AQUATIC RESEARCH

AQUATIC RESEARCH
E-ISSN 2618-6365

Aquat Res 6(4), 245-252 (2023) • https://doi.org/10.3153/AR23023 Research Article

Coccidian infestation in cultured common pandora (*Pagellus erythrinus*)

Tülay AKAYLI, **Çiğdem ÜRKÜ**

Cite this article as:
Akaylı, T., Ürkü, Ç. (2023). Coccidian infestation in cultured common pandora (*Pagellus erythrinus*). *Aquatic Research*, 6(4), 245-252. https://doi.org/10.3153/AR23023

Istanbul University, Faculty of Aquatic Sciences, Department of Aquaculture and Fish Diseases, Istanbul, Turkiye

ORCID IDs of the author(s):
T.A. 0000-0003-2375-2224
Ç.Ü. 0000-0003-0381-9321

Submitted: 19.07.2023
Revision requested: 27.07.2023
Last revision received: 20.08.2023
Accepted: 21.08.2023
Published online: 23.08.2023

Correspondence:
Çiğdem ÜRKÜ
E-mail: yavuz. curku@istanbul.edu.tr

Available online at
http://aquatres.scientificwebjournals.com

ABSTRACT

Coccidia is a spore-forming obligate intracellular protozoan parasite that causes disease in many fish species. This study aimed to diagnose a parasitic disease case that affected a common pandora (*Pagellus erythrinus*) with a high mortality rate. The samples prepared from the internal organs of the diseased fish and the gills and muscle tissues were examined parasitologically using histological methods, a light microscope, and Transmission Electron Microscopy (TEM). No parasites or parasitic formations were found in the wet mount preparations. The presence of parasitic spores (1-1,5 x 0.3-0.7 µm) was detected towards the intestinal tissue between the intestinal microvilli of fish in the electron microscopy study. On the other hand, histological examination showed that a cystic structure full of spores (sporocyst) 550-750 µm in size developed in the abdominal muscles of the infected fish. At the same time, there were no such structures in the intestines. As a result, since the parasite spores observed in the diseased fish are very small, they settle in the cell and pass through the intestines by forming cysts in the abdominal muscles. They were identified as Coccidian *sp.* because of their similar morphology to those of the parasites in the Coccidian group. Coccidian infestation was detected in this fish species for the first time in this study. However, detecting the spores' entrance through the fish's intestines in the early stage and observing a small number of sporocyst structures suggest that the disease is in the development stage.

Keywords: Fish, *Pagellus erythrinus,* Parasite, Coccidian, TEM, Histology

Introduction

Common pandora (*Pagellus erythrinus*), belonging to the Sparidae family, is a valuable species for mariculture in the Mediterranean region (Le Breton, 1999; Klaoudatos et al., 2004). Fish are successfully produced at the hatching stage in some marine aquaculture enterprises on Turkey's Aegean coastline. In the last decade, the worldwide aquaculture production of common pandora has progressively dropped from 197.00 tonnes to 0.04 (FAO, 2021). Therefore, more studies are needed to improve the production of this species in offshore net cage systems. To achieve this successful aquaculture production, it is necessary to take into account some important disadvantages such as low growth performances, decreased quality of flesh, increased ratio of mortality, susceptibility to diseases, and skin colouration properties that do not match the wild specimens (Divanach et al., 1993; Başçınar, 2004; Cascarano et al., 2021).

The Apicomplexa phylum consists of unicellular and mostly parasitic sporozoan parasites. Coccidian is spore-forming obligate intracellular protozoan parasites, which are members of the Apicomplexa group and are usually found in the intestinal tissues of freshwater and marine fish (Steinhagen et al., 1990; Molnar, 2006; Shrestha, 2022). This parasite group has a complex life cycle that includes different stages (sporogony, merogony and gamogony) and produces resistant sporocysts in different parts of organs and tissue within a single host (Dyková and Lom, 1981; Harding and Frischknecht, 2020; Shrestha, 2022). However, piscine coccidia is intracellular organisms of epithelial tissues (intestine, gallbladder, swim bladder, kidney tubules and liver) according to their developmental sites and life cycles (Molnar, 2006; Sitjà-Bobadilla et al., 2016).

Coccidiosis in cultured fish is usually a chronic infection with gradual mortality. These parasites live intracellularly in the intestines of fish, adversely affecting the fish's immune system. They cause direct death, slow development, high sensitivity to opportunistic pathogens and low resistance to stress (Molnar, 2006; Sitjà-Bobadilla et al., 2016; Shrestha, 2022). The main endoparasitic protozoans that cause coccidiosis in cultured gilt-head sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) belong to *Eimeria, Goussia* and *Cryptosporidium* species. (Sitjà-Bobadilla and Alvarez-Pellitero, 2002; Gjurčević et al., 2017; Fioravanti et al., 2020). In Türkiye, *Eimeria merlangi* (Özer et al., 2012) and *E. sardinae* species have been reported in the intestines of whiting fish (*Merlangius merlangus*) caught by fishing (Özer et al., 2014).

There are structurally diverse life cycle stages and host-parasite interfaces in fish coccidian (Molnar, 2006; Sitjà-Bobadilla et al., 2016; Fioravanti et al., 2020). Scientists are investigating in detail the complex structures of this type of parasite formed during their intracellular development and the mechanism of the spores, which are the perfect invasion machines of Apicomplexans, with the electron microscope (Steinhagen et al., 1990; Molnar, 2006; Dogga et al., 2015; Sitjà-Bobadilla et al., 2016). Identifying coccidian infestation at the early stage is very important for preventing and treating the disease. Nevertheless, there is limited information about the mechanisms of transportation of the parasite's early stages (Steinhagen et al., 1990; Molnar, 2006; Cascarano et al., 2021; Shrestha, 2022).

The current study aimed to investigate the presence of intracellular spore-formed protozoan parasites that affect the gut and muscle tissues of naturally infected cultured common pandora (*Pagellus erythrinus*) by electron microscopy and histological methods.

Materials and Methods

Sampling

Moribund common pandora (BW 45-100 *g*) were detected on the surface of the cages at the aquaculture farm located on the Türkiye Aegean Sea coast, which recorded 45% mortality. Samples for bacteriology, histopathology and electron microscopy were taken from six diseased fish.

Bacteriology

The internal organs (spleen, liver and kidney) of the fish samples (n=6) were inoculated into Trypticase Soy agar (TSA) containing 1.5% NaCl. Routine bacteriological laboratory methods and an API 20E rapid diagnosis kit were used to determine the biochemical properties of isolated bacteria.

Electron Microscopy

The gut tissue samples (n=6) were prepared for transmission electron microscopy (TEM). Prefixation of the gut samples was carried out in 3% glutaraldehyde solution buffered with 0.1 M sodium cacodylate, and they were post-fixed in 2% OsO_4 and dehydrated in a series of ethanol treatments. Afterwards, the gut samples were passed through propylene oxide and embedded in an open. Ultrathin sections were cut, double-stained using uranyl acetate (Watson, 1958) and Reynold's lead citrate (Reynolds, 1963), and examined under an electron microscope (TEM).

Histology

Processing of tissue samples from the liver, kidney, spleen, gills, gut, and abdominal muscle for histopathology was performed after they were fixed in 10% buffered formalin. After the routine processing of the tissues for histology was performed, 5 µm sections prepared from paraffin blocks were stained with haematoxylin and eosin (H&E) (Bruno et al., 2006).

Results and Discussion

Clinical Findings

In the moribund fish samples, haemorrhages on the pectoral fins and supraorbital region and swelling in the abdominal region drew attention externally (Figure 1a). Adiposity in internal organs and paleness in the liver colour were observed internally (Figure 1b).

Bacteriological Findings

In the general bacterial examination, no bacterial colonies were isolated from the inoculation made from most fish samples. Solely, *Aeromonas* spp. were recovered and identified from the liver of only one fish sample.

Electron Microscope Findings

In the detailed examination performed with transmission electron microscopy (TEM), 3 oval-shaped parasitic spores trying to migrate to the host were found between the microvilli in the hindgut of the diseased fish (Figure 2a). It was noted that the average size of these intracellular spores was between 1-1,5 x 0.3- 0.7 µm and that they headed for the intestinal tissue of the host. The oval-shaped parasitic and pyriform-like infective spores reaching the intestinal tissue were detected in another tissue section (Figure 2b). This infective sporoplasm increasing in size was observed trying to migrate to the host cell. In Figure 2c, it was observed that an active infective spore penetrated the intestinal epithelial cell and tried to extrude its content into the cytoplasm of the host cell. In the same example, a parasitic spore, which completed this process, lost its structure, and started to melt, was detected.

By the data obtained from the electron microscope examination, because parasitic spores in the intestinal sections of diseased fish were very small and they tried to migrate to the body of the host through the intestinal tract, they were determined to show similarities to the general characteristics of the obligate parasites in the *Coccidian* group (Pasnik et al., 2005).

Therefore, these intracellular parasite spores were identified as *Coccidian* sp.

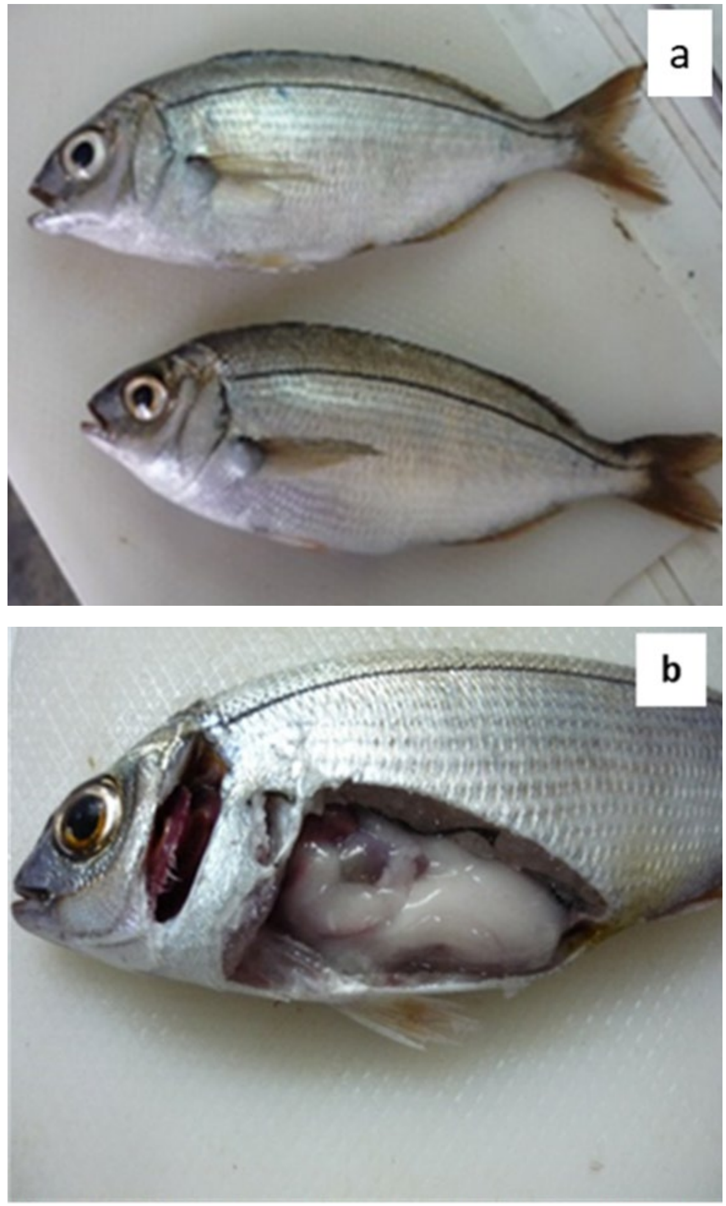

Figure 1. External (a) and internal (b) view of diseased fish

Histological Findings

No parasite or parasitic formations were detected in the histopathological examination of various internal organs such as the kidney, liver, intestine, spleen, and gills of diseased fish. However, while the general histologic appearance of the intestinal tissue of the examined fish was normal (Figure 3a), the presence of vacuolar degeneration and diffuse hemorrhagic foci within the tissue was noted in the detailed examination with a higher magnification (Figure 3b).

Moreover, it was observed that fish had intense tissue loss in the abdominal muscle tissue, and the amount of adipose tissue increased significantly due to necrosis. Furthermore, a sporocystic structure in the ellipsoidal sporophorous vesicle, 550-750 µm in size, was noted in this region (Figure 4a). Numerous parasitic spores ranging between 1.5 x 20 µm in size were observed in the cyst (Figure 4b).

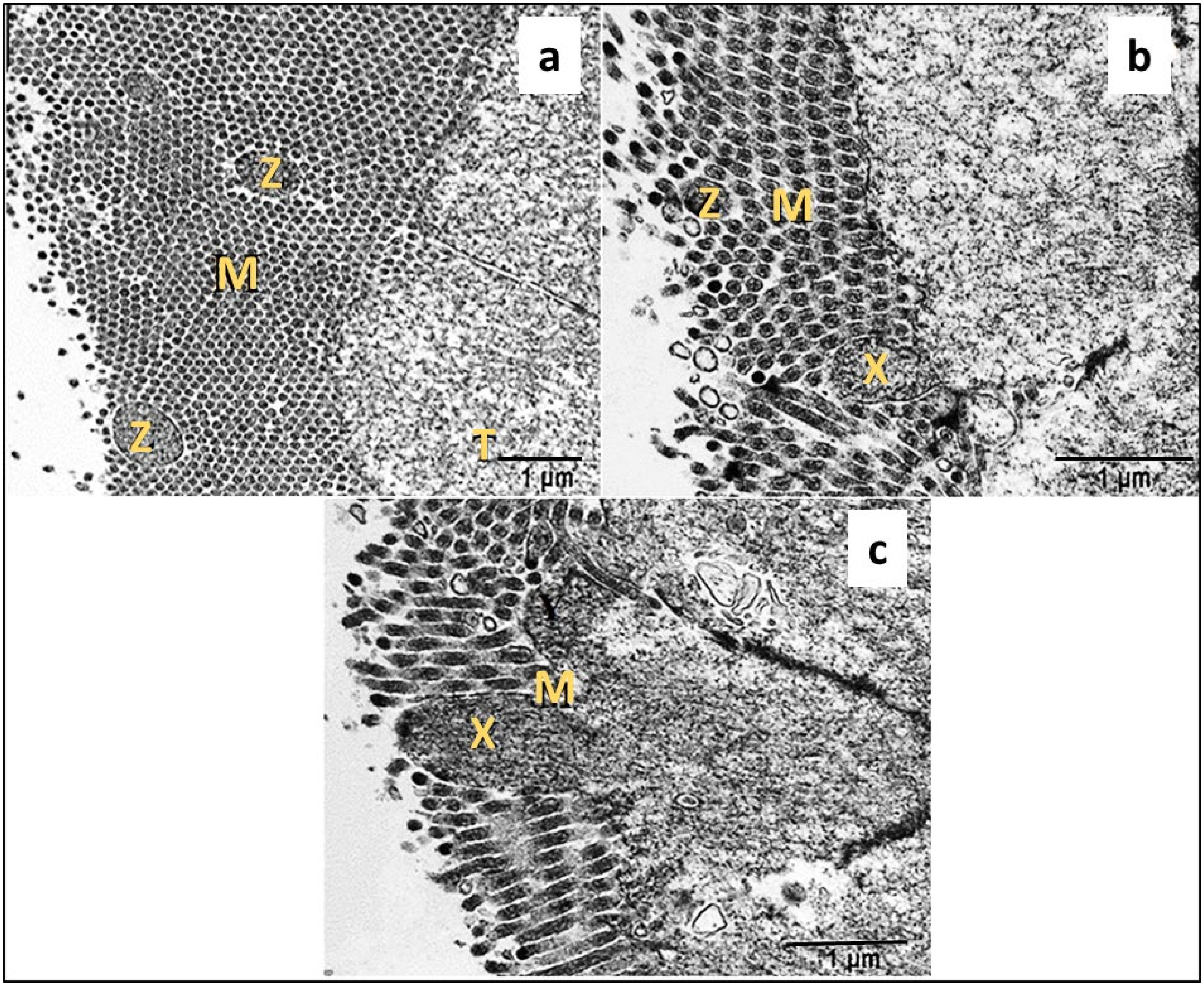

Figure 2 (a, b, c). The appearance of motile parasite spores between the transverse and longitudinal sections of the intestinal villi of fish (TEM). (**M:** microvillus, **Z:** parasitic spore, **X:** pyriform-like infective spores, **Y:** the appearance of spore entering the tissue, **T:** intestinal epithelial cell membrane)

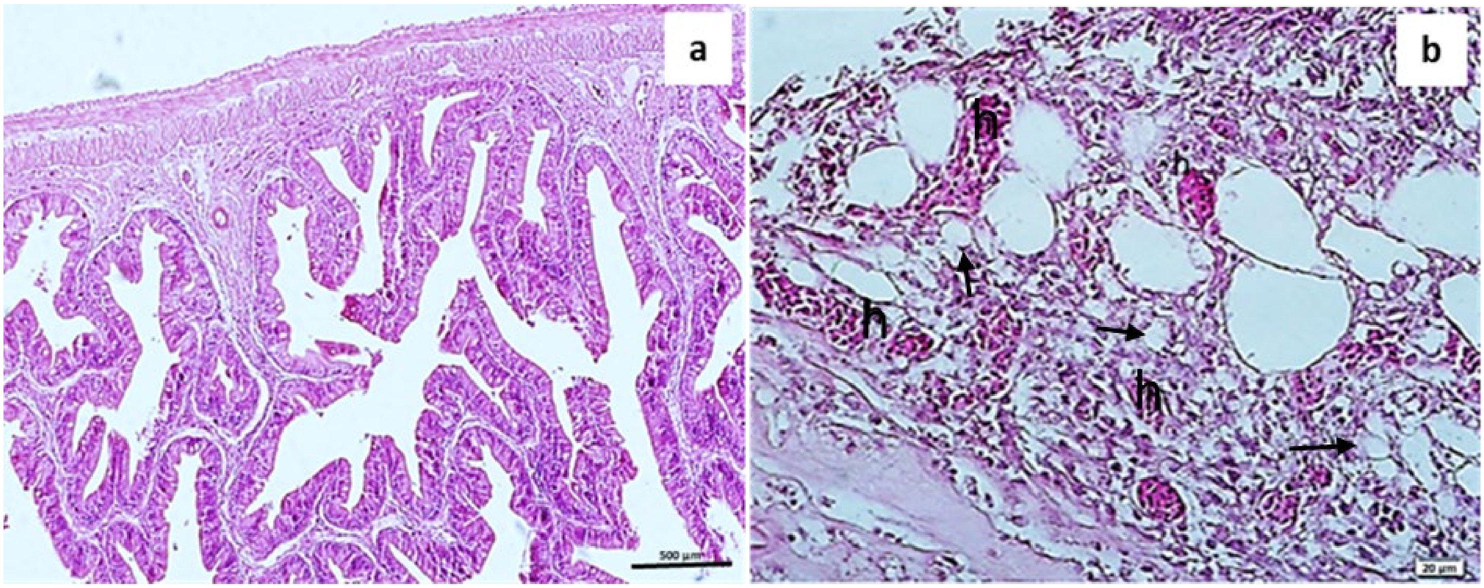

Figure 3. **(a)** General view of intestinal tissue of fish, **(b)** vacuolar degeneration (arrowed) and diffuse hemorrhagic foci (h) within the tissue

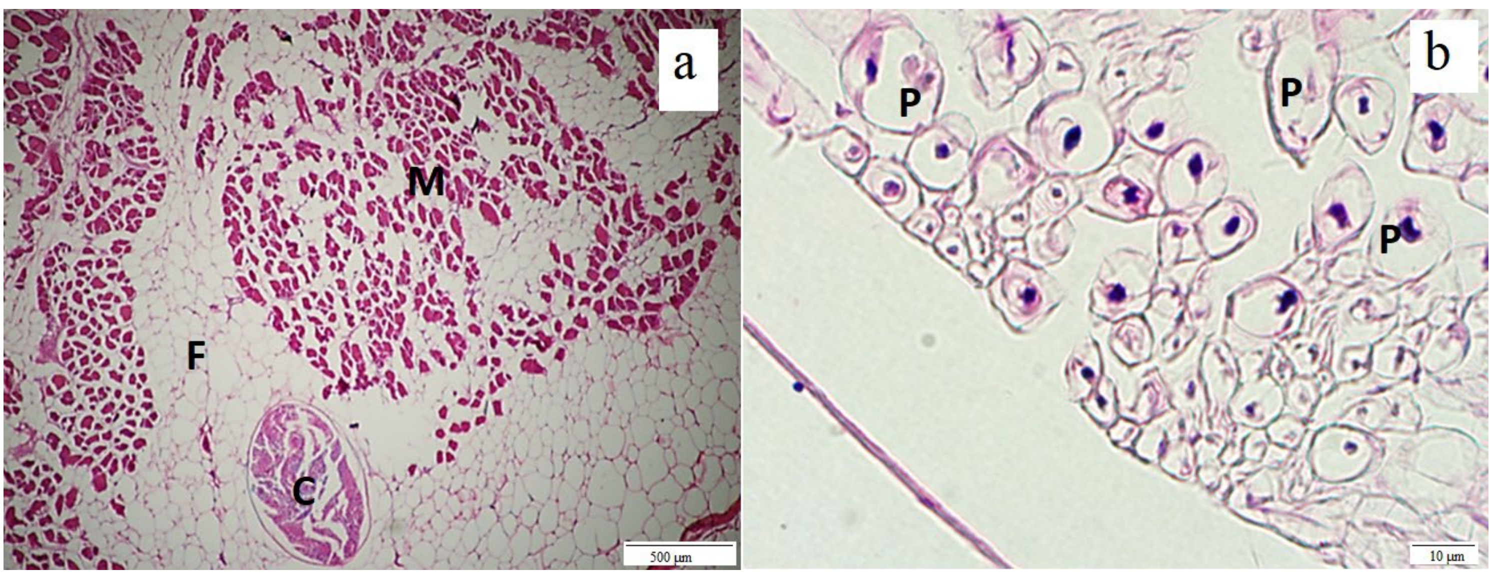

Figure 4. **(a)** General view of the sporocystic structure (C) in the abdominal muscle tissue of the diseased common pandora, (**b**) Appearance of numerous parasitic spores inside the cyst (C). (**M:** muscle, **F**: fatty tissue, **C**: sporocyst, **P:** parasitic spore)

As a result of the histological examination, it was concluded that the sporocystic structure containing numerous spores in the muscle tissue of diseased fish is similar to the sporocystic formations caused by the coccidian parasites in the muscle (Gjurčević et al., 2017).

Knowing the early stages and host penetration mechanisms of coccidian parasites is very important for the progression of infestations (Steinhagen et al., 1990; Molnar, 2006; Cascarano et al., 2021; Shrestha, 2022). Consequently, the growth rate of cultured fish reduces; which negatively affects the productivity of fish farms by causing high mortality in infected fish in advanced stages (Alvarez-Pellitero, 2004; Fioravanti et al., 2020; Shrestha, 2022).

Coccidia that is pathogenic to marine and freshwater fish do not have a distinct locomotion organelle, but their motile spores move forward with gliding movement and then enter the living cell with its apical complex (apicoplast) (Dyková and Lom, 1981; Harding and Frischknecht, 2020). The researchers examined the ultrastructural features of the apical complex and spores using electron microscopy to identify the parasites. Apicoplast was found to be present in the genera *Eimeria*, *Goussi*, *Toxoplasma* and *Sarcocystis* but not in *Cryptosporidium* (Molnar, 2006; Harding and Frischknecht, 2020). It has been reported that species such as *Eimeria sparis*, *E. dicentrari, E. bouixi*, *Goussia sparis* and *Cryptosporidium molnari* were found in the intestinal tissue of gilt-head sea bream and European sea bass cultured in Mediterranean countries and caused infestation (Alvarez-Pellitero, 1995; Gjurčević et al., 2017; Fioravanti et al., 2020).

Coccidian parasites have a complex life cycle with three primary stages: sporogony, merogony and gamogony. The sporogony phase occurs in the fish's external environment when the sporozoites enter the fish's gut. Coocidian parasite spores that enter from the intestines of fish transform into trophozoites in a vacuole structure that surrounds them in the intestinal tissue at the next stage. These cells become self-similar and multinucleated schizonts after the schizogony (asexual multiple division) stage. The cells developing in the schizont turn into an orange slice-shaped merozoite, and the merogony stage begins (Steinhagen et al., 1990; Dogga et al., 2015, Shrestha, 2022). In an experimental *Cryptosporidium molnari* infestation in gilt-head sea bream, all three stages were observed in different internal organs (Sitjà-Bobadilla and Alvarez-Pellitero 2002; Sitjà-Bobadilla et al., 2016). Yang et al. (2016) reported that *C. molnari* in koi fish (*Cyprinus carpio*) also causes severe granulomatous inflammatory lesions in the gills, liver, spleen, kidneys, and intestines of the fish. The size of coccidian sporozoites causing infection in fish is approximately between 0,5-1.5 µm x 20 µm (Sitjà-Bobadilla et al., 2016; Gjurčević et al., 2017; Harding and Frischknecht; 2020). This study determined that the parasitic spores of 0,3-1,5 x 0.5-30 µm in size enter the intestine. Infective parasitic spores passed through the microvilli, developed in this stage, and reached the intestinal cell by penetrating the host cell membrane. The patterns exhibited by the spores detected in the fish samples are very similar to the life cycle of coccidian parasites (Bruno et al., 2006; Harding and Frischknecht, 2020).

The present study determined using electron microscopy and histological examination techniques that parasite spores passed from the intestine to the fish, and cysts formed (550-750 µm in size) in the muscle tissue in the next stage. In the cyst, numerous parasitic spores ranged between 3.5 x 5 µm in size. Moreover, the studies performed with the electron microscope revealed that coccidian parasite spores migrate to the host cell through the intestinal microvilli. In contrast, the histological examination of the tissue sections revealed that the spores progress towards the abdominal muscles and form a sporocystic structure. As indicated by other researchers, the present electron microscope study revealed that the parasitic spores, which are found in the surrounding aquatic environment of the diseased common pandora and enter through the anus, pass through the intestines and reach the fish via anal gavage (Steinhagen et al., 1990; Molnar, 2006; Dogga et al., 2015; Sitjà-Bobadilla et al., 2016). The variation of the parasite localisation among *Eimeria*, *Goussia* and *Cryptosporidum* species made identifying this coccidian species difficult. The cystic structure was not observed in the intestinal tissue of the infected fish, but local hemorrhagic foci and vacuolisation were noted. However, the cystic structure developing in the muscle tissue containing many nuclei and oval-shaped spores indicates that it is in the schizont-forming stage (Steinhagen et al., 1990; Dogga et al., 2015; Shrestha, 2022).

Conclusion

Light and electron microscopy and histological techniques are widely preferred to describe coccidian parasites' taxonomy, life cycle and developmental forming. The very small and oval-shaped parasite spores in the intestines of the diseased common pandora (*Pagellus erythrinus*), which formed sporocysts in the abdominal muscles, were identified as *Coccidian* sp. However, since the ultrastructural properties and histological findings in the current investigation covered only some of the life stages of the sporozoan parasite in detail, a complete identification could not be made. We can only suggest that the intracellular parasite has a low capability to spread infection in the host cell and therefore develops at the onset of the disease. These findings will help expand our understanding of coccidian-induced diseases of the fish.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The local ethic comittee report was received in İstanbul University, Animal Experiment Local Ethic Committee Report at 01/7 report number and 13.012.2008 report date.

Funding disclosure: The Istanbul University Research Projects Unit project., supported this study project number 2637.

Acknowledgments: -

Disclosure: -

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