Laboratory investigation of two IHNV infected farms in Denmark

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Cover photo: Rainbow trout juveniles experimentally infected with IHNV, affected fish showing skin darkening, exophthalmia and loss of balance

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Content

1. Background and rationale

On 18th of May 2021, the Danish Food and Veterinary administration (DVFA) declared the first outbreak of Infectious Haematopoietic Necrosis (IHN) in a Rainbow trout fish farm located in Stouby northeast of Vejle.

The disease is listed in the OIE Aquatic code as well as listed in Commission delegated regulation (EU) 2020/689 and categorized as a list C disease according to Commission implementing regulation (EU) 2018/1882.

After the first detection, an epidemiological investigation was conducted. This has involved all the contact farms, and also all put and take lakes, which have received or delivered fish to the infected farms, are under investigation.

The initial sampling by DVFA on the first sites and contact farms were conducted in a period where water temperatures were below 14 °C as it is demanded by the legislation on sampling and testing of fish farms for demonstrating freedom of infection with IHNV (Commission delegated regulation (EU) 2020/689).

Overall, the diagnostic testing of the fish farms under suspicion was conducted between May and June 2021. For each site, 30 individual fish were collected by qualified personnel at DVFA. Sample processing and testing in the laboratory was done according to the diagnostic manual provided by the EURL for fish diseases, but using a modified RT-qPCR probe as described by Hoferer et al., 2019.

Currently (January 2022) the infection with IHNV has been confirmed in eight fish farms and three put and take lakes (https://foedevarestyrelsen.dk/Dyr/Dyresygdomme/dyresygdomme/IHN/Sider/IHN---Aktuel-situation.aspx).

Danmark has withdrawn the status as IHN free country, further measures for disease control are under discussion.

The farms addressed in this report (identified as site A and site B) are located in at Holsted in Vejen Municipality

According to the legislation sampling for surveillance of infection with IHNV shall be conducted at water temperatures below 14°C. It was therefore debated if IHNV could still be detected at higher temperatures during summer. This study was therefore initiated to assess the capability of IHNV detection at higher water temperatures and assess how confident we could be in testing fish staying at temperatures above 14°C.

2. Aim of the study

In order to monitor the kinetics of the infection in the fish farm along with the effect of increased water temperature on detection of IHNV, two follow up samplings were designed and conducted by the NRL for Fish Diseases. Fish samples have been collected on June 24th and July 22nd 2021, respectively. In the period of time covered by the follow up sampling, temperatures have been fluctuating between 12° to 20°C.

3. Materials and Methods.

3.1 Initial investigation for tracing infection – DFVA

The sampling by DVFA of site A was done the 31.05.2021, and received at the lab of DTU Aqua on 01.06.2021. Heart, spleen and kidney from the 30 fish collected were divided in three pools of 10 fish in test tubes with EMEM. Along with test tubes containing organs , the heads of the corresponding fish were shipped cool in plastic bags marked fish 1-10, 11-20 and 21-30 matching the labelling on the tubes.

At the laboratory, the brain was sampled in pools of five fish. So from a group of 10 fish, one pool of organs in EMEM and two pools of brain were obtained.

Both organs (heart, spleen and kidney) and brain pools were homogenized by mortar and pestle and clarified by centrifugation. The supernatant was then used for inoculation in EPC cells and for RNA extraction and testing for IHNV by RT-qPCR. The RT-qPCR used for detection of IHNV was the current method described in the Diagnostic manual, but using the probe described by Hoferer et al., 2019.

The sampling at site B was performed the 28.05.2021 and received at the lab of DTU Aqua on 28.05.2021. The 30 fish collected were divided in three pools of 10 fish each containing heart spleen, kidney and brain in EMEM for virological examination. The same diagnostic methods and laboratory procedures were applied as for site A.

3.2 Sites

The two aforementioned sites (site A and B) have been inspected again on June 24th 2021 and on July 22nd 2021. Due to summer weather conditions, water temperature has increased, and is reported to fluctuate between 12 to 20 degrees Celsius during the period covered by the samplings. Temperature fluctuatons of the river system were recorded by the meteorological station located about 20 km downstream from the 2 farms.

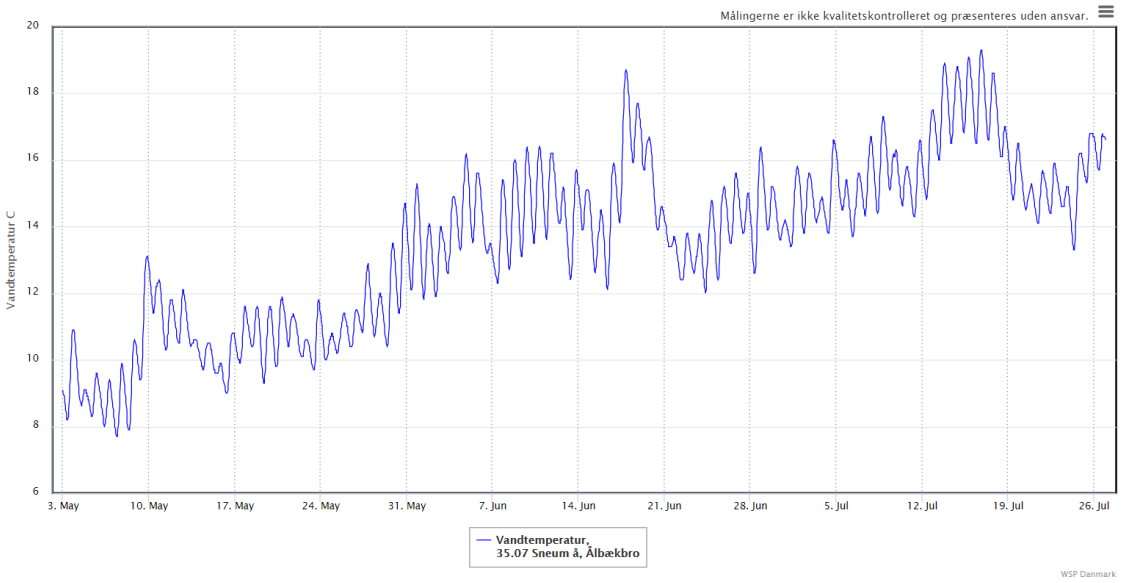

Fig. 1. Water temperature recorded in the river system during the sampling period. (https://vandportalen.dk/plotsmaps?days=84&end=26072021&id=10798-0-0)

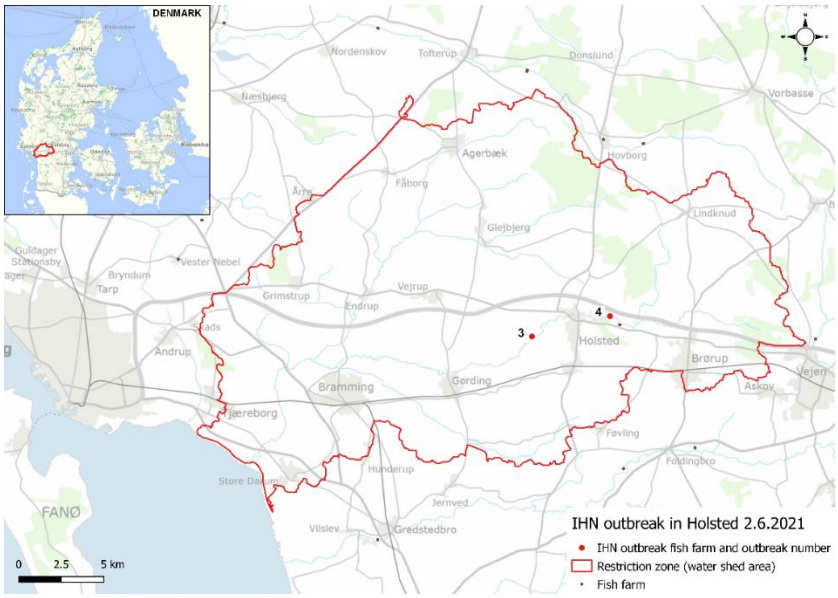

Fig.2 Geographical placement of the farms used in this study. Sites are marked as “3” and “4” corresponding to B and A respectively. Source: DFVA website (

https://www.foedevarestyrelsen.dk/english/Animal/AnimalHealth/Animal%20diseases/IHN/PublishingImages/holsted%20zonekort%20020621.png

3.3 Follow up sampling

At both sites, fish are farmed in earth ponds connected in parallel to the river.

At each farm, 30 fish were collected following the guidelines of the diagnostic manual, targeting fish showing clinical signs.

The 30 specimens were divided into six pools of five fish each. If clinically affected fish were found, it was noted which pool they were included in.

From each group of five fish:
- brain tissues were pooled into a tube with EMEM
- heart spleen and kidney were pooled into a tube with EMEM

In total 12 samples were collected for testing for the presence of IHNV.
In addition, the kidney of 10 fish were sampled for bacteriology.

The same procedure was conducted during the second sampling, with the exception that from each individual fish two further subsamples were collected in RNA later:
- one subsample in RNA later from each of the 30 fish containing small pieces of heart, kidney and spleen tissue
- one subsample in RNA later from each of the 30 fish containing a small piece of brain

This additional sampling was done to investigate the pooling effect on the detection of the pathogen and its prevalence.

3.4 Virological examination

All samples collected in EMEM have been treated according to the procedures described in the Diagnostic Methods and Procedures for the Surveillance and Confirmation of Infection with VHSV and IHNV https://www.eurl-fish-crustacean.eu/fish/diagnostic-manuals/ihn
Briefly, 24 hours after collection, tissues were homogenized by mortar and pestle and clarified by centrifugation (at 2 °C to 5 °C at 2000 to 4000 x *g* for 15 minutes).

An aliquot of the supernatant was collected for RNA extraction and tested by RT-qPCR as specified in the procedures for testing the first sampling. Another aliquot was incubated with antibiotics and inoculated on EPC and BF-2 cell culture for viral isolation.

Upon appearance of CPE, the isolate was identified by an ELISA detecting VHSV, IHNV and IPNV antigen

3.5 Bacteriological examination

The swabs collected from the kidney for bacteriological examination were streaked onto agar plates (Blood agar and TYES agar) for isolation of bacterial pathogens.

4. Results

4.1 Results resume

Site / sampling	RT-qPCR PO	RT-qPCR Brain	Cell culture PO	Cell culture brain	Bacteriology
Site A - DFVA	3/3 +ve	5/6 +ve; (1 susp)	3/3+ve	3/6 +ve	N/A
Site A – 1st FU	2/6 +ve ; (1 susp)	1/6 +ve; (2 susp)	2/6 +ve	0/6 +ve	1/10 F.psych.
Site A – 2nd FU	0/6 +ve	0/6 +ve	0/6 +ve	0/6 +ve	1/10 F.psych 1/10 A.salm.
Site B - DFVA	3/3 +ve	6/6 +ve	3/3 +ve	6/6 +ve	N/A
Site B- 1st FU	6/6+ve	6/6+ve	6/6+ve	6/6+ve	3/10 Y.ruck 2/10 F.psych.
Site B- 2nd FU	3/6 +ve	2/6 +ve	3/6 +ve	1/6 +ve	4/10 Y.ruck 3/10 F.psych

The table present a resume of the findings. In each cell the number of positive pools is reported.
In addition if suspected pools were detected they are added in brackets.
FU = Follow up
PO= pool of organs (kidney, spleen, heart)
Name of pathogenic bacteria is shortened, *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, *Yersinia ruckerii*

4.2 Initial investigation for tracing infection- DVFA

4.2.1 SITE A

The presence of IHNV was detected in all three organ pools and in five of the six brain pools by RT-qPCR, the last brain pool gave a suspect result.

IHNV was isolated on cell cultures and identified by ELISA in all pools of organ material and furthermore in three of six of the brain pools.

4.2.2 SITE B

IHNV was detected in all samples by RT-qPCR, and the virus was isolated on cell cultures and identified by ELISA in all pools.

4.3 Follow up samplings

Water temperature recorded during the sampling on June 24th was 13.5 °C and 14.5 °C on July 22nd. It was reported that temperature would fluctuate between 12°C and 18°C during the day, depending on weather conditions.
Thirty fish were collected at the two sites on both occasions.

4.3.1 First follow up sampling on June 24th

At site A, where only rainbow trout are present, 30 fish were collected. Clinical signs of disease were observed only in two of the fish sampled. These specimens were included in pool number 6.

At necropsy, no specific remarks.

At site B, both rainbow trout and golden trout (both *Oncorhynchus mykiss*) are present. The sampling consisted of 15 rainbow trouts and 15 golden trouts. Three pools of five fish from each species were made and kept separately. At this site, many fish showed clinical signs, predominantly abnormal swimming behavior and apathy.
At necropsy, petechial bleedings and hemorrhages were observed in 9 of 30 specimens in both internal organs and swim bladder.

4.3.2 Second follow up sampling on July 22nd
At site A, where only rainbow trout are present, 30 fish were collected. Disease signs (skin lesions) were observed only in two of the fish sampled. One freshly dead fish was collected in the sampling. These specimens were included in pool number 1.

At site B, both rainbow trout and golden trout (both *Oncorrhynchus mykiss*) are present. The sampling consisted of 25 rainbow trout and 5 golden trout. A total of six pools of five fish were made and kept separately. At this site, some fish showed clinical signs, predominantly abnormal swimming behavior and apathy.
At necropsy, petechial bleedings in gills and swim bladder and enlarged spleens were observed (two of five of the golden trout specimens, and five of 25 rainbow trout specimens).

4.4 Laboratory investigation

4.4.1 SITE A

First follow up sampling June 24th

At site A, *Flavobacterium psychrophilum* (the causative agent of Rainbow Trout Fry Syndrome) was isolated from one of the kidney swabs.
IHNV RNA was detected by RT-qPCR (weakly positive) in:

- Pool 2 organs (heart spleen kidney)
- Pool 3 brain
- Pool 6 organs (heart spleen kidney)

Suspect results were recorded for:

- Pool 2 brain
- Pool 4 organs (heart spleen kidney)
- Pool 5 brain

IHNV was isolated on EPC cell culture and identified by ELISA in:

- Pool 2 organs (heart, spleen, kidney)
- Pool 6 organs (heart, spleen, kidney)

Second follow up sampling July 22nd

At site A, *F.psychrophilum* was isolated from one of the kidney swabs and *Aeromonas salmonicida* (the causative agent of furunculosis) from another of the kidney swabs.

Two pools of organs yielded suspect signal for IHN, but when testing the individual fish composing that pool no positive signal was recorded. The cell culture isolation is negative and thereby the suspect signal is considered non-specific.

4.4.2 SITE B

First follow up sampling June 24th

At site B, *Yersinia ruckeri*, the causative agent of Enteric Red Mouth Disease, was isolated from the kidney swabs in three of 10 fish sampled (one golden trout and two rainbow trout).

From two of 10 fish (one golden trout and one rainbow trout, neither of them positive for *Y. ruckeri*), *F. psychrophilum* was isolated.

IHNV RNA was detected in all 12 samples collected, both the six samples consisting of pools of organs and the six consisting of pools of brain.

IHN virus was isolated on EPC cell culture and identified by ELISA in all samples as well.

Second follow up sampling July 22nd

At site B, *Y. ruckeri* was isolated from the kidney swabs of four of 10 fish sampled (one golden trout and three rainbow trout).

From three of 10 fish (two golden trout and one rainbow trout, neither of them also being positive for *Y. ruckeri*), *F. psychrophilum* was isolated.

IHNV RNA was detected in three of the six pools of organs collected, and in two of the six pools of brain.

Three pools of organs and 1 pool of brains tested positive by ELISA when inoculated on EPC cell cultures.

5. Discussion

The aim of this diagnostic exercise was to investigate how time and temperature of the water would affect the capability of detecting IHN virus in infected farms.

As reported by the farmers and recorded by the meteorological station, temperature in the river of interest is not a steady parameter and there are significant fluctuations during the day. The water temperature recorded at the first follow up sampling was just below 14°C, although farmers reported fluctuations up to 20°C during the day. The second follow up sampling was conducted on July 22nd, starting July 1st the meteorological station recorded temperature constantly above 14°C with peaks up to 20°C.

Three to four weeks after the initial detection, it was still possible to detect IHN virus both by isolation on cell culture and by RT-qPCR. Already at this stage, some interesting difference between the two sites, were observed. Although it was still possible to detect and isolate the virus from both sites, site A had relatively lower number of positive samples when compared to site B.

With the second follow up sampling this difference became more remarkable. Whereas at site A it was no longer possible to detect the virus, at site B the reduction was of lower magnitude, and detection still reached 66% positive samples, when both organs and brain are included.

There are considerable changes in the number of positive samples at the individual farm over time. This is more evident in site A, where all three pools were positive at the first sampling conducted by DVFA on May 31st , only two out of the six pools of organs were positive for IHNV in late June and one month after in July the virus was no longer detected.

At site B it is also possible to observe a reduction of the relative number of the positive pools per sampling. All three pools were positive at the sampling conducted by DVFA on May 31st, and all 12 pools (six pools of organs plus the corresponding six pools of brains) were positive at the first follow up sampling, while only three of six pools of organs and one of six pools of brains where positive at the second follow up sampling.

These differences in relative number of the positive pools between the farms can be influenced by several factors such as the initial viral load at each site, the prevalence of the infection, and the ability to clear the virus from the infected population. The latter one can be affected, among others, either by difference in age groups or stocking conditions and farm environment. The reduction in the relative number of positive samples is also affected by the movement restrictions that both sites were subjected to. Since no fish could be introduced into the farm, it is expected that the infection resolve after a time period, as the virus does not encounter new naïve hosts to infect and further propagate.

In relation to this perspective, one of the main factors as mentioned before that may explain the difference between the two farms, is the biomass stocked at these sites, which is significantly higher in site B. Fish density, as a factor that significantly contributes to maintain the infection ongoing in a population, has also been described experimentally (Ogut and Reno 2004).

This persistence of infective viral particles in a farmed population at a farm which did not introduce new batches of fish for the period of this investigation, confirm the importance of culling the infected stocks as soon as possible, to prevent further spread of the virus into the water catchment.

Regarding the effect of pooling, this was further investigated in the second follow up sampling where samples from individual fish were tested and compared to the Ct value obtained by testing the pooled sample. The data obtained so far show that one individual fish, included in a pool with four negative fish is sufficient to let the pool sample yield a positive result. In order to address the weak single observed in the pooled samples with Ct's above 38, the individual fish samples composing the pool, were tested and all yielded negative results. This finding was further corroborated by the negative results obtained by inoculating the pool sample on cell culture.

Notably, targeted surveillance schemes do not take into account the overall health status of the farm. This, however, may have important implications in the diagnostic flow and detection of listed diseases, since increased mortality could be ascribed to production pathogens (non notifiable) or technical failures. Therefore it has to be remarked that the presence of other bacterial pathogens, reported on both sites and with higher prevalence in the last follow up sampling, may be regarded as a risk for misdiagnosis. The findings in the current investigation show that production diseases might mask infection with IHNV.

From the current data, there is no indication that the temperature increase and its daily fluctuation has "*per se"* reduced the capability of detecting IHNV by the sampling and diagnostic procedures given in the diagnostic manual. The observed apparent reduced prevalence of the virus over time was expected as a natural function of the disease kinetics in a farm, where it was foreseen that the disease would resolve faster at higher summer temperatures than in colder water. Conversely, the combination of factors as water temperature, time after introduction of the virus, fish density, and incomplete diagnosis for the presence of other fish pathogens may affect the prompt detection of a listed pathogen at a fish farm. It is therefore important to underline that increased mortality at a fish farm should be investigated starting by ruling out the presence of listed pathogens according to the methods given in the diagnostic manuals provided by the EURL for fish diseases.

6. Conclusion:

It was still possible to detect the presence of IHNV in fish at water temperatures above 14 degrees C.
No apparent reduction in the capability of detecting the virus was observed at temperatures above 14°C and the observed reduction in relative number of positive samples was attributed to infection kinetics at the farms and not the temperature.

Reference

Hoferer, M., Akimkin, V., Skrypski, J., Schütze, H., Sting, R., 2019. Improvement of a diagnostic procedure in surveillance of the listed fish diseases IHN and VHS. J. Fish Dis. 42 (4), 559–572. https://doi.org/10.1111/jfd.12968.

Commission Delegated Regulation (EU) 2020/689 of 17 December 2019 supplementing Regulation (EU) 2016/429 of the European Parliament and of the Council as regards rules for surveillance, eradication programmes, and disease-free status for certain listed and emerging diseases (Text with EEA relevance) (Text with EEA relevance)

Commission Implementing Regulation (EU) 2018/1882 of 3 December 2018 on the application of certain disease prevention and control rules to categories of listed diseases and establishing a list of species and groups of species posing a considerable risk for the spread of those listed diseases (Text with EEA relevance.)

Ogut H, Reno PW (2004) Effects of fish density on spread of infectious hematopoietic necrosis virus (IHNV) in rainbow trout, *Oncorhynchus mykiss*. Isr J Aquacult Bamid 56:218–225