Mortality outbreak by perch rhabdovirus in European perch (Perca fluviatilis) farmed in Italy: Clinical presentation and phylogenetic analysis

MORTALITY OUTBREAK BY PERCH RHABDOVIRUS (PRV) IN EUROPEAN PERCH (*PERCA FLUVIATILIS*) FARMED IN ITALY: CLINICAL PRESENTATION AND PHYLOGENETIC ANALYSIS

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ABSTRACT

This work reports a mortality outbreak, occurred in 2015 and affecting juveniles of European perch (*Perca fluviatilis* L.) farmed in Italy. Perch Rhabdovirus (PRV) was detected by viral isolation and biomolecular investigations. Phylogenetic analysis clustered our isolate into genogroup B, which also includes PRV isolates from *Perca fluviatilis* identified in France (2004-2009); diagnostic investigations also revealed opportunistic bacteria (*Aeromonas hydrophila*) and parasites (*Chilodonella piscicola*). Since, occasionally, PRV has been reported in the natural environment, which is often a source of eggs and broodstock for farms, it could be possible that both similar France and Italian isolate were imported from a same place elsewhere and have a common origin. Improving biosecurity measures (batch control) and disinfection of egg strings with an iodine based solution helps prevent apparent vertical transmission of PRV.

KEYWORDS: Perch Rhabdovirus; *Perca fluviatilis*; Rhabdoviridae; Phylogenetic analysis

Among all known viral diseases in aquaculture, Rhabdoviroses have the most important impact, based on their pathogenicity, wide host-spectrum and worldwide diffusion. *Rhabdoviruses* (family *Rhabdoviridae*) contain a single molecule of linear, negative-sense RNA (Talbi et al., 2011). The International Committee on Taxonomy of Viruses (ICTV) divided the family *Rhabdoviridae* in eighteen different genera; among these, the genera *Novirhabdovirus, Vesciculovirus, Sprivivirus* and *Perhabdovirus* contain viruses infecting both marine and freshwater fish. *Perhabdovirus*, a new genus of fish rhabdoviruses, includes three important species: *Perch Perhabdovirus* (PRV), *Anguillid perhabdovirus* (AngRV) and *Sea trout perhabdovirus* (STRV). PRV infecting European perch (*Perca fluviatilis*) is of particular interest since its impact is increasing with the farm intensification in Europe and North America.

The first case of PRV pathogenic for perch was reported in the early 1980 in France, in wild *Perca fluviatilis* (Dorson et al., 1984). Outbreaks of PRV infection were also reported in Denmark, Ireland, Germany, Norway, and Switzerland (Jørgensen et al. 1993; Dorson et al., 1984; Dannevig et al., 2001; Bigarrè et al., 2017; Wahli et al., 2015). More recently, PRV was detected for the first time in Finland in grayling fry (*Thymallus thymallus* L.) (Gadd et al., 2013).

In 2015, a batch of juveniles of European perch (*Perca fluviatilis* L.) from a broodstock group composed of internal production and one bought abroad in another farm, were stocked in tanks in a commercial perch farm located in Italy. Juveniles showed abnormal swimming behavior 3 days after stocking and cumulative mortality reached 95% in a week. Clinical signs were rather aspecific, with respiratory distress, swimming on the water surface and looping and they gave rise to concerns over the possibility of a transmissible disease. A sample of 30 fish were submitted to parasitological, bacteriological and virological examinations, according to standard laboratory procedures. For virological analysis monolayer cell cultures of bluegill fry (BF-2) and epithelioma papulosum cyprinid (EPC) cell lines were used. The 24-well plates were incubated at 15±2°C. Cultures were daily inspected for the occurrence of cytopathic effect (CPE). For PRV biomolecular detection, total RNA was extracted both from pooled juvenile and from 350 µl of cell culture supernatant as previously described by Caruso et al. (2014). Reverse transcription (RT) was carried out using High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) in a total volume of 20 µl per reaction. To detect PRV in samples, the primers from Gadd et al. (2013) were used to amplify the L gene. PCR was performed using Platinum™ Taq DNA Polymerase (Invitrogen) in a final volume of 25 µl containing 2.5 µl of 10× PCR Reaction Mix, 500 nM of forward and reverse primer, 200 µM of dNTP, 500 µM of $MgCl_2$, and 1U/reaction of Taq DNA polymerase and 2.5 µl of cDNA. The PCR conditions were as follows: initial step of 10 min at 95°C, 30 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 2 min, followed by a final extension step of 15 min at

72°C. PCR products were visualized by gel electrophoresis and sequenced with both the forward and reverse PCR primers using ABI - PRISM Big Dye Terminator v.3.1 cycle sequencing kit. Subsequently, for phylogenetic analysis, primers pairs oPVP116 and oPVP118 (Talbi et al., 2011), and oPVP126 (Talbi et al., 2011) and Rha-G-seqR2 (Gadd et al., 2013) were used to obtain the complete viral G gene sequence in two overlapping fragments. The expected amplicon size for primers oPVP116&oPVP118 was approximately 1400 bp, while for primers oPVP126&Rha-G-seqR2 was 676 bp. Purified DNA was used for sequencing both strands with an ABIPRISM Big Dye Terminator v.3.1 cycle sequencing kit. The consensus sequence was determined by the alignment of forward and reverse strand sequences of each genetic region in a single alignment using SeqMan and sequence similarities were calculated using MegAlign (Lasergene package, DNASTAR Inc.). Multiple sequence alignment was performed with BioEdit version 7.0.5.2 software using CLUSTAL W. MEGA version 7 was used for p-distance matrices calculation and phylogeny inference according to the Neighbor-Joining method. The nucleotide substitution model was Kimura two-parameters. The robustness of the hypothesis was tested in 1.000 parametric bootstrap analyses.

Differential diagnosis with other common viral pathogens was also carried out. Parasitological analyses allowed to detect *Chilodonella piscicola* in the gills with mild to severe infection intensity. Bacteriological analysis allowed the isolation of *Aeromonas hydrophila*.

Virus isolation revealed CPE at the second passage both on BF-2 and EPC cells. CPE consisted in large area of monolayer destruction in BF-2 cell line, and multifocal syncytia in EPC. Scientific literature describes the possibility to evaluate the CPE of Rhabdovirus in many cell cultures (Lorenzen et al., 1999) but in this case, EPC and BF-2 cell lines have been successfully used for viral isolation resulting particularly suitable for Rhabdoviruses isolation according to Bigarrè et al. (2017). Afterwards, biomolecular methods carried both on pooled juvenile fry (screening) and the supernatant of EPC and BF-2 cells allowed the identification of PRV showing a 399 bp band corresponding to the expected length, even if weaker in pooled fry. Differential diagnosis with other common viral pathogens was negative. Phylogenetic analysis (Figure 1) based on the complete sequence of the glycoprotein G gene (1.560 nts) revealed that our isolate clusters into genogroup B, which includes other PRV isolates from *Perca fluviatilis* identified in France (2004-2009) that share 98.6-99.2% similarity with our sequence at the glycoprotein G gene. Genogroup B also includes one isolate from the Baltic Sea and PRV sequences 196 identified in other host species, like *Salmo trutta* L. (Baltic Sea, 2009), *Thymallus thymallus* (Finland, 2002-2010) (Gadd et al., 2013). However, all these sequences have relatively lower similarities (95.3-96.9%). Interestingly, the only other Italian isolate (27V10) clusters within genogroup A.

Molecular data showed high identity of the Italian PRV isolate with a France strain; in a previous work, Talbi et al., (2011) also showed a striking genetic similarities between perch rhabdoviruses and isolates from other European countries and various ecological niches, most likely reflecting the circulation of viruses through fish trade as well as putative transfers from marine to freshwater fish; indeed, PRV is not a problem only connected to fish farming since, occasionally, it has been reported in the natural environment, which is often a source of eggs and broodstock for farms. It could be possible that both similar France and Italian isolate were imported from a same place elsewhere in Europe and have a common origin. Diagnostic investigations also revealed the presence of *Aeromonas hydrophila*, an opportunistic pathogen widely distributed in freshwater environment (Austin & Adams, 1996; Burr et al., 2012), and the parasites *Chilodonella piscicola* in the gills; these microorganism may have had a minor role in the onset of symptoms with a secondary pathogenic role to the PRV infection. No quarantine or biosecurity measures (such as isolation of new stock) were applied in the Italian farm; since most percid Perhabdoviruses reported to date originate from wild animals introduced into farms (Dorson et al., 1984; Dannevig et al., 2001; Ruane et al., 2014), careful disinfection of egg strings with an iodine based solution helps prevent apparent vertical transmission of PRV.

Following observed results, it could be useful to insert the research of PRV in monitoring plans, above all for trading purpose. Despite the increasing interest on the impact of rhabdoviruses in percid farming, few data are still available in literature and the diversity of the viral populations is still poorly investigated (Talbi et al., 2011). Therefore, it is important to acquire epidemiological data, through reporting PRV outbreaks, which allow to add and share more clinical and molecular information. Moreover, additional information on genetic diversity, transmission routes and alternative hosts for perch rhabdoviruses are needed to understand their evolution; improving knowledge on PRV epidemiology will lead to better prevent and control the disease both in farmed and in wild perch populations.

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AUTHORS' CONTRIBUTIONS

MP and MLF coordinated this study. CC and AG wrote the paper; CC, RP and PP performed the molecular and virological analysis. SP performed typing PCRs, sequencing and phylogenetic analyses. AG and MLF carried out the sampling, bacteriological and parasitological examination. PLA, LM, MLF and MP critically read the manuscript. All Authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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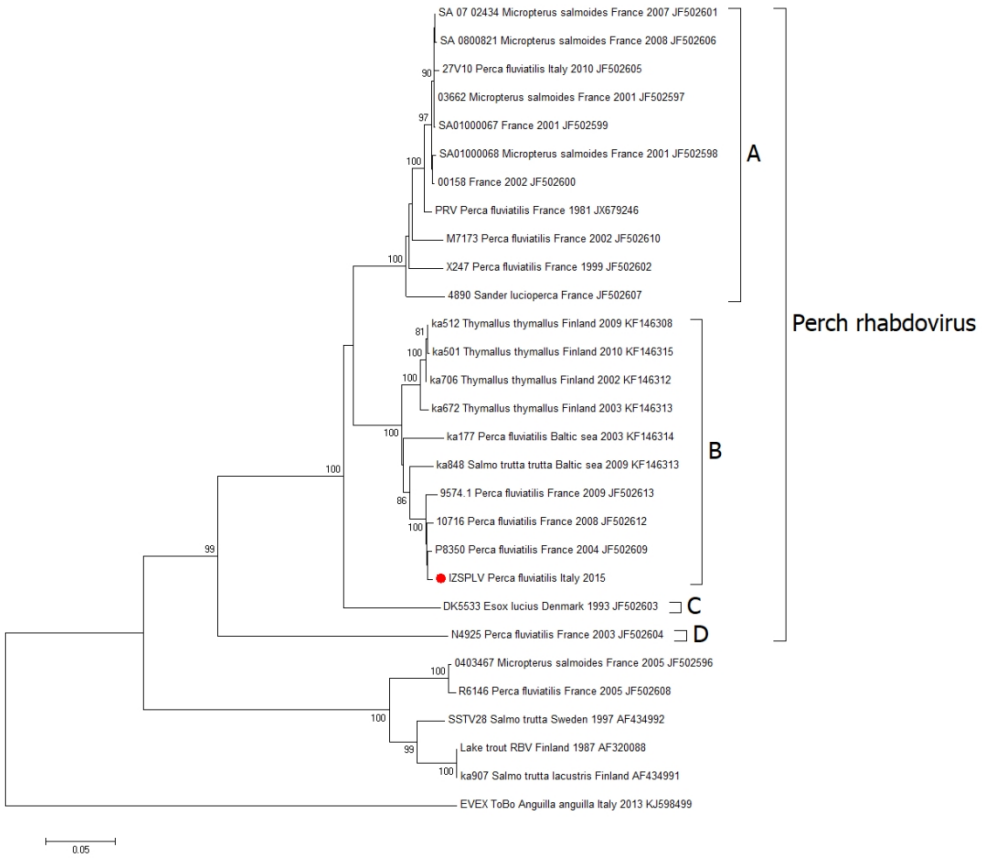

Figure 1 Phylogeny inferred by Neighbor-Joining analysis obtained by an alignment of 1,560 nucleotides, covering the glycoprotein (G) gene region of PRV. The phylogenetic tree includes the PRV isolate of this study (marked by the red circle; GenBank acc. No. MK360920) and sequences available in GenBank. Host species, country of origin, collection year and accession number are indicated for each sequence. Eel Virus European X (EVEX) was used as outgroup. Bootstraps (1,000 replicates) values > 80 are shown at the internal nodes. The length of each pair of branches represents the distance between sequence pairs. The scale bar represents the percentage of nucleotide differences.