Utility of the viral neutralisation test for detection of antibodies to infectious haematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Screening of infectious haematopoietic necrosis virus (IHNV) antibodies was conducted at an infected rainbow trout farm during an eleven month period. Blood samples were collected from brood fish and the virus neutralisation test (VNT) carried out to detect IHNV antibodies. The results were interpreted, together with conventional laboratory diagnostic methods, using virus isolation on cell culture and molecular methods. Out of 178 samples collected, 121 (67. 9%) were detected as positive for IHNV antibodies with a titre ≥ 1:80. In June, out of 99 fish, 71.7% tested positive; in September out of 58 fish 70.7% tested positive, while in April out of 21 fish, 42.9% tested positive. During the entire study period, IHNV was detected only once in one pooled sample collected from fingerlings. This research confirmed that the VNT is a useful method for surveillance of IHNV and as an additional method for diagnostic purposes. Since it is not necessary to sacrifice the fish, this method is especially suitable for IHNV surveillance of wild fish.

Introduction

The infectious haematopoietic necrosis virus (IHNV) is an enveloped RNA virus belonging to the genus *Novirhabdovirus* in the *Rhabdoviridae* family (ICTV, 2013). The viral genome is non-segmented, negative-sense and a single stranded RNA.

Infectious haematopoietic necrosis (IHN) is a serious disease found in salmonid fish. Epizootic outbreaks are common in hatcheries in Europe, North America and Asia, where losses among juvenile fish can reach up to 90% of production (Bovo et al., 1987). Mortality depends on the species and the age of the fish, the virus strain and the environmental conditions. Clinical signs of the disease are lethargy, exophthalmia, darkening of the body, pale gills and haemorrhages in the skin and eyes (Wolf, 1988). Further studies on the immune response to IHNV in rainbow trout (*Oncorhynchus mykiss*), have demonstrated the differences in the efficacy of serological techniques for the detection of specific antibodies to IHNV (Vestergaard Jørgensen et al., 1991). The serological tests for the surveillance of IHNV could have advantages compared with the virus isolation methods,

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especially during summer, when the water temperature is high and consequently there is a decreased possibility to detect the virus during the latent period of the disease. After the infection, the IHNV immune response is lower in cold water than when the temperature is above 15°C and after 4 - 6 weeks antibodies can be detected (Zhuang et al., 1992; Fregeneda-Grandes et al., 2009).

IHNV can usually be isolated during the short period after the infection; however the humoral antibody response can be detected up to one year after the infection (Enzmann et al., 2010). However, fish antibodies are less stable than mammalian antibodies and some diagnostic methods can give false results (St-Hilaire et al., 2001).

The utility of the virus neutralisation test (VNT), indirect immunofluorescent test (IIF) and enzyme linked immunosorbent assay test (ELISA) has already been described (Ahne et al., 1993; Hattenberger-Baudouy et al., 1995; Lorenzen and LaPatra, 1999; Knuesel et al., 2003). Vestergaard Jørgensen reported that the IIF test was the most sensitive method for the detection of IHNV antibodies (Vestergaard Jørgensen et al., 1991). It was reported that the IIF test detects antibodies against all viral antigens, while the VNT method detects only neutralising antibodies that react with the viral glycoprotein (Schyth et al., 2012). All the IHNV isolates form a single sero-group and consequently, the specific neutralising antibodies neutralise all genotypes of IHNV (Johansson et al., 2009, Engelking et al., 1991). The IHNV infection has been present in Slovenia since 1993, however, only a few outbreaks have been officially diagnosed in recent years (Jenčič et al., 2002). The phylogenetic analysis of 17 IHNV Slovene isolates showed that 13 of them belong in two subgroups (M-Eur-1 and M-Eur-2), while four isolates were clearly separated from other Slovenian isolates, but within European subgroup M-Eur-2 (Grilc Fajfar et al., 2011). With regard to actual relevant Slovene legislation, all hatcheries must be tested for the presence of IHNV once per year. Fish are also tested for viral infections in the case of suspicion of viral disease. Laboratory diagnostic methods are applied according to the OIE and EU standards (Commision Decision 2001/183/EC).

The aim of this study was to test the usefulness of serology in an IHNV positive farm, and the implementation of the VNT into the laboratory procedure, together with the interpretation of results for IHN monitoring purposes. In the future we would like to monitor IHNV infection in open waters without sacrificing fish, to define the IHNV carrier status in wild fish populations.

Materials and methods

Fish farm

The research study was carried out over an eleven month period, starting in June and finishing in April the following year, and was conducted on an IHNV positive rainbow trout farm utilising brood stock located in spring water. The water temperature was between 7.8°C and 14.8°C. In the investigated farm the initial presence of IHNV was detected from ovarian and seminal fluid samples as part of the Slovenian Annual Decree. Prior to the removal of infected fish to assist the eradication procedure, an IHNV antibody detection study was conducted and the serology results were compared to conventional laboratory diagnostic methods using virus isolation on cell cultures and RT-PCR.

Collection of sera and tissue samples

Blood samples were collected three times: in June (n=99), September (n=58) and April (n=21). The samples were collected from brood fish (300g) using the caudal vein puncture method. Fish were anesthetised using 0.4 ml/l of 2-phenoxyethanol (Fluka, USA) and, after blood sampling, the fish were released back into the water. The collected samples were placed into a cooling box (2-8°C) and immediately transported to the laboratory where they were allowed to clot overnight.

The following day, the blood samples were centrifuged at 1000 x *g* for 10 min, the complement in the serum samples was inactivated by heat treatment at 45°C for 30 min and the samples were then stored at -20°C until laboratory testing.

In addition, 25 fingerling size fish, weighting between 5 and 50 g, were randomly sampled for viral diagnostics during each visit for blood sampling, as well as in January. Fish were sacrificed by an overdose of the aforementioned anaesthetic. At the time of sampling the temperatures were 14.8°C, 10.8°C, 7.8°C and 7.8°C, respectively. Tissue samples, approximately 5 g, (kidney, spleen, heart and brain) from five fish were pooled into a single pool; June (n=5 pools), September (n=5 pools), January (n=5 pools) and April (n=5 pools). The samples were placed in a sterile tube (50 ml) containing 20 ml of the transport medium (cell medium RPMI 1640 with 1% antibiotics-antimycotics, Gibco®, Life Technologies, USA). Samples were transported to the laboratory using a cooling box (2-8°C).

Virus neutralisation test (VNT)

The protocol for the implementation of the VNT was kindly provided by Dr. Jeanette Castric (AFSSA, Plouzane, France). Minor modifications consisting of the adjustment of the titres of the neutralisation virus were necessary in order to generate a clear cytopathic effect in the negative control cells within 4-5 days and the addition of complement, titrated on EPC cells (*Epithelioma papulosum cyprini*, ATCC® Number: CRL-2872™) for cytotoxicity. During the assay, the complement was kept on ice. The complement was obtained from the serum of IHNV-free rainbow trout and prepared in a cell culture medium in a final dilution 1:160 v/v. VNTs were performed following an endpoint technique conducted in cell culture microtitre plates. Using a U-shaped well-bottomed microtitre plate and pre-dilutions of the test sera, positive and negative controls were prepared in the cell culture medium starting with a 1:20 to a final dilution 1:640 v/v. Thirty microliters of the complement dilution (fish serum prediluted 1:20) was added in the appropriate wells and into the wells for the control of cytotoxcycity. Finally, 30µl IHNV, isolate SLO1/97, with Genbank acc. number GU571159 and a viral dilution containing about 600 $TCID_{50}$ was added. The microtitre plates were incubated for 24 h at 4°C. In 96 well flat-bottomed microtitre plates (TPP, Switzerland) 30µl of cell culture medium was distributed in each well of the plate. A transfer of 50µl of virus/serum mixture was made from the U-shaped bottom microtitre plate wells to the corresponding flat-bottomed microtitre wells of the plates containing EPC cells. Plates were incubated at 15°C for 4-6 days in a humidity incubator with 5% CO_2. The cells were then fixed with 10% formaldehyde and coloured with a crystal violet solution in ethanol (1.3g crystal violet in 100ml ethanol and added to 400ml distilled water). The neutralising titre (ND_{50}) was expressed as the

inverse of the initial serum dilution giving 50 % neutralisation of the reference IHNV compared to the control virus. A sample was considered positive if it had an antibody titre ≥ 1:80. The lowest tested final serum dilution was 1:40 and the highest was 1:320.

Virus isolation on cell culture

Fish tissue homogenates (kidney, spleen, heart, brain) were prepared as described previously (OIE; 2013) and processed for IHNV isolation on *Epithelioma papulosum cyprini* (EPC) and *Blue gill fry* (BF-2) cell cultures according to the protocol described in the Manual of Diagnostic Tests for Aquatic Animals (OIE; 2013). Microtitre plates were incubated at 16°C ± 1°C and observed daily for cytopathic effect (CPE) for 7 days, with two additional sub cultivation steps performed at days 7 and 14 if CPE was not observed. In the case that a cytopathic effect was identified, the tissue supernatant from the cell culture was discarded and IHNV was confirmed using a commercial indirect immunoperoxidase test kit (IIP test) produced by Bio-X Diagnostics (Jemelle, Belgium).

Extraction of RNA, RT-PCR

Total RNA was extracted from the IHNV infected cells using a commercial QIAamp® Viral RNA Mini Kit (Qiagen, Germany). The extraction procedure was performed following the manufacturer's instructions.

Primers E1 and E3n were selected from the genome region encoding the G-protein (Emmenegger et al., 2000). The RT-PCR was performed as previously described (Grilc Fajfar et al., 2011), and specific RT-PCR products (532bp) were detected by using 1.8% agarose gel electrophoresis.

Results

During the eleven month research period, the rainbow trout in the infected farm did not show any clinical signs of lethargy, darkening of the body, abnormal swimming, exophtalmia and hemorrhagies in the skin. In addition, no increased mortality on farm was observed during the research period.

In June, out of 99 fish, 71.7% tested positive for an IHNV antibody response; in September out of 58 fish, 70.7% tested positive, while in April 42.9% of samples tested positive (Table 1). Out of the 178 samples collected, 121 (67.9%) were detected as positive for the IHNV antibodies with a titre ≥ 1:80.

From 100 fingerling fish, samples were collected and tissues were checked for the presence of IHNV. All the fish collected in June, September and April were negative by virus isolation and RT-PCR. In January, one pool sample out of five was IHNV positive by both virus isolation and RT-PCR.

Discussion

Infectious haematopoietic necrosis is a serious viral disease of salmonids which is causing significant biological and economic losses in fish farming worldwide. Therefore, within the OIE and EU it is a notifiable disease and it should be controlled and eradicated where possible. The approved diagnostic methods for the detection of IHNV and VHSV (Viral haemorrhagic septicaemia virus) are virus isolation on cell lines defined by the Manual of Diagnostic Tests for Aquatic Animals and the EU Commission Decision 2001/183/EC. In recent years, molecular methods with good sensitivity and specificity have been developed and introduced for IHNV detection.

Table 1. IHNV antibodies detected by VNT; samples were collected from rainbow trout brood fish at three time points during an 11 month period on a known IHNV infected Slovenian fish farm.

Month	Temperature of water (°C)	No. of tested blood samples	Number of positive samples	% of positive samples	No. of positive samples (%) and titres	
June	14.8	99	71	71.7 %	3 (3.0%)	1:80
					17 (17.2%)	1:160
					51 (51.5%)	≥ 1:320
September	10.8	58	41	70.7 %	11 (19.0%)	1:80
					8 (13.8%)	1:160
					22 (37.9%)	≥ 1:320
April	7.8	21	9	42.9 %	1 (4.8%)	1:80
					3 (14.3%)	1:160
					5 (23.8%)	≥ 1:320

During, and for a short time after, the clinical phase of an IHN outbreak, it is possible to confirm the presence of IHNV by virus isolation or RT-PCR. However, detection of IHNV is largely impossible outside this time period. Fish that recover from the disease only excrete the virus for a short time, while the IHNV antibodies are present for much longer periods (Knuesel et al., 2003). Therefore, serological assays for detecting specific antibodies could be suitable diagnostic methods as was successfully proved in this study.

Serological tests for the detection of fish antibodies to viral infections are not commonly used in fish viral diagnostics. Before using VNT for the detection of specific IHNV antibodies, the titration of a complement is required. In order to avoid false negative results the complement was titrated for cytotoxicity, starting with the dilutions from 1:2 to 1:2048. An appropriate volume of a self-prepared complement from IHNV- and VHSV free trout sera was added to each serum dilution.

The IHNV antibodies were detected at all 3 sample time points when water temperatures were between 7,8–14.8°C. However, detection of IHN virus by cell culture isolation and RT-PCR only occurred at one time point from 1/5 pools screened, when the water temperature was low (7,8°C) in January.

The traditional OIE and EU prescribed method for monitoring IHNV is reliable in confirming a suspected infection during an IHN disease outbreak when clinical symptoms are present. However, it is more difficult to demonstrate IHNV infection in fish that have overcome the disease (Thorburn, 1996). Clinical outbreaks of IHN occur when the water temperature is below 14°C, which is usually during winter in Slovenia, however in the investigated fish farm the water temperature slightly exceeded this limit for a few summer months. Although the temperature was relatively low during the entire period of this research study, and the fish farm was only visited four times, IHNV was only detected at one sample time point.

However, using a serological test, antibodies to IHNV were found throughout the entire eleven month research period. The presence of IHNV-specific antibodies in the fish population is therefore a good indicator of a previous IHNV infection (Vestergaard Jørgensen et al., 1991; LaPatra et al., 1993; Lorenzen and LaPatra, 1999). In this study, the authors confirmed that rainbow trout can produce specific and highly functional antibodies that are able to neutralise IHNV *in vitro*. The VNT test for IHNV serology needs many controls: control of cells, control of virus titre used in tests, complement control and serum toxicity control. Additionally, a trained and experienced person is needed to read and interpret the VNT.

This study confirmed that the serological VNT assay was a suitable and comparable complementary diagnostic method for the control of IHN. Serological methods are able to detect the infection even when the clinical signs of the disease are not present and it is difficult to detect IHNV using virus isolation in cell culture, or even by RT-PCR. It is also important to emphasise that it is not necessary to sacrifice the fish for the analyses which is especially relevant for surveillance of wild fish.

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