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CASE REPORT

Pathobiological Findings of Epitheliocystis in Giant Grouper (*Epinephelus lanceolatus*) in Taiwan

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A B S T R A C T

A Giant groupers (*Epinephelus lanceolatus*) farm in southern Taiwan displayed symptoms of hypoxia with mortality around 3.5%. Wet mount examination revealed mottled gills with abundant oval-shaped cysts as the main characteristic lesions. Sporadic occurrence of diffusely distributed white nodules in the kidney was also observed. Through histopathological examination, basophilic, cytoplasmic cysts were found diffusely scattered in the secondary lamellae. The contents of the cysts produced a dark magenta hue under Macchiavello stain, indicating the presence of chlamydia-like organisms. Focal to multifocal granulomatous, necrotic lesions were observed in the kidney and spleen, and were the result of *Vibrio* spp. and *Photobacterium* spp. infection. DNA samples were extracted from the gills and the resulting product was a 298 bp fragment with a nucleotide sequence that was 98% homologous with *Simkaniaceae* spp. As the result, the final diagnosis was epitheliocystis with secondary bacterial infection.

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INTRODUCTION

Epitheliocystis is a common disease of fish around the world, but cases in giant groupers (*Epinephelus lanceolatus*) have not been formally reported yet. To date, despite failures to isolate the organism *in vitro*, the causative agent of epitheliocystis is classified as a chlamydia-like organism (CLO) on the basis of ultrastructural morphology (Grau and Crespo, 1991) and DNA sequencing of this organism (Kumar *et al*., 2014). The most common symptom of infected fish was associated with oxygen insufficiency, and the most characteristic lesion of epitheliocystis is hypertrophy of the epithelia of the gills with distinctive cytoplasmic inclusion bodies, which contain abundant basophilic granules. The morbidity and mortality are variable depending on different factors, such as species, climate, and fish density or age (Draghi *et al.*, 2004). Mortality of epitheliocystis in cultured fish may range from 4% to 100% (Kim *et al.*, 2005).

History and clinical examination: A population of about 3,400 stepwise *Epinephelus lanceolatus* was maintained at an indoor fish farm in southern Taiwan. The population was divided into two 30 m^3 ponds with 1,500 fish and one 10 m^3 pond with 400 fish supplied with constant ambient 25°C non-circulating seawater. Since 25th October 2013, growth rates were retarded and fish appeared lethargic. Hyperpigmentation was observed as well as a tendency of fish to congregate near the air-water interface (Fig. 1, A and B). Deaths occurred at a rate of 2-12 fish per day. Formalin bath (30 ppm, 1-2 h), amoxicillin (40 mg/kg/d, per oral, 3-5 d) and florfenicol (10 mg/kg/d, per oral, 3-5 d) were used as treatment, but the situation did not improve (Fig. 2).

Wet mount microscopy of the gill filament revealed abundant, transparent, round to oval-shaped cysts (Fig. 1C), which were about 14 to 70 µm in diameter. On average, 23 cysts could be calculated under a moderate magnification (100×) view (Fig. 1D).

Gross lesions: The fish were transported under refrigeration to NCHU within one day after death. They were about 30 cm in total length and appeared emaciated (Fig. 3A). All of the fish displayed multifocal pale patches distributed throughout the gills, giving them a mottled appearance that was also accompanied by increased mucus secretion. Two of the 10 fish also exhibited small numbers of pinpoint hemorrhages in the gills (Fig. 3 B and C). Thirty percent of the submitted fish had white to yellow, pinpoint nodules scattered throughout the posterior portion of the kidney.

Histopathology findings: The most significant histopathological finding was the presence of cytoplasmic inclusions in the epithelial cells diffusely scattered in the secondary lamellae of the gills with mild chronic progressive necrotizing bronchitis. At low magnification, basophilic, round to oval-shaped cysts encapsulated by epithelial cells were observed. Secondary lamellae were fused and necrotic in differing levels of severity and accompanied by mucus hypersecretion (Fig. 4A). At higher magnification, the infected epithelial cells appeared variably enlarged and solid with well-distributed cytoplasmic inclusions composed of dense, uniform-sized fine basophilic granules. Packing of the cytoplasm with these granules displaced the nuclei to the borderline of cells (Fig. 4B). Macchiavello staining in this case provided a clearer distinction between the epithelial cysts and the gill filaments (Fig. 5B). A positive result of the Macchiavello stain was perceived as dark magenta to maroon dyeing (Fig. 5C), indicating that the agents in the epithelial cysts were CLO or Rickettsia-like organism (RLO). Some magenta, granular substance, which was similar to the characteristic contents of epithliocysts, was distributed in multiple cells of the secondary lamellae. This substance was considered to be the pathogens that were transmitted from the cysts in the initial stage (Fig. 5D).

Severe necrotizing, granulomatous nephritis of the posterior kidney was seen in 30% of the submitted fish. Multiple areas of necrosis, along with heterophil infiltration with bacterial colonies located in the center of the necrotic lesion, were noted. Similar to the kidney, necrotic and granulomatous splenitis was observed.

Etiology identification: Tissue homogenates were prepared from gill samples of the submitted fish. The primer pairs 16SIGF and 16SIGR used in the detection of Chlamydiales from gill samples have been previously described (Kumar *et al*., 2014). In this case, the primer pair amplified a 298 bp fragment from the gills (Fig. 6). Sequenced the fragment, the nucleotide sequences were similar to *Simkaniaceae* bacterium with 98% homogenicity.

Blood agar and thiosulfate-citrate-bile salts-sucrose (TCBS) agar were used for bacterial isolation from the kidney, and produced 2 different colonies, *Vibrio harveyi*, and *Photobacterium damselae* subsp. *damselae*. Isolated bacteria were cultured with antibiotic discs on Müller-Hinton agar at 25°C for 18 h. The results revealed that *P. damselae* subsp. *damselae* was susceptible to nalidixic acid, florfenicol, ceftiofur, and enrofloxacin, while *V. harveyi* was susceptible to nalidixic acid, florfenicol, lincospectin and enrofloxacin.

Treatment: *P. damselae* and *V. harveyi* are autochthonous inhabitants of aquatic ecosystems. In the present case, we considered the causative agent of the epitheliocystis as the primary pathogen, and the bacterial infections as secondary. Therefore, it was recommended that the owner use oxytetracycline (OTC) based on the results of the antibiotic susceptibility assay and the fact that OTC has been the most common drug used to treat epitheliocystis in other reported cases. OTC was mixed in the feed at a dose of 50 mg/kg/d for 3-5 consecutive days. Deaths declined after the medication was administered for two cycles of the recommended usage with a one-day interval between the two cycles (Fig. 2). 10 days later, no deaths were observed.

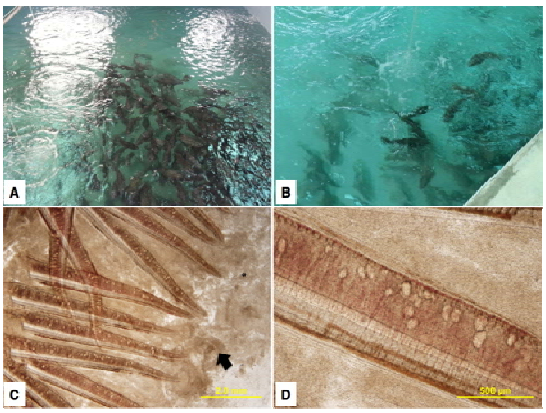

Fig. 1: A & B) The fish were congregating near the surface of the water and air pump; C) Wet mount demonstrated mucus hyper-secretion of the gill (arrow), bar=2.0 mm and D) oval-shaped cysts, bar=500µm.

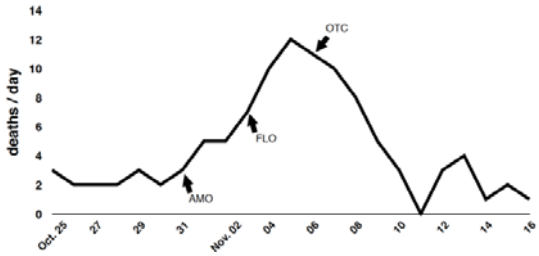

Fig. 2: Numbers of dead fish during the disease outbreak. Treatment started with amoxicillin (AMO), florfenicol (FLO) and oxytetracycline (OTC) on various days.

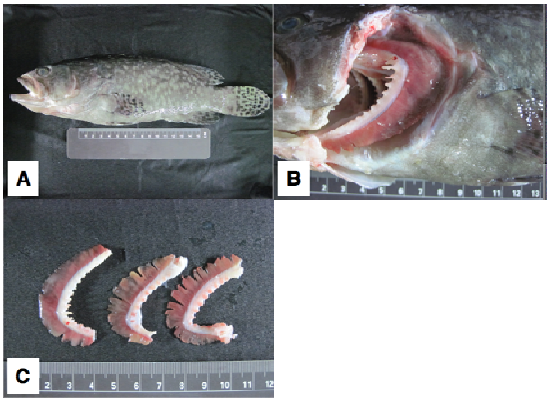

Fig. 3: A) Emaciation, mucus hypersecretion of the body surface; B and C) Mottled appearance of gills with multiple pale plaques and increased mucus secretion in every submitted fish.

DISCUSSION

The final diagnosis of the present case was epitheliocystis with secondary autochthonous bacterial infection. There have been sporadic cases of epitheliocystis infections in southern Taiwan in the past few years without formal report. The similarities of those cases to this case indicate the disease may be endemic in giant groupers in Taiwan.

In this case, all of the clinical signs were associated with hypoxia. The fish were congregating near the air-water interface and air pump, contradicting the usual behavior of this demersal fish. General causes of hypoxia syndrome include environmental hypoxia, gas bubble disease or parasitic

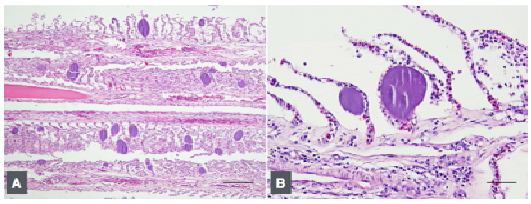

Fig. 4: Abundant basophilic oval-shaped inclusion cysts in the secondary lamella. Gill, H&E; A) bar=200 µm; B) bar=50 µm

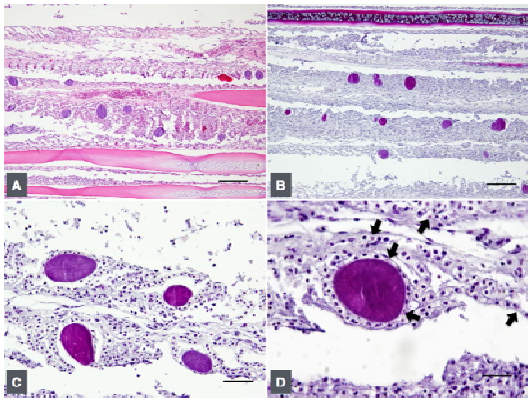

Fig. 5: Compared to H&E stain (A) and Macchiavello stain (B) distinguished the cysts from gill tissue more clearly. bar=200 µm. C) bar=50 µm and D) Light magenta granular materials (arrow) located in some epithelia of the gills. Macchiavello stain, bar=25 µm.

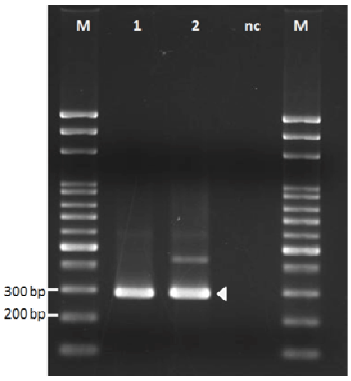

Fig. 6: Agarose gel electrophoresis of PCR products. M: 100 bp DNA ladder; lanes 1 and 2: test samples; nc: negative control. Arrowhead indicates 298 bp PCR products.

infections of the gill. This case had a low morbidity rather than the nearly 100% morbidity caused by environmental hypoxia (Noga, 2010). Furthermore, parasitic infections and gas bubble disease can be easily diagnosed under wet mount microscopic examination.

The diagnosis of epitheliocystis was made based on wet mount microscopy and pathological examination. Lymphocystis should also be considered in the differential diagnosis of cases of epitheliocystis in marine fish (Toenshoff *et al*., 2012). Moreover, epitheliocystis infects mostly epithelial cells while lymphocystis affects dermal fibroblasts. In order to identify further the cytoplasmic organism, gills were subjected to PCR and DNA sequencing. The outcome indicated that the organism shared 98% homology with *Simkaniaceae* bacterium, which belongs to the order Chlamydiales.

Though Chlamydiales and *Rickettsiae* are Gram-negative bacteria, Gram stain does not differentiate them from the cellular material with which they are usually associated. Giemsa and Macchiavello stains are commonly used to detect Chlamydiales in impression smears and paraffin sections (Sachse, 2014). In Macchiavello stain, organisms are dark red to magenta against the blue background of the tissue, if the organisms are chlamydia or rickettsia. The dark magenta produced after Macchiavello staining of the cysts in this case corresponded to CLO (Luna, 1993).

The organisms identified through microbial analysis were *P. damselae* subsp. *damselae* and *V. harveyi*, which induced necrotic nephritis, splenitis and liver necrosis (Vaseeharan *et al*., 2007). Although multifocal necrosis of the kidney and spleen could be seen in the case, due to the low proportion of this lesion in the total number of dead fish, this was suspected to be secondary infection rather than the primary cause. Moreover, the results of the antibiotic susceptibility assay support this viewpoint.

Conclusions: Chronic infection and enlargement of cysts in the gills led to oxygen insufficiency and the clinical signs associated with it. Stress brought on by this chronic infection resulted in secondary bacterial infection. However, treatment with OTC after the diagnosis of epitheliocystis proved to be very effective. Giant grouper is a species that possesses very high economic value. Therefore, implementation of a disease surveillance system was suggested to prevent future outbreaks. Both normal and ill fish should be checked for the presence of gill cysts on a regular basis.

Authors' contribution: HKC and YLT diagnosed the cases and wrote the report for this case. KPL performed the molecular biology examination. YCW isolated and identified the bacteria. JHS, SLH and CCL provided guidance on the case and the report. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

REFERENCES

Draghi A, Popov VL, Kahl MM, Stanton JB, Brown CC *et al*., 2004. Characterization of "*Candidatus* Piscichlamydia salmonis" (order *Chlamydiales*), a Chlamydia-like bacterium associated with epitheliocystis in farmed atlantic salmon (*Salmo salar*). J Clin Microbiol 42: 5286-5297.

Grau A and Crespo S, 1991. Epitheliocystis in the wild and cultured amberjack, *Seriola dumerili* Risso: ultrastructural observations. Aquaculture, 95: 1-6.

Kim DJ, Park JH, Seok SH, Cho SA, Baek MW *et al*., 2005. Epitheliocystis in Carp (*Cyprinus carpio*) in South Korea. J Vet Med Sci, 67: 119-120.

Kumar G, R Mayrhofer, H Soliman and M El-Matbouli, 2014. Novel Chlamydiales associated with epitheliocystis in grass carp (*Ctenopharyngodon idella*). Vet Rec, 172: 47.

Luna LG, 1993. In: Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts. American Histolabs, Inc., Publications Division, Gaithersburg, MD, pp: 236.

Noga EJ, 2010. Problem 1: Environmental hypoxia. Noga EJ. In: Fish disease: diagnosis and treatment, 2nd Ed. Wiley-Blackwell, Iowa, USA, pp: 81-87.

Sachse K, 2014. Avian chlamydiosis. In: Manual of diagnostic tests and vaccines for terrestrial animals 2014. Vol.1, OIE, Sec 2.3 AVES, Ch 2.3.1, pp: 1-13.

Toenshoff EB, Kvellestad A, Mitchell SO, Steinum T and Falk K, 2012. A Novel Betaproteobacterial Agent of Gill Epitheliocystis in Seawater Farmed Atlantic Salmon (*Salmo salar*). PLoS ONE, 7: e32696.

Vaseeharan B, Sundararaj S, Murugan T and Chen JC, 2007. *Photobacterium damselae* subsp. *damselae* associated with diseased black tiger shrimp *Penaeus monodon* fabricius in India. Lett Appl Microbiol, 45: 82-86.