Review

INFECTIOUS PANCREATIC NECROSIS OF SALMONID FISH - DISTRIBUTION AND LABORATORY METHODS FOR DIAGNOSIS

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ABSTRACT

One of the fastest growing branches of the national livestock breeding in Bulgaria is aquaculture production. The development of aquaculture production is directly related to the health of the cultivated fish and indirectly to the health of the wild fauna. Economically, breeding can be severely complicated by the appearance of infections especially those ones with viral etiology. The most serious problem for Bulgarian trout producers over the last decade is Infectious pancreatic necrosis (IPN) which is a highly contagious viral disease of young salmonid fish. It is caused by a virus (IPNV) that belongs to the family *Birnaviridae*. The IPNV has been isolated from many geographic regions around the world with different environments and a variety of aquatic animals. In Bulgaria, the IPNV was confirmed 13 years ago. There are several cases with heavy mortality rates every year. Thus, the aim of this review was to extend knowledge of IPN, and to present methods of its control.

Key words: Infectious pancreatic necrosis, *Aquabirnavirus*, salmonid fish, control

INTRODUCTION

One of the fastest growing branches of the national livestock breeding in Bulgaria is aquaculture production (1). Our country is relatively rich in natural freshwater resources suitable for the rearing of warm and cold water fish species (2). The most preferable cold water species are salmonids such as Brown trout (*Salmo trutta*) and perfectly adapted to our conditions Rainbow trout (*Oncorhynchus mykiss*). Another salmonid that was successful introduced is Brook trout (*Salvelinus fontinalis*). In the recent years, several closed recirculating systems have been created to grow Atlantic salmon (*Salmo salar*) and Coho salmon (*Coho salmon*).

For the first time, the disease is described as catarrhal enteritis due to a poor nutrition of young trout up to 10 g (16). Later on, the clinical signs were described by Wood in 1955 for North American cases (38), but these

clinical signs were already noted in the United States in 1920 (39). In Europe, it was reported to have occurred in the 1960s in France (9) and shortly after that, there were notes about it from different European countries. There are 649 registered fish farms in Bulgaria. 105 of them, breed and cultivated salmonids – approximately 16 %. The districts with significantly more salmonid farms than others are Smolyan, Blagoevgrad, Sofia and Pazardzik – more than 87 % of all (106).

The development of aquaculture production is directly related to the health of the cultivated fish and indirectly to the health of the wild fauna. Economically, breeding can be severely complicated by the appearance of infections especially those ones with viral etiology which lead to global losses of around 20% of production (4). To present, there are over 100 viruses that are pathogenic to fish. They belong to 14 families: Herpesviridae, Iridoviridae, Adenoviridae, Birnaviridae, Rhabdoviridae, Paramyxoviridae, Coronaviridae, Picornaviridae, Retroviridae,

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Reoviridae and others (5). Under Directive 88/2006/EC (93) and Decision 1554/2015/EC (87) as implemented in Bulgaria by Regulation 17/2008 mandatory testing is necessary only for viral diseases (“listed diseases” compulsory subject when an outbreak of infection appears). They cause the greatest and destructive damage, the most difficult to manage, and treatment with known chemotherapeutics is not possible.

The most serious problem for Bulgarian trout producers over the last decade is the infectious pancreatic necrosis (IPN) (3).

The aim of this paper is to summarize and present the information about the distribution, prevention, and control of IPN and the nature of causative agent IPNV. Retrospective view at available data from first reports to present should indicate why the main problem in our salmonid aquaculture farming is precisely IPNV.

DISTRIBUTION

In the IPNV survey of 2017, Rimstad (6) stated that the virus was widespread all over the world:

The IPN occurred in Europe – the main producer of trout – in Belgium, areas of the former Czechoslovakia, Finland, Luxembourg, Spain, Serbia, Montenegro (7), Denmark (8), France (9), Germany (10), Italy (11), Norway (12), Sweden (13), and the United Kingdom (14). IPNV is present in most areas of North America - Canada (15, 7); United States (7). Central and South America the virus was isolated from Mexico (95), Chile (96). The authors reported occur from Asia – Japan (16) through China (17) to Turkey (18). Only one author points out IPNV is also present in Russia (97). In the southern hemisphere, IPN was reported in Australia (7), Tasmania (19), New Zealand (7, 20). South Africa (7) and ocean open waters: (16, 17, 21-24).

From Bulgarian aquaculture farms the IPN virus was isolated for the first time in 2006 at the “Sini hancheta” farm, Smolyan District. Thereafter, it was isolated in 2007 while after 2009 the number of outbreaks increased (**Figure 1**). Eighteen outbreaks were registered to 2014 (1).

Figure 1. Distribution of IPNV in Bulgaria to 2014 by river catchments (personal data)

ETIOLOGY

• Classification

Birnaviridae family includes four genera with double-stranded RNA – *Aquabirnavirus*, *Avibirnavirus*, *Blosnavirus*, and *Enthomobirnavirus* (25). IPNV is the type species of genus *Aquabirnavirus*. Serologically and biochemically related viruses in this genus induce a variety of disease syndromes in taxonomically diverse host groups, which also include aquatic invertebrates (7).

• Morphology

The genus *Aquabirnavirus* includes icosahedral, double-stranded (ds), bi-segmented RNA viruses with a non-enveloped capsid, measuring 60 nm in diameter. Genome segment A encodes all the structural (VP2 and VP3) and non-structural proteins whereas Segment B encodes the viral RNA-dependent RNA polymerase (VP1) (29).

- Biological features

Based on the genome analysis of multiple strains of IPNV, two serogroups are distinguished: A - with 9 serotypes and B - with 1 serotype (29, 30). Initially, two strains - Ab and Sp, which are different from each other and from strain VR299, were isolated in Denmark from the rainbow trout (30, 98).

At the present, IPN and IPN-like viruses are classified into the already mentioned serogroups A and B. All strains of A are pathogenic to fish (28). Serotype A1 covers most of North America-specific strains while A6 - A9 strains are mainly isolated in Canada. The four remaining types from A2 to A5 are isolated in Europe.

Serogroup B contains a non-pathogenic for fish TV-1 (*Tellina virus*) (30, 31).

- Genomic structure

The viral RNA contains two linear segments A and B. Segment A has a large ORF (open reading frame) that codes a 106 kDa polyprotein (NH₂-pVP2-VP4-VP3-COOH) and a small ORF that binds the amino terminus of the VP5 polyprotein size 17 kDa. This polyprotein is unstructured, anti-apoptotic (34) and in the assembling phase of the virus, it forms: pVP2 - the protein precursor VP2 (large capsid protein), VP4 (protease function), and VP3 (small capsid protein) forms complexes with genomic RNA in the capsid of the virus (34). Segment B codes VP1-RNA-dependent RNA polymerase, which is both free in the viral particles and covalently linked to the viral genome (35).

A phylogenetic sequence analysis based on amino acids of segment A (protein VP2) indicates that there are seven IPNV genotypes separated by geographic and serological characteristics. Differences between genotypes vary between 18-30% and show wide antigenic diversity (28, 32). The two serogroups, accepted in general, did not respond to a virus-neutralization cross reaction but the author (30) described a serotype TV-1, pathogenic for bivalve mollusks of the genus *Tellina* (31), showing antigenic similarity to two strains of IPNV.

The virus may survive at a temperature of about 10°C for 7 months (89), several years at -20°C (some strains are sensitive to freezing and thawing, especially if the environment they come into has low pH). It is resistant to exposure to ether, chloroform and glycerol. It is inactivated by chlorine, iodophors and UV radiation.

EPIZOOTOLOGY

The disease affected predominantly young salmonids – the fried trout and Atlantic post-molts (99). Clinical signs vary and include behavioral changes as spiral swimming, exophthalmos, open operculums bilaterally, anorexia (36).

Worldwide epizootic studies have shown that IPNV is actively circulating, surviving in a variety of conditions, and is isolated from a variety of vectors and sources - wildlife fish, clams (exploring sedimentary samples around Spain's fishing farms) and even food pellets even after (physicochemical) treatment (38).

From IPNV first isolation in the United States, it seemed that infections were limited to such salmonid fish (42). Then, the current theory that IPNV infects only salmonids has been revised when the virus was isolated from healthy white sucker – freshwater Cypriniform fish (*Catostomus commersoni*) (43) and from Japanese eel (*Anguilla japonica*) (98). The known hosts range to present includes fish from more than 30 families. Some important for our region non-salmonids are: *Cyprinus carpio*, *Carassius aurata*, *Abramis brama*, *Esox lucius*, *Anguilla Anguilla*, *Barbus barbus*, *Astacus astacus*, *Danio rerio*, *Mytilus edulis*, *M. galloprovincialis*, *Hirudo medicinalis*, etc. Most of them have been experimentally infected. Domesticated hosts except salmonids are *Limanda limanda*, *Seriola quinqueradiata*, *Psetta maxima*, and *Hyppoglossus hypoglossus* (6).

The complete epizootic cycle of the virus has not yet been fully clarified. It is not possible to establish why viral antigen is detected in areas where there are no fish farms - rivers, lakes, dams or seawater with no possible vectors. According to Souter (39), detection of the virus in the internal organs of 229 samples of Arctic charr (*Salvelinus alpinus*) harvested at the beginning of the breeding season in various habitats - the delta and upstream of the Mackenzie River and the rivers on the Yukon territory, where there are almost no humans and no aquaculture.

The virus is transmitted horizontally and vertically. It is proven for salmonids but in non-salmonids – farmed turbot or halibut which are susceptible, there is not enough research (40, 41, 49). Vertical transmission is intraovarian - via infected eggs or seminal fluids (42).

The optimal temperature of virus replication varies and depends on the host and the strain. Juvenile brook trout (*Salvelinus fontinalis*)

infected with North American strain had maximum mortality at 10° C but the challenge with VR 299 cause the similar mortality at 15.5°C (105).

After an experimental infection, Molloy et al., demonstrated (2013) that *Mytilus edulis* has the ability to accumulate and transmit IPNV (99). Other researchers (102) observed viable virus in the digestive gland, muscle, gonads and rectum tissue at 333 day after the experimental injection of IPNV into the adductor muscle of scallop.

Swanson et al., after an experimental infection trial of IPNV using fry trout reported data about 2 days latent period after the inoculation in which the virus can be detected in the water. After these 2 days, there is a period of 2-4 days through which the fish manifest a clinical sign. This

incubation time depends on the environmental and internal factors (101).

DIAGNOSIS

CLINICAL AND PATHOLOGICAL SIGNS

Diagnosis is based on clinical signs, laboratory examination, and case history.

Clinical signs can be seen only in small fish up to 10 g, most often 0.1-3 g (43). Fish weighing approximately 50-100 g, mortality at IPN is about 38-45%. At this stage of development, there are physiological changes that cause both stress and decreased immunity (45, 46).

Predisposing factors for increasing the virulence of IPNV are age, stress and water temperature. External signs are non-specific, but can be used as a guide: blackening of the body (hyperpigmentation), exophthalmia, ascites (47) as shown in **Figure 2:**

Figure 2. External signs of IPN: A-*post mortem*; B-*in vivo* (personal data).

Less common - lesions or petechiae, especially in the area of gills. The gills are anemic (47). The gross pathology of the infected young fish may include an empty

stomach and intestinal tract. The pyloric caeca may have petechiae and the body cavity may contain milky exudate (**Figure 3**).

This signs are presented in **Figure 3:**

Figure 3. Gross anatomy pathology *in situ* of pyloric caeca with apical hemorrhage (personal data).

Anorexia, frequent pseudo-fecal portion and atypical swimming behavior can also help diagnosis. Movements are often uncoordinated - periods of lethargy can be followed by fast and atypical swim (for example, rotating like a corkscrew). A clinic sign may be missing, but high mortality is an indication for a possible infection with IPNV (36, 37).

Some researchers have seen the same signs demonstrated by non-trout fish species infected with IPN-like viruses (30,49,50,51). According to Evensen (52), in the acute phase of the infection mortality can reach 100%. In young fish, it can be restricted by increasing immunity and modifying environmental factors (53). Marine fish, infected with pathogenic aquarium viruses, can have clinical signs as abdominal ascites and/or spinal deformity or may have no clinical signs. Laboratory testing only shows the presence of viral antigen in tissues (54, 55).

No clinical signs have been reported so far in the presence of *Tasmanian aquabirnavirus* (TAB). Changes can only be seen in histological examination (19).

Fish from the elderly technology group may have the virus, but they do not show any clinic or pathoanatomical alterations. Even though they continue to release excretions containing IPNV, which is a potential cause of horizontal transmission of the infection (41). The titer of viral particles from the elderly survived fishes may vary from non-detectable to relatively high

(56). Occasionally, in the *lamina propria* cells of the asymptomatic Atlantic salmon – this cells so-called "McKnight cells" - necrotizing histiocytes can be seen (60).

Histopathological changes may include hemorrhage in the liver, swollen respiratory epithelium in gills, massive necrosis in acinar cells with vacuolar degeneration of pancreatic cells. The cells have a pyknotic nucleus and karyorrhexis (102). Since clinical signs are non-specific and pathological changes are not certain, the final diagnosis depends on the selection of the exact virus detection method or viral antigen detection method. There are two possibilities. The first one is direct evidence in tissues that have responded to infection or tissue processing.

The pathoanomic finding is unclear. There may be lesions or haemorrhages in the apical part of the pyloric. Observed under a microscope, with immunohistochemical methods and by appropriate use of staining, you can see necroses in pancreatic cells (acini) and hematopoietic kidney cells (48).

Fish weighing over 10-12 g and older than 6 months are resistant to disease. The carriers are asymptomatic but periodically virus ejection with feces and gonads product maintenance the infection in susceptible fish (103, 104).

LABORATORY METHODS

The second is to cultivate a supernatant derived from them on an appropriate cell cultures and subsequent identification.

One of the most accurate direct methods is the electron microscopy of tissue slides.

- Cultivation and serological methods

A standard for proving the presence of viral antigen in salmonids is object of regulatory and

it's described in MDTAA (61). The sampling tissue from the correct organ (spleen, anterior kidney, heart, encephalon, ovarian or seminal fluids) and inoculation of obtained by grinding supernatant into cell cultures (**Figure 4**).

Figure 4. A-EPC – normal monolayer; B-CPE – after 48 h of inoculation with IPNV (personal data).

In case of small fry sampling is from whole fish or from the viscera including a kidney – its depend on body length (87). Different cell lines - CHSE-214, BF2, EPC, RTG-2, FHM are used (59-61). When there is a cytopathic effect (CPE) – Figure 8 methods that can confirm it are usually serological - ELISA, IFAT, VN, immunoperoxidase, flow cytometry, immunodot-blotting, immunostatic fibroblast protein A (62). These methods are sensitive, specific and fast and are suitable for routine diagnosis in the acute phase of the disease as well as suspected latent carriers but they require at least two weeks. They are confirmed as a standard for several reasons:

- the virus is confirmed 100%, as it is in a relatively high titre in the precursor tissues - spleen; immunogenic part of the kidney, and brain; (63);
- there is no latent phase during which IPNV cannot be isolated and cultivated;
- the cell lines used are highly sensitive (60);
- requires a prolonged period of time during which CPE may be initiated in several ways - titration, change of CC, addition of enzymes, etc. (64-66);
- ELISA is a suitable confirmatory method (67), as well as IFAT (68) - both methods have come into practice and can be combined with more advanced molecular methods if needed (69, 62).
- Molecular methods

The use of molecular methods as stand-alone or as confirmation of aquabirnaviruses, found via other methodologies, began in the early 1990s.

Protocols for virus confirmation are developed in the CC (70-74). Developments are made with the prevalence of amplification methods, but in 2004, Alonso et al. (75) developed an in situ hybridisation (ISH) method for detecting the virus in infected BF-2, EPC and CHSE-214 CC, which however was not widely used.

Reverse transcriptase polymerase chain reaction - RT PCR and RT qPCR - real-time reaction is much more popular.

Various variants of RT PCR are now widely used to demonstrate IPNV. The first protocol was developed by Lopez-Lastra in 1994 (76). In 1995 (72) and 2001 (74) various modifications were developed to confirm isolates from cell cultures. Later, Barlic-Maganja et al. (77) and Shivappa et al. (78) use their protocols to prove the virus. In a comparative study of 5 serological methods and conventional RT PCR (62), the authors demonstrated that the most sensitive and suitable for routine diagnosis methods of virus detection in tissues (in the acute phase of IPN development) are molecular. Protocols for nested RT PCR were developed (79, 80). Real-time reverse transcriptase polymerase chain reaction (RTqPCR) was used for the first time successfully for fish viruses successfully by Dhar et al. (80). They used fluorescent staining with SYBR Green I. Thereafter, the use of the TaqMan probe was proved for different viral fish pathogens (82). The first RTqPCR-based screening based on the use of a probe is made by Orpetveit et al. (83) in Norway. According to the authors, the method is 100 times more sensitive than the conventional one but a major

disadvantage of molecular methods is confirming the presence of a viral antigen regardless the virus is alive or not.

DIFFERENTIAL DIAGNOSIS

Many of the observed clinical signs in the acute phase of the infection such as open gills, exophthalm, darkening of the skin surface, ascites, enlarged spleen, chaotic movements are similar to those of other diseases - VHS, IHN, ISA (84, 85) mass infestations, myxosis, bacterial infections, especially in fish about 100 g (86).

The laboratory standard, including inoculation on a suitable cell line, identifies the virus only after a serological examination of the supernatant collected from the wells of the plaque with CC. Since IPNV is believed to be worldwide spread, the OIE recommends adding antiserum to the inoculant supernatant in the CC in the screening of VHS and IHN. The purpose is to prevent CPE due to the presence of IPNV. RT PCR or RT qPCR is recommended as a parallel method for fast identification (87).

CONTROL AND PREVENTION

IPNV is extremely resistant to most disinfectants because of the vertical transmission. Its virulence decreases significantly when heated to 55-60° C, ozone, formalin, drying, and extreme pH values below 2 and above 12 (89). This makes it especially dangerous for susceptible risk groups of fish in neighboring farms - located along the catchment area or just close to it. Transmission can also be carried out on the import of feed material, inventory, etc. (90).

To prevent the spread of IPN and to reduce damage, it is necessary to strictly comply with hygiene and regulatory requirements:

1. Control over the movement of the material, live fish and facilities in the country and in individual farms to be strict (91).
2. The import of fertilized caviar shall be carried out by disease-free farms. Caviar must have a certificate of origin from a disease-free farm or a negative result for virus-carrying by an accredited laboratory.
3. In case of a disease outbreak, implement the current Biosecurity Plan.
4. It is recommended to grow more resistant species of salmonids (87, 91).
5. The losses can be limited by increase of temperature of water. It depends on IPNV strain and fish species (40).
6. Vaccination (of uterine herds) with appropriate vaccines should be done. At the present, several commercial vaccines are commercially available. The application is different: oral (107), by immersion (applied into 3 g. fish body weight

(108), intramuscular (5 g) (109) or intraperitoneal injection (*i.p.* – 10-35 g) (108), by modulating Qtl regions of the genome (92). Aquaculture vaccination in Bulgaria is forbidden. (93). Some pharmaceutical company as Intervet-International BV, Merck Animal Health, Microtek International, CDV, Pharmaq AS, Centrovet, Aqua Health Ltd, provide commercial vaccines.

CONCLUSION

Viral fish diseases are considered as the most important in Bulgaria. The IPN occurring increased since 2006.

The IPNV has a wide distribution in the most major salmonid-farming countries. In our geographical region it is present in a freshwater salmonid farms of our neighbours, as well as in all Balkan countries. It is present in the most countries with which we trade and import fertilizer eggs. Vertical transmission could be avoided by import from farms, free of IPNV or by laboratory test of eggs – before being hatched. Horizontal transmission can be done through a variety of organic vectors in which it can persist such as Black Sea shellfish, fish eating birds, wild salmonid, other wild and cultured fish populations, and mechanical vectors such as facilities, people in contact with infected fish, etc.

Field diagnosis is difficult since the clinical signs are nonspecific and a point of reference can be only age, body darkening pigmentation, and uncharacteristic swimming behaviour. Accurate and reliable laboratory methods are available. The gold standard is virus isolation in specific cell cultures and subsequent identification by serological techniques.

In recent years, molecular methods have become more sensitive and specific. RT PCR and RTqPCR have become methods of rapid routine diagnosis, but a major drawback is demonstrating the existence of a viral antigen without clarifying whether virus is alive and is it possible to develop. Therefore, the importance is sampling with great precision from predilection tissue, stored and transported according to accepted procedures.

Knowledge of the molecular features of the IPN virus, an interaction: “IPNV-host”, “IPNV-fish cultured population”, “IPNV-wild fish population” and “IPNV-other fish pathogens”, assumes a deep understanding of the nature of IPNV and its behavior. There is not enough data of the epizootiological cycle but it is clear that the economic impact of IPN

is high. It causes devastating losses of each affected farm. Good laboratories, veterinary and farming practices as well as legislative and executive authorities need up-to-date information for effective control and prevention.

ABBREVIATION

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
IPN	infectious pancreatic necrosis
IPNV	infectious pancreatic necrosis virus
VHS	viral hemorrhagic septicemia
IHN	infectious hematopoietic necrosis
ISA	infectious salmon anemia
CC	cell culture
EPC	epithelioma papulosum cyprinid cell line
BF2	bluegill fry 2 cell line
CHSE-214	chinook salmon cell line
RTG2	rainbow trout gonad tissue cell culture
FHM	fathead minnow cell line
CPE	cytopathic effect
PCR	polymerase chain reaction
RT PCR	reverse transcriptase polymerase chain reaction
RTqPCR	quantitative reverse transcriptase polymerase chain reaction
ELISA	enzyme linked immunosorbent assay
IFAT	indirect immunofluorescence test
UV	ultraviolet light

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