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First molecular identification of Spironucleus salmonis (Diplomonadida) from diseased rainbow trout Oncorchynchus mykiss in Poland

LESZEK GUZ, KRZYSZTOF PUK

Institute of Biological Bases of Animal Diseases, Sub-department of Fish Diseases and Biology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Akademicka 12, 20-033 Lublin, Poland

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Guz L., Puk K.

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Summary

The aim of this study was the isolation of diplomonads from the digestive tract of infected rainbow trout (Oncorhynchus mykiss) and the molecular identification of Spironucleus species from samples. Samples were collected from a total of 40 fish (0.8 g ± 0.1) from a commercial farm in Pomorskie Voivodeship, Poland. Polymerase chain reaction (PCR) and a partial sequence analysis of the 18S ribosomal gene were used to identify Spironucleus species. The PCR of the 18S rDNA yielded a 705 bp DNA band on agarose gel, and a sequence analysis of the DNA confirmed the isolate as Spironucleus salmonis. This is the first molecular identification of an isolate of S. salmonis in Poland. Further studies are needed to determine the prevalence of the parasite in this species of fish in other locations and to investigate the impact of the parasite on the total fish population.

Keywords: Spironucleus salmonis, fish, PCR

Diplomonad flagellates occur in shellfish, crustaceans, amphibians, and fish (freshwater and marine) (25). The flagellates are found in the gut lumen, less commonly in the skin, or the infection can be systemic. Diplomonad genera include *Hexamita*, *Octomitus*, *Giardia* and *Spironucleus* (15, 16), and constitute a clade known as *Fornicata*. *Spironucleus* species are particularly important in aquaculture, as they can cause outbreaks of systemic infection in farmed fish. They are presumed to have a very wide range and geographical distribution (25). Using transmission electron microscopy, 5 species of piscine diplomonads have currently been recognized: *S. barkhanus*, *S. salmonicida*, *S. salmonis*, *S. torosa*, and *S. vortens*. *S. salmonis* has caused massive outbreaks of systemic infection in farmed Norwegian Atlantic salmon (*Salmo salar*), grayling (*Thymallus thymallus*), and Arctic char (*Salvelinus alpinus*), as well as in Chinook salmon (*Oncorhynchus tshawytscha*) in British Columbia (7, 8, 11, 13, 14, 18). Transmission is extremely high, especially in the crowded environments of fish farms, and mortality in experimental infections approaches 100% (5, 6).

Despite numerous reports on cases of spironucleosis in different species of fish in Poland (3, 23, 24), the causative species have not been identified by DNA techniques. In the present study, *S. salmonis* from feral rainbow trout was identified by a molecular technique.

Material and methods

Spironucleus salmonis was collected from a total of 40 individual rainbow trout (*Oncorhynchus mykiss*) (0.8 g ± 0.1) from a commercial farm in the Pomorskie Voivodeship, Poland. The fish were killed by a sharp blow to the head. Live flagellates from the intestine of the fish were observed with a Jenaval (Carl Zeiss Jena) light microscope. Size measurements were made by using the Olympus Cell software on digital micrographs of formalin-immobilized specimens.

The intestine was removed, cut open and preserved in 96% ethanol. DNA isolation was carried out with the DNA AX Stool Spin kit (A&A Biotechnology Gdynia, Poland). The PCR reaction for *S. salmonis* was carried out using a pair of primers, Salmonis-1f (5'-TTG TGT ACG AGG CAG TGA CG-3') and Salmonis-4r (5"-CGA TCC ATG GAA ATT GAT CC-3'), which amplify a fragment of the conserved SSU rDNA gene with a length of 705 bp (4). Amplification mixtures were heated for 5 min at 95°C, then subjected to 35 cycles (95°, 55° and 72°C for 45 sec. each), heated for 4 min at 72°C and cooled to 4°C.

PCR results were evaluated by agarose gel electrophoresis using Midori Green DNA Stain (Nippon Genetics,

Düren, Germany) in parallel with a 100 bp DNA ladder (A&A Biotechnology Gdynia, Poland).

The PCR product was purified using Gel-Out columns (A&A Biotechnology Gdynia, Poland) following the manufacturer's protocol. The DNA sequence was determined on both strands using the same primers employed for PCR at a DNA sequencing core facility (Genomed S.A., Warsaw, Poland). DNA sequences were assembled and edited using ClustalW (20) alignments with published 18S rRNA gene sequences for *Spironucleus* spp. from the National Centre for Biotechnology Information Gene Bank. Phylogenetic analyses were conducted by MEGA4 software (19).

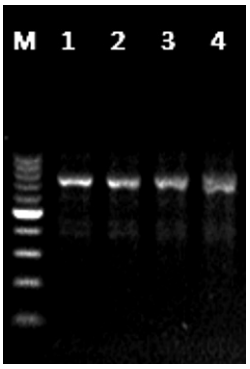

Fig.1. PCR amplification of a partial sequence of *S. salmonis* 18SrDNA gene (product size 705 bp) from rainbow trout digestive tract samples
Explanations: Lane M – molecular weight marker = 100 bp; lanes 1-4 correspond to PCR products from examined samples

Results and discussion

Intestinal diplomonad infections in farmed trout can be associated with morbidity and mortality (4). In Poland, spironucleosis in fish is reported, but poorly characterized (3). In the present study, diseased fish exhibited progressive emaciation, lethargy, weakness, anorexia, excretion of stringy faeces and increased mortality. Live flagellates from the fish intestines typically moved rapidly in straight lines with sudden changes of direction. The cytoplasm of the isolates appeared homogeneous and had no visible inclusion bodies or vesicles. Moreover, cysts were observed in faecal samples. Previous studies have likewise observed cysts *in vivo* (2, 12) and *in vitro* (22), but have failed to detect cysts in the faeces of fish infected with *S. salmonis* (8, 9, 21) and *S. vortens* (26).

Samples from fish known to be positive for *Spironucleus* spp. were analysed by PCR with two primers, Salmonis-1f and Salmonis-4r, which produced bands of predicted sizes. *S. salmonis* DNA was detected in the digestive tracts of the sick fish (Fig. 1). The sequences of *S. salmonis* products with a length of 705 bp obtained in the PCR showed a high similarity (99-100%) to the sequence of the *S. salmonis* 18S rDNA gene listed in the GenBank (Fig. 2). The assay

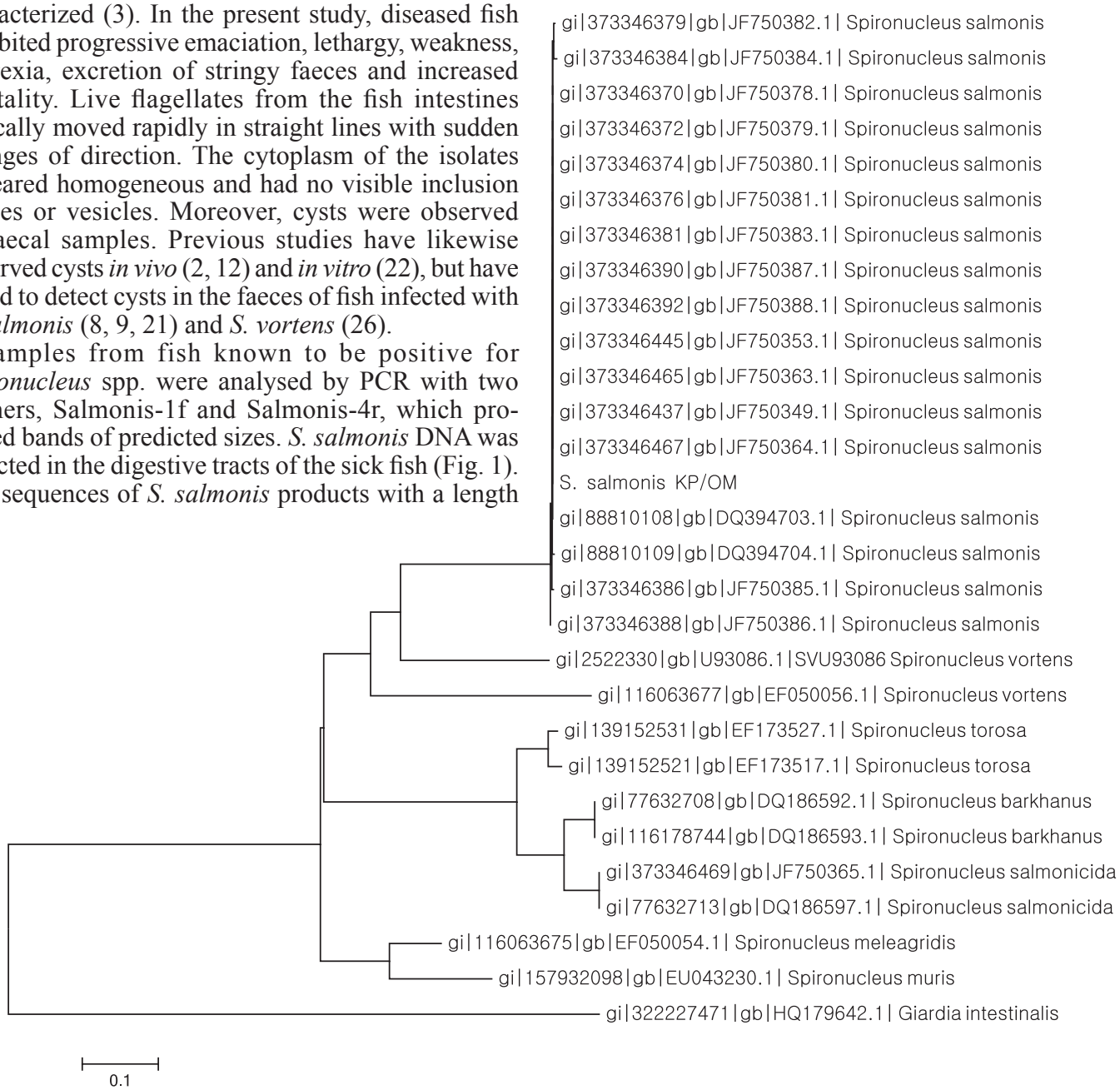

Fig. 2. *Spironucleus salmonis* KP/OM phylogenetic position. A neighbor-joining analysis of a selection of diplomonad taxa based on 705 positions of the small subunit rRNA gene

using the Salmonis-1f and Salmonis-4r primers did not produce any bands in samples from fish known to be negative for *S. salmonis* (data not shown). To the best of the authors' knowledge, this study reports the first molecular detection of *S. salmonis* in rainbow trout from Poland.

The most common chemotherapeutic means of eradicating human and veterinary diplomonad infections is metronidazole (17, 21). In our laboratory, the fish were experimentally treated with metronidazole (25 mg/kg of body weight, *p.o.*), administered every 24 h for 5 days. The therapy led to a significant improvement in the health status of the fish 72 h after application of the drugs. Complete recovery of the infected fish was observed after 5 days of treatment. The results of a control PCR test performed three weeks after the treatment had been completed were negative for the presence of *Spironucleus* genetic material in the intestines of the fish. However, in 1998 European Council Regulation 613/98/EEC (10) banned the use of metronidazole on outdoor European fish farms because of its environmental side effects, as well as cytotoxicity and genotoxicity in fish (25). Excessive use of metronidazole in fish can damage the kidneys and other internal organs (1). Thus the search for new agents to combat spironucleosis is crucial.

In conclusion, this is the first molecular identification of *S. salmonis* in Poland. It seems that intensive surveys are needed to determine the prevalence of *S. salmonis* in different regions of Poland.

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Corresponding author: dr hab. Leszek Guz, Akademicka 13, 20-950 Lublin, Poland; e-mail: leszek.guz@up.lublin.pl