Surveillance of viral fish diseases in the Czech Republic over the period January 1999 – December 2006

S. Reschova, D. Pokorova, J. Hulova, P. Kulich, T. Vesely

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: The present study reports the results of virological survey in fish with an emphasis on the diseases included in the Czech National Legislation. Monitoring of fish both without symptoms and with a manifested disease was performed within this virological surveillance. Between January 1999 and December 2006, viral haemorrhagic septicaemia virus (VHSV), infectious haematopoetic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) were surveyed by the National Reference Laboratory for viral diseases on fish. Among of 3789 sample pools processed, VHSV was detected in seven cases (6× in *Oncorhynchus mykiss,* 1× in *Salmo trutta m. fario*), IHNV was detected in seven cases (6× in *Oncorhynchus mykiss,* and 1× in *Esox lucius*) and IPNV was diagnosed once in *Oncorhynchus mykiss.* In four cases, the viral agents were detected in ovarian fluid from broodfish without any clinical symptoms. In the remaining cases the virus was isolated from tissue homogenates. Our results show that ovarian fluid is valuable material for the detection of viral agents during the monitoring of fish diseases.

Keywords: viral diseases; VHSV; IHNV; IPNV; monitoring; fish

Viral agents represent a significant proportion of the losses caused by pathogenic microorganisms in aquaculture. The etiological agents causing the highest losses in the European aquaculture include viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) belonging to the genus *Novirhabdovirus* within the Rhabdoviridae family and infectious pancreatic necrosis virus (IPNV) belonging to the genus *Aquabirnavirus* within the family Birnaviridae (Ariel and Olesen, 2002).

The VHSV and IHNV both induce similar clinical signs in susceptible fish, viraemia with subsequent destruction of internal organs, impairment of osmotic balance, edema and haemorrhages, in adipose and muscle tissue. These clinical signs are a consequence of the high virus multiplication in endothelial cells of blood capillaries, haematopoietic tissues and nephron cells. Externally, the fish may exhibit skin haemorrhages and/or exophthalmia (Wolf, 1988; Vesely et al., 2004).

IPNV persists for a long time in the aquatic environment, and can easily be introduced to rearing facilities with susceptible hosts (Wolf, 1988). IPNV can also be vertically transmitted (Bootland et al., 1991). Because of these reasons, a rapid and accurate diagnosis is critical for controlling the spread of the virus.

The diseases of husbandry animals including aquaculture have been classified according to seriousness, spread and diagnostic methods available (OIE, 2006). Within the European Union member states, animal diseases were classified into three categories (91/67/EEC, 1991). The legislations of the Czech Republic (No. 166/1999 Coll. and later amendments, 2003) follow the EU legislations in terms of monitoring of VHS, IHN and infectious salmon anaemia (ISA). Unlike the EU legislation, it is also compulsory to monitor IPN in the Czech Republic.

Due to high virulence and seriousness of the above mentioned diseases, prevention of the spread of these viruses in farms is crucial (Antychowicz et

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al., 2006; Ariel and Olesen, 2002). One of the essential prerequisites for reaching this aim is monitoring populations not only of salmonid fish, but also other species known to be susceptible to these viral agents. The examination methods are generally based on the protocols published by OIE (OIE, 2006). The basic examination methods of fish tissue include the virus isolation on sensitive cell lines and virus identification when a cytopathic effect (CPE) is manifested. These protocols are equally used for routine monitoring, for diagnostic purposes in case an infection is suspected, and for testing fish breeds after import or before export.

The present study reports the results obtained during the recent eight years of surveillance and diagnosis of fish viral diseases at the National Reference Laboratory for viral diseases in fish (serving the State Veterinary Administration) of the Czech Republic.

MATERIAL AND METHODS

Sample collection

Samples originated either from routine surveillance or from fish with clinical signs submitted to our laboratory (diagnostic samples). Altogether 3 789 pools of samples were processed.

In the case of routine monitoring, the farms maintaining fish susceptible to classified infections provided samples for monitoring twice a year from October to May. The samples were collected at least four months apart and when the water temperature was below 14°C. Thirty fish were sampled each time and sample pools of up to ten individuals were prepared. In the case of farms with broodstock, pooled samples of ovarian fluid were also included. In the case of more than 6 cm long fish, tissue samples included heart, spleen and head kidney. Fish less than 6 cm long were processed and pooled as a whole after removal of head and tail fin.

In the case of diagnostic samples from diseased fish (mortality, non-typical behaviour, haemorrhagies, exophthalmus etc.), the fish were processed essentially in the same way but usually in a lower numbers.

Preparation of tissue homogenates

Organs or parts of fish were homogenised in a mortar with sterile sea sand, supplemented with Eagle's medium TRIS MEM (minimal essential medium, Sigma, USA), pH 7.6, enriched with 10% FBS (foetal bovine serum, GIBCO) and centrifuged at 4°C in the JOUAN CR 3i centrifuge, rotor T40, 5 000 × g, for 15 min. The supernatant was incubated overnight at 4°C with the addition of antibiotics (100 IU/ml of penicillin and 100 µg/ml of streptomycin) and afterwards used for virological testing on cell lines.

Isolation of viruses on tissue cultures

For virus isolation, two 24-h cell lines were prepared in a parallel setup: RTG-2 (rainbow trout gonads) (Wolf and Quimby, 1962) and EPC (epithelioma papulosum cyprini) (Fijan et al., 1983). The examination was performed in 24-well plates (NUNC). Cell lines were inoculated with at least two serial tenfold dilutions of the samples and incubated at 15°C for seven days. The cell lines were monitored by microscopy every day for the occurrence of CPE. If no CPE was observed, the cultures were frozen, thawed and subcultured on new cells for additional seven days. If CPE was not observed after subcultivation, the samples were considered negative. Samples inducing CPE were further examined for identification of viruses.

Virus identification

For virus identification, sandwich ELISA assays were used (Rodak et al., 1988, 1993). The wells of the microtitration plate (GAMA, Czech Republic) were coated with polyclonal swine antibodies specific for VHSV, IHNV or IPNV in a coating buffer (0.05M carbonate – bicarbonate, pH 9.6) and incubated overnight in a humid chamber at 4°C. The following day, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST). A 50 µl volume of a diluting solution (1% lactalbumin hydrolysate in PBST, pH 7.6) was dispensed in each well and a equal volume of supernatant from CPE positive cell cultures was added. After 60 min incubation at 37°C and a subsequent wash, primary specific rabbit antibodies were added. After an additional incubation and washing, peroxidase conjugated secondary antibody (HRP-SwARIgG, DAKO, Denmark) was added and visualization of the enzymatic reaction was enabled by pipetting 100 µl of the substrate solution (H_2O_2 and 3,3',5,5'

tetramethylbenzidin in 0.1M acetate buffer, pH 5.8) to each well. The reaction was stopped after 10 min by the addition of 100 µl 1M H_2SO_4 and optical density of the substrate was read by spectrophotometry at 450 nm (SLT Spektra, Austria). A sample was considered positive when the value of its average OD reading was higher than 0.1 and equaled or exceeded the double value of the average absorbance for the negative control antigen. Each ELISA test included positive and negative controls. Positive controls included ref. isolates of VHSV (CAPM V-553), IHNV (CAPM V-611) and IPNV (CAPM V-513) grown on EPC cell lines. Non-infected EPC cell supernatant was used as negative control.

Electron microscopy

Electron microscopy was performed on a representative number of cell cultures displaying CPE. Samples were negatively stained by osmium tetroxide and examined using a Philips 208 electron microscope at 18 000 × magnification and an accelerating voltage of 90 kV.

RESULTS

During the period between 1999 and 2006, altogether 3 789 samples were processed (Table 1). Tissue homogenates were analyzed in 2 525 cases while 1 264 samples were of ovarian fluid origin. Among these samples, VHS virus was detected seven times, IHN virus seven times and IPN virus once.

Isolates of VHSV were detected in the period from 1999 to 2004 with a maximum in 2000, when three outbreaks were occurred. Six isolates were from *Oncorhynchus mykiss* and one was from *Salmo trutta m. fario*. The virus isolations were successful in the first passages in all seven cases (Table 2). Positive samples included tissue homogenates as well as ovarian fluids. The virus was isolated only once during the routine surveillance. The remaining positive cases were derived from diseased fish, despite the fact that the number of diagnostic samples was nearly 10 times lower than the number of surveillance samples.

In the case of IHNV, its identification in rainbow trout in 2001 was the first detection in the Czech Republic. Since then 2001, the occurrence of this virus has remained, although the frequency is low. IHNV was isolated 6 times from *Oncorhynchus mykiss* and in one case from *Esox lucius*. Except for the sample from *Esox lucius,* for which a second passage was required, the virus isolation was successful in the first passage. As for IHNV, positive samples included also tissue homogenates and ovarian fluid but the IHNV was more frequently isolated during routine surveillance compared to VHSV (Table 2).

IPNV was isolated only once in rainbow trout fry in 2001. The virus was isolated from a tissue homogenate and a second cell culture passage was required for its detection. The sample originated from diseased *Oncorhynchus mykiss*.

The presence of viruses was in all cases confirmed by ELISA and also by electron microscopy (Table 3, Figure 1 and 2).

Table 1. The numbers of examinated fish samples within the monitored period

Year	Farm (*n*)	Pool of organ homogenate (*n*)	Pool of ovarian fluid (*n*)	Total	Viral isolate		
					VHSV	IHNV	IPNV
1999	56	261	114	375	1	0	0
2000	60	288	114	402	3	0	0
2001	63	348	162	510	1	2	1
2002	88	442	198	640	0	2	0
2003	114	354	169	523	1	0	0
2004	133	325	158	483	1	0	0
2005	119	263	164	427	0	1	0
2006	83	244	185	429	0	2	0

Table 2. Detection of viral agent VHSV and IHNV within the monitored period

Year	Species	Isolate	Samples	Reason for examination
Viral haemorrhagic septicaemia (VHS)				
1999[1]	*Oncorhynchus mykiss*	CAPM V 553	organ homogenate	mortality
2000[2]	*Oncorhynchus mykiss*	V 560	organ homogenate + ovarian fluid	mortality
2000[3]	*Oncorhynchus mykiss*	V 574	organ homogenate	mortality
2000[4]	*Salmo trutta m. fario*	CAPM V 610	organ homogenate	mortality
2001[5]	*Oncorhynchus mykiss*	CAPM V 624	organ homogenate	mortality
2003[6]	*Oncorhynchus mykiss*	CAPM V 626	ovarian fluid	monitoring
2004[7]	*Oncorhynchus mykiss*	CAPM V 628	organ homogenate	mortality
Infectious haematopoietic necrosis (IHN)				
2001[1]	*Oncorhynchus mykiss*	CAPM V 629	ovarian fluid	monitoring
2001[2]	*Oncorhynchus mykiss*	V 696	ovarian fluid	monitoring
2002[3]	*Oncorhynchus mykiss*	CAPM V 611	organ homogenate	monitoring
2002[4]	*Oncorhynchus mykiss*	CAPM V 625	organ homogenate	mortality
2005[5]	*Esox lucius*	V 1010	organ homogenate	monitoring
2006[6]	*Oncorhynchus mykiss*	CAPM V 639	organ homogenate	mortality
2006[7]	*Oncorhynchus mykiss*	CAPM V 638	organ homogenate	mortality

CAPM = Collection of animal pathogenic microorganisms, Veterinary Research Institute, Czech Republic

Table 3. Summary of positive detections of viral agents of fish diseases

Virus	1st passage	2nd passage	ELISA result			Electron microscopy
			IHN	IPN	VHS	
VHSV[1]	+	+	–	–	+	*rhabdovirus*
[2]	+	+	–	–	+	*rhabdovirus*
[3]	+	+	–	–	+	*rhabdovirus*
[4]	+	+	–	–	+	*rhabdovirus*
[5]	+	+	–	–	+	*rhabdovirus*
[6]	+	+	–	–	+	*rhabdovirus*
[7]	+	+	–	–	+	*rhabdovirus*
IHNV[1]	+	+	+	–	–	*rhabdovirus*
[2]	+	+	+	–	–	*rhabdovirus*
[3]	+	+	+	–	–	*rhabdovirus*
[3]	+	+	+	–	–	*rhabdovirus*
[5]	–	+	+	–	–	*rhabdovirus*
[6]	+	+	+	–	–	*rhabdovirus*
[7]	+	+	+	–	–	*rhabdovirus*
IPNV	–	+	–	+	–	*birnavirus*

[1 to7]identification mark used in Table 2 and 3

Figure 1. Electron microscopy: rhabdoviral particules (VHSV) isolated from *Oncorhynchus mykiss*

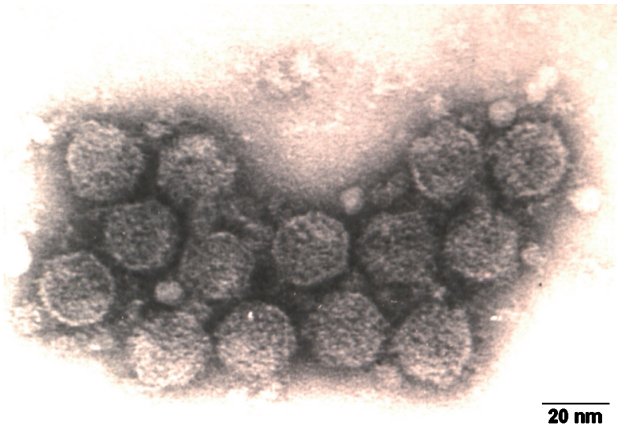

Figure 2. Electron microscopy: birnaviral particules (IPNV) isolated from *Oncorhynchus mykiss*

DISCUSSION

The aim of this study was to obtain an overview of the presence of VHSV, IHNV and IPNV in Czech aquaculture during the period 1999–2006 (Table 1). In the year 1999 and 2000, only farms producing rainbow trout (*Oncorhynchus mykiss)* and brook trout (*Salvelinus fontinalis*) intended for export were monitored. Since 2001, facilities producing brown trout (*Salmo trutta m. fario)* for the restocking of free waters have been included in the survey as well as the production breeds. Farms producing other than the above mentioned fish susceptible to some of the classified infections have also been included in the survey since 2002.

The number of pooled samples varied between 375 and 640 per year. These variations were mainly due to varying activities of hatcheries producing the fry for restocking free waters. As far as susceptibility is concerned, rainbow trout is known to be highly susceptible to viral diseases (Reno, 1999). The reported high sensitivity of rainbow trout to viral infections is in agreement with the findings of our study, 70% of all virus-positive tissue samples within the diagnostic material originated from rainbow trout.

VHSV was first isolated in Denmark (Jensen, 1965), using monolayers of fish cell cultures. Electron microscopic techniques identified the causative agent as a rhabdovirus. The primary aquaculture fish species susceptible to VHS at all age-stages is the rainbow trout. VHS is enzootic in most countries of continental Europe (Raja-Halli et al., 2006) and has also been identified in the USA (Meyers and Winton, 1995; Gagne et al, 2007). De Kinkelin et al. (1985) attributed a 20–30% loss of the total European rainbow trout aquaculture industry to VHS.

The impact of VHS on the rainbow trout production is less serious in the Czech Republic. Only seven sites were found with VHSV isolation during the inspected period. The distribution of these findings was maximal (three isolations) in 2000 and has been decreasing since then. This is probably reflecting the long careful monitoring of VHSV in the Czech Republic and also its geographical location. The low number of sites with virus positive fish corresponds to the situation in Switzerland which is also an inland country where during the surveys in 1984/85 and 2000/01, virological examination of 1 769 pool samples resulted in only eight VHSV isolations (Knuesel et al., 2003).

Historically, the geographical range of IHN was limited to the western parts of North America (Wolf, 1988), but the disease is now also present in continental Europe (Arkush et al., 1989; Jorgensen et al., 1991; Knuesel et al., 2003). High levels of the virus are shed from infected juvenile fish. Adult

fish are generally asymptomatic carriers that may shed the virus in their sexual products at the time of spawning (OIE, 2006).

This finding is also supported in our study. IHNV was isolated from ovarian fluid specimens in two cases in 2001, which were the first isolations in the Czech Republic. These positive samples originated from rainbow trout which were sent for routine control purposes. In the year 2002 two new isolates of IHNV were detected. The first one was from a rainbow trout without any clinical manifestation of disease and the second one from a rainbow trout showing signs of disease. In 2006, two virus-positive cases were also found, both from rainbow trout with clinical signs of disease. The question is whether it is tendency or only randomness caused by low numbers.

The susceptibility of nonsalmonid fish species to the VHSV has been widely reported (Meier et al., 1986; Skall et al., 2005). Since it is known that fish surviving a VHS infection can become carriers, it is suspected that wild stocks play an important role in the epizootology of the disease (Meier et al., 1994). A different situation occurs for IHNV, which seems to affect salmonid fish only. Initially, pike was mentioned as a susceptible fish (OIE, 1995), but later on the susceptibility was not confirmed and this species was omitted from the list (OIE, 2006). Our contribution to this controversy is detection of IHNV in tissue homogenates from pike without clinical signs which were sent in for routine control purposes.

IPNV is a highly contagious virus causing the mortality of fry in several cultured fish species. From its first isolation in the USA in 1957 and throughout the 1960s, IPNV was detected in a number of different salmonid species (Hill and Way, 1995) and as well as in a wide range of non salmonid fish species (Ahne, 1978; Reno, 1999).

Czech salmonid farms were sampled for IPNV as part of a pathogen monitoring programme. The incidence of IPN seems to be only occasional, as we isolated IPNV in only one case during the surveyed period. Moreover, none of the other non salmonid samples included in this study revealed presence of IPNV. This indicates a low prevalence of IPNV in the Czech Republic.

The situation in the Czech Republic concerning IPN (disease of especially young fish) could also be documented from the portion of ovarian fluid samples which has been increasing from about 30% (114/375) in 1999 to more than 40% (185/429) in 2006. In total, 1 264 pooled samples of ovarian fluids were examined over the period 1999 to 2006 without any isolation of IPNV.

All data collected during the surveillance programme indicated that the viruses were not consistently present in the same location. Furthermore, new outbreaks observed during the surveillance programme appeared to be related to other farms. From our results we conclude that the uncompromising eradication method (killing all the fish from an infected farm and disinfection of the plant before restocking) that is applied to virus-positive farm sites is a suitable tool to reduce outbreaks of viral disease.

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Corresponding Author:

Tomas Vesely, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 112, fax +420 541 211 229, e-mail:vesely@vri.cz