Wild Arctic char *Salvelinus alpinus* and trout *Salmo trutta*: hosts and reservoir of the salmonid pathogen *Spironucleus salmonicida* (Diplomonadida; Hexamitidae)

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ABSTRACT: *Spironucleus salmonicida* is a diplomonad flagellate known to cause systemic infections in farmed salmonids. In northern Norway, outbreaks of spironucleosis in farmed Atlantic salmon *Salmo salar* have been a recurring problem. Common to all these outbreaks was the origin of smolts: all came from the same farm. In the present study, wild Arctic char *Salvelinus alpinus* and brown trout *Salmo trutta* were sampled from the lakes used as a water source for the smolt supplier. In addition, smolt and three-spined sticklebacks *Gasterosteus aculeatus* were sampled from the smolt farm. Bile and intestinal contents from the sampled fish were examined by light microscopy and PCR. *Spironucleus salmonicida* was identified in both wild Arctic char and brown trout from the lakes used as water sources by the smolt farm, suggesting that the farmed fish were exposed to this pathogen before transfer to the sea. *Spironucleus barkhanus* and *Spironucleus salmonis* were also identified in the sampled fish. The present study also demonstrated that infections with multiple *Spironucleus* species are present in wild salmonids. No indications of disease related to diplomonad infections were observed in the wild fish, suggesting that wild salmonids are reservoir hosts of *Spironucleus salmonicida*.

KEY WORDS: Co-infection · Farmed Atlantic salmon · Fish disease · Hatchery · Protozoa · Spironucleosis · SSU rDNA

INTRODUCTION

Diplomonad flagellates of the genus *Spironucleus* may cause severe systemic infections in both ornamental and farmed fish. Systemic spironucleosis in farmed salmonids may cause mass mortalities and huge economic losses as the fish are unsuitable for human consumption (Mo et al. 1990, Paull & Matthews 2001). The first case of systemic spironucleosis (reported as hexamitosis) in farmed Atlantic salmon *Salmo salar* was reported from a marine fish farm in northern Norway in November 1989 (Mo et al. 1990). In the following years, additional outbreaks were observed (Poppe et al. 1992, Poppe & Mo 1993), and hexamitid flagellates indistinguishable from *Hexamita salmonis* (current valid name *Spironucleus salmonis*) were identified by light microscopy in smears and imprints from visible lesions from a wide range of different organs (Poppe & Mo 1993). In 1991, an outbreak of systemic spironucleosis (hexamitosis) was observed in farmed chinook salmon *Oncorhynchus tsawytscha* in Canada (Kent et al. 1992). The infectious agent was identified by light microscopy as *H. salmonis*.

Electron microscopy was initially considered essential for identification of species belonging to the genus *Spironucleus* (Poynton & Sterud 2002). Sterud et al. (1997) presented a scanning and transmission
electron microscopy (SEM and TEM) study of hexa- 
mimid flagellates from farmed Atlantic salmon with 
systemic infection where ultrastructural data showed 
that the hexamitid flagellate causing systemic dis- 
ease in farmed Atlantic salmon was different from 
H. salmonis. The ultrastructural features of the stud- 
yed hexamitid flagellate were consistent with those 
of the genus Spironucleus. However, this flagellate 
could be clearly distinguished from other species 
within this genus; thus the species Spironucleus 
barkhanus was established. The same study also 
showed that S. barkhanus was present in the intesti- 
tine and gall bladder of grayling Thymallus thymal- 
lus. Grayling was chosen as the type host of S. bar- 
khanus as the infection was endemic and highly 
prevalent in these fish (close to 100%), compared to 
the more sporadic outbreaks in farmed Atlantic 
salmon. Spironucleus barkhanus was later described 
as an apparent commensal in the gall bladder of wild 
Arctic char Salvelinus alpinus (Sterud et al. 1998), 
and some years later S. barkhanus was also identified 
as the cause of spironucleosis in farmed Arctic char 
(Sterud et al. 2003). Populations of Arctic char in 
lakes connected to the sea by rivers or streams may 
contain both resident and anadromous fish. Migra- 
tory fish make short migrations to the sea during the 
summer months for feeding and were considered to 
be the source of the infectious agent causing sys- 
temic spironucleosis in farmed salmonids (Sterud et 
al. 1998). A study of an outbreak of systemic spironu- 
cleosis in farmed Arctic char showed that the parasite 
from the gall bladder of wild Arctic char was geneti- 
cally very different from the isolates causing sys- 
temic disease in the farmed fish (Jørgensen & Sterud 
2004). The differences in the small subunit ribosomal 
RNA (SSU rRNA) gene were of such magnitude that 
the conspecificity of these isolates was questioned 
(Jørgensen & Sterud 2004). A more comprehensive 
study included several new parasite isolates obtained 
from both wild and farmed salmonids with a wide 
geographic distribution (Jørgensen & Sterud 2006). 
In addition, nucleotide sequence data from the 
SSU rRNA, α-tubulin and glutamate dehydrogenase 
genes were analysed and lead to the redescription 
of the pathogenic isolate infecting farmed salmonids as 
S. salmonicida. The cause of systemic spironucleosis 
in marine farmed salmonids was thus finally identi- 
fied as S. salmonicida. However, the wild and ‘pri- 
mary’ host of S. salmonicida was never identified 
(Jørgensen & Sterud 2006).

Since the first outbreak of the disease in 1990, spo- 
radic cases of the disease have been registered at the 
Norwegian Veterinary Institute. All Atlantic salmon 

farms in Norway that have experienced systemic 
spironucleosis have received smolts from the same 
smolt farm. Fish from this farm, and fish from the 
water source of this farm, have previously been 
examined for the presence of diplomonad flagellates, 
but were never found to be positive (T. T. Poppe pers. 
obs.). To further explore the possibility of a reservoir 
of S. salmonicida in the 3 lakes used as water sources 
for this smolt farm, wild fish were sampled and 
analysed for the presence of diplomonad flagellates.

MATERIALS AND METHODS

Sampling

Wild brown trout Salmo trutta and wild Arctic char 
were caught by angling and gill nets in the Store 
Måsvatn, Helvetjavrri and Landersfjordvatnet lakes, 
indicated as localities A, B and C in Fig. 1, respec- 
tively. These lakes are used as water sources for the 
smolt farm (referred to as Company A) suspected to 
have delivered smolts infected with Spironucleus 
salmonicida. Sampling was carried out from 7 to 
31 July and from 15 to 19 September 2008. Fish were 
killed by a blow to the head and examined in the lab- 

oratory at the smolt farm. In total, 20 trout and 48 
Arctic char were sampled in the 3 lakes (see Table 1 
for more detailed information). Furthermore, 240 
Atlantic salmon smolts were sampled from fresh- 
water tanks and 1 saltwater tank at the farm. In 
addition, 12 three-spined sticklebacks Gasterosteus 
aculeatus were sampled from several freshwater 
tanks at the farm. All fish were kept at 4°C in a 
fridge (up to 8 h) until full necropsy. In addition to 
bile, digesta and mucus of the intestine, organs that 
showed signs of pathology were examined by light 
microscopy (100 to 400×) for the presence of diplo- 
monad flagellates. Positive samples were preserved 
in 96% ethanol for molecular analyses. See Table 1 
for a complete list of samples, hosts and sample local- 
ities. A subset of the fish sampled at the smolt farm 
and wild fish caught in September were only 
subjected to molecular analysis (see Table 1).

DNA extraction

Aliquots of 1 ml ethanol-preserved bile or intestinal 
 content were centrifuged for a minimum of 5 min 
at 3000 × g. The pellets were resuspended in phos- 
phate-buffered saline (PBS), centrifuged for 5 min 
at 3000 × g and resuspended in 200 µl PBS. The
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QIAamp DNA Stool Mini Kit (Qiagen) was used for ‘intestinal’ samples, while the NucleoSpin Tissue kit (Macherey Nagel) was used to extract DNA from lesions/abscesses and bile samples. For intestinal samples, DNA was eluted in 100 µl of the supplied Buffer AE (Qiagen). Samples extracted using the Nucleospin kit (Macherey Nagel) were eluted in 200 µl of the supplied Buffer EB.

**PCR, cloning and sequencing of the SSU rDNA**

All samples positive for hexamitid flagellates by light microscopy were analysed by PCR using 2 nested PCR protocols. The general eukaryotic primers ERIB 1 (5'-ACC TGG TTG ATC CTG CCA G-3') (Fiala 2006) and EUKB (5'-ATC CTT CTG CAG GTT CAC CTA C-3') (Medlin et al. 1988) were used in the first reaction. The primer set Spiro-1f (5'-AAG ATT AAG CCA TGC ATG CC-3') and Spiro-2r (5'-GCA GCC TTG TTA CGA CTT CTC-3') was used in nested reactions for amplification of *Spironucleus* spp. The primer set Spironucleosis-1f (5'-TCA TTT ATC AGT GGT TAG TAC ATG C-3') and Spironucleosis-2r (5'-TTT ACC CCT AAC CAC GAC AAG-3') was used in nested reactions for amplification of *S. salmonicida* (Jørgensen & Sterud 2004). The nested PCR protocols were used for increased sensitivity.

The 50 µl reaction mix contained 5 µl of template DNA, 500 µM of each primer, 200 µM of each dNTP, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-Cl (pH 9 at room temperature) and 2.5 units of Taq DNA polymerase (GE Health). The following thermal parameters were used to amplify the SSU rDNA fragments: initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 45 s. After 35 cycles an extension step at 72°C for 4 min was included to ensure complete elongation of all products. The nested reaction contained 5 µl of the first reaction as template DNA and used the thermal cycling conditions as described above, except that the annealing temperature was set to 56°C for 45 s.

PCR products obtained using primers Spiro-1f and Spiro-2r from samples H1 and H5 were cloned into the plasmid vector pCR 2.1-TOPA using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer’s instructions. All samples were plated out on Luria-Bertani (LB) plates containing ampicillin (50 µg ml⁻¹) and incubated at 37°C overnight. From each cloning reaction, 24 colonies were chosen and screened for the presence of transformants with inserts of the correct size using PCR and the primers M13 forward (5'-TGT AAA ACG ACG GCC AGT-3') and M13 reverse (5'-CAG GAA ACA GCT ATG ACC-3'). The M13 PCR reaction was incubated at 94°C for 10 min to lyse the cells and inactivate nucleases. The annealing temperature was set to 56°C for 45 s, all other settings were as described above.

All PCR products were purified using the Nucleospin Extract II (Macherey Nagel) according to the manufacturer’s instructions, and sequenced on both strands using the PCR primers and the internal sequencing primers for the SSU rDNA fragments: Spiro-3f (5'-CAT TGG GTA ATY TYC GCC CCT-3'), Spiro-4r (5'-GAY TCY GGA GAV TGR GCA YGA G-3'), Spiro-5f (5'-STY TCC GTC AAT MCY TTM AAG TTT C-3') and Spiro-6r (5'-AAG RYT GAA ACT TKA ARG KAT TGA CCG-3'), as previously described (Jørgensen & Sterud 2004). All products were sequenced using DyeIT™ chemistry (GE Health) and analysed on a MegaBACE 1000 sequence analysis.
system (GE Health) according to the manufacturer’s instructions. PCR products from positive clones were sequenced using primers Spiro-4r and Spiro-5f only.

Sequence identification

The raw sequence data were manually edited using the Vector NTI software package (Invitrogen) and included in a BLAST search at the National Centre for Biotechnology Information (NCBI) for identification. *Spironucleus* sequences were subsequently aligned against SSU rDNA sequences from *S. salmonicida*, *S. barkhanus* and *S. salmonis* retrieved from GenBank (accession numbers U93083, DQ186576, DQ394704, respectively). Alignments were manually read to check for misalignments before they were subjected to Neighbor-Joining (NJ) analyses using MEGA4 (see Fig. 2). The NJ analysis was conducted using the maximum composite likelihood substitution model, uniform rates among sites and complete deletion of positions with gaps and missing data. Bootstrap support analysis (1000 replicates) was used to estimate the confidence of the nodes within the resulting topologies.

RESULTS

A total of 320 fish (68 wild salmonids, 12 sticklebacks and 240 farmed smolts) were sampled and examined for the presence of diplomonad flagellates. All 17 wild fish samples positive by light microscopy (obtained in July) were subjected to molecular analyses. A total of 14 of these fish were PCR positive. In wild fish sampled in September (only subjected to molecular analyses), 2 of 33 fish were PCR positive for diplomonad flagellates (see Tables 1 & 2 for more detailed information). All obtained SSU rDNA sequences were subjected to NJ analysis together with known SSU rDNA sequences representing *Spironucleus salmonicida*, *S. barkhanus* and *S. salmonis* (GenBank accession numbers U93083, DQ186576, DQ394704, respectively). Three major assemblages representing the species *S. salmo- nicida*, *S. barkhanus* and *S. salmonis* were recovered (bootstrap support >96%) by the NJ analysis (Fig. 2a). The genetic variation between sequences within the assemblages was on average 0.5%. Detailed information on parasite species identified, host records and target organs is provided in Fig. 2 and Table 2. In total, *S. barkhanus* was found in 30% of trout and 2.1% of Arctic char. *S. salmonis* was found in 4.2% of Arctic char and 33.3% of three-spined sticklebacks. *S. salmonicida* was found in 15% of trout and 8.3% of Arctic char.

Analyses of obtained SSU rDNA sequence data (GenBank accession numbers JF750347 to JF750365, Fig. 2, Table 2) showed that *Spironucleus salmonicida* was found in fish from all 3 lakes, while *S. barkhanus* and *S. salmonis* were found only in the Landersfjordvatnet and Helvetjavrri lakes (Table 2). *S. salmonicida*, *S. barkhanus* and *S. salmonis* were found in both wild brown trout and wild Arctic char. All 3 species were present in samples from the intestine, while only *S. barkhanus* was found in samples from bile (Table 2, Fig. 2). The 2 independent nested PCR reactions, employing *Spironucleus* general and *S. salmonicida*-specific primers (in second round reactions), generated sequence data from different *Spironucleus* species in single samples of intestinal content from 2 fish (HJ1 and HJ5). Furthermore, a dual infection was identified in sample LFV 10 (Table 2, Fig. 2a) where *S. salmonicida* and *S. barkhanus* were found in the intestine and gall bladder, respectively. The PCR products from samples HJ1 and HJ5, generated using general *Spironucleus* primers, were subjected to cloning. The obtained sequences have been deposited in GenBank under

<table>
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Table 1. Fish sampled for *Spironucleus* spp. in 2008. Sample localities, number of samples, and number of positive samples by light microscopy (July only) and PCR (July and September)
Fig. 2. Phylogenetic analyses of *Spironucleus* species isolated from fish sampled in the northern Norwegian lakes Helvetjavrri (HJ), Landersfjordvatnet (LFV) and Store Måsvatn (SM), and three-spined sticklebacks from the smolt farm (SB). (a) Neighbor-Joining (NJ) analysis based on 752 positions (positions with gaps deleted) of the small subunit ribosomal RNA gene (SSU rDNA) of directly sequenced PCR products. Sequences are deposited in GenBank under accession numbers JF750347 to JF750365. (b) NJ analysis based on 473 positions (positions with gaps deleted) of the SSU rDNA of cloned PCR products from samples HJ1 and HJ5. Sequences obtained from clones are deposited in GenBank under accession numbers JF750366 to JF750388. For both analyses, bootstrap support values above 70% are indicated at the nodes. All sequences are presented with sample IDs according to sample locality, clones are also listed with numbers. Sequences obtained from samples isolated from bile are indicated with an asterisk, all other sequences were obtained from samples of intestinal digesta/mucosa. Sequences retrieved from GenBank are indicated with species names and accession numbers. The scale bars represent number of nucleotide substitutions per site.
acquisition numbers JF750366 to JF750388. Six and 19 clones with an insert of correct size were obtained for HJ1 and HJ5, respectively. All PCR-positive clones were sequenced, and subjected to NJ analysis with sequences representing *S. salmonicida*, *S. barbatus* and *S. salmonis* included (as described above). The cloned sequences clustered with *S. salmonis* and *S. salmonicida* with strong bootstrap support (Fig. 2b), demonstrating the presence of a co-infection of *S. salmonicida* and *S. salmonis* in these samples.

A total of 240 smolts from the farm (both sampling periods) were also examined; 120 fish were obtained from freshwater and 120 fish from saltwater. No diplomonad flagellates were detected by light microscopy or by PCR. *Spironucleus salmonis* was identified in samples of intestinal content from 4 of 12 three-spined sticklebacks taken from freshwater tanks at the farm.

**DISCUSSION**

**Infection source**

In December 2007, an Atlantic salmon farm situated in the Alta fjord experienced an outbreak of spironucleosis in fish supplied by Company A. Fish from another supplier (Company B) were also kept at the same location. The smolt from the 2 suppliers were transferred to sea at the same time of year and kept in adjacent net cages. At slaughter, 100 and 77 fish originating from Company A and Company B, respectively, were sampled. Spironucleosis was diagnosed in 25% of the fish from Company A, while no signs of spironucleosis were seen in fish from Company B (data not shown). The present study has demonstrated that *Spironucleus salmonicida* is present in both wild Arctic char and wild brown trout in the 3 lakes used as water sources for Company A. *S. salmonicida* was not found in any of the fish sampled at the smolt farm (Company A). However, based on all available information, it is likely that fish that developed spironucleosis were infected before being transferred to the sea. A possible explanation may be that smolt were not infected by the parasite in this particular season; this is supported by the fact that there were no reports of systemic spironucleosis in these fish after they were transferred to the sea. Furthermore, not all stocks of Atlantic salmon smolt supplied by Company A have developed spironucleosis, indicating that the problem is sporadic, making the parasite hard to detect in smolts from the farm.

*Spironucleus salmonicida* was found in all 3 lakes (Fig. 1) used as water sources for Company A. The largest of these 3 lakes (Store Måsvatn) is connected by streams and rivers to several other lakes on the mountain plateau Finnmarksvidda. It is thus probable that the parasite is present in other lakes on Finnmarksvidda. Several smolt-producing companies use lakes on Finnmarksvidda as their water source, thus Company A is probably not the only smolt producer exposed to *S. salmonicida*. The diagnosis of spironucleosis in farmed Arctic char in Vesterålen (Norway) (Jørgensen & Sterud 2004) and in farmed chinook salmon in Canada (Kent et al. 1992) also substantiate the notion of a wide geographic distribution of *S. salmonicida*. Moreover, spironucleosis has also been reported from wild chinook salmon in Lake Ontario (Meseck et al. 2007). This parasite was not identified to species level; however, a systemic infection was caused by a diplomonad flagellate probably belonging to the genus *Spironucleus* (Meseck et al. 2007). Most of the fish studied were sampled during the spawning season, suggesting that the problem is connected to the presumed immunocompromised state of salmonids during spawning. Thus, the fact that only farmed Atlantic salmon from a few suppliers develop spironucleosis can possibly be explained by their reduced general health status combined with exposure to the parasite.

**Dual Spironucleus infections in wild salmonids**

Dual infections were detected in 3 fish by both cloning and nested PCR with 2 sets of second round primers (see ‘Materials and methods’). Samples HJ1 and HJ5 were selected for cloning based on ambiguities in the sequence obtained initially. In individual PCR reactions, the general primers (Spiro-1 and Spiro-2) amplified *Spironucleus salmonicida* and *S. salmonis* both together and independently, as detected by sequence analysis. The *S. salmonicida*-specific primers generated *S. salmonicida* sequence data only from the same samples. Samples HJ1 and HJ5 probably contained equal amounts of target DNA from *S. salmonicida* and *S. salmonis*. By chance, 1 of the 2 species may sometimes be overrepresented in individual PCR reactions, while on other occasions, amplicons from both species would be produced in equal amounts, causing ambiguities in the generated sequence data. The number of dual infections in our material may be significantly higher than detected, as only 2 samples (HJ1 and HJ5) were cloned. In addition, infections with all 3 *Spironucleus* species in
individual fish may also be present in our material even though they were not detected.

In some samples, only short sequences could be obtained. Intestinal samples from fish may be a challenge for downstream molecular applications due to inhibitors (Wilson 1997). This is supported by the fact that some samples identified as positive by light microscopy did not amplify by PCR (Table 1). However, this could also be due to the presence of novel diplomonad species with SSU rDNA sequences that do not match the primers used.

Implications

The present study is the first report of wild salmonid hosts and potential reservoirs of Spironucleus salmonicida in the intestine of wild brown trout and wild Arctic char. Jørgensen & Sterud (2004, 2006) have previously studied diplomonad flagellates in wild Arctic char using molecular methods. However, only flagellates isolated from bile were included and thus only S. barkhanus was identified. The present study indicates that S. barkhanus is the only diplomonad that can be found in the gall bladder (as well as in the intestine) of salmonid fish in Norway, while S. salmonicida and S. salmonis are restricted to the intestine. Thus, S. salmonicida and S. salmonis may have been present in the fish studied by Jørgensen & Sterud (2004, 2006), but were missed due to the lack of intestinal samples. Furthermore, all previous reports and descriptions of intestinal diplomonad flagellates in salmonid fish could comprise at least 3 different species: S. barkhanus, S. salmonicida and S. salmonis, or a mixture of these species. Infections with multiple species/genotypes in individual hosts have also been demonstrated for Giardia, a close relative of Spironucleus (Read et al. 2004, Sprong et al. 2009, Cacciò & Sprong 2010). Together, these highlight the need for the use of molecular techniques, including cloning, when studying diplomonad flagellates from fish or other animals.

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