VETBIONET

Veterinary Biocontained facility Network for excellence in animal infectiology research and experimentation

Deliverable D7.4

Improved fish model for relevant pathogens in aquaculture

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A number of methods of infectiology was defined in this deliverable for the majority of fish species of interest to the European aquaculture sector. The focus was on serious viral diseases for which good infectiology protocols are critical for the characterization of virulence and for the establishment of control strategies. Five partners developed challenge methods in their respective fish species: Atlantic salmon (*Salmo salar*) for MS, Rainbow trout(*Oncorhynchus mykiss*) and pike (*Esox lucius*) for ANSES, INIA, INRAE and PIWeT, European sea bass (*Dicentrarchus labrax*) for IZSVe, Carp (*Cyprinus carpio*) for PIWeT. The route of infection, isolates, animal husbandry parameters, sampling regimes and methodologies were optimized for each host species. The following methodologies were determined for successful experimental infection based on immersion (or bath) or intramuscular (i.m.) routes of infection but also by contact (Rainbow trout with pike)

Host	pathogen	route
Atlantic salmon	Salmon Pancreatic Disease Virus - SPDV	bath
Trout	Infectious Pancreatic Necrosis Virus - IPNV	bath
Trout	Viral Haemorrhagic Septicaemia Virus - VHSV	bath, i.m.
Pike	Viral Haemorrhagic Septicaemia Virus – VHSV Viral Hematopoietic Virus - IHNV	Bath Contact
Carp	Spring Viraemia of Carp Virus - SVCV	bath
Sea bass	Betanodavirus NNV	bath, i.m.

In addition, a novel approach of monitoring the progress of the disease (pathogen replication dynamic) and the host response was established for aquatic vertebrate: the same individual is monitored through the non-lethal collection of small amounts of blood. In combination with a reporter cell-based assay, the viremia can be measured from the same animal, giving a robust resolution and statistical power to experimental infections experiments. In this deliverable, two models of individual monitoring of viremia were established in salmonids. One of them was

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applied to vaccine efficacy testing. In term, these approaches will contribute to the reduction of animal uses in some infectiology procedures.

Objectives:

The objective of this deliverable is to improve salmonid and cyprinid infectiology models for refined monitoring of both disease progression and host response. It concerns the following host/pathogen models: Salmon Pancreatic Disease Virus (SPDV) infection model in Atlantic salmon *Salmo salar* with respect to the use of minimal invasive methods (MS); the improvement of the current IPNV (Infectious Pancreatic Necrosis Virus), VHSV (Viral Haemorrhagic Septicaemia Virus), KHV (Koi Herpes Virus), SVCV (Spring Viraemia of Carp Virus) and NNV (Nervous Necrosis Virus) infection models in rainbow trout *Oncorhynchus mykiss*, carps *Cyprinus carpio* and sea bass *Dicentrarchus labrax* by the use of genetically and phenotypically characterized virus strains and/ or by expanding the portfolio of immunological evaluations (INIA, ANSES, PIWET, IZSVe). The potential of pike to spread VHSV and IHNV and in particular to contaminate salmonids was also investigated using cohabitation studies. Next to these live animal models, a previously established reporter cell line (RTG-P1) will be used to study the early viremia kinetics of SPDV and possibly other fish pathogens (MS, INIA, ANSES, PIWET, IZSVe, INRA), using results from Task 8.5 on immunoassays for the detection of viremia in native or inactivated serum samples that will be obtained in the infection trials outlined above.

Rationale:

To understand virulence and to be able to implement control strategies, robust experimental infection methods must be optimized for each of host species and pathogenic agents. The route of infection, sampling regime, animal husbandry, monitoring strategies are important parameters to optimize in order to replicate the disease in the laboratory.

Teams involved:

INRAE, INIA, ANSES, PIWET, IZSVe

The large number of species of interest for the fish farming industry in Europe implies a large effort used to optimize infectiology protocols. This deliverable describes the integrated work of 5 partners with expertise in health of aquatic vertebrates. A number of infection protocols for important viral diseases were established or improved. In addition, a novel approach of monitoring, in the same individual, both the host response and pathogen replication dynamic, was implemented for two of the host/pathogens models. This non-invasive

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methodology may have some benefit in the 3Rs (reduction of animal use). The report list the results obtained for each host/pathogens combination.

3.1. Salmo salar and Salmon Pancreatic Disease Virus (SPDV)

A novel individual monitoring approach was established for fish for a number of salmonid infectiology models. Combined with a cell-based assay for viremia, this methodology allows to robustly describe the host response and pathogen replication kinetics using a limited number of animals. It was developed in Atlantic salmon in relation to DNA vaccination and infection with Salmon Alphavirus (SAV), causative agent of Pancreatic Disease (PD). The results are detailed in Collins et al. (2021).

3.2. Oncorhynchus mykiss and Viral Haemorrhagic Septicaemia Virus (VHSV)

A similar approach as *Salmo salar*/SPDV was developed for *Oncorhynchus mykiss* in relation to infection with VHSV (pilot study) and DNA vaccination (linked to a VetBioNet TNA Prof. Niels Lorenzen). The viremia test (Collins et al., 2021) was adapted to VHSV (isolate VHSV7.71) and used to monitor the viral replication after experimental infection via intramuscular injection. A pilot study demonstrated the feasibility of the method (Figure 1). In addition, the individual immune response could be followed by qPCR on the blood cells (Figure 2) for which an optimal method of sampling and RNA purification was established. The pilot experiment was carried out over 9 days in 12 animals. The TNA project was carried out in two phases, a first phase of DNA vaccination with several experimental constructs followed by a second phase of infection (same method as in the pilot experiment). Viremia could be used to characterize the intensity of infection and its decrease as a correlate for vaccine efficacy in a similar way as described by Collin et al., 2021.

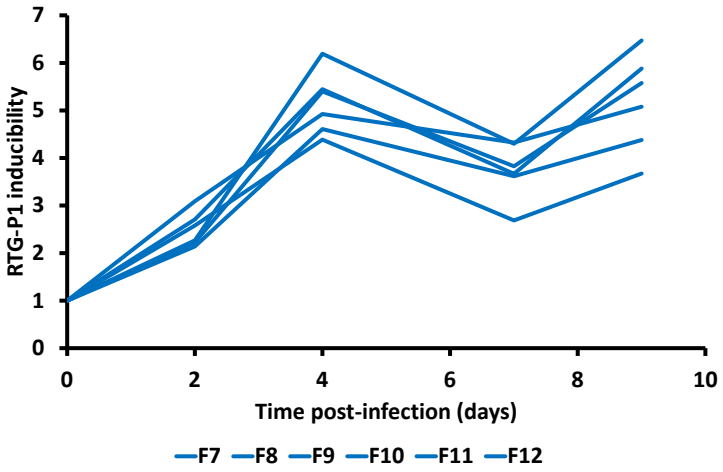

Fig 1. Individual viremia kinetics on individual rainbow trout infected with VHSV. Data represent the luciferase inducibility measured in the reporter cell line RTG-P1 after incubation with plasma samples collected over the course of infection from 6 animals.

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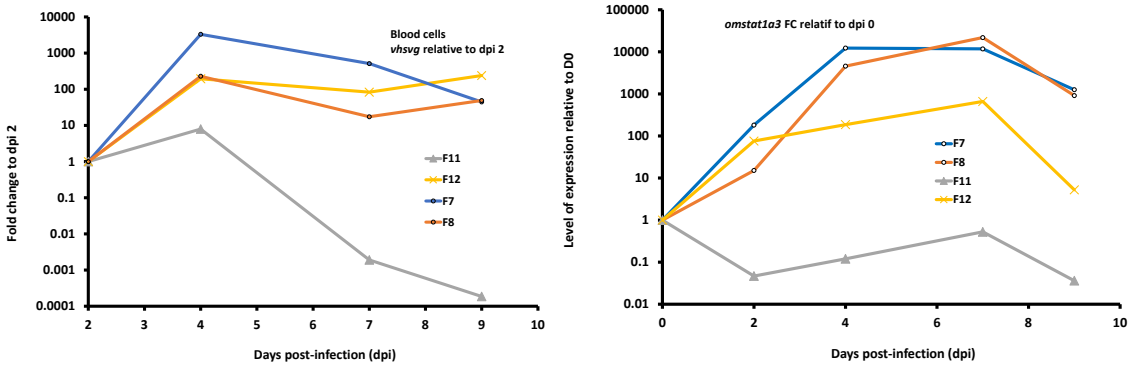

Fig 2 (left) *vhsv g* gene expression level in blood cells of same animals during course of infection. Data are fold changes relative to dpi 2. (right); *stat1a3* gene expression levels in blood cell of same animals during the course of infection. Data are fold changes relative to dpi 0.

Experimental bath infection protocol has also been optimized in rainbow trout with the VHSV DK-3592 strain to monitor mortality. The infection was performed by bath exposing fish of approximately 5 g for 1 h to $5x10^5$ virus/ml. The mortality rate obtained was around 60-80%. This updated protocol has been effectively used to test the efficacy of different vaccines and immunostimulants.

An experimental system allowing viral contamination of pike (*Esox Lucius*) was developed in order to investigate the role of this species as a potential vector for VHSV and IHNV in trout. Although susceptibility of pike to VHSV was confirmed, especially in early stage of development, pike could be contributing to the persistence of VHSV but also IHNV in adapted hydro-geographic areas and, potentially, to recurrent infection events in salmonid farms. VHSV and IHNV were isolated from several pike *Esox lucius* samples collected from a pond near the salmonid farms of interest. All VHSV isolates analyzed were 100% identical to each other concerning their partial glycoprotein gene sequences. VHSV pike strain OO128-25 belonged to the Ia genotype and shared 99.1 to 99.5% nucleotide (nt) identity with strains recently isolated from the farms and their virulence was tested in rainbow trout. IHNV pike strain OO121-8, European genotype, appeared to be different from strains from France characterized since the first isolation in 1987. All isolates from pike were highly virulent for rainbow trout.

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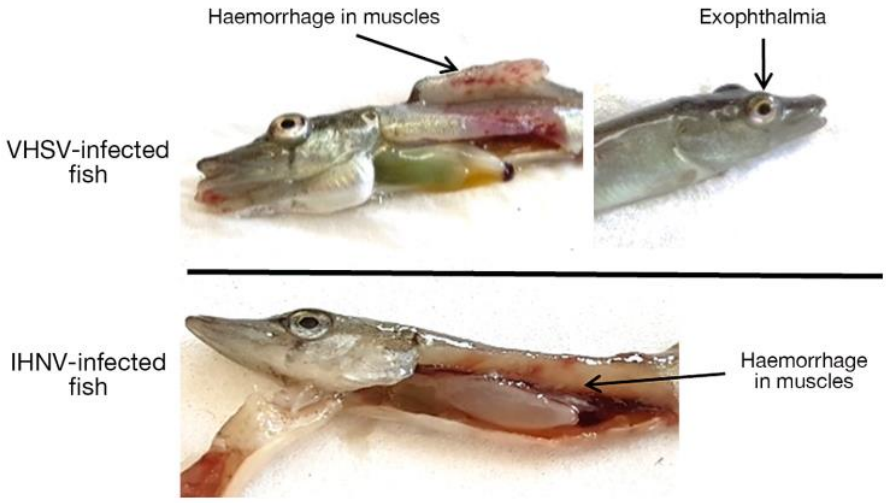

Fig 3. Exophthalmia and muscle hemorrhage in pike infected with IHNV and VHSV 8 days post-infection.

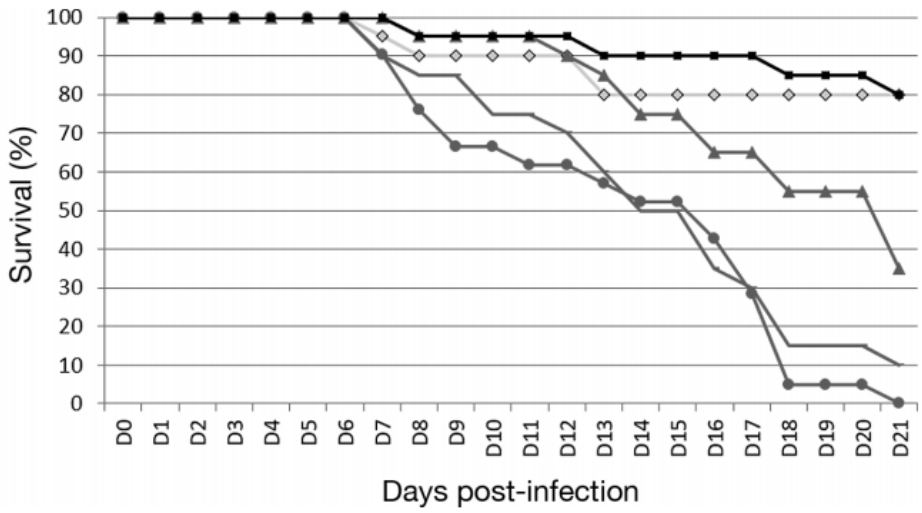

Fig 4. Mortality kinetics obtained for juvenile pike after immersion infection with VHSV 23/75 (positive control; gray line without symbol) or OO128-25 (gray triangle) and IHNV 11198 (positive control; black circle) and 00121-9 (black square) strains. Control conditions are represented by diamonds. Mortality was monitored daily for 3 weeks on a total of 20 fish/condition.

Several experimental infections of pike at different physiological stages were done: OO128-25 induced 65% mortality in pike fingerlings whereas only weak mortality was observed with OO121-8, despite characteristic symptoms in infected fish (Fig 3 and 4). High levels of specific antibodies to VHSV and IHNV were detected in adult pike in the absence of clinical signs. Infection of rainbow trout in contact with experimentally VHSV- or IHNV-infected pike fingerlings suggest a horizontal transmission. These results suggest that pike could act as a reservoir for VHSV and IHNV in the wild, providing additional evidence to explain viral persistence and resurgence in certain areas (Cabon et al., 2020; Louboutin et al., 2018).

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To expand the portfolio of immunological assays available, an ELISPOT to quantify the number of B cells secreting VHSV-specific IgMs was developed. B cells secreting VHSV-specific antibodies can be detected in spleen or head kidney after the infection.

Bath challenge with one hour contact between 12-30g trout and cell-grown VHSV (genotype Ia – $2.7.10^6$ $TCID_{50}$) in 200 l tanks was carried out. Kidney, liver, spleen, heart, gill, brain from fish were collected to the RNA Later separately at 1, 3, 7, 9, 11, 15, 18, 21 and 28 ? dpi and stored in -80°C to further analysis. Total RNA from each tissue was extracted by using Total RNA Mini following the manufacturer protocol. Real Time RT-PCR was carried out with specific primers pairs. The viruses presence was observed from first day to the 28 day post infection in all internal organs studied and the infection caused high mortality.

3.3. Rainbow trout (Oncorhynchus mykiss) and Infectious Necrotic Pancreatic Virus (IPNV)

Cell-based viremia test was evaluated for this model but no significant induction of the reporter cell line could be detected. qPCR was also not able to detect the presence of the virus in blood cells.

Experimental infection of rainbow trout with IPNV was carried out. At days 3 and 7 kidney, spleen, pyloric caeca and intestine samples were collected for RNA isolation. Different biological samples were collected from those experiments. The viremia in inoculated fish were analysed using the RTG.P1 cell line. Although there is a clear induction of luciferase when using poly I:C, no induction was observed with sera from IPNV fish and viremia could not be detected by RT-PCR either.

Rainbow trout blood leukocytes were infected with IPNV *in vitro* and used to study the transcriptional response of different leukocyte subsets to the virus by single cell transcriptomics. A transcriptomic profile of IPNV-infected vs control cells, for the different blood leukocyte subsets (B cells, T cells, NK cells, monocytes…) has been established and will be published in the next months.

To expand the portfolio of immunological assays available, an ELISA to monitor IPNV-specific IgMs was established.

Bath challenge with one hour contact between 12-30g trout and cell-grown IPNV (serotype Sp – $1.3x10^8$ $TCID_{50}$) in 200 l tanks was carried out. Kidney, liver, spleen, heart, gill, brain from fish were collected to the RNA Later separately at 1, 3, 7, 9, 11, 15, 18, 21 and 28 dpi and stored in -80°C to further analysis. Total RNA from each tissue was extracted by using Total RNA Mini following the manufacturer protocol. Real Time RT-PCR was carried out with specific primers pairs. The viruses presence was observed from first day till the 28 day post infection in all internal organs.

3.4. European Sea bass (Dicentrarchus labrax) and Betanodavirus

A selection of 9 reassortant RGNNV/SJNNV betanodaviruses were genetically characterized and checked for presence of virulence markers. These strains were used for infecting European Sea Bass in order to better investigate RGNNV/SJNNV viruses' virulence in this non-target species. Two SJNNV strains and 1 RGNNV strain were used as low and highly virulent control respectively. Infection was performed on groups of 54 juveniles sea bass

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(5-6 g) by intramuscular injection with 0.1 ml of each virus. The experiment lasted 28 days. Brain samples from dead fish as well as from 10 survivors per group were collected. Samples were tested by quantitative real time PCR targeting RNA1 gene. Results confirmed, as expected, that the two SJNNV viruses were substantially non-pathogenic for sea bass (0 and 1.8% mortality), while the RGNNV caused 34.5% cumulative mortality. The reassortant strains generally possessed low pathogenicity for sea bass causing mortality ranging from 2% to 15.7%. Only one viral strain (132/05) caused 26% mortality, resembling that of the pathogenic RGNNV. These results were statistically supported. All RGNNV/SJNNV survivors tested positive to the real time PCR and were similar in RNA1 copy numbers. Statistical elaborations of survival curves allowed the distinction of reassortant strains (RG/SJ) into three classes of virulence:

- High (isolate 132 /05)
- Intermediate (isolate 292 -1.2/09 and 292 -7.8/09)
- Low (all other RG/SJ strains)

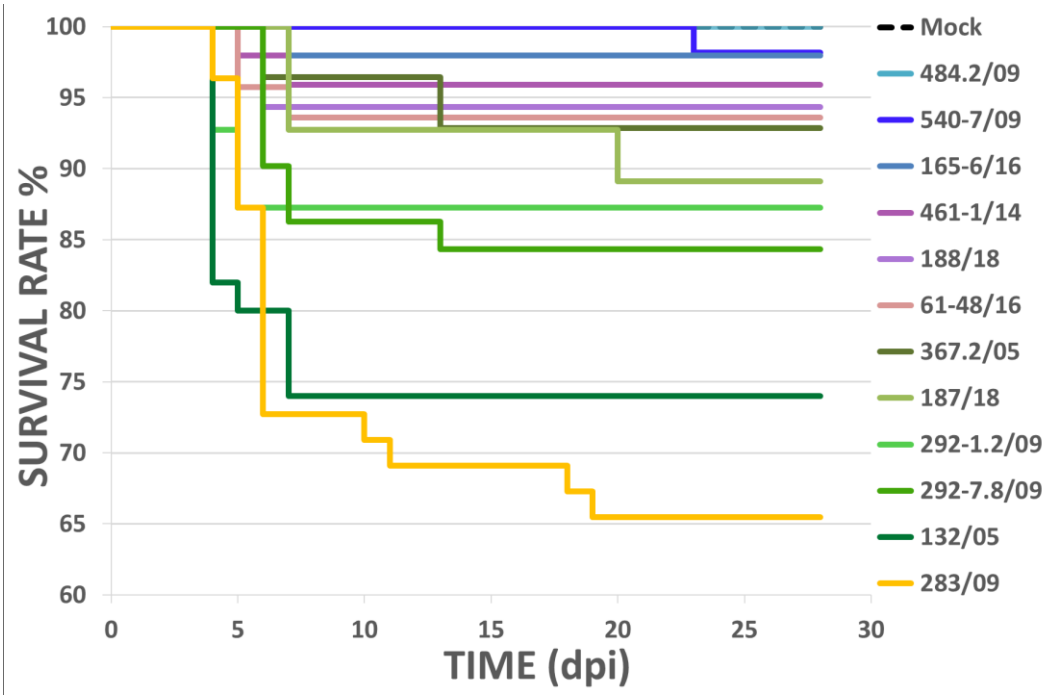

Fig. 5 Kaplan-Mayer survival curves of European sea bass infected intramuscularly with different betanodaviruses (9 RGNNV/SJNNV genotypes, 2 SJNNV genotypes named 484.2/09 and 540-7/09, 1 RGNNV genotype named 283/09). Analysis performed with the non-parametric Wilcoxon-Breslow -Gehan test (p -value <0.05 ;95 %

Bath challenge with 3 reassortant viruses representative of the different virulence classes previously observed was performed. Groups of 54 juvenile European sea bass (*D. labrax*) of 4-5 g were bath challenge with 10^4 $TCID_{50}$/ml of each viral strain for 4 hours at 25°C and 25 ‰ salinity. One group was mock infected and used as negative control, another group was infected with RGNNV, a known pathogenic genotype, as positive control. The experiment lasted 28 days and mortality was monitored daily. Brain samples from dead fish as well as from

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10 survivors per group were collected. Samples were tested by quantitative real time PCR targeting RNA1 gene.

Results showed that sea bass are susceptible to viral infection with reassortant strains as showed by viral load detected in survivors' brains. Mortality by bath ranged from 3.7% to 7.4% according to the viral strain. The positive control groups infected with RGNNV showed 18.5% mortality. These results confirm that the RGNNV/SJNNV strains, which are highly pathogenic for sole and sea bream, appear to pose a low to moderate risk of causing clinical disease in sea bass. Due the generally low pathogenicity of these strains, the intramuscular injection appears the best infection route the highlight small changes in virulence.

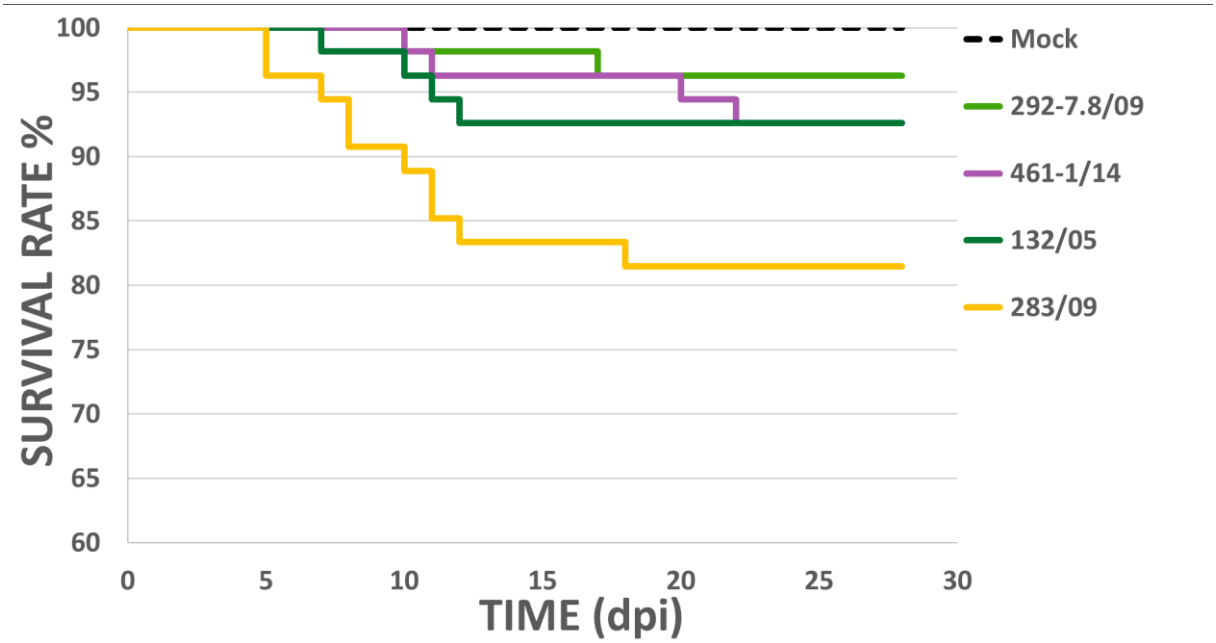

Fig. 6 Kaplan-Mayer survival curves of European sea bass infected by bath with different RGNNV /SJNNV betanodaviruses.

Previous studies identified putative markers of virulence on the RNA2 gene (coat (coat protein) of reassortant RG/SJ RG/SJ strains (Moreno et al., 2019). Our findings partially confirm previously reported result, but suggests the possibly existence of additional virulence markers: 5 new variable aa positions within RNA2 coding region. Interestingly, the five amino acid residues are exposed externally to the structure of the P-domain protein. Moreover, in the c-terminal portion of the non -structural protein B2 (RNA 3 gene) some amino acid substitutions are observable in a proline-rich region.

Viral genome in the brain of survivors at 28 days post challenge was quantified and it was not different from the amount of that present in the positive control group brains despite the route of infection (intramuscular or bath) . This indicates that the reassortant viruses replicates in the brain and survivors could act as healthy carrier for other species, highlighting the risk poses by fish polyculture.

Results were presented at the 20th International conference on diseases of fish and shellfish held online on 20-24 September 2021.

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As previously observed with IPNV, low luciferase activity was measured at 20° and 25 °C after RTG-P1 infection with Betanodavirus (RGNNV, SJNNV and bioth reassortant strains strains). It was concluded that Betanodavirus possess reduced capacity to induce *mx* gene, potentially indicating interferon suppression capability.

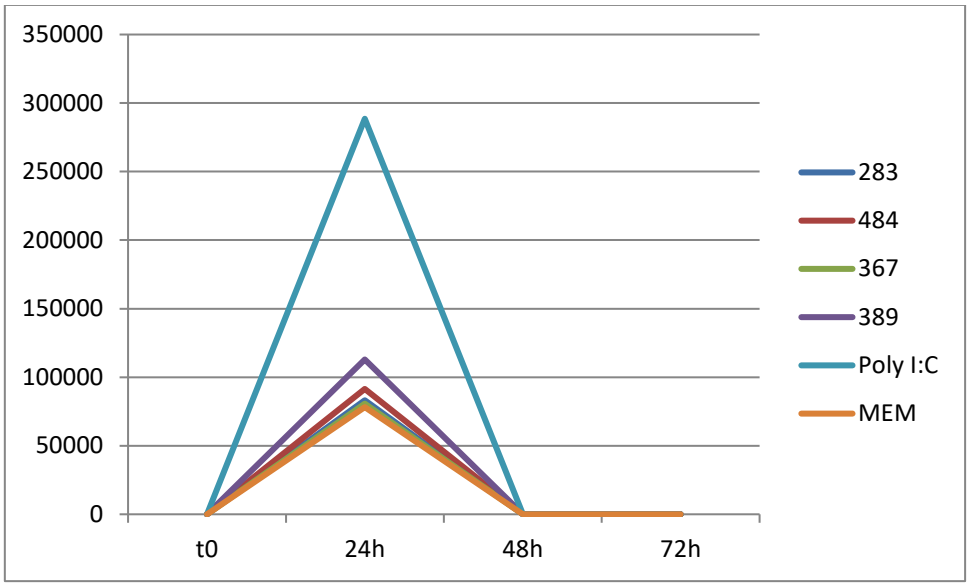

Fig. 7 Luciferase inducibility (RLU on y-axis) measured in the reporter cell line RTG-P1 after incubation at 20°C with different genotype of Betanodvirus at different time points (0-24 and 48 hours post infection). Poly I:C was used as positive control, while the sterile MEM was used as blank. Carp (*Cyprinus carpio*) and Spring Viremia of Carp Virus (SVCV)

Forty five (45) juvenile healthy common carps were used to compare the process of infection of SVCV isolates belonging to genogroups Ia and Id occurring in Poland. One isolate from each genogroup was chosen. From genogroup Ia we selected KF98371 isolate from 2011 year, from slaskie voivodship. This isolate was more similar to Asian isolates than to European. From genogroup Id we used isolate KF983466 from 2000 year, from zachodniopomorskie voivodship. This isolate was more similar to other Polish and European isolates. These two isolates were obtained in different years and come from distant fish farms in Poland. We used the EPC cell line to cultivate both viruses and check their titre. Infected cell line was stored in 21°C. Cultivation was continued until the cytopathic effect was evident. Then, the cell culture supernatant was used to infect fish.

The pathogenicity and optimal parameters for infection of selected SVC virus isolates was evaluated in carp fry weighing 8 - 60 g. Fish were exposed to virus suspension trough bath immersion at a water temperature of 12 °C and observed to check any differences in the course of infection between different isolates.

Fish were divided into four groups in the following order:

Group I - infection 45 min. isolate 11/14573 belonging to genogroup Ia
Group II - infection 45 min. SVC-3 isolate belonging to genogroup Id
Group III - infection 120 min. isolate 11/14573 belonging to genogroup Ia
Group IV - infection 120 min. SVC-3 isolate belonging to genogroup Id.

An additional group was a control group, with healthy carps.

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The fish from groups I-IV were infected with two different isolates of the spring viremia of carp virus, but with a similar titre. Kidney, liver, spleen, heart, gills, brain were collected on days 1, 4, 7, 9, 14, 17, 21, 24, 28 after infection from three fish from each group. Genetic material (RNA) was isolated from the 192 samples in total and used to perform reverse transcription, followed by Real Time PCR including a previously prepared genetic construct to calculate the number of viral copies in the tested samples.

The Id isolate appears to be more pathogenic than the analysed Ia virus isolate, because already on the first day after infection it was present in all organs tested and its presence was found in the tested samples up to 24 days after infection. The presence of group Ia virus on the first day after infection was found only in the gills with a shorter 45-minute infection and in the kidney, liver, spleen and gills after a 120-minute infection. Fish infection in a water bath for 120 minutes, provides better results compared to an infection that lasted 45 minutes with both the Id group isolate and the Ia group isolate.

The sequencing of the whole genomes of these two viruses from different genogroups was carried out. Primers for sequencing were obtained by using Primer 3 software. Primers amplified genes: G - glycoprotein, L - RNA dependent RNA polymerase, M - matrix protein, N - nucleoprotein. Reverse transcription was carried out in following conditions: 50°C - 30 min, 95°C - 15 min., 45 cycles in 94°C - 15 sek., 60°C - 40 sek. The sizes of the RT-PCR products were as expected: 1053 bp of N gene, product of 924 bp of P gene, product of 700 bp of M gene and product of 1344 bp of G gene. Product 3364 bp of L gene was divided on smaller parts: 913 bp, 605 bp, 905 bp and 951 bp. Specificity of the obtained products was verified by sequencing at the Genomed Company, Warsaw, Poland. Sequences were compared and differences between KF983466 isolate and KF98371 isolate were studied. Percent of identity between sequences was about 90 %. The results suggest that G gene is the more variable, than other genes, so it is a good model for characterization of SVC viruses. The observed differences involved single mutations - substitutions.

Continuous work on the improvement of the reliability and efficiency of the SVCV experimental challenge in common carp or the zebrafish model; testing of novel zebrafish infection models (incl. FV3, an iridovirus causing potentially fatal infections in farmed and wildlife fish, amphibians and reptiles) was carried out.

The multiplicity of fish species in aquaculture complicate the development and improvement of infectiology methods. In this deliverable, the 5 partners (INRAE(MS), IZSVe, INIA, PIWeT, ANSES) have investigated a range of fish species/viral pathogen models covering most of the aquacultured species in Europe as well as one important species in ponds extensive production's. The existing infection methods have been refined and novel method for monitoring the host response/pathogens replication established.

The following infectiology models have been developed:

Host	pathogen	route
Salmon	SPDV	bath
Trout	IPNV	bath

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Trout	VHSV	bath, i.m.
Pike	VHSV, IHNV	Bath, contact
Carp	SVCV	bath
Sea bass	Betanodavirus	bath, i.m.

Individual monitoring was established for two salmonid models and combined with reporter cell-based viremia tests, allowing less animals to be used in virulence studies or vaccine efficacy testing. The reporter cell-based viremia test was evaluated with other viral pathogens with mixed results.

Cabon J, Almeras F, Baud M, et al (2020) Susceptibility of pike Esox lucius to VHSV and IHNV and potential transmission to rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 139:175–187. https://doi.org/10.3354/dao03474

Collins, C., Lester, K., Del-Pozo, J., Collet, B., 2021. Non-Lethal Sequential Individual Monitoring of Viremia in Relation to DNA Vaccination in Fish-Example Using a Salmon Alphavirus DNA Vaccine in Atlantic Salmon *Salmo salar*. Vaccines 9, 163. https://doi.org/10.3390/vaccines9020163 .

Louboutin L., Cabon J., Almeras F., Baud M., Pallandre L., Langlois Y., Dupire A., Morin T. Pike, a “discreet vector” of Viral Hemorrhagic Septicemia virus (VHSV) and Infectious Hematopoietic Necrosis virus (IHNV)? in AQUA Congress, Montpellier, France, 25-29 August 2018.

Biasini L., Toffan A., Marsella A., Abbadi M., Buratin A., Pascoli F. Pathogenicity of different betanodavirus RGNNV/SJNNV reassortant strains in European sea bass. (Poster) 20° International conference on Disease of fish and shellfish. Held online 20-24/9/21.

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