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DETECTION OF CO-INFECTION IN DISEASED GUPPY (*POECILIA RETICULATA*): *TETRAHYMENA* SP., *MYXOBOLUS* SP. AND OPPORTUNISTIC BACTERIA

Cigdem Urku[1*], Mustafa Kemal Meydan[2]

[1] University of Istanbul, Faculty of Aquatic Sciences,
Department of Fish Disease, Istambul, Turkey
[2] ADEC Aquarium and RAS Turnkey Systems, Milas, Turkey

Abstract

The guppy (*Poecilia reticulata*) is one of the world's most popular freshwater aquarium fish. The present study aims to determine the cause of mortality in guppies obtained from the ornamental fish farm in Istanbul province and to detect tissue damage. Externally diseased guppies showed sloughing scales and large ulcerative skin lesions on the body surface. Bacteriological inoculation from internal organs was made onto Tryptic Soy Agar (TSA). After incubation, isolated bacteria were identified as *Aeromonas hydrophila* and *Vibrio parahaemolyticus* according to their physiological, morphological, and API 20E profile. As a result of the parasitological examination, *Tetrahymena* sp. and *Myxobolus* sp. were found on the skin and intestinal lumen of the diseased guppy, respectively. Histopathologically, cytoplasmic vacuolation in the liver, degeneration, and necrosis of the tubular epithelium and granuloma-like structure in the kidney, vacuolization of epithelial cells in the intestinal lumen, hyperplasia of the gill and *Tetrahymena* sp. between necrotic muscles were observed. Interestingly, *Myxobolus* sp. spores were only detected in the intestinal lumen. Co-infection of guppies was determined to cause severe disease with high mortality and tissue damage. The synergistic effects of pathogens may cause more damage to diseased guppies. This report is the first detailed report containing new histopathological findings of tetrahymeniosis and the first detection of *Myxobolus* sp. in the gut of diseased guppy in Turkey.

Key words: *Aeromonas hydrophila,* guppy, histopathology, *Myxobolus sp., Tetrahymena sp., Vibrio parahaemolyticus*

[1*] Coresponding Author: curku@istanbul.edu.tr

DETEKCIJA KOINFEKCIJE KOD OBOLELIH GUPIJA (*POECILIA RETICULATA*): *TETRAHYMENA* SP., *MYXOBOLUS* SP. I OPORTUNISTIČKE BAKTERIJE

Cigdem Urku[1], Mustafa Kemal Meydan[2]

[1] Univerzitet Istanbul, Fakultet nauke o vodama,
Katedra za bolesti riba, İstanbul, Turska
[2] ADEC Akvarijum i RAS sistemi ključ u ruke, Milas, Turska

Kratak sadržaj

Gupi (*Poecilia reticulata*) je jedna od najpopularnijih slatkovodnih akvarijumskih riba širom sveta. Cilj ovog istraživanja je da se utvrdi uzrok smrtnosti gupija sa ornamentalne riblje farme u provinciji Istanbul i detektuje nastalo oštećenje tkiva. Eksterno oboleli gupiji manifestovali su opadanje krljušti i velike ulcerativne kožne lezije na površini tela. Bakteriološka inokulacija iz internih organa urađena je na tripton soja agaru (TSA). Nakon inkubacije izolovane bakterije su identifikovane kao *Aeromonas hydrophila* i *Vibrio parahaemolyticus* na osnovu fiziološkog, morfološkog i API 20E profila. Parazitološko ispitivanje dokazalo je prisustvo *Tetrahymena* sp. i *Myxobolus* sp. na koži odnosno intestinalnom lumenu obolelih gupija. Histopatološki su uočeni citoplazmatska vakuolacija u jetri, degeneracija i nekroza tubularnog epitela i strukture slične granulomima u bubrezima, vakuolizacija epitelnih ćelija u intenstinalnom lumenu, hiperplazija škrga i *Tetrahymena* sp. između nekrotičnih mišića. Interesantno je da su spore *Myxobolus* sp. detektovane samo u intestinalnom lumenu. Ustanovljeno je da koinfekcija gupija izaziva težak oblik bolesti sa visokom smrtnošću i oštećenjem tkiva. Sinergijski efekat patogena može izazvati još veća oštećenja kod obolelih gupija. Ovaj rad predstavlja prvi detaljan izveštaj koji sadrži nove histopatološke nalaze tetrahimenioze i prvu detekciju *Myxobolus* sp. u crevima obolelog gupija u Turskoj.

Ključne reči: *Aeromonas hydrophila,* gupi, histopatologija*, Myxobolus sp., Tetrahymena sp., Vibrio parahaemolyticus.*

INTRODUCTION

Ornamental fish production is a profitable industry in the world. The global ornamental fish trade is approximately US$ 15–30 billion per year (Evers et al., 2019) with 10 % average annual growth (Whittington and Chong, 2007; Dey, 2016). In recent years, it was estimated that 1,471 marine ornamental fish and more than 4,000 species of ornamental freshwater fish were traded globally each year (Sharon et al., 2014). There are 30 predominantly traded freshwater species, of which guppy (*Poecilia reticulata*) and neon tetra (*Paracheirodon innesi*) account for 25% of the market by volume and more than 14% of the total trade value (Dey, 2016; Evers et al., 2019). Guppy, which is native to Trinidad Island and the northern part of South America, lives in natural waters at 24-30 °C and has tolerance to a wide range of salt concentrations. The most popular and widely traded guppy has about 300 subspecies (Sharon et al., 2014).

Tetrahymena is a protozoan parasite, which is the causative agent of tetrahymeniosis or 'guppy killer disease' in tropical aquarium fish, which causes severe economic losses in commercial guppy farms worldwide (Sharon et al., 2014). The species can settle in the scale pockets of the fish and feed on the tissues in that region, as well as penetrate the tissue and settle in the internal organs (Imai et al., 2000). Although it is defined as an invasive pathogen, it can easily cause secondary infection (Sobecka et al., 2012). The first report of tetrahymeniosis in Turkey/Black Sea Region was reported in the guppy (Kayis et al., 2019), and histopathological analyses were not used to detect the tissue damage. Later studies also detected this parasite in *Cichlasoma nigrofasciatum* and *Labidochromis caeruleus* (Kayış et al., 2013).

Myxozoans consist of a species-rich lineage of endoparasitic cnidarians with complex life cycles and have invertebrate and vertebrate hosts in marine and freshwater environments (Okamura et al., 2018). Although *Myxobolus* sp. has been reported in a few aquarium fish, such as Oscar and goldfish fish (Nematollahi et al., 2016), there are limited reports on the detection of this parasite in the guppy (Segovia Salinas et al., 1991; Mohammed et al., 2020).

Most bacterial diseases of ornamental fish result from a combination of factors ranging from environmental conditions, stress, and host susceptibility to pathogen virulence and cause economic losses at every step of the production (Wildgoose, 2001). Known as opportunistic bacteria, *Aeromonas sobria*, *A. hydrophila*, *Shewanella putrefaciens*, *Plesiomonas shigelloides*, *Vibrio cholerae*, *V. parahemalyticus*, *Pseudomonas* spp., are frequently isolated from diseased ornamental fish (Carnevia et al., 2013; Sicuro et al., 2020; Urku, 2020).

The present study aims to determine the cause of high mortality in dis-

eased guppies obtained from the ornamental fish farm in İstanbul province and to detect tissue damage. This report is the first detailed report containing histopathological findings of tetrahymeniosis and the first detection of *Myxobolus* sp. in the foregut of the diseased guppy in Turkey.

MATERIAL AND METHODS

Fish Samples

Ten diseased guppies were obtained from an aquarium fish-rearing farm in Istanbul/Turkey. There was a 30 % mortality in diseased guppies. The aquarium water temperature and pH values in the are 25-26 ºC, and 7, respectively.

Bacteriological Examination

Samples were taken from the liver and kidney of diseased guppies. They were inoculated onto Tryptic Soy Agar (TSA). Petri plates were incubated during 24-48 h at 24-25 °C. The isolates (n=15) recovered from guppy were identified by using the conventional bacteriological method (Gram staining, cell morphology, motility, oxidase and catalase activity, oxidation fermentation test, growth on Thiosulfate Citrate Bile Sucrose (TCBS) medium, vibriostatic test -O129/10,150) and API 20E (Buller, 2004; Austin and Austin, 2016).

Parasitological Examination

The presence of external parasites was investigated by examining fresh samples from the gills and skin of the diseased guppy under a light microscope. After the autopsy, samples taken from the intestine and stomach were also examined for internal parasites (Buchmann, 2007).

Histopathological Examination

After the ventral incision, whole fish (n=5) were immediately fixed in 10% phosphate-buffered formaldehyde, dehydrated with a graded series of ethanol, cleared in chloroform, and embedded in paraffin wax according to standard histological procedures. Paraffin blocks were cut into serial sagittal sections (4-5 µm) with a microtome (Leica RM2125, Germany) and stained with Mayer's hematoxylin and eosin (H&E) and also ZN (Ziehl-Neelsen). Slides were examined under an Olympus BX51 light microscope equipped with an Olym-

pus DP72 digital camera. The width (µm) and length (µm) of detected parasites were measured using ImageJ software version 1.36 (Roberts et al. 2012).

RESULTS

Clinical Findings

Ten diseased guppies exhibited externally large hemorrhagic skin lesions extending to the muscles on the body surface, eroding fins, and loss of the scale (Figure 1).

Figure 1. Diseased guppy: Extending to the muscles pronounced hemorrhagic whitish lesions (arrowed) and loss of the scales

Bacteriological Findings

After incubation of liver and kidney swabs, two different colonies were observed on TSA. All isolated bacteria (n=15) were motile, Gram-negative basil, oxidase and catalase positive, fermentative, and resistant to O129/10. Five isolates were susceptible to 0129/150 and formed green colonies on TCBS (Thiosulfate Citrate Bile Sucrose) medium. According to conventional bacteriological test results, some isolates were identified as *Aeromonas* sp. (10); others were identified as *Vibrio* sp. (n=5). In addition, they were defined as

Aeromonas hydrophila (Ah) (n=10) and *Vibrio parahaemolyticus* (Vp) (n=6) with their 304712757 and 410710457 API web profile numbers, respectively.

Parasitological Examination Findings

As a result of the external parasitological examination, *Tetrahymena* sp. was found intensively on the skin of diseased guppy. The parasite (55-35 µm) with macro (12,35 µm) and micronuclei (4,362 µm) had a pear-like body structure covered with cilia (Figure 2a). The presence of any parasite was not detected in the internal parasite examination.

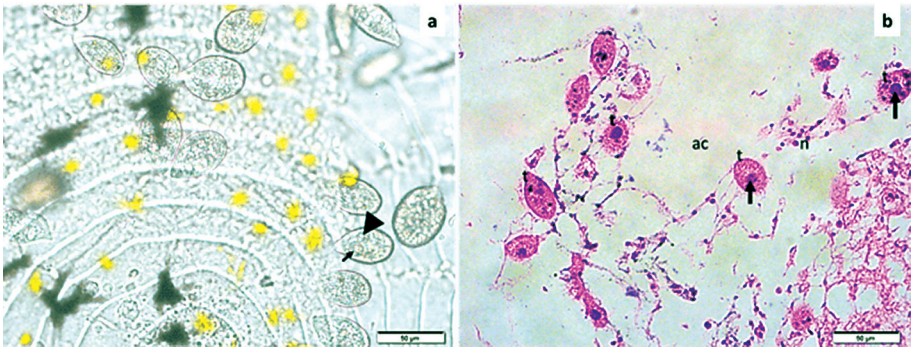

Figure 2. (a) *Tetrahymena* sp. (t) detected on the skin (b) abdominal cavity (ac) (H&E) of the diseased guppy (arrowhead: micronucleus, arrowed: macronucleus, n: leucocytes)

Histopathological Examination Findings

Necrosis of the tubular epithelium and granuloma-like structure and periglomerular and tubular edema in the kidney (Figure 3a, b), cytoplasmic vacuolation and eosinophilic cell infiltration around the necrotic hepatocytes cells (Figure 3c), hemosiderin deposits in the spleen (Figure 3d) (Figure 3c), hyperemia, eosinophilic cell infiltration, and hyperplasia of the gill filaments (Figure 4a, b) were observed. *Tetrahymena* sp. between muscle cells (Figure 5a), pseudobranch (Figure 5b), spinal cord, abdominal cavity (Figure 2b), even between enterocytes (Figure 6a) and around the kidney (Figure 3b), and esophagus (Figure 6d) were noted. Although the presence of any parasite was not detected in the internal parasite examination from the lumen of the intestine and stomach, interestingly mature *Myxobolus* sp spores (35-29 µm) were observed in the foregut lumen of the diseased guppy (Figure 6b). Vacuoliza-

tion was noted in intestinal epithelial cells (Figure 6c). In addition, acid-fast positive bacterial cells were not observed in ZN-stained preparations.

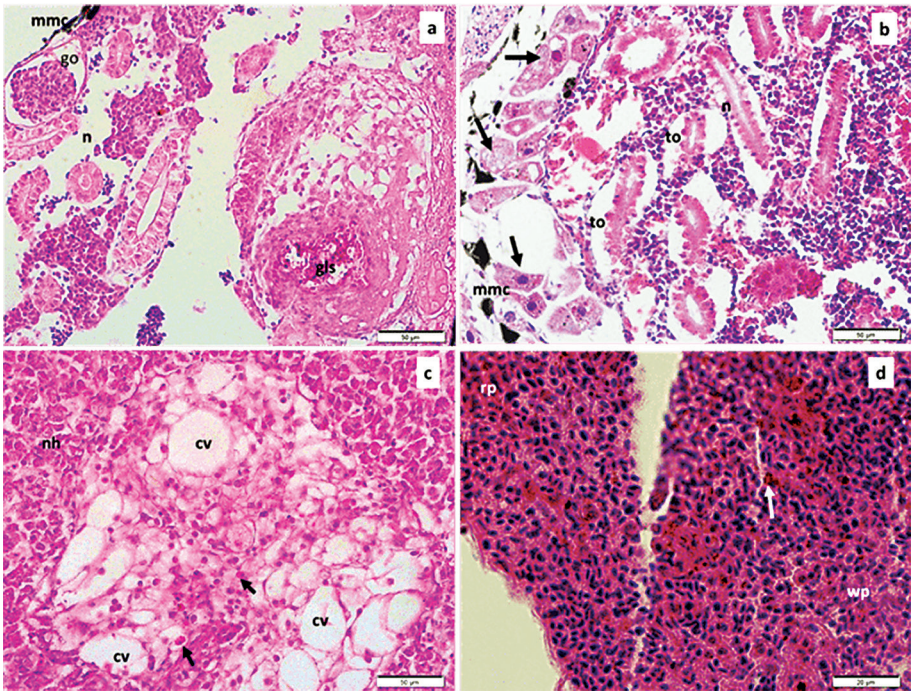

Figure 3. Granuloma-like structure (gls) and discharge of haemopoietic tissue (n), periglomerular (go), tubular (to) edema and melanomacrophage centers (mmc) (a, b); notice *Tetrahymena* sp. (arrowed) around the kidney tissue (b); cytoplasmic vacuolation (cv), and eosinophilic cells (arrowed) among the necrotic hepatocytes (nh) (c); hemosiderin deposits in the spleen (d) (H&E)

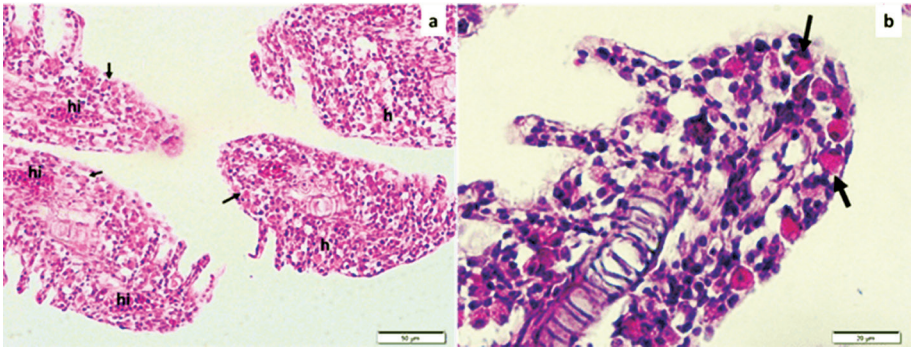

Figure 4. Hyperplasia (h), hyperemia (hi), and eosinophilic cell infiltration (arrowed) in the gill tissue (a, b) (H&E)

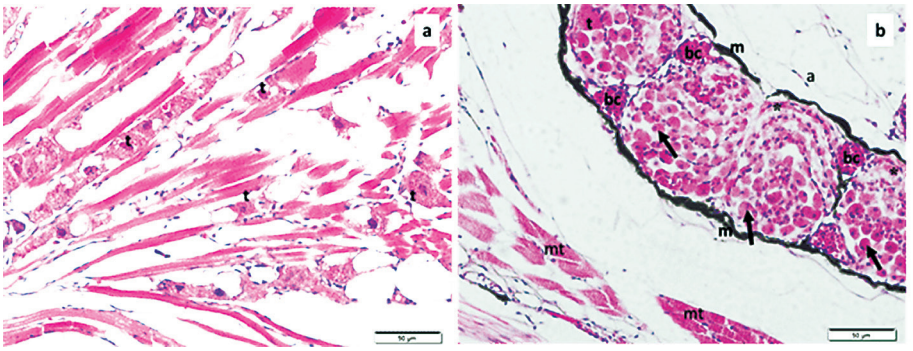

Figure 5. Parasite (t) showed a characteristic pyriform shape penetrating the connective tissue of the muscle cells; note that many parasites are between the muscle fibres with no evident inflammatory response (a); hypertrophy in pseudobranch cells (arrowed) rich in capillary blood vessels (bc); notice *Tetrahymena* sp. (t) between the cells (b) (H&E)

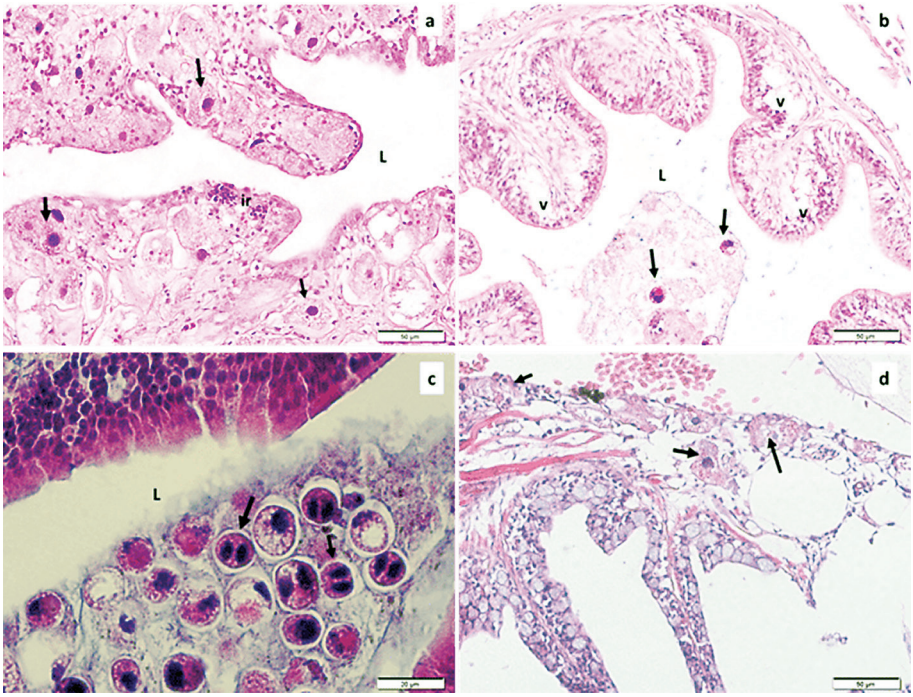

Figure 6. *Tetrahymena* sp. (arrowed) in the midgut, note that many parasites are among the enterocyte with inflammatory reaction (ir) (a) and esophagus (d); vacuolization (v) of epithelial cells of the intestinal lumen of foregut (b); Presence of spores of *Myxobolus* sp. (arrowed) in the foregut (c). (L: lumen) (H&E)

DISCUSSION

This study revealed a co-infection caused by invasive parasites such as *Tetrahymena* sp. and *Myxobolus* sp., and the bacteria *A. hydrophila* and *V. parahaemolyticus*, which were detected as disease agents in the diseased guppy.

Tetrahymeniosis results in parasite invasion of skin, muscle, and even internal organs in many aquarium fish species. Although the main clinical finding of tetrahymeniosis is lethargy or showing characteristic white skin lesions, this parasite causes systemic disease. The disease affects many fish species, and compared to other aquarium fish, guppies are the most susceptible to this disease (Sigh and Buchmann 2002; Leibowitz and Zilberg, 2009; Sharon et al., 2014). In this study, in addition to white skin lesions, which are the main symptoms of the disease, large hemorrhagic skin lesions extending to the muscles on the body surface, eroding fin, and loss of the scale were detected in the fish. This clinical structure, especially hemorrhagic skin lesions, may be caused by the isolated bacteria such as *A. hydrophila* and *V. parahaemolyticus* accompanying the co-infection in the present study.

Myxobolus infection may be coelosoic, most often in the gallbladder and urinary bladder, or histozoic, targeting the skin, muscles, gills, or digestive tract (Lom and Dykova, 2006). Many myxobolus species have been reported to cause significant pathological problems such as reduction of epithelial area of the gill, myocarditis of the heart, deformation, displacement, retraction, and compression of capillaries of the gill lamellae and skin nodules (Mathews et al., 2020). In ornamental fish species, it has been reported that *Myxobolus* spp has been detected in the gills of Oscar and goldfish (Nematollahi et al., 2016). Additionally, *Myxobolus nuevolonensis* has been detected in the bones at the base of the tail, dorsal and anal fins in the molly (*Poecilia mexicana*) and guppy (*P. reticulata*) (Segovia Salinas et al., 1991). As reported by Rasouli et al. (2022) in goldfish, *Myxobolus* sp. was only detected in the intestinal lumen of diseased guppy in this study. Histologically, the parasite sporoplasm, could not be detected in all organs of diseased guppy.

Motile Aeromonas Septicemia (MAS), caused by *A. hydrophila* and characterized by hemorrhagic septicemia, is generally characterized by the presence of body surface lesions and hemorrhages, eroded fins, sloughing scales (Anjur et al., 2021). Although clinical findings vary among fish species in vibriosis caused by *V. parahaemolyticus*; skin and muscle are the tissues most affected by the pathogen bacteria. It has been reported to cause black skin lesions in goldfish, deep dermal ulceration, scale loss in tilapia, and ulcerative skin lesions in guppy (Kiani et al., 2016; Adnan Al-Darwesh et al., 2016; Zaher et al.,

2021). In addition, these two bacteria (*A. hydrophila* and *V. parahaemolyticus*) are zoonotic. Therefore, it is essential for aquarists' health.

Tetrahymena invades the host through the skin, reaching the blood vessels and spreading systemically. The organs most affected by the parasite are the gills, heart, kidney, liver, pancreatic tissue, intestinal wall, gonads, and peritoneal cavity (Leibowitz and Zilberg, 2009). It has been reported to cause an intense inflammatory reaction in the tissues where the parasite is localized in the koi and goldfish (Sharon et al., 2014). This study determined that the parasite was not localized in the heart tissue and did not cause a very strong inflammatory reaction in the tissues it penetrated. In addition, it has been observed that the parasite is most densely localized on the skin and around the internal organs such as the kidney and gut.

Although it was reported that *Tetrahymena* sp. was not detected in the intestinal epithelium and lumen (Sharon et al., 2014), parasites localized intensively between the enterocytes drew attention in the present study. Although Imai et al. (2000) did not report any pathological changes in the intestinal mucosa and submucosa, vacuolization was detected in the intestinal epithelial cells of diseased guppy in this study.

The kidney is the most important hemopoietic tissue of fish. Although the presence of the parasite around the kidney tissue was reported, the histopathological changes caused by the *Tetrahymena* sp. in kidney tissue were not described in previous reports (Leibowitz and Zilberg, 2009; Sharon et al., 2014). It destroys tissue cells during parasite migration. In this study, the granuloma-like structure was detected for the first time. Despite the reports that granuloma-like structures in guppy fish are common histological finding in Mycobacterium infections (Areechon et al., 2001), the presence of acid-fast positive bacteria was not detected in ZN stained preparations of tissue samples taken from diseased guppy examined in this study.

In general, *Tetrahymena* sp. is believed to accumulate around the blood vessels in the liver of mollies and guppies, having a unique attraction mechanism to these areas. The parasite causes detachment of hepatocytes (Sharon et al., 2014). Granulomas and an increased number of melanomacrophage centers are in the liver of infected fish (Herbert and Graham, 2008; Urku et al., 2018). Although parasites were not detected around the blood vessels in the liver of the fish examined in the present study, cytoplasmic vacuolation and eosinophilic cells among the necrotic hepatocytes were detected. The appearance of a vacuole in the liver is often a parameter of histological liver damage. However, the granuloma reported by Herbert and Graham (2008) was not detected in the liver tissue.

In aquarium fish such as guppy, molly, or angelfish, inflammatory responses are not evident, and the presence of parasite-associated leukocytes was not detected in these fish species (Sharon et al., 2014). This study did not detect the inflammatory reaction in the dermis layer, where the parasite is most intensive; however, leukocytes in the abdominal cavity and inflammatory reaction in the mucosa epithelium were detected.

As a result, the granuloma-like structure pathologically detected in the kidney tissue is not due to mycobacteriosis or myxobolosis; it has been determined that *Myxobolus* spp is found only in the intestinal lumen and not in other tissues and organs. Additionally, acid-fast bacteria were not detected in ZN-stained tissue samples. Therefore, it can be thought that this pathological change is caused by *Tetrahymena* sp.

CONCLUSION

The present report demonstrated that *Tetrahymena* sp. forms a port entrance for opportunistic bacteria such as Aeromonas and Vibrio accompanied co-infection. In addition, it was histologically determined that the co-infection was associated with *Myxobolus* sp. originating from the live feed. Therefore, disinfection is highly recommended when live feed is used in ornamental fish farms. The synergistic effects of pathogens (invasive parasites and opportunistic bacteria) may histopathologically cause more tissue damage to diseased guppies.

Author's contribution:

CU made contributions to the conception and design of the study, involved in data collection and drafting of the manuscript. MKM provided resources and laboratory work. All authors read and approved the final manuscript.

Competing interests:

Authors declare that they have no competing interests for a work presented.

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