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Short Communication

Oncogenic papillomavirus-like particles in Angelfish (*Pterophyllum scalare*) from Iran

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Introduction

The incidence of neoplasia is not a specificity of human tissues and is not even restricted to mammals. Notably, it is a common occurrence in piscine species -the largest group of vertebrates- with over 27,000 species (Claver and Quaglia, 2009). Compared to mammals, malignant neoplasms with or without metastasis are seldom reported in fish (Hugh Ferguson, 2006; Grizzle and Goodwin, 2010), which could be due to a different antitumor immunity in fish (Bubanovic and Najman, 2005). Fish neoplasms are often benign (da Rocha *et al.*, 2017). In general, benign neoplasms are well-differentiated neoplasms that represent a slow rate of growth and have been demonstrated as a cohesive expansive mass that is often delineated by a fibrous capsule (Cotran *et al.*, 1999). Cutaneous neoplasm -also known as skin tumor- is a rare and frequently benign soft tissue neoplasms, however, recurrence is common in cases of incomplete surgical excision (Groff, 2004). The most recent WHO classification for skin tumors (Gershon, 2021) categorizes fibromas and their

precursor lesions, totally benign, based not only on their clinical and histomorphological characteristics but also on their molecular profile and genetic fingerprint. Fibromas are characterized as discrete, raised, homogeneous, encapsulated, white to pale tan or pink, firm to hard tumors of variable size with a narrow (pedunculated) or broad base that may be firmly attached at the base or may appear as a loosely attached tag of tissue and may not always be covered by epithelium. It is rarely identified in fishes (Ferguson, 1989, Roberts, 2001). Papilloma generally is a benign neoplasm of epithelioid cells that both higher and lower vertebrates could engage with (Peters and Watermann, 1979; Hedrick *et al.*, 1990; Rahmati-Holasoo *et al.*, 2015). It is known as the most prevalent skin neoplasms in wild and reared fish species and their appearance features are described as discrete, soft to firm, slightly to prominently raised , Single or multiple, papillary growths with a presence as flat to nodular cutaneous plaques (Roberts, 2001; Chong, 2022). Papillomaviruses as infectious agents of the family *Papillomaviridae* suspected in association with several of these proliferative diseases in a wide range of vertebrates, alike in piscine species(López-Bueno *et al.*, 2016; Tisza *et al.*, 2020; Labella *et al.*, 2019; Surján *et al.*, 2021; Kraberger *et al.*, 2022). It is worth mentioning that, an odontomas/fibromas retroviral-induced tumor (Rahn *et al.*, 2004; Claver and Quaglia, 2009) and a fibroma tumor previously recognized in *Pterophyllum spp* (Coffee *et al.*, 2013; Vergneau-Grosset *et al.*, 2017). Given that the detections of various types of viruses in fish tumor lesions were severely reported (Anders and Yoshimizu, 1994). To our knowledge, this is the first report of oncogenic virus detection that has a striking resemblance to papillomavirus particles associated with fibropapilloma tumor of angelfish in Iran.

Materials and methods

Specimen collection

This study was performed on 100 specimens of 1 to 2 years angelfish (*Pterophyllum scalare*) (72% male and 28% female) belonging to an ornamental fish reproduction farm in Gilan province, which delivered alive to "Virology laboratory for fish, of Inland waters aquaculture research center, Bandar Anzali, Gilan, Iran" with visible lips hyperplasia, for diagnostic examinations. To perform a necropsy, fish were anesthetized with clove (*Syzgium aramaticom*) powder which was diluted in water (0.7 g. L^{-1}). The routine tissue collection was performed from the main organs (lips, liver, kidney, and spleen) for histopathological examinations.

Physicochemical assessment

After observing the abnormal signs in fish, the water samples of the studied reproduction farm were immediately collected. The collection assortment,

preservation, and analysis of the main ions of water samples were carried out through standard methods explained by the EPA (Nelson, 2003).

Histopathological examinations

Tissues were excised and placed in 10% buffered formalin solution in conical tubes. After 24 hours, formalin was refreshed. Tissues were dehydrated in ethanol gradient of 50, 70, 90, and 100 percent of concentration and cleared with chloroform then embedded in paraffin. The sections of 5 µm were prepared from tissue samples using a rotary microtome(Leitz 1512, Germany) and stained with hematoxylin and eosin (H&E) and also Masson's trichrome (MT). Afterward, sections were examined by light microscope (Nikon Ci-L plus, Japan) according to the standard procedures (Bancroft and Gamble, 2008).

Electron microscopy

Sections were prefixed with 2.5% glutaraldehyde (TAAB laboratories-3 Minerva, Calleva park, Aldermaston, Berks, RG78NA, England–EM grade) in 0.1 M phosphate buffer saline (PBS, pH 7.2) for 2 h at 4°C. The electron microscopy procedure was performed in Rastak Lab (Tehran, Iran) as follows: prepared sections were washed three times in the PBS (10 min for each time). After washing, they were post-fixed in 0.5% osmium tetroxide (TAAB laboratories-3 Minerva) in the same buffer at room temperature for 1 h. After washing in the PBS for three times (10 min for each time), the samples were dehydrated in ascending alcohol series, acetone, acetone-resin mixture (50/50) and finally embedded in TAAB embedding resin (TAAB laboratories-3 Minerva) and polymerized in 60°C for 48 h. Fifty nm ultra-thin sections were then prepared by Lika Ultracut R (Lika, Wetzlar, Germany), placed on 300 mesh copper grid and double stained with 20% uranyl acetate (BDH Laboratory Chemicals Division, England, No. 0148860) in pure methanol (E. Merck, D-6100 Darmstadt) for 45 min and in Reynolds solution (lead nitrate and sodium citrate; Reynolds, 1963) for the same time. Finally, the samples were examined with the transmission electron microscope (EM208S, PHILIPS, Netherlands) at the accelerating voltage of 100 kV.

Results and discussion

Physiochemical characteristics of water

The water source of the surveyed ornamental farm was obtained from deep wells. As mentioned in Table 1, the consequences of hydrochemical assessments of water samples, declared the normal condition for examined water samples, in a way that neither endocrine-disrupting nor health-threatening rate of measured parameters resulted (Table 1).

Macroscopic and microscopic examinations

Grossly, abnormal cutaneous hyperplasia was clearly observed in all 100 freshwater angelfish, with the

characteristics of smooth prominent or cauliflower-like swellings, consistency, relatively crisp and brittle lips. Furthermore, there weren't any signs of existing abnormalities on the other parts of the skin. It should be mentioned that no abnormal swelling or metastases were detected in the internal organs during the necropsy. The diameter of the nodular tumors varied between 0.5 cm × 0.5 cm to 1cm × 1 cm. The color of the investigated tumors varied from light pink to cream. In addition, tumors were in constant contact with the surrounding skin tissue (Fig. 1).

Table 1: Consequences of hydrochemical assessments of water samples

Parameter	Value	Parameter	Value
Temperature (°C)	21	K^+ (mg/L)	1.97
Electrical conductivity (ms/cm)	0.71	Total hardness (mg/L)	246
pH	8.10	Cl^+ (mg/L)	147
Turbidity (F.T.U)	28	Nitrite (NO_2^-) (mg/L)	0.001
Dissolved Oxygen (mg/L)	11.3	Nitrate (NO_3^-) (mg/L)	0.037
Ca^{2+} (mg/L)	61	Sulfate (SO_4^{2-}) (mg/L)	30.9
$Mg2^+$ (mg/L)	23	Phosphate (PO_4^{3-}) (mg/L)	0.055
Na^+ (mg/L)	25.5	K^+ (mg/L)	1.97

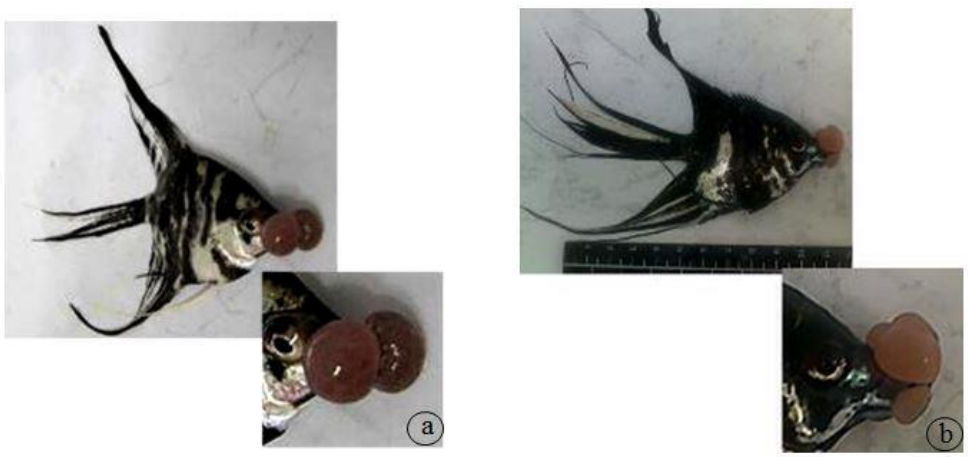

Figure 1: Gross pathology of *Pterophyllum. scalare*. (a, b) represent typical epidermal fibropapilloma on the jaws.

In the microscopic study, prominent squamous cell hyperplasia was observed with increasing epidermal thickness. The vacuolization and necrosis of cells were clear in the malpighian layer. In the dermis layer, the proliferative fibroblasts were seen in a way that the arrangement of fibroblast cells was bundled. Additionally, thickness, folding and swelling, and vascularization were also observed. The Hypodermis contained thick edematous fibrovascular stroma and fibroblastic cells were severely proliferated, which was considered as the tumor stem. Mason trichrome-specific staining showed blue collagen fibers that resulted in fibroblast activities. The tumor cells showed in reddish-brown (Fig. 2).

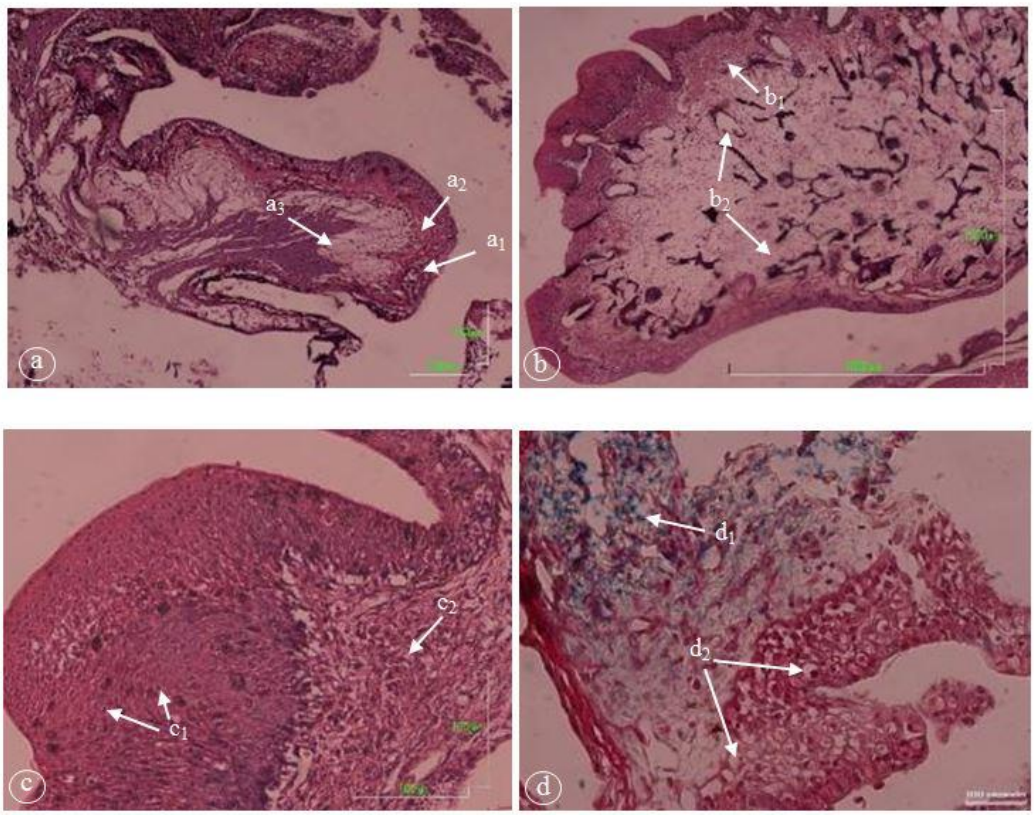

Figure 2: (a) A fibropapilloma tumor; (a_1) the hyperplasia of epidermis that vacuolization of epidermal cells showed with arrow; (a_2) the swelling and folding of abnormally proliferated dermis; (a_3) the stem of tumor, fibroblasts proliferated intensely (H&E, Bar: 100 µm). (b_1) vacuolization of malpighian cells; (b_2) The dermis area was severely edematous vascularization and budding was also observed (H&E, Bar: 1000 µm). (c) Hyperplasia of squamous cell carcinoma, the thick fibrovascular stroma of epithelium; (c_1) the swollen nuclei of activated fibrocytes which indicates collagen production and tumor appearance; (c_2) necrosis and abnormal proliferation of malpighian layer (H&E, Bar: 100 µm). (d) Hyperplastic mass of lips skin; (d_1) green-blue points illustrate collagen fibers, (d_2) Conjunctival squamous papilloma and epithelioid cells with pleomorphic nuclei and vacuolated cells were observed (MT, Bar= 100 µm).

Virus morphogenesis

Examination of ultrathin sections of virus-infected tissues by transmission electron microscopy (TEM), showed aggregation of virus particles. In some infected cells, virions existed within both the cytoplasm and nuclei (Fig. 3a). Similar to the virions of the family *Papillomaviridae*, studied virions were seen as non-enveloped and capsomeres arranged in icosahedral symmetry which could be observed as hexagonal two-dimensional arrays in the size range of approximately 50 to 70 nm (Fig. 3d).

In this study, histological analyses demonstrated that the lesions in *P. scalare*, which are mainly observed in the skin of fish in spring, are cutaneous hyperplasia that represented characteristics of both fibroma and papilloma, which is called fibropapilloma. Therefore, this study is the first report of fibropapilloma of neoplastic transformation in tissues of angelfish in Iran.

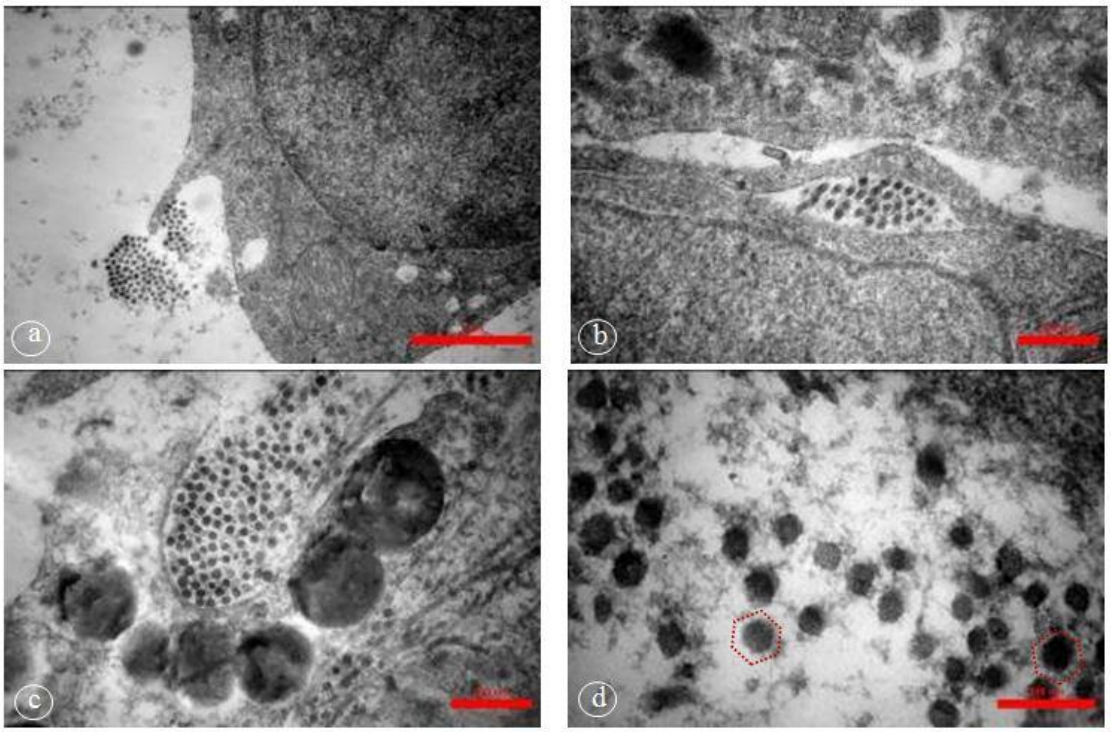

Figure 3: Electron micrograph of the cytoplasm and nuclei of enlarged cells. Many Inclusions are present in the cytoplasm that contain densely staining virus-like particles; (a to c) the aggregations of virus-like particles presented in various magnifications; scale bars refer 1µ, 500 nm, and 500 nm respectively. (d) the two-dimensional hexagonal characteristics of some papilloma virus-like particles outlined with red dashes; Bar=200nm

The most revealed reason for tumor manifestation in fish are infectious agents. Due to the visibility and ease of sampling, virus-associated tumors of the skin have been studied extensively (Bowser and Casey, 1993). Considering that thirteen proliferative neoplasms have been identified as diseases associated with the presence of retroviruses or retrovirus-like particles in fish species (Quackenbush *et al.*, 2010), based on provided electron micrograph evidence of this research, the main features of *Retroviridae* family including spherical envelope, diameter ranging between 100 to 200 nm, heterogeneous morphologies and polymorphic capsids of mature ones and distinct doughnut shape morphology of immature ones which capsomeres covered by the surface membrane (Zhang *et al.*, 2015) was not observed. Conversely, the electron microscopic observations of the present study suggested the presence of papillomavirus-like particles in examined angelfish. Papilloma in *P. scalare* is histologically similar to the papilloma seen in Brown bullhead (*Ameiurus nebulosus*) and White sucker (*Catostomus commersonii*) (Smith, 1989; Poulet *et al.*, 1994; Premdas *et al.*, 1995). Although the papilloma in the present study showed different developmental stages, none of the specimens examined were invasive or metastatic, indicating that the tumors were benign.

Inflammation in papilloma can be a progressive factor for papilloma growth (Smith, 1989; Premdas *et al.*, 1995). In the present study, no leukocytes were observed in the histological sections of angelfish. To prevent neoplasms development in the aquatic environment, filtration, frequency of water exchanges, disinfection, and the employment of broodstocks free of oncogenic agents are recommended.

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