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Detection of piscine orthoreoviruses (PRV-1 and PRV-3) in Atlantic salmon and rainbow trout farmed in Germany.

Running title: PRV-1 and PRV-3 in salmonids in Germany.

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Summary

Piscine orthoreoviruses (PRVs) are emerging pathogens causing circulatory disorders in salmonids. PRV-1 is the etiological cause of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*), characterized by epicarditis, inflammation and necrosis of the myocardium, myositis and necrosis of red skeletal muscle. In 2017, two German breeding farms for Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), respectively, experienced disease outbreaks with mortalities of 10% and 20%. The main clinical signs were exhaustion and lethargic behavior. During examinations, PRV-1 in the salmon and the PRV-3 in trout were detected for the first time in Germany. Further analyses also indicated the presence of *Aeromonas salmonicida* in internal tissues of both species. While PRV-1 could be putatively linked with the disease in Atlantic salmon, most of the rainbow trout suffered from an infection with *A. salmonicida* and not with PRV-3. Interestingly, the sequence analysis suggests that the German PRV-3 isolate is more similar to a Chilean PRV-3 isolate from Coho salmon (*Oncorhynchus kisutch*) than to PRV-3 from rainbow trout from Norway. This indicates a wide geographic distribution of this virus or dispersal by global trade. These findings indicate that infections with PRVs should be considered when investigating disease outbreaks in salmonids.

Keywords: piscine orthoreoviruses, PRV, PRV-1, PRV-*Om*, PRV-3, heart and skeletal muscle inflammation, HSMI, *Aeromonas salmonicida*.

Introduction

Piscine orthoreovirus (PRV) is an emerging pathogen causing circulatory disorders in salmonids. PRV-1 was the first piscine orthoreovirus described and originally found to be associated with the disease heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*) in Norway (Palacios et al., 2010, Finstad et al., 2012). PRV-1 is ubiquitous in the sea phase of farmed Atlantic salmon, thus questioning its etiological role in HSMI. However, the causal relationship was later verified (Wessel et al., 2017). The histopathologic changes of HSMI are characterized by epicarditis, inflammation and necrosis of the compact layer of the myocardium, myositis and necrosis of red skeletal muscle (Kongtorp et al., 2004). In Atlantic salmon, an accumulated mortality of 20% has been reported, but most often it is considerably lower. Since its first discovery, PRV-1 has been detected in several salmonid species from the *Salmo* and *Oncorhynchus* genera in multiple

locations, including Northern Europe, Canada, USA, and Chile (Garver et al., 2016, Marty et al., 2015, Kibenge et al., 2013). Recently, a subtype of PRV, PRV-*Om,* was detected in rainbow trout (*Oncorhynchus mykiss*) in Norway. This variant showed about 85% nucleotide identity to PRV-1 on the S1 segment. Similar to PRV-1, PRV-*Om* was also associated with an HSMI-like disease (Olsen et al., 2015). A closely related viral strain was also detected in Coho salmon (*Oncorhynchus kisutch*) in Chile (Godoy et al., 2016). Coho salmon is also susceptible to another subtype of PRV detected in Japan, PRV-2, causing the erythrocytic inclusion body syndrome (EIBS) (Takano et al., 2016). In line with the nomenclature proposed by Dhamotharan et al., (2018), we use PRV-3 to name the PRV described from rainbow trout (previously named PRV-*Om*).

PRV infections could be involved in reduced performance of infected fish (Morton et al., 2017, Lund et al., 2017). Therefore, PRV can be considered as a threat to aquaculture and the aquatic environment. German aquaculture, with a significant production of rainbow trout and conservation programs for Atlantic salmon in several river systems, is potentially vulnerable to the impact caused by these virus infections. The common trading of fertilized eggs and live animals, both intra- and inter-continentally, makes the potential spread of aquatic viruses rapid and broad (Crane and Hyatt, 2011). Therefore, we decided to evaluate the prevalence of piscine orthoreoviruses in German salmonid populations with indications of circulatory system failure.

PRV-1 replicates in cardiomyocytes and erythrocytes (Finstad et al., 2012, Finstad et al., 2014). Currently, it is not possible to cultivate the virus in cell culture monolayers: Thus, laboratory tests for conducting surveillance and diagnostics mostly rely on specific reverse-transcriptase quantitative PCR (RT-qPCR) protocols (Palacios et al., 2010, Olsen et al., 2015) supported by immunohistochemistry. In 2017, a two-step RT-PCR based screening for PRV-1 and PRV-3 was initiated at the Fish Disease Research Unit, University of Veterinary Medicine in Hannover, Germany. Additional viruses (salmonid alphavirus type 2, SAV-2, piscine myocarditis virus, PMCV and Atlantic salmon calicivirus, ASCV) were included in the examinations due to their associations with myocardial diseases (Wiik-Nielsen et al., 2016).

Material and Methods

Outbreak 1: In 2017 a German farm specialized in breeding salmonids (Atlantic salmon, tiger trout *Salmo trutta* × *Salvelinus fontinalis* and rainbow trout) experienced a disease outbreak followed by mild chronic health problems spread over a period of two to three months (March till May) in Atlantic salmon and tiger trout

kept in fresh water. This led to cumulative mortalities estimated as being 5 to 10% of the affected stocks. Affected fish showed signs of exhaustion, reduced mobility and lethargic behavior. During necropsies, sporadic bleedings in the muscles and the pericardium were observed. Samples from these fish were examined for the presence of viral infection by cultural methods as described below. Since these analyses gave negative results, one Atlantic salmon and five tiger trouts (*Salmo trutta* × *Salvelinus fontinalis*) were sampled for molecular diagnostics of virus infection. Over a period of six months post the initial outbreak, further Atlantic salmon individuals were included in this analysis. The complete panel of specimen collected for laboratory investigations consisted of n=34 Atlantic salmon (with a body length of 17 - 52 cm) and n=5 tiger trout (with a body length of 20 - 21 cm) (Table 1). Furthermore, wild Atlantic salmon (n=4; with a body length of 62 – 101 cm) captured in rivers in western Germany were also included in the analyses.

Outbreak 2: In the same year, a rainbow trout farm in central Germany experienced a disease outbreak in early summer, lasting for two weeks and affecting all age groups excluding the fry. Diseased fish showed darkening of the skin, lethargic behavior and the mortality rate reached 20% in affected stocks. The internal clinical signs were similar to those seen in Atlantic salmon from Outbreak 1, with hemorrhaging in the muscles and the heart. Eight diseased fish from the group subjected to final ongrowing (16 months old), with a body length of 15-31cm, were sampled for analysis (Table 2).

In both cases, the fish samples were subjected to classical bacteriologic and virologic culturing test, and analyzed for the presence of nucleic acids of PRV-1, PRV-3, SAV-2, PMCV and ASCV as described below.

Tissue samples (heart, spleen, kidney, head kidney) were collected into RNA*later* from all fish and total RNA was extracted from 50 mg of selected tissue using TRI-Reagent (Sigma) in accordance with the manufacturer's instructions. cDNA was transcribed from 900-1000 ng total RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA samples were diluted 1:20 with nuclease-free water prior to end-point or quantitative PCR analysis. The end-point assays were performed using the KAPA2G Robust Hot Start PCR kit (Sigma) in accordance with the manufacturer's instructions. The reaction contained: 1× KAPA2G buffer A, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 U of KAPA2G Robust Hot Start Polymerase, 5.0 µl 20× diluted cDNA and nuclease-free water to a final volume of 20 µl. The amplification program included an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 60 s. Primer sequences are listed in Supplementary Table 1. After electrophoretic

separation in a 1% agarose gel containing 1x Gel Red (Biotium) fluorescent DNA stain and visualization in 302-nm UV light, the PCR products were used for Sanger's direct sequencing performed by LGC Genomics (Berlin, Germany). The obtained nucleotide sequences, 787 bp of the L1 segment of PRV-1 and 371bp of the PRV-3 segment S1, were aligned with piscine orthoreovirus sequences accessible in GenBank using tools available at www.phylogeny.fr (Dereeper et al., 2008). MrBayes phylogeny (Bayesian inference) was used with Markov Chain Monte Carlo parameters set-up to 10 000 generations, with tree sampling every ten generations and burn-in of 250 trees sampled.

Quantitative PCR was performed in duplicate using the Maxima SYBR Green 2 × mastermix (Thermo Fisher Scientific) in a Stratagene Mx3005P cycler (Agilent). The reaction mix contained: 1× Maxima SYBR Green mastermix (with 10 nM of ROX), 0.2 µM of each primer (sequences in Supplementary Table 1), 5.0 µL of 20× diluted cDNA and nuclease-free water to a final volume of 20 µL. The amplification program included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. A dissociation curve was performed at the end of each run. For the RT-(q)PCRs analyses, positive controls were obtained by courtesy of Dr. Hilde Sindre from the Norwegian Veterinary Institute and of Dr. Peter Steinbauer from Tiergesundheitsdienst Bayern, Germany. In the qPCR for aeromonads 16S rRNA a plasmid-based quantification of total gene copy numbers was performed as described earlier (Adamek et al., 2013).

From fish showing prominent clinical signs (n=10 Atlantic salmon, n=10 tiger trout from Outbreak 1 and n=10 rainbow trout from Outbreak 2), the spleen, head kidney and brain were pooled and collected in tissue culture medium (Eagle's minimum essential medium containing 10% foetal bovine serum, 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin). After homogenization, samples were inoculated into RTG-2 and EPC cell culture monolayers for detection of VHSV, IPNV, and IHNV in accordance with the "General Procedures for Virology" indicated in the AFS-FHS Blue Book (Ganzhorn and LaPatra, 1994) and EU guideline (European Commission, 2015). Additionally, spleen, liver, and kidney samples collected from the same fish were inoculated into blood agar plates incubated at 15 °C or 25°C for 48h and observed for bacterial growth. This was followed by identification of bacterial colonies with the API 20 NE biochemical test kit (Biomerieux).

Three macroscopically affected hearts from Atlantic salmon were collected three-six months post the initial outbreak (See Table 1), fixed for histology in 4% buffered formaldehyde, dehydrated and embedded in

paraffin wax. Sections were cut, stained with hematoxylin–eosin, and examined with a light microscope (AxioPhot, Zeiss).

Results and Discussion

Virologic examination of samples from both outbreaks gave negative results in cell cultures, ruling out infections with VHSV, IHNV and IPNV. This also excluded any other virus which would be capable of replicating on RTG-2 or EPC cells (Ganzhorn and LaPatra, 1994). Also, the RT-PCR assays for PMCV, SAV-2 and ASCV tested all samples negative (See Tables 1 and 2). Only PRV-1 and PRV-3 were detected in Atlantic salmon and rainbow trout, respectively.

From the 34 farmed Atlantic salmon screened, seven were positive for PRV-1 (20.5% prevalence). The virus was not found in four sampled wild salmon (Table 1). The Cq values from 25.44 to 32.31 in PRV-1 positive fish, suggested a moderate to low load with virus genome. A fish with the highest load of virus genome was sampled after the initial outbreak of the disease, while individuals collected at later time-points harbored a lower load of virus genome or gave a negative result (See Table 1). As shown in earlier infection studies, clinical HSMI is related to high virus load early in the disease progression (Finstad et al., 2014, Wessel et al., 2017), while fish may become asymptomatically persistent carriers of the virus at later stages. Microscopically, only three fish were evaluated: one PRV-1 positive and two PRV-1 negative. A mild myocarditis of compact myocardium, often located near the border of the spongy myocardium of the ventricle, was noticed in one PRV-1 positive (Supplementary Figure 1) and one of the PRV-1 negative fish. The second fish negative for PRV-1 showed mild epicarditis (data not shown), indicating that the cause of the heart pathology remained uncertain. PRV-1 was the only virus detected in the affected Atlantic salmon. However, further analyses revealed that the clinical signs displayed by the fish could have a dual etiology related to bacterial coinfection, which could influence the patho-histologic evaluation. While during initial sampling the only one Atlantic salmon sampled was positive for PRV-1, with the highest virus load recorded in the whole screening, several tiger trout from the initial sampling and Atlantic salmon individuals collected at further time-points were PRV-1 negative and instead seemed to have suffered from a systemic infection with *Aeromonas salmonicida ssp. salmonicida*, which was found during routine bacteriologic diagnostics (Hellmann, unpublished observation). The bacterial infection was confirmed by RT-qPCR analysis for aeromonads 16S rRNA. With this RT-qPCR we were able to demonstrate a high bacterial load

in some internal tissues in individual fish specimens (See Table 1): for instance, at initial sampling two clinically most affected tiger trout harbored 1,425,534 and 95,072,800 copies of aeromonads rRNA, respectively. Interestingly, in samples from all but one PRV-1 positive Atlantic salmon the amounts of bacteria were very low (9 to 980 copies of aeromonads rRNA) and thus most likely cannot be associated with the clinical signs of infection observed in these individuals. Therefore, we hypothesize that PRV-1 could be the causative agent of the initial disease outbreak in Atlantic salmon. The virus persisted in the population, while some Atlantic salmon acquired an infection with *A. salmonicida.* This could have been spread by tiger trout raised at the same farm because some of the tiger trout harbored a very high bacterial load during initial sampling (See Table 1). At this time, the bacterial load in Atlantic salmon was low (See Table 1). The presence of a small amount of aeromonads rRNA in all samples is in line with earlier results showing that *A. salmonicida* DNA was commonly found in blood of Atlantic salmon (Mooney et al., 1995). Nonetheless, it could also be related to a contamination during sample collection, since aeromonads are ubiquitous in the microbiome associated with the fish's skin or gills. However, it is also possible that PRV-1 and *A. salmonicida* infections existed in parallel in Atlantic salmon from the start of the outbreak and a coinfection with both pathogens was the cause of the initial health problem in the farm.

Nevertheless, this is the first confirmation of PRV-1 in Germany. In a phylogenetic analysis of the L1 sequence fragment, the German isolate (GenBank ID: MG770489) showed the highest similarity to the Norwegian isolate NOR 2012-V3621 (See Figure 1A). Taking into account the widespread distribution of this virus in farmed and wild populations of Atlantic salmon (Garver et al., 2016, Marty et al., 2015, Kibenge et al., 2013), the detection of this virus in Atlantic salmon in Germany is not surprising. The results from a much wider screening performed in fish from Norwegian rivers showed that the virus could be found in 13.4% of wild Atlantic salmon, and in 24.0% of salmon cultured for the enhancement of natural stocks (Garseth et al., 2013). A screening performed in British Columbia (BC, Canada) indicated a prevalence of PRV-1 of 1.7 – 100% in virus positive populations (Marty et al., 2015). Similarly, in the neighboring states of BC, in Washington and Alaska (USA), the prevalence of infection ranged from 2% to 73% in 25 PRV positive stocks, with Coho and Chinook salmon (*Oncorhynchus tshawytscha*) being the species found most frequently positive for PRV-1 (Purcell et al., 2018). Importantly, PRV-1 infection of salmonids may cause heart pathology which may impact migration success to their reproduction sites as recently suggested from studies on Pacific salmonids in Canada (Morton et al., 2017). In this recent research study, a negative correlation was observed between the amount of PRV-1 positive

Pacific salmons (*Oncorhynchus* spp.) at a particular spawning site and the difficulty to reach this site by upriver migration. Although speculative, these results could indicate that a PRV-1 infection might pose a challenge to the migratory and reproductive success of infected individuals (Morton et al., 2017). Reduced hypoxia tolerance and cardiac performance has been shown for fish with experimentally-induced HSMI (Lund et al., 2017). The possible influence of PRV-1 infection on the migration success of returning anadromous salmonids should be monitored and modeled more closely for the German populations.

While PRV-1 seems to be common in several salmonid populations, much less is known about the prevalence of PRV-3 in salmonid aquaculture. In a recent experimental infection, the virus presence was strongly associated with the development of heart pathology in rainbow trout (Hauge et al., 2017). The detection of this virus in two of eight rainbow trout tested from the second farm, with Cq values of 23.22 and 26.44 in heart samples, confirms the presence of this virus in the trout population in Germany. This is the third country (after Norway and Chile) where PRV-3 has been described (Cartagena et al., 2018, Olsen et al., 2015). Interestingly, the phylogenetic analysis of segment S1 sequence suggests that the German PRV-3 (GenBank ID: MG770490) is more similar to the Chilean PRV-3 isolate from Coho salmon than to PRV-3 from rainbow trout from Norway (See Figure 1B). The latest phylogenetic studies on PRV-3 suggest that the newly detected isolates from Germany, Denmark, Scotland and Italy and Chile belong to a different clade (PRV-3b) than the PRV-3 isolate from Norway (PRV-3a) (Dhamotharan et al., 2018). Taken together, these results indicate that global trade with live salmonids may play a role in distributing this virus. However, at the current stage with a limited testing for PRV-3 we cannot exclude the hypothesis of potential wild reservoirs for the virus in endemic salmonid populations (Dhamotharan et al., 2018).

PRV-3 was not the only pathogen present in clinically affected rainbow trout during Outbreak 2. Results from bacterial cultivation suggested a systemic infection with *Aeromonas salmonicida* ssp. *salmonicida* in this case as well (Flamm, unpublished observation). The RT-qPCR analysis for aeromonads 16S rRNA confirmed a high bacterial load in hearts and spleens of six of eight fish with 18,200 – 38,104,887 copies of aeromonads rRNA (Table 2). Combined, the findings of bacterial cultivation and qPCR strongly suggested that furunculosis and not PRV-3 could be the main cause of the clinical signs and the losses observed in the rainbow trout of this case. After the initial detection of PRV-3 we screened eight additional rainbow trout farms from four federal states of Germany and this resulted in the detection of PRV-3 one further farm in 2018, in a different state than the initial

detection (data not shown). This result could indicate wide distribution of PRV-3 in Germany and suggests that further studies of prevalence are needed.

The PRV-1 and PRV-3 screenings presented in our study covered only a limited amount of animals. Furthermore, due to coinfections with *A. salmonicida,* the contribution of the viral infection to disease was more challenging to assess. Therefore, at this point, the epidemiologic importance of these viruses is difficult to evaluate for the German salmonid aquaculture and for the natural populations of Atlantic salmon. However, these findings confirm the large geographic distribution of piscine orthoreoviruses. Worldwide, aquaculture of salmonids is a significant source of animal protein for human consumption. Atlantic salmon and rainbow trout are the most popular salmonid species with a production of 3.14 million metric tonnes worldwide in 2015 (FAO, 2017) . Additionally, due to their high market value of nearly $15 billion, the aquaculture of these fish species is important for the gross domestic product of several countries, including Norway and Chile (FAO, 2017). Piscine orthoreoviruses infection and diseases caused by them, could pose a challenge to the sustainable development of salmonid aquaculture worldwide. In this context, our findings should imply an increase in the awareness of PRV-1- and PRV-3-associated diseases in Atlantic salmon and rainbow trout populations. European diagnostic laboratories for aquatic animal health should include these infections in the diagnostic pool when investigating unexplained disease outbreaks in salmonid fish species.

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Conflict of interest statement:

There is no potential conflict of interest.

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.**Figures:**

Figure 1. Phylogenetic analysis of: (A) the nucleotide sequence encoding: the segment L1 PRV-1 from Germany (DH_747072017, GenBank ID: MG770489), Norway (NOR2012-V3621, GenBank ID: KY429945), Canada (B5690, GenBank ID: KX851983; B7274, GenBank ID: KX851982; BCJ31915_13, GenBank ID: KT429740; BCJ19943_13, GenBank ID: KT429730) and USA (WSKFH12_14, GenBank ID: KT429750), the tree was rooted using PRV2 (GenBank ID: LC145610) as outgroup; (B) the nucleotide sequence encoding the segment S1 of the sigma 3 protein of PRV-3 isolates from Germany (DH773072017; GenBank ID: MG770490), Norway (F445-2013NOR, GenBank ID: LN680851) and Chile (C10/P2.2, GenBank ID: KX844962; C10/P3.1, GenBank ID: KX844961; C10/P3.2, GenBank ID: KX844960; C10/P4.2, GenBank ID: KX844959; VT12202013-CGA-2013-3, GenBank ID: KU131595; C10/P1.1, GenBank ID: KX844965; C10/P1.2, GenBank ID: KX844964; VT12202013-CGA-2013-5, GenBank ID: KU131596) and PRV-1 isolate from Norway (1195Aaroy2007, GenBank ID: HG329893), the tree was rooted using PRV-2 (GenBank ID: LC145616) as outgroup. MrBayes phylogeny (Bayesian inference) was used with Markov Chain Monte Carlo parameters set-up to 10 000 generations, with tree sampling every ten generations and burn-in of 250 trees sampled. The branch length is proportional to the number of substitutions per site. Branch supporting values are indicated with the number.