewanellosis. Our results highlighted the importance of integrated disease management and enhanced biosecurity in aquaculture.

ACKNOWLEDGMENTS

Express our gratitude to the staff of the Faculty of Veterinary Medicine at the University of Kufa, particularly those in the Department of Pathology and Fish diseases and the Department of Microbiology. Also thank the owners of fish farms for facilitate the samples collection.

NOVELTY STATEMENT

The study was undertaken to ivestigate secondary bacterial infections associated with the initial outbreak of Koi Herpesvirus (KHV) disease in Common Carp (Cyprinus carpio L.) in Al-Najaf Province, Iraq. This outbreak has resulted in significant economic losses and presents a considerable public health concern.

AUTHOR'S CONTRIBUTIONS

Ali. M. and Ismael Raheem have planned carried out the experiments Both of them took the lead in writing the manuscript.
Ahzan. K., Murtadha Abbas. and Saif. Al AQBI contributed to samples preparation and interpretation of the results.
Ali. M. and Ismael Raheem took the lead in writing the manuscript.
All authors provided critical feedback and helped shape the research, analysis and manuscript.

Conflict of Interest

The authors declare no conflicting interests.

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