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RESEARCH ARTICLE

Outbreak of Swim Bladder Inflammation Caused by *Sphaerospora dykovae* in Koi (*Cyprinus carpio koi*) in Taiwan

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A B S T R A C T

SBI has had a severe economic impact on the fish industry in central Europe, but the present study is the first to describe an outbreak of SBI in Koi in Taiwan, even in Asia. A group of 7-month-old koi (*Cyprinus carpio koi*) showed abnormal swimming behavior and abdominal distension with a fatality rate of 40%. Smear examination of swim bladders showed numerous identical basophilic spherical organisms. Gross examination revealed severe adhesion of abdominal organs and erosion, enlarged of the swim bladder. Severe inflammation of the submucosal layer of the swim bladder and large numbers of pansporocysts and zygospores were noted microscopically with Hematoxylin and Eosin stain, Giemsa and modified Grocott's methenamine silver (GMS) stain. The nucleotide sequence of the pathogens isolated from the fish samples had a 99% homology with *Sphaerospora dykovae*, the pathogenic agent in swim bladder inflammation (SBI).

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INTRODUCTION

Swim bladder inflammation (SBI) in fingerlings of carps (Cyprinidae) is an acute and severe disease which has a considerable economic impact. Seasonal outbreaks have been reported in central Europe, especially Hungary and Czech Republic. In the published studies, the prevalence of SBI was about 50% in general, and the mortality rates can reach 100%. With younger carp, considerably lower incidence rates have been reported (Holzer *et al.*, 2014; Gómez *et al.*, 2015). Though SBI is a common fish disease in Europe, the disease has not been reported in Taiwan, nor in other Asian countries.

MATERIALS AND METHODS

History of the disease outbreak in the fish farm: The farm had five hundred koi (*Cyprinus carpio koi*), which were approximately seven months old, were maintained indoors. In December 2014, the fish showed abnormal swimming behavior, with abdominal distension, and subsequently died. The morbidity rate of the fish in the farm was 50% (250/500) and the fatality rate was 40% (100/250). The owner submitted five of the ill fish to the animal disease diagnostic center in National Chung Hsing University (ADDC-NCHU) for diagnosis.

Laboratory analysis: The submitted fish was necropsied and sampled. Swim bladder, spleen, and gill were smeared on glass slides and stained with Diff-Quik solution (Jackson *et al.*, 2013). Some of the tissues were sampled for histopathology exam with Hematoxylin and Eosin stain (H&E stain), Giemsa stains (Stout *et al.*, 2013) and modified Grocott's methenamine silver stain (GMS stain) (modified from Luna, 1992; Ma *et al.*, 2013;) (Deparaffinize; 4% chromic acid for 1 hour; wash and rinse in distilled water (DW); 1% sodium sulfite for 1 minute; wash in DW; stain with working methenamine silver solution (3% hexamethylenetetramine, 5% silver nitrate, 5% borax in DW) in 60℃ for 1 hour; rinse with DW; 0.5% gold chloride for 3-5 minutes; wash the slides with 5% hypo and used light green (0.2 g light green, 0.2 ml glacial acetic acid, 100 ml DW) 1 minute as counter stain; air dry the slides.). Blood agar and MacConkey Agar were used for bacterial isolation from the swim bladder and effusion of the body cavity.

Pathogen identification: For pathogen identification, kidney, and gill samples. SphF and SphR were used as the primer to 18S rDNA of *Sphaerospora* spp. in goldfish, which has been published before (Molnár *et al*., 2010; 2012; Holzer *et al.*, 2013; Liu *et al.*, 2016). We also used specific primer of koi herpes virus (TKf and TKr, El-Matbouli and Soliman, 2011; Meyer *et al.*, 2012; Monaghan *et al*., 2014) and spring viremia of carp virus (SVCV F1 and R2; Wolf, 1988; Dikkeboom *et al.*, 2004; López-Muñoz *et al.*, 2010) that have been used to screen the submitted fish.

RESULTS

In appearance, all of the submitted fish showed swollen abdomen (Fig. 1A). Several organs showed severe adherence to each other (Fig. 1B) and abundant bloody effusion had accumulated in the body cavity. Lesions were typically characterized by a swollen, tympanic, and opaque swim bladder, and the kidney was swollen in appearance (Fig. 1C). Serosae of swim bladders, both of anterior and posterior chambers, were massively hemorrhagic with several festering foci. The distended chamber contained copious clear, yellowish and mucinous effusion with a little suspension. The inner surface of the swim bladders was thickened, while the swim bladder wall was gelatinous and hemorrhagic (Fig. 1D).

In the smears of swim bladders, abundant clusters of organisms could be observed. There were 2 different forms of those organisms. One was soroplasm-like (Fig. 2A), and the other was pansporocyst-like, comprising 4 to 6 basophilic and ellipse nuclei (Fig. 2B). The histopathology revealed that swim bladders had severe edema, necrosis, and hemorrhage from the submucosa to serosa (Fig. 3A). Abundant pansporocysts were found, which packaged 12 to 25 basophilic zygospores within an tissue homogenates were prepared from swim bladder, eosinophilic capsule. The pansporocysts were about 25-40 μm in diameter, and had infiltrated the incrassate submucosa and muscular layer with scattered macrophages and lymphocytes (Fig. 3B). Large amount of zygospores were not only located within a cyst but also dispersed within the muscular and submucosal layer of the swim bladders (Fig. 3C). Many organisms measuring 20 μm in diameter and possessing characteristically symmetrical water drop-shaped nuclei were found in the same area. They were considered to be mature myxospores (Fig. 3D). All of the cystic structures of the organisms could be clearly distinguished from the normal swim bladders tissues by Giemsa stain (Fig. 3C, D). In GMS stain, the organisms were stained black, which could be distinguished from the green background in the swim bladder and kidney (Fig. 4A, B). The pyriform nuclei were stained black by GMS, which should be the polaroplast of the myxospores, could be observed clearly under high power view (Fig. 4C). Polaroplast is the characteristic structure for microsporidia (Amigo, 1996). The histopathology of kidneys revealed aggregation of numerous basophilic cells in the renal interstitial tissues. These round cells had hollow or light purple plasma and the marginalized nuclei resembled the symmetrical nuclei of the organisms in the swim bladder (Fig. 3E, F).

The primer pair amplified a 588 bp fragment product from all of the samples (Fig. 5). The nucleotide sequences were similar to *Sphaerospora dykovae*, with 99% homogenicity (Bartošová *et al.*, 2013). Tests were negative for the specific primer of koi herpes virus and spring viremia of carp virus. *Aeromonas jandaei* was isolated from swim bladder and effusion of the body cavity. The bacteria were greatly susceptible to florfenicol in the antibiotic susceptibility assay.

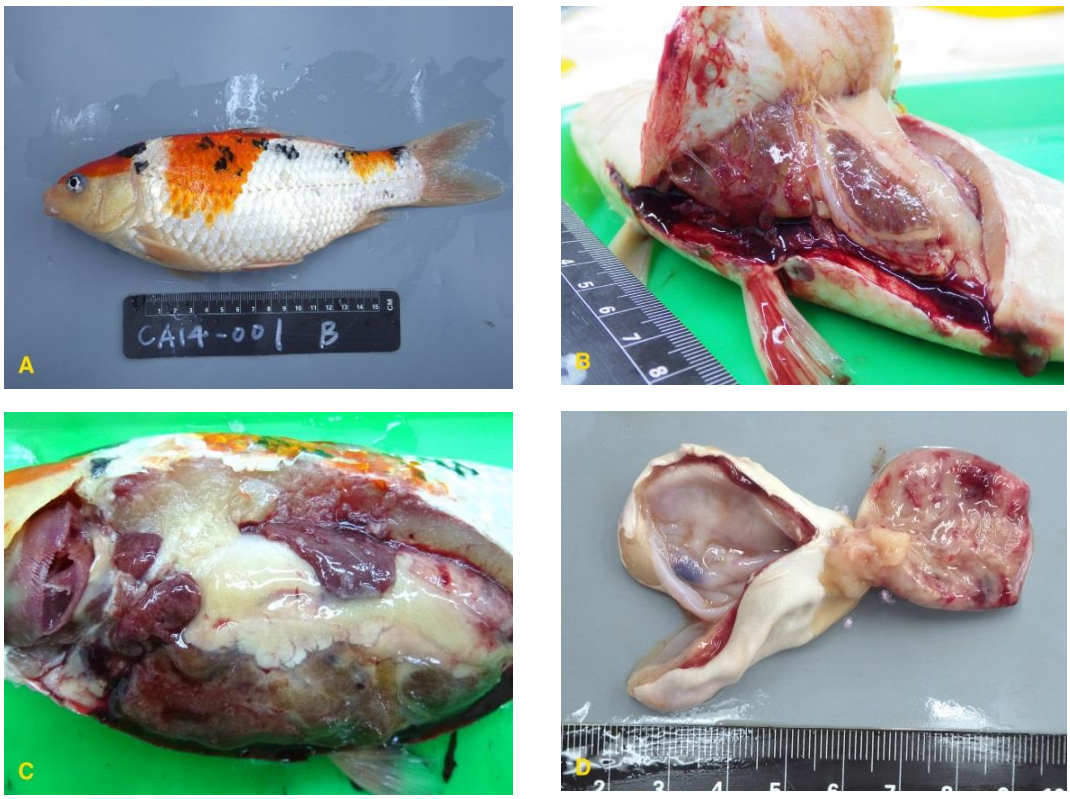

Fig. 1: Gross lesions of the submitted fish. A) Enlarged abdomen. B) Adhesion of multiple organs. C) Swollen kidney and swim bladder. D) Severe erosion and hemorrhage of swim bladder.

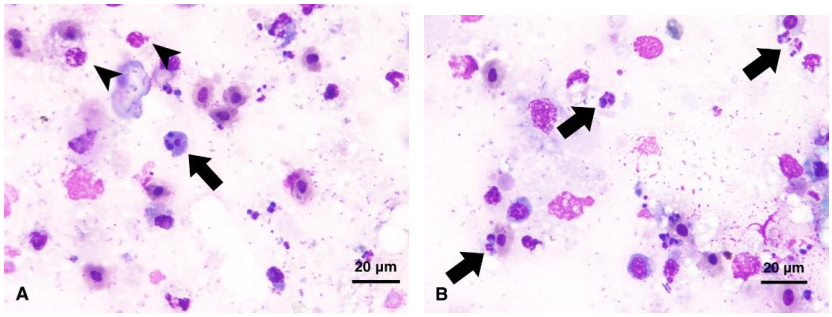

Fig. 2: Smear of the swim bladders (Diff-Quik, 100X). A) Soroplasm (arrow) and pansporocyst (arrow head). (B) Abundant clustered zygospores (arrow).

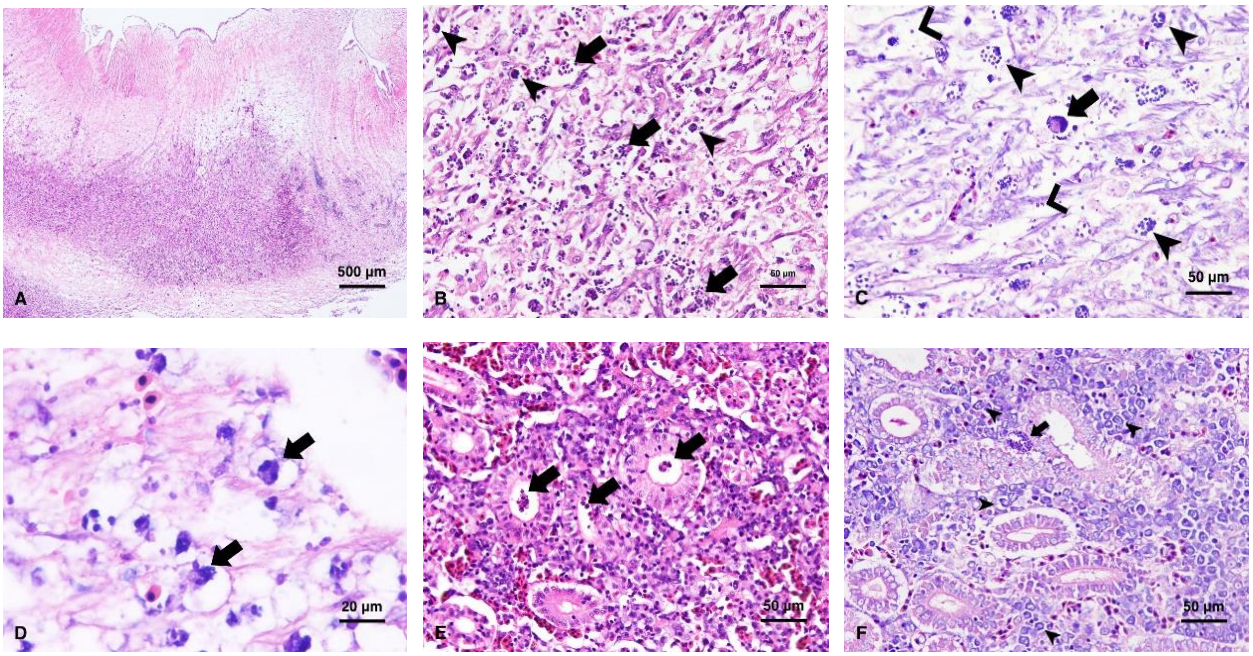

Fig. 3: Histopathology of the submitted fish. (A-D are swim bladders; E-F are kidneys). A) Severe inflammation of submucosa to serosa (H&E, 40X). B) Abundant pansporocyst (arrow) and a few myxospores (arrow head) (H&E, 400X). C) Pansporocyst (arrow head) and the spread of zygotes (short arrow head). Mature myxospore (arrow) (Giemsa, 400X). D) A myxospore with symmetrical pyriform nuclei (arrow) (Giemsa, 1000X). E) Zygotes (arrow) (H&E, 400X). F) Abundant plasmodia were clustered in the interstitium of kidney (arrow head), with a few pansporocysts (arrow) (Giemsa, 400X).

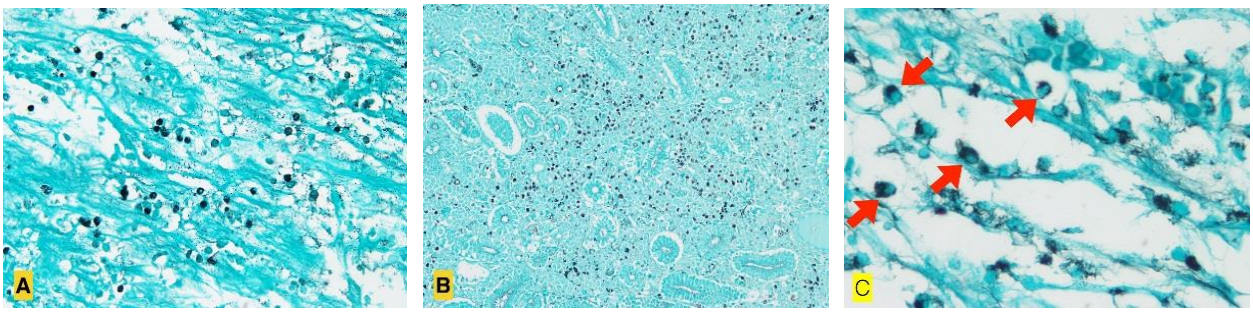

Fig. 4: The results of GMS stain in swim bladder (A, 200X) and kidney (B, 100X) of the fish. (C) At high magnification, the polaroplast of the organisms were stained black, which was in a "V" shaped (arrow) (GMS, 1000X).

DISCUSSION

According to the Taiwan Ornamental Fish Association, the total export value of Taiwan's ornamental fish has increased very rapidly since 2005. Furthermore, the export of koi from Taiwan accounted for 4% of the national ornamental fish export value in 2011 (Council of Agriculture, Taiwan, 2016). The SBI must be eradicated as soon as possible to reduce its impact on koi farms.

Outbreaks of the SBI only occur when the water temperature is in the range of 16-22℃ (Holzer *et al.*, 2014). Therefore, the disease is not suited to tropical climates, which explains why SBI had not occurred in Southern Asian countries before. The life cycle of *S. dykovae* includes two intermediate hosts, Annelida and carp. There are two stages in the *S. dykovae* life cycle during which spores are infective, namely, the myxospore and actinospore stages (Holzer *et al*., 2013). Proliferation of *S. dykovae* in the swim bladder, renal tubule, collecting duct, and glomerulus results in considerable tissue damage (Feist *et al*., 2006; Jirků and Bartošová, 2014).

To date, clinical syndromes and epidemiology are still the main criteria used to provide a tentative diagnosis of SBI. Diagnosis can be confirmed by smear, histopathology, and sequence analysis based on 18s rDNA (Pees *et al*, 2010; Holzer *et al.*, 2013; Fiala *et al*., 2015).

In our study, the fine structure of the organisms can be observed much more remarkable than other stains in modified GMS stain. Therefore, we considered that the GMS stain should be a much better morphology method to diagnosis the disease. The plasmodia of *S. dykovae* could not be readily distinguished from the interstitial cells of the koi kidney. However, the symmetrically pyriform nuclei could be observed clearly at high magnification in GMS stain. In contrast to previously studies (U-taynapun *et al.*, 2012; Molnár and Eszterbauer, 2015), we found a negligible number of organisms in the lumen of the renal tubule.

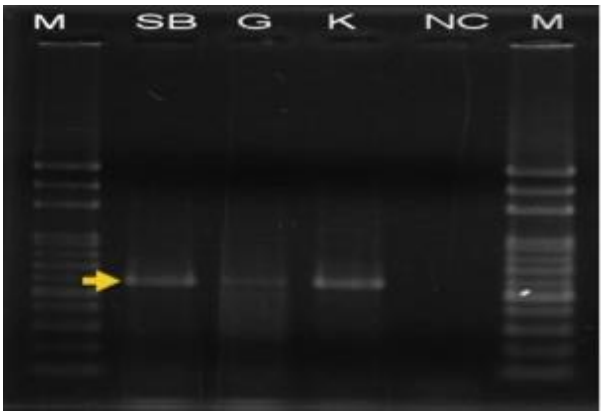

Fig. 5: 588 bp was detected in the swim bladder (SB), gill (G), and kidney (K). Negative control (NC). Marker (M).

Conclusions: The present study is the first reported outbreak of SBI in koi (*Cyprinus carpio koi*) caused by *S. dykovae* in East Asia. This study is also the first study to compare the morphology of H&E, Giemsa and GMS stain of SBI.

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Author's contribution: HKC, YAC, CCL are designed the study and executed the experiment. YCW isolated the pathogen and identified it. YLT help us to revise the manuscript. WFC, JHS, SLH and CCL Lin are advisor of the study.

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