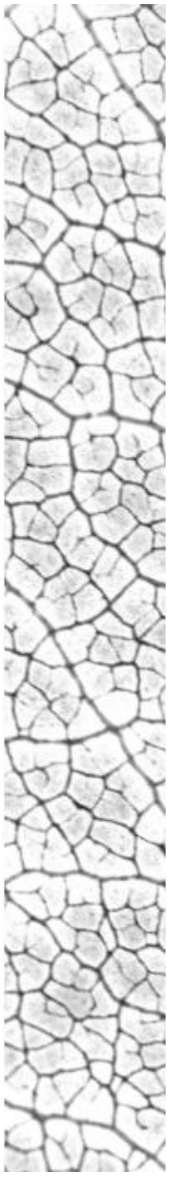

EurAsian Journal of BioSciences
Eurasia J Biosci 14, 1965-1971 (2020)

Koi herpesvirus disease outbreak in Iraq

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Abstract
Iraqi fish breeding industry suffered a severe fatal outbreak in September and October 2018, where the high mortality rate was observed in Iraqi fish farms especially that depended on cage system breeding. The current study was conducted to investigate and diagnose the phenomena of high mortality in September and October 2018. Twenty-five affected common carp fishes were collected from five fish farms use cages system (five fishes each) on Euphrates River banks. Affected fish samples were analyzed via gross. Histopathology, PCR for viral detection, bacteriology and mycology techniques. The high mortality rate was observed in the all affected farms, which reach to 80% of farms fishes. Grossly, hemorrhage patches in skins of affected fishes were observed. Congestion and hemorrhage were observed in liver, kidneys, spleen and muscle. Also, erosion of gills filament with presence of white to gray patches in affected gills were observed. Histopathology results showed necrosis lesion in gills, liver and kidneys cells with presence of hemorrhage. The qPCR results for virus detection revealed that all tested samples were positive for Koi Herpes virus (KHV). The bacteriology results showed presence of five gram negative bacteria including three species of Aeromonas, Raoultella ornithinolytica and Shewanella putrefaciens. The mycology results showed the presence of *Aspergillus fumigatus* and *Mucor* spp. In conclusion, according to results the high mortality outbreak in fish that occurred in September and October 2018, Iraq was due to Koi Herpes virus infection with presence of secondary bacterial or fungal infection.

Keywords: 2018 fishes outbreak in Iraq, Common carp, Koi Herpes virus, Bacterial secondary infection

Al-Salih H, Kane AM, Al-Dabhawi AH, Al-Rufaii HM, Mansour AO, Samaka HM (2020) Koi herpesvirus disease outbreak in Iraq. Eurasia J Biosci 14: 1965-1971.

INTRODUCTION

Iraqi fish breeding industry suffered a severe fatal outbreak in September and October 2018, where the high mortality rate was observed in Iraqi fish farms especially that depended on cage system breeding. Fish breeding in Iraq has recently shown an increase in quality and quantity in breeding techniques, which accompanied by the establishment of new fish ponds using new breeding techniques including breeding in cages using banks of rivers (Al-Mahmood et al. 2018). Common carp Common carp (*Cyprinus carpio*) is a widely cultivated freshwater fish, originated from Eastern Europe and Central Asia. This fish species has been domesticated and introduced worldwide into freshwater environments for aquaculture (Rathore and Kumar, 2012). Common carp considered the most common type of fish bred in Iraq, where the common carp has several properties including the rapid rate of weight gain as well as resistance to many pathogens made it a suitable choice for breeding in the aquatic environment of Iraq (Kitto et al. 2013).

In 1998, a koi viral disease outbreak was first noticed in cultured common carp in Israel and USA (Hedrick et al. 2000, Pokorova et al. 2005) and in Germany in 1997-1998 (Bretzinger et al. 1999). This disease has been termed as Koi herpes virus disease (KHVD), and the disease causing agent was identified as Koi herpes virus (KHV) also named as *cyprinid herpesvirus 3* (CyHV-3) (Klafack et al. 2019). Koi virus causes a high rate of mortality in carp aquaculture to reach 80% losses (Klafack et al. 2019; Ugwu, et al, 2017).

Together with carp pox virus (*Cyprinid herpesvirus 1*), goldfish herpesvirus (*Cyprinid herpesvirus 2*), and eel herpesvirus (*Anguillid herpesvirus 1*), koi virus classified as a member of the genus *Cyprinivirus* in the family, *Alloherpesviridae* (Waltzek et al. 2009; Reichert et al. 2019). The primary target of KHV is respiratory epithelial cells of the gill lamellae, and release of virions from infected epithelial cells resulted in a systemic infection

Received: July 2019
Accepted: April 2020
Printed: June 2020

Fig. 1. Photographs of *Cyprinus carpio* fish farms A) Common carp fish farms that used the cages system on Euphrates river. B) Dead fishes were floated in cages, the daily mortality

affecting the kidney, spleen, heart, brain and liver (Pikarsky et al. 2004; Miyazak et al. 2008; Rahmati-Holasoo et al. 2016; Panicz et al. 2019). The current study was conducted by an academic specialist team in faculty of veterinary medicine, University of Kufa to investigate and diagnose the phenomena of high mortality in September and October 2018 using the gross, histopathology, bacteriology, mycology and PCR techniques.

MATERIALS AND METHODS

Twenty-five affected common carp (Cyprinus carpio) fishes were collected from five fish farms use cages system (five fishes each) on Euphrates River banks in the area extended between the Al-Hindiya barrage and Al-Kufa barrage.

Gross and Histopathology

Upon arrival, case history was documented and fishes were examined grossly to determine any gross pathological changes, immediately after necropsy. For histopathology, gills, kidneys and liver tissue samples were collected and fixed with 10% formalin for 48 hours. Tissue samples were processed for dehydration and embedded in paraffin wax. Tissue samples were sectioned at 4 µm in thickness and mounted on glass slides. The tissue slides were stained with eosin and hematoxylin stain following the standard procedure.

Virus Detection

Gills, kidneys and liver samples were collected and inoculated on FTA card and send to European reference laboratory for fish disease and crustacean, technical aquatic resources, national institute of diseases, University of Denmark, Denmark. The Quantitative PCR technique were used for virus detection. The following qPCR assay were used: Forward primer (KHV-86f): 5′-GACGCCGGAGACCTTGTG-3′; Reverse primer (KHV-163r): 5′- CGGGTTCTTATTTTTGTCCTTGTT-3′; and probe (KHV-109p): 5′-FAM-CTTCCTCTGCTCGGCGAGCACG-3′. Cycling conditions: one cycle of 95 °C at 15 minutes, followed by 40 cycles of 94 °C at 15 seconds and 60 °C for 60 seconds. Negative template controls and positive controls were included on each plate run (Official Journal of the European Union, L 247/1 2015; Manual of Diagnostic Tests for Aquatic Animals 2019).

Bacteriology

The swaps were collected from gills, kidneys and liver. Each swap was inoculated into nutrient broth and incubated at 37^{o} C for 24 hours, then sub-cultured into blood and MacConkey agars. The suspected colonies were selected and inoculated on triple sugar iron agar for biochemical identification tests using VITEK-2 device. The test was conducted following the manufacturer protocols (MacFadin 2000).

Mycology

Gills samples were collected and stained with Lactophenol Cotton Blue (LPCB) and fluorescent CFS stains. The gills samples were prepared and cultured in Sabouraud's dextrose agar (SDA) media for fungal detection.

RESULT

The high mortality rate was observed in the all affected farms, which reach to 80% of farms fishes. The team observed that water levels in Euphrates River was very low comparing with previous years at same time period (**Fig. 1**). The fishes breeding conditions in cages was disorganized where the fish was crowded (the fish breeding rate > 50 kg/m^{3}). Also, the space between the cages was close (less than 50 cm) (**Fig. 1**). Floated dead fishes were observed daily in all selected farms, where the mortality rate reached 75% of affected farms (**Fig. 2**).

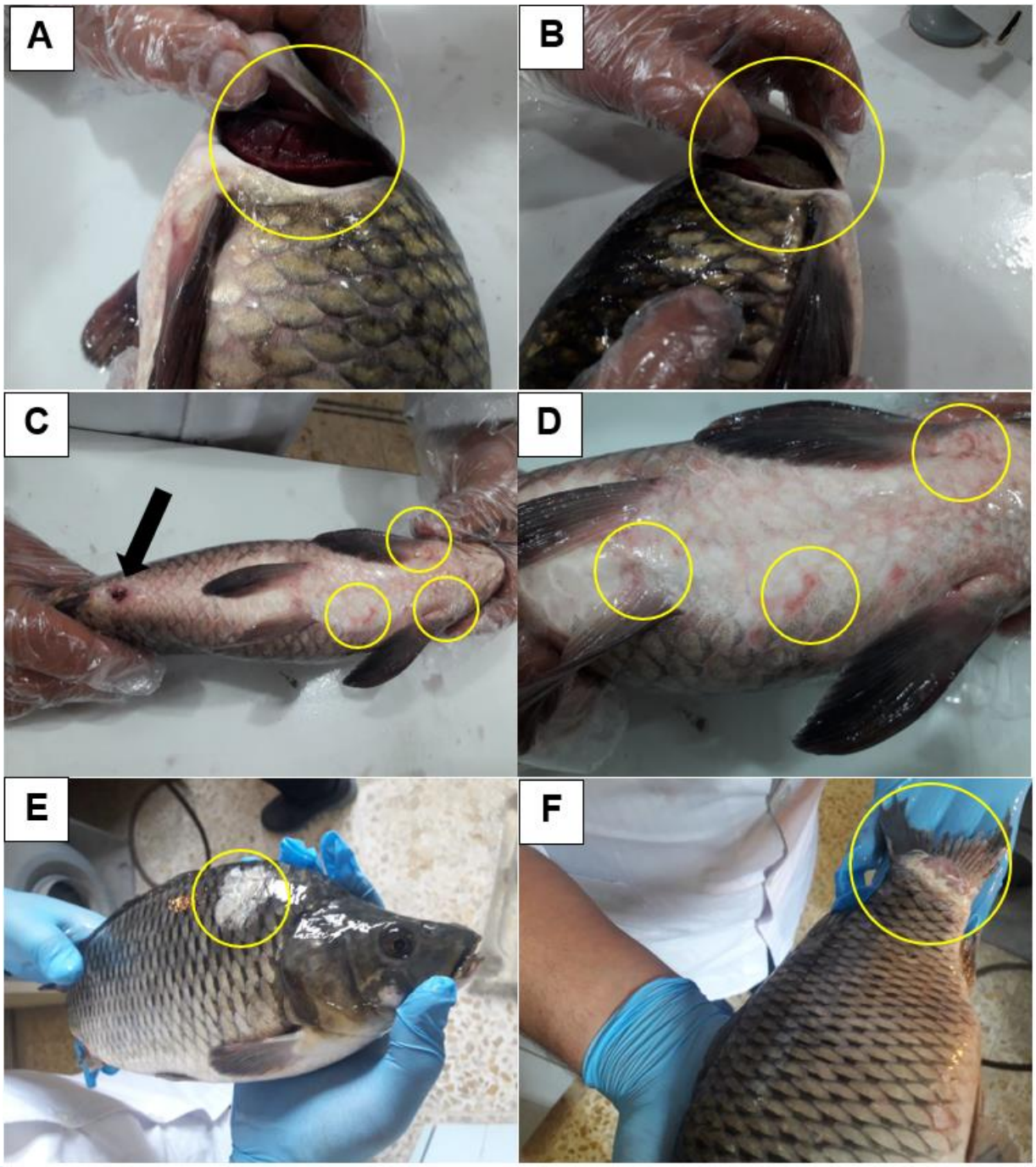

Fig. 2. Photographs of *Cyprinus carpio* fish. **A)** G ills of affected fish was severely congested (yellow circle). **B)** Necrotized gills was observed in affected fishes, where the erosion of gills filament with presence of white to gray in color patches (yellow circle) in affected gills were observed. **C& D)** Hemorrhage patches (yellow circles) were observed in abdomen area that extended from caudal fin to gills area. Note the congested anus area (black arrow). **E)** Erosion and sloughing skin were observed in some affected fishes with presence of white patches in lateral areas (yellow circle). **F)** Erosion of caudal fin (yellow circle), where the caudal fin was eroded with sloughing of fins trunk.

The gross pathology examination showed hemorrhage in skins of left and right lateral with presence of hemorrhage patches in abdomen area extending from the gills area to caudal fins of affected fishes. After necropsy liver, kidneys and spleen were enlarged and congested with presence of hemorrhage on the muscles (**Fig. 3**). Necrotized gills was observed in affected fishes, where erosion of gills filament with presence of white to gray patches in affected gills were observed. Also, gills hemorrhage and/or congestion were observed in certain affected fishes. Skin lesion was also observed, where white to gray color lesion with presence of erosion skin layers were observed.

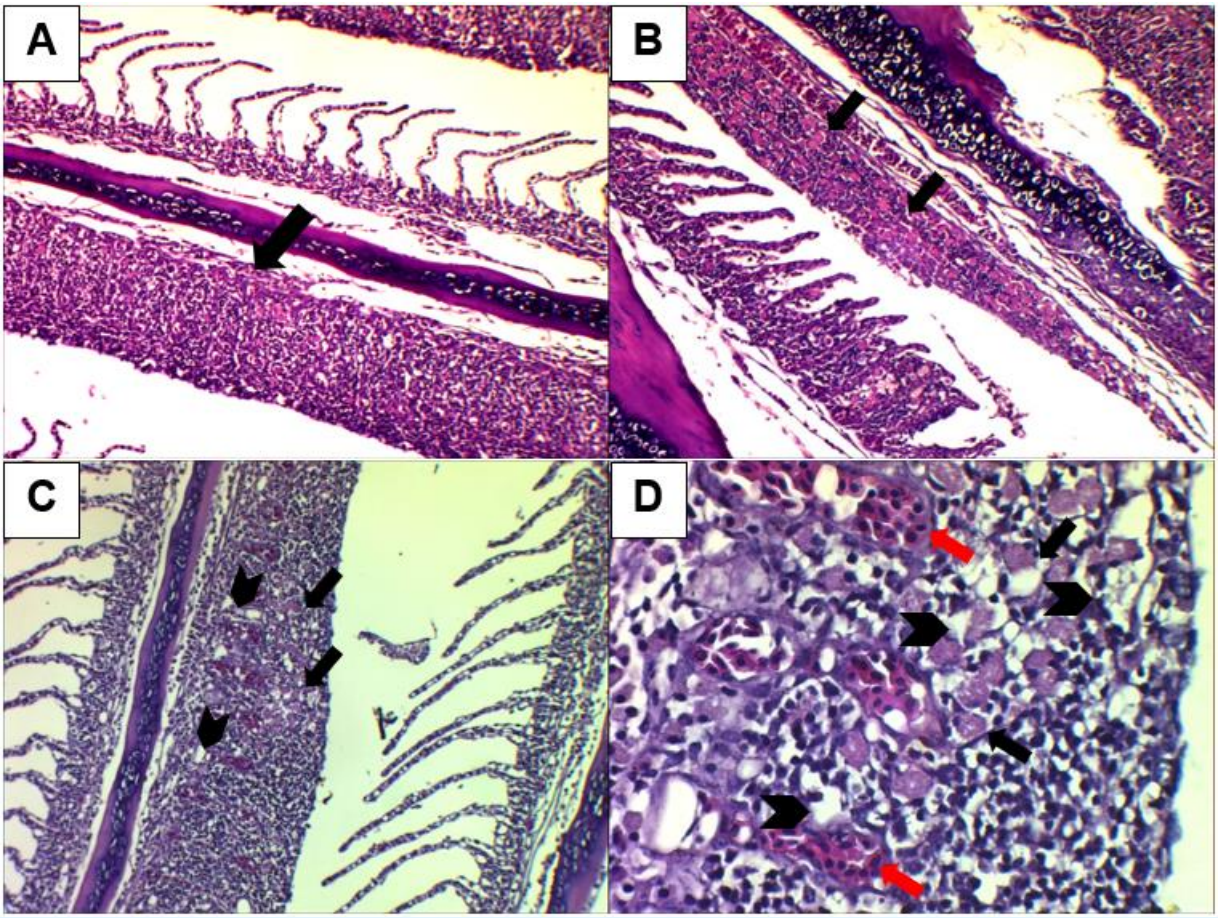

Fig. 3. Photomicrograph of gills of *Cyprinus carpio* fish. **A)** Hyperplasia of epithelial cells (black arrow) of secondary lamella led to absence of normal architecture of secondary lamella comparing with other side of lamella which showed a normal lamella architecture. **B)** Hyperplasia of epithelial cells of secondary lamella was observed. Coagulative necrosis of epithelial cells (black arrows) of secondary lamella. **C and D)** Coagulative necrosis of epithelial cells (black arrows) of secondary lamella led to forming a spaces (arrow heads) in affected areas with blood vessels congestion (red arrows) were observed. H&E. A, B and C: x100, D: x400.

Sloughing of scales in necrotized skins especially in both lateral sides (**Fig. 3**).

Histopathological results revealed a necrotic lesion in gills, where the necrosis in epithelial cell of secondary lamella was observed, leading to destruction of normal gills architecture. Also, hyperplasia of epithelial cells of primary and secondary lamella led to absence of spaces between lamella was observed in certain cases (**Fig. 4**). In kidney, the necrosis of renal tubules epithelial cells leaving a spaces in kidney parenchyma was observed. Hemorrhage within the hematopoietic tissue of kidney and surrounding the renal tubules also observed (**Fig. 4**). The necrosis of hepatocytes of liver was observed, where pyknosis, karyohexis and/or karyolysis of hepatocytes was observed. Infiltration of mononuclear inflammatory cells mostly lymphocytes was observed in hepatic sinusoids and in blood vessels lumens (**Fig. 4**). The qPCR results for virus detection revealed that all tested samples were positive for *cyprinid herpesvirus 3* (CyHV-3) or called Koi Herpes virus (KHV).

The bacteriology results showed presence of five gram negative bacteria including three species of Aeromonas (Aeromonas Sobria, Aeromonas hydrophila, Aeromonas caviae), Raoultella ornithinolytica and Shewanella putrefaciens (**Table 1**). The mycology results showed the presence of on strain of *Aspergillus fumigatus* and two strains of *Mucor* spp. In mycology results, the stained samples did not show any fungal filaments, and the samples that cultured in SDA media showed presence stains of Aspergillus fumigatus and Mucor spp.

DISCUSSION

In last decade, the aquaculture industry was expanded using many types of breeding including fish ponds, cages systems or close system led to cover the local market need. However, the fish breeding industry in Iraq was concerned about the quantity of production rather than the standard breeding conditions including the rate of fishes breeding or quality of feed. The cage system in fish breeding was significantly increased using

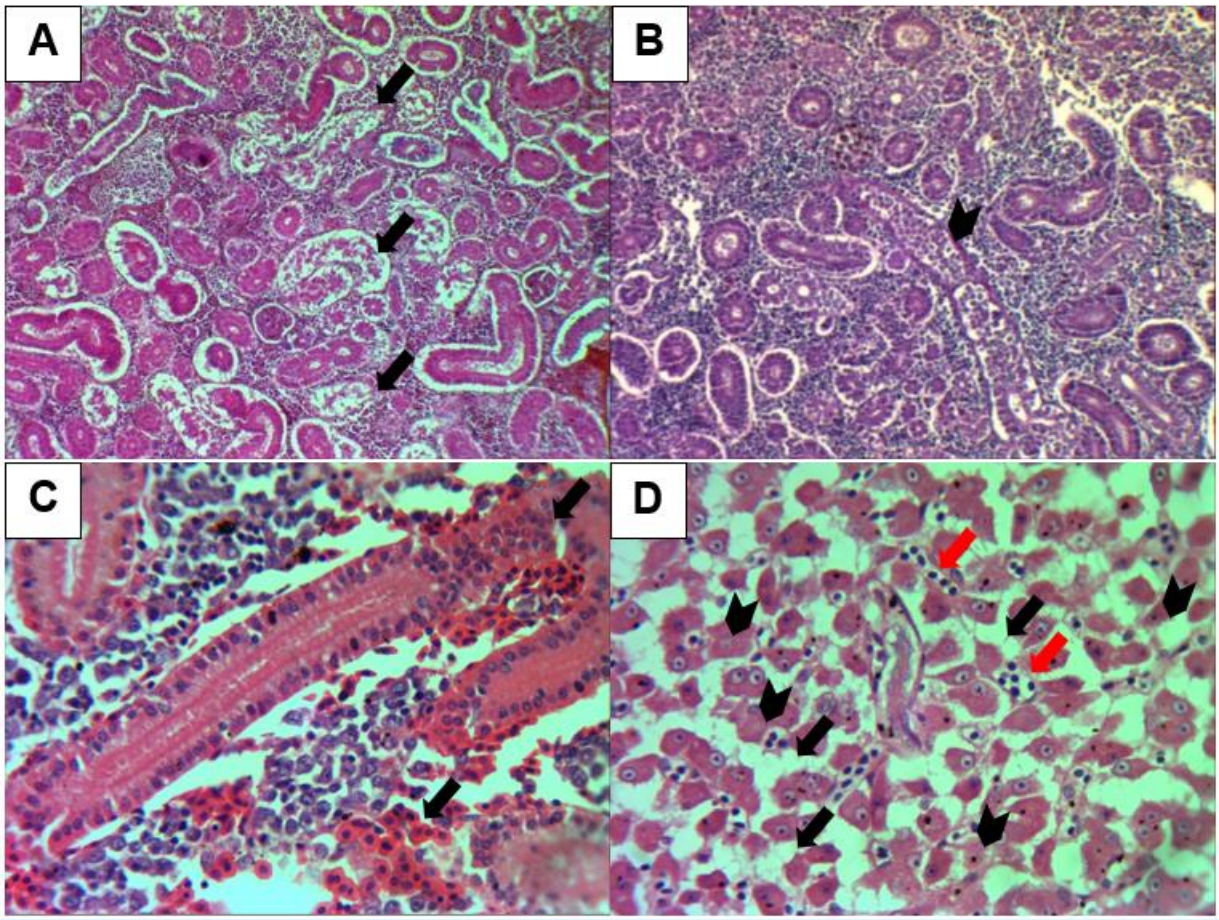

Fig. 4. Photomicrograph of kidneys and liver of *Cyprinus carpio* fish. **A and B)** Necrosis of epithelial cells of renal tubules led to forming an empty space (black arrows) or filled with RBC and inflammatory cells (arrow head) in kidney parenchyma. **C)** necrosis of epithelial cells of renal tubules with presence of hemorrhage (black arrows) was observed. **D)** necrosis of hepatocytes of liver led to forming a spaces (black arrows) within the affected areas, also pyknosis, karyorrhexis and karyolysis (arrow heads) of nucleus of hepatocytes was observed. Infiltration of lymphocytes (red arrows) in affected area was noted.

Table 1. Bacteriology and mycology results for each farm

Farms	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Aeromonas	2	2	1	3	1
Raoultella ornithinolytica	1	0	1	1	0
Shewanella putrefaciens	1	0	1	1	0
Aspergillus fumigatus	1	1	2	2	1
Mucor	0	1	1	1	1

the banks of Tigris and Euphrates rivers. But, the cage system fish breeding in Iraq was mostly not authorized and disorganized, where the fish bred in bad conditions including high rate of breeding per m^3 or the short spaces between each cage. In summer 2018, Iraq suffered from water crisis and extended to autumn 2018, that happened due to severe shortage of water levels in both rivers Tigris and Euphrates. The water crisis of summer 2018 was followed by high fatal outbreak in fish breeding industry led to huge loss in fish's numbers, affecting all fish breeding industry. The present study was conducted to investigate and diagnose the causes of the fatal outbreak in fish breeding industry in September and October 2018. According to PCR results, all tested samples were positive for koi virus. Also the gross and histopathology results resembling in hemorrhage in internal organs and on the skin with presence of necrosis in kidneys, liver and gills, which observed in previous cases of koi virus outbreaks (Pikarsky et al. 2004; Panicz et al. 2019) confirming PCR results.

However, the water crisis in Euphrates River accompanied with bad conditions of fish breeding worked as predisposing factors for koi virus outbreak. Where, the previous studies about koi virus documented that the koi virus outbreaks appeared with certain circumstances that cause a stress in bred fishes including low water levels or bad breeding conditions (Lin et al. 2017).

The bacteriology biochemical tests showed that the fish's samples were contained a certain of gram negative bacteria including three species of Aeromonas, Raoultella ornithinolytica and Shewanella putrefaciens. The Aeromonas spp. Infection may cause necrotizing

lesion in skin or enterotoxaemia in fishes (Praveen et al. 2016). Raoultella ornithinolytica It is commonly found in fish, water and soil as nonpathogenic agent for fish (Hajjar et al. 2018; Mooraki, and Sedaghati, 2019). Shewanella putrefaciens rarely considered as a serious pathogenic agent in fish, however Shewanella putrefaciens may cause a lesion in skin and gut areas of fishes (Koziñska and Pekala, 2004; Paździor, 2016). The mycology results showed that all tested samples were contained several stains of Aspergillus fumigatus and Mucor spp, which usually observed in water of rivers and Aspergillus fumigatus can act as pathogenic agent in fish when immunity system of fish suppressed or stressed (Wang et al. 2014).

However, the lesions in the current case was hemorrhage of skin and internal organs and gills, kidneys and liver necrosis, which considered the main characteristic lesion in koi virus infections (Pikarsky et al. 2004; Panicz et al. 2019). Therefore, the viral infection considered the primary infection and the bacterial infection considered a secondary infection, especially the bacteria species that diagnosed in the recent case cannot cause a severe fatal outbreak as in the recent case.

In conclusion, according to results the high mortality outbreak in common carp fish that occurred in September and October 2018, Iraq was due to Koi Herpes virus infection with presence of secondary bacterial or fungal infection.

ACKNOWLEDGEMENT

The current study was conducted by academic staff of Faculty of Veterinary Medicine, University of Kufa. I would like to thank all staff of Pathology and Poultry diseases Department in the Faculty of Veterinary Medicine faculty, University of Kufa. Also, I would to thanks all owner of fish farms for them cooperation with the team for supplying the samples and introducing the assistant.

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