Vol. 111: 165–171, 2014
doi: 10.3354/dao02766

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 30

NOTE

First outbreak of sleeping disease in Switzerland: disease signs and virus characterization

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ABSTRACT: Sleeping disease is a contagious disease mainly of freshwater farmed rainbow trout, caused by salmonid alphavirus (SAV) Subtype 2. Here we describe the first case in Switzerland. Pathological changes ranged from acute pancreas necrosis to more chronic lesions with complete loss of exocrine pancreas and simultaneous degenerative, inflammatory and regenerative heart and muscle lesions. The partial sequencing of SAV *E2* and *nsp3* genes placed the Swiss SAV variant within the Subtype 2 clustering together with freshwater isolates from UK and continental Europe. Although mortality stayed low, growth rates were significantly reduced, making the disease economically relevant.

KEY WORDS: Sleeping disease · Salmonid alphavirus · SAV · Subtype 2 · Pathology · Trout · Phylogenetic analysis

INTRODUCTION

Sleeping disease (SD) is a contagious disease mainly of freshwater farmed rainbow trout (Boucher & Baudinn-Laurencin 1994, Boucher et al. 1995, McLoughlin & Graham 2007, Lester et al. 2011). The syndrome has many similarities to pancreas disease (PD), a serious condition of farmed Atlantic salmon and rainbow trout in seawater (Munro et al. 1984, Taksdal et al. 2007). Recent studies have shown that SD and PD are caused by the same virus identified as salmonid alphavirus (SAV), in the family *Togaviridae* (Weston et al. 1999, 2002). Six SAV subtypes have been classified so far. SAV Subtypes 1, 2, 4, 5, and 6 have been isolated from fish affected by PD in Ireland and Scotland (Weston et al. 2005, Fringuelli et al. 2008, Lester et al. 2011). SAV Subtype 3 has only been found in Norway (Hodneland et al. 2005, Weston et al. 2005, Karlsen et al. 2006, Fringuelli et al. 2008). Recently, a marine SAV Subtype 2 was detected in Atlantic salmon in Scotland (Fringuelli et al. 2008) and Norway (Hjortaas et al. 2013). Although now isolated from Atlantic salmon in the seawater phase, the majority of outbreaks due to SAV Subtype 2 occur in freshwater rainbow trout causing SD (Castric et al. 1997, Fringuelli et al. 2008). SD has been observed in France for many years (Boucher & Baudin-Laurencin 1994), and subsequently it was diagnosed in Croatia, Italy, Spain, Germany, England and Scotland (Branson 2002, Graham et al. 2003, McLoughlin & Graham 2007, Vardic Smrzlić et al. 2013). All sizes and ages of freshwater rainbow trout are susceptible; however, mortality mainly occurs in fingerlings. Older fish may be infected without showing any clinical signs of disease. In natural outbreaks, mortality can vary between 3 and 22% (Graham et al. 2003). There is no evidence to date that surviving fish become long-term carriers.

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The most characteristic sign of the disease is the unusual behaviour of the fish, which stay on their side at the bottom of the tank. Histological examinations of SD-affected fish reveal a chronological appearance of lesions (Graham et al. 2007). Initially, necrosis of exocrine pancreas appears shortly before degeneration of heart muscle fibres. Later, skeletal muscle degeneration, necrosis and inflammation develop. The red muscle damage is particularly severe in the late stage of the disease and is probably responsible for the clinical signs of disease (Boucher et al. 1995, Boucher & Baudin-Laurencin 1996, Kerbart Boscher et al. 2006).

Here we report the first case of SD in Switzerland. Clinical and pathological signs are reported. The molecular characterization of the virus is shown, including the relationship with SAV from fish farms in other European countries.

MATERIALS AND METHODS

In June 2013, a fish farmer reported rainbow trout lying on their side at the bottom of the tank. The fish originated from imported eggs from a fish farm in Denmark (free of notifiable diseases and sleeping disease). At a weight of about 15 g, fish were moved to a part recirculation system with 90% spring water and 10% creek water. After about 3 wk, the signs mentioned above became obvious. A sample of 10 rainbow trout was sent alive to the Centre for Fish and Wildlife Health for pathological investigation. Time schedule of disease signs and sampling is shown in Fig. 1. Fish were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS-222®, Argent Chemical Laboratories). Each trout was measured, followed immediately by a standard necropsy. Fish were examined for changes in external and internal organs. For parasitological examination, a skin scraping, a gill sample and the intestinal content were investigated by light microscopy. On 3 animals a bacteriological examination of liver, spleen and kidney was performed using blood agar plates (Bio Merieux) and bromothymol blue–lactose–agar plates. To examine for *Flavobacteria*, samples of gill and spleen were cultivated on specific agar plates (Anacker & Ordal 1959). For virological examination, tissues from the gill, heart, head kidney, brain and pyloric caeca including pancreas of 5 fish were pooled as one sample. Antibiotic solution was added (1:10). Following a homogenisation of the tissue the solution was centrifuged at 1200 × *g* and 4°C for 20 min. Half of the supernatant was further diluted with antibiotic solution (1:10). Both dilutions were incubated on bluegill fry (BF-2) and epithelioma papulosum cyprini cells (EPC) for 2 passages each of 7 d at 15°C. Gill, heart, kidney, liver and pyloric caeca including pancreas from several fish were removed and fixed in 10% buffered formalin for histopathological examination. Fixed samples were paraffin embedded, sections were cut, stained with haematoxylin-eosin (H&E) and examined by light microscopy.

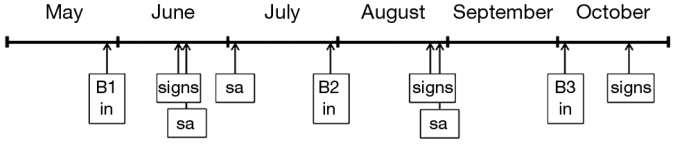

Fig. 1. Time line (2013) representing the chronology of introduction of fish in the part recirculation system, time point of first recognition of clinical signs of disease on farm and time point of sampling for diagnostics. B(n) in: number of the batch (Batch 1, B1; Batch 2, B2; Batch 3, B3) introduced into the recirculation system; signs: first recognition of disease signs on farm; sa: sampling for examination

In July 2013, 9 animals originating from the same batch as the previous one were sent for necropsy (Fig. 1). Additional to the methods mentioned above, muscle tissue was fixed in 10% buffered formalin for histopathological examination

Because of the diagnosis of secondary parasitic and bacterial infections in these animals, different baths with disinfection agents against *Ichthyophthirius multifiliis* and *Flavobacteria*, were performed on the fish farm without success. The surviving fish were killed. The system was disinfected and was kept empty for 14 d.

A second batch of fingerlings with average weight 25 g (eggs from Denmark) was then introduced to the same system (Fig. 1). In August 2013, 4 wk after introduction, 13 of these fish were examined in the same way as described above (Fig. 1). Additionally, samples of gills, heart, head kidney, brain and caeca pylorica including pancreas were placed in a RNA stabilization reagent, RNA*later*® (Ambion), and nucleic acid extraction was performed using the RNeasy Mini Kit® (Qiagen) following the manufacturer's instructions. The nucleic acid yield was determined by spectrophotometry using the NanoDrop photometer (Witec AG). A conventional reverse transcription polymerase chain reaction (RT-PCR) specific for SD virus (SDV) was performed according to Villoing et al. (2000a,b). Primers for the amplification of a 284 base pair (bp) fragment were derived from the published SDV nucleotide sequence (Villoing et al.

2000a; GenBank accession number AJ238578). As a positive control, RNA of cultured SAV 3, from the Norwegian Veterinary Institute, Oslo, Norway, was used. The PCR-amplification products were analyzed by agarose gel electrophoresis and amplification products were visualized using SYBR Green I staining (Molecular Probes). To test the specificity of the PCR and to exclude other differential diagnoses, namely infectious pancreatic necrosis virus (IPNV), a RT-PCR specific for IPNV was performed according to Taksdal et al. (2001). As a positive control, RNA of cultured IPNV was used.

For verification and further characterization of the virus, tissue samples were examined at the Norwegian Veterinary Institute in Oslo. Total nucleic acid from 4 tissue samples from 13 animals collected in RNA*later*® (Ambion), was then extracted using the automatic extraction instrument NucliSense® easyMag™ (bioMérieux bv). The samples were analysed for the presence of SAV by real-time RT-PCR (Villoing et al. 2000a,b), and characterization of the virus was performed by partial sequencing of SAV *E2* and *nsp3* genes as described by Hjortaas et al. (2013). Maximum likelihood phylogenetic analysis was based on multiple alignments of 357 nucleotides of the *E2* gene obtained in this study together with reference sequences from GenBank representing all 6 SAV subtypes. The analysis was performed using PhyML (v. 3.00) software (http://phylemon.bioinfo.cipf.es/phylogeny.html) with GTR plus Gamma model for nucleotide substitution. Clustering support was assessed through 100 bootstrap replicates. Graphic presentation of the phylogenetic tree was performed in MEGA5 Software (Tamura et al. 2007). The nucleotide sequences obtained in this study were deposited in GenBank with accession numbers HG937642 and HG937643.

Furthermore, 2 samples, each containing a pooled tissue homogenate from gill, heart, head kidney, brain and pyloric caeca from 5 fish obtained at the last sampling, were inoculated on Chinook salmon embryo (CHSE-214) cell monolayers for virus isolation and identification by an indirect immunofluorescence test (IFAT) according to the procedure described by Jansen et al. (2010).

A third batch of fingerlings, each at about 30 g, was introduced in October 2013 (Fig. 1). Again, after 3 to 4 wk, typical signs started (Fig. 1). Fish were transferred to the flow-through system, and feeding was stopped for a few weeks. Mortality and growth rate were recorded, but no further diagnostic work was conducted.

RESULTS

Clinical signs

In June 2013, approximately 3 wk after the first fish group entered the farming system, about 60% of fish (15 g) showed typical signs of SD, i.e. lying on their side at the bottom of the tank. However, when fish were disturbed, they were able to swim. Mortality at that stage was low (<1%). Water temperature was around 16°C, and the fish were still feeding. About 14 d later, mortality increased and became massive (up to 15% d^{-1}) due to secondary infections with *Ichthyophthirius multifiliis* and *Flavobacteria* resulting in 100% mortality within 3 to 4 wk.

In August, 3 to 4 wk after the second batch of fingerlings entered the system, the disease signs started again. The mortality was around 0.2% d^{-1} for about 4 wk, in total about 8%. The fish were then moved to a flow-through system. Feed was stopped for 3 wk and then started again at a lower rate. During the subsequent 2 mo, the fish did not gain weight, but some of the affected fish group recovered while growth was still retarded in the remainder.

The third batch of fingerlings that was introduced in October 2013 also developed typical SD signs after 3 to 4 wk. After transfer to the flow-through system and a period without feeding, the mortality was continuously low (approximately 2%), but the growth rate was retarded.

Necropsy

At necropsy, all fish sampled in June and July (Batch 1) were in good body condition, and the length varied between 12 and 19 cm. Parasitologically, a mild to moderate infestation with *I. multifiliis* on the skin was diagnosed. Bacteriological examination revealed the presence of single *Flavobacteria* on gills and in spleen. Histologically, different lesions were visible in individual animals from the same samplings. In some animals, the pancreatic acinar cells were completely replaced by necrosis, oedema and single inflammatory cells. These lesions were interpreted as acute change. In other animals, the exocrine pancreatic tissue was completely lost (Fig. 2a) with pancreatic ducts and endocrine pancreas being unaffected. There were no signs of fibrosis. In these animals, multiple cardiomyocytes, mainly in the spongy layer of the ventricle and atrium, showed signs of degeneration (Fig. 2c). In these areas multiple myocytes showed enlarged

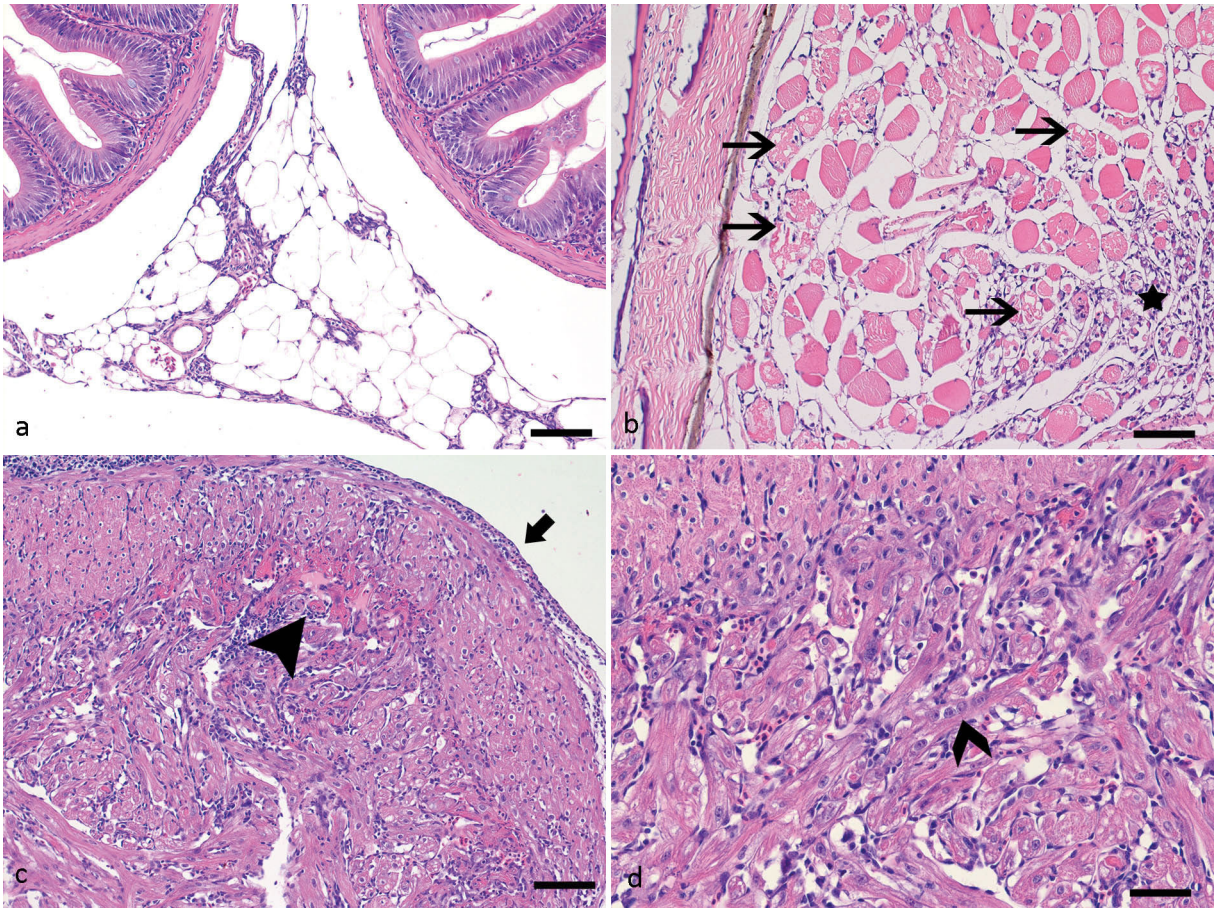

Fig.2. (a) Pancreatic acinar cells were completely lost with pancreatic ducts being unaffected; scale bar = 25 µm. (b) White muscle tissue showed multifocal degeneration (arrows) and mild infiltration with mainly lymphocyte-like cells (star); scale bar = 50 µm. (c) Multiple cardiomyocytes, mainly in the spongy layer, showed signs of degeneration (arrowhead); the epicardium was infiltrated with small to moderate amounts of inflammatory cells (arrow); scale bar = 25 µm. (d) Multiple myocytes showed multiple nuclei (open arrowhead); scale bar = 50 µm. H&E stain

nuclei (megakaryosis) or multiple nuclei, indicative of regeneration (Fig. 2d). These changes were interpreted as subacute to chronic change. The degenerative lesions were often accompanied by inflammation with moderate numbers of lymphocyte- and plasma-like cells and less macrophage-like cells in the myocardium and lower numbers of inflammatory cells in the epicardium (Fig. 2c). This stage was classified as chronic. In animals examined in July, white muscle tissue showed multifocal muscle fibre degeneration and mild infiltration with mainly lymphocyte-like cells (Fig. 2b).

In animals sampled in August (Batch 2) there were subacute and chronic changes, with almost no degenerative changes present in pancreas and heart. Based on the clinical and histological findings, the presumptive diagnosis of sleeping disease was made.

Virology

No cytopathic effect (CPE) was observed after inoculation of tissue homogenates onto BF-2, EPC and CHSE-214 cell cultures. The examination of CHSE-214 cell cultures by IFAT gave negative results.

A conventional SDV-specific RT- PCR revealed a single prominent band with a molecular weight of 284 bp in the samples of both diseased fish and the positive control. From fish collected in August, SAV was detected in 3 of 4 samples using a SAV-specific real-time RT-PCR. Cycle threshold values indicated high amounts of virus in one sample. From that sample, fragments of *E2* (488 bp) and *nsp3* (439 bp) genes were amplified and sequenced. The initial nucleotide blast of the sequences showed that the SAV variant detected in rainbow trout in Switzerland was closely

related to SDV (Fig. 3). These findings were further confirmed by phylogenetic analysis which placed the Swiss SAV variant within the Subtype 2 clustering together with freshwater isolates from UK and continental Europe. IPN-specific RT-PCR revealed no signal in diseased fish (data not shown).

DISCUSSION

Here we report the first detection of SD in Switzerland. SAV was identified in tissues from rainbow trout with clinical signs and microscopical changes typical for SD. The detection of SAV was confirmed by sequence analyses showing that the virus was closely related to other freshwater isolates of SAV Subtype 2 previously detected in continental Europe and the UK.

Fish presented with typical signs of an ongoing acute to subacute disease. However, in histology, the majority of fish examined showed chronic changes with inflammation and regeneration. This is in accordance to other reports stating that clinical signs usually are attributed to changes in the red muscle tissue that develop late in the disease process (Boucher et al. 1995, Boucher & Baudin-Laurencin 1996, Kerbart Boscher et al. 2006). In our case study, clinical signs of disease were present 3 to 4 wk after fish were transferred to the affected fish farming system, earlier than reported in previous studies. A possible explanation for this could be higher water temperature in the current outbreak compared with other reported outbreaks. In our study, water temperature was 16°C or above whereas Graham et al. (2007) reported temperature ranging from 8 to 14.5°C. Furthermore, in natural outbreaks of PD in Norwegian

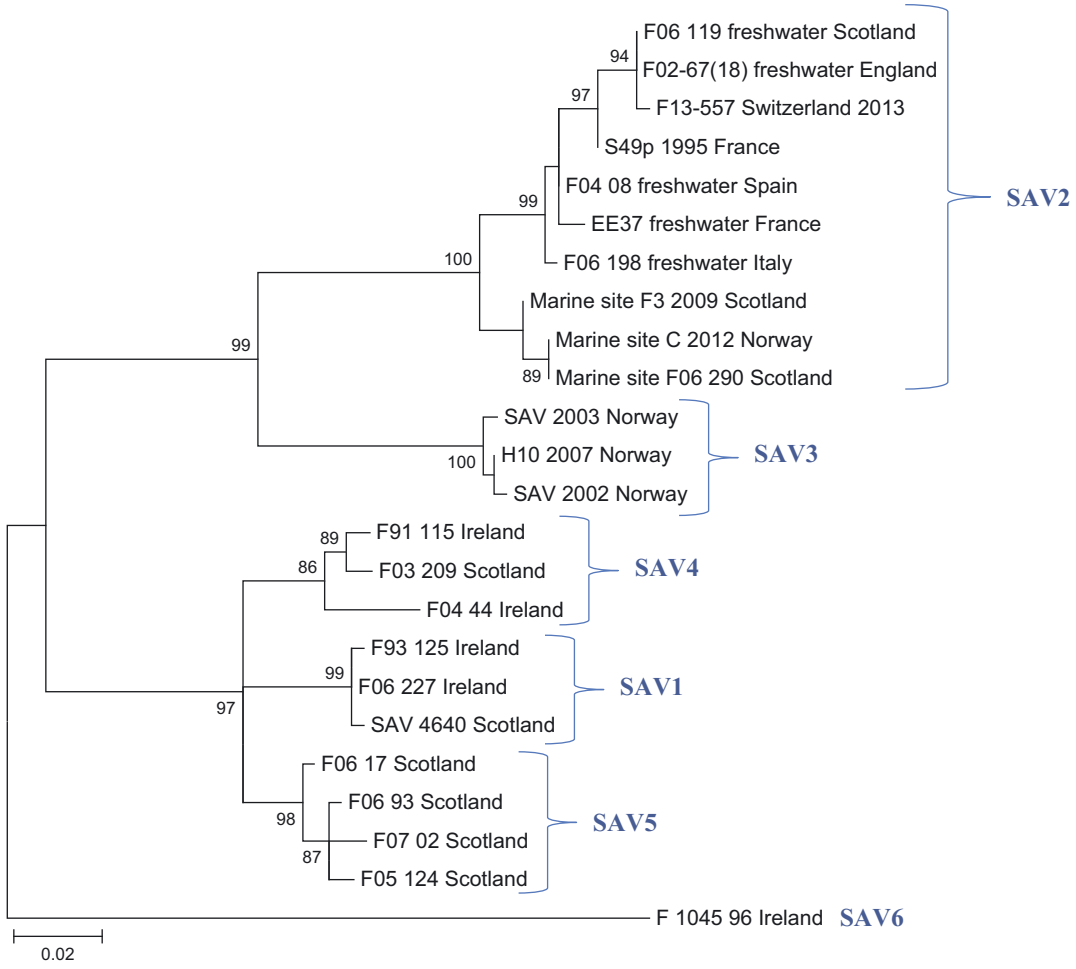

Fig. 3. Phylogenetic tree showing the relationship between salmonid alphavirus (SAV) F13-557 Switzerland 2013 and representative sequences from each of the 6 established subgroups of SAV. The tree was constructed using maximum likelihood and the general time reversible model for nucleotide substitution. Clustering support was assessed through 100 bootstrap replicates

salmon farms, Stene et al. (2014) showed that sea temperature influenced incubation time. Cohorts exposed to lower sea temperatures had significantly longer incubation periods than cohorts infected when the sea temperatures were higher (Stene et al. 2014).

Virus cultivation was unsuccessful, similar to earlier experiences with primary culture of SAV from infected fish (Christie et al. 1998, López-Dóriga et al. 2001, Graham et al. 2003, 2008, Petterson et al. 2013). Possible causes as low viral loads, virus neutralizing antibodies and/or defective virus particles in the samples have been suggested (Graham et al. 2010, Petterson et al. 2013). Challenges associated with primary isolation of SAV are also in accordance with our unpublished data from attempts to cultivate the virus from several outbreaks of PD in Norway. Nevertheless, the negative results of cell culture examinations on a range of cell types strengthens the view that other cultivable fish pathogenic viruses, including IPNV-virus, did not contribute to the problems in the fish farm. The origin of SAV in the case presented here remains unknown. After the first outbreak of disease, the virus possibly became established at the site, and disinfection methods were probably insufficient.

The fairly high mortality that initiated the diagnostic examinations in this case was probably correlated to *I. multifiliis* or *Flavobacteria*. The influence of stress to increase mortality induced by *I. multifiliis* infestations is well known (e.g. Ewing et al. 1982, Davis et al. 2002). After these secondary stressors were removed, mortality declined and stayed around 2 to 8%. However, growth rate was still reduced, indicating that SD is of significant economic importance for fish farmers.

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Editorial responsibility: Mark Crane, Geelong, Victoria, Australia

Submitted: February 18, 2014; Accepted: May 13, 2014
Proofs received from author(s): August 19, 2014