Bulgarian Journal of Veterinary Medicine (2004), 7, No 2, 129–135

DETECTION OF INFECTIOUS PANCREATIC NECROSIS (IPN) VIRUS IN RAINBOW TROUTS IN BULGARIA

V. CHIKOVA & D. ILIEVA

National Diagnostic and Research Veterinary Medical Institute,
Sofia; Bulgaria

Summary

Chikova, V. and D. Ilieva, 2004. Detection of infectious pancreatic necrosis (IPN) virus in rainbow trouts in Bulgaria. *Bulg. J. Vet. Med.*, **7**, No 2, 129 –135.

Epizootological studies on the prevalence of infectious pancreatic necrosis (IPN) in Bulgarian trout farms were carried out between 1993 and 2000. Whole alevin or organ homogenates (pyloric caeca, spleen, kidneys, brains) of rainbow trouts with typical clinical signs of IPN were virologically investigated. Eight field IPN viral strains were isolated on indicatory cell cultures RTG-2, BF-2 and EPC identified by means of the neutralization test and the indirect fluorescent antibody test. The comparisions to reference antisera and hyperimmune sera obtained by us showed that six viral isolates belonged to the Sp serotype whereas the other two – to the Ab serotype of the IPN virus.

Key words: indirect fluorescent antibody test (IFAT); infectious pancreatic necrosis virus (IPNV), neutralization test (NT)

INTRODUCTION

The intensification of rainbow trout breeding, the extensive exchange of eggs and fry fish among trout farms and their import from other coutries are real preconditions for the appearance and spreading of infectious pancreatic necrosis (IPN) in the rainbow trout populations in our country.

The reported data (Wolf *et al.*, 1960; Malsberger and Cerini, 1963) evidenced the viral etiology of the disease. The etiological agent (IPN virus) belongs to the Birnaviridae family (Dobos and Roberts, 1983). The isolates show several antigenic differences and belong to three serotypes: Sp, Ab and VR 299 (Dorson and Torchy, 1981; Ahne *et al.*, 1989; Melby *et al.*, 1994; Hill and Way, 1995).

The diagnostic studies for detection of the IPN virus are based on direct isolation in indicatory cell cultures followed by serological identification (Jorgensen, 1974; Swanson and Gillespie, 1981; Agius *et al.*, 1982; Hill and Way, 1995).

The impact of the IPN virus on Bulgarian fish breeding industry is suggested by sporadic epizootological and clinical observations, but it is not detected by reliable virological methods.

The importance of the problem for trout breeding and the need of screening surveys in order to depict the epizootological status in our country necessitated the performance of the present studies.

MATERIALS AND METHODS

Extent of studies

Epizootological and virological studies of 154 pathological specimens (whole alevin or organ homogenates of pyloric caeca, spleen, kidneys and brains) of rainbow trouts with typical clinical signs, originating from trout farms located in different regions of the country, were carried out.

Cell cultures

Stable cell culture lines RTG-2, EPC and BF-2 were used. The cells were cultivated on a synthetic nutrient medium Eagle MEM supplemented with 10% foetal calf serum and antibiotics at 25 °C.

Viral strains

In the comparative studies, three referent IPN viral strains (2 from the Sp serotype and 1 from the Ab serotype) obtained from the National Reference Laboratory of Fish Diseases, Padua, Italy, were included.

In cross-over serological studies, a heterologous strain of the trout viral haemorrhagic septicaemia virus (VHS-F_1) was also used.

Hyperimmune sera

Specific anti IPN-Sp and anti IPN-Ab sera were obtained following hyperimmunization of rabbits with the referent strains following a specific schedule (Jorgensen, 1974).

The specificity and activity of obtained hyperimmune sera (HIS) were tested by titration in neutralization test (NT) performed with homologous viruses.

Isolation and cultivation

Cell cultures (24–48 h) with a well-formed monolayer, inoculated with virus-containing material and left for adsorption at 14 °C for 60 min were used for infection. A maintenance nutrient medium supplemented with 2% foetal calf serum was added and cell cultures were incubated at 14 °C. They were daily monitored for the development of a characteristic cytopathic effect (CPE) for 7–10 days. When the result was negative, a second blind passage was made and the CPE was examined again.

Identification of viral isolates in cell cultures

The investigation and identification of isolates was done according to the procedures of the NT (Hill and Way, 1995). Six isolates homologous to the reference IPN-Sp viral strain (Sm/93; Is/99; Is/3; Is/4; Is/5 and Is/6) and two isolates, homologous to the reference IPN-Ab viral strain (Sm/00 and Sm/02) were included in the experiments. The presence and the location of IPN viral antigen in infected cell cultures was performed by the indirect fluorescent antibody test (IFAT) (Jorgensen, 1974). For comparative studies, reagents prepared by us and standard kits (Bio–X, Belgium) were utilized.

RESULTS AND DISCUSSION

After the virological studies on pathological specimens of fingerling trouts originating from trout farms in various regions of the country, 8 viral strains were isolated.

The early changes in cell cultures EPC were observed 48 and 72 hours after the infection with pathological material, as compared to non-infected EPC cell cultures (Fig. 1 and 2). Infected cells lost their characteristic fusiform shape. The CPE was characterized by the appearance of enlarged, rounded "luminous" cells (Fig 3a), that were transformed into clus-

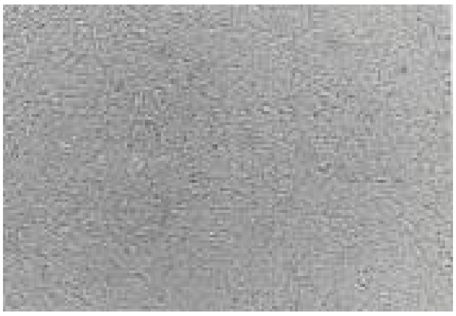

Fig. 1. Stable EPC cell line. Control non-infected tissue. Magnification ×125.

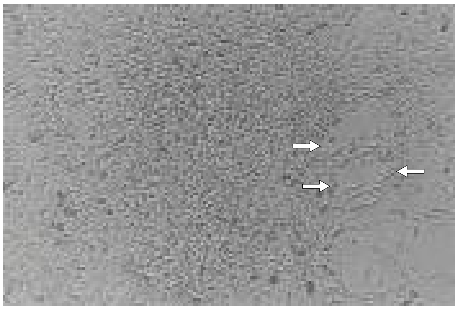

Fig. 2. Stable EPC cell line 48 hours after infection with an IPN viral strain (Sm/02 isolate). Part of infected cells lose their characteristic fusiform shape (arrows). Magnification ×125.

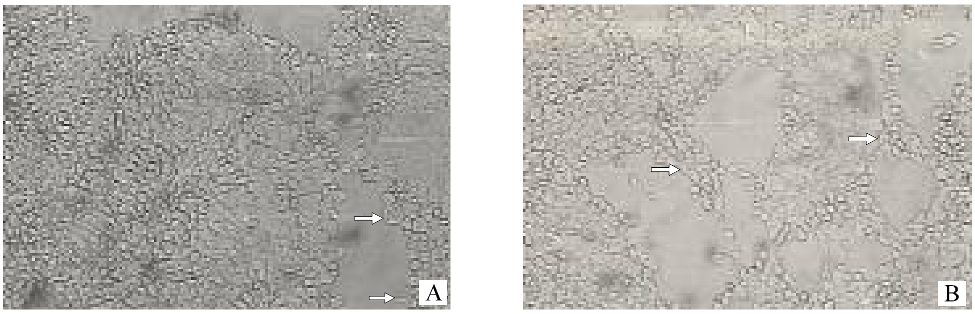

Fig. 3. EPC cell culture 72 hours after infection with an IPN strain (Sm/02 isolate). Enlarged, rounded “luminous” cells are observed (A), transformed into clusters of big balloon-like cells (arrows) (B). Magnification ×125.

ters of big balloon-like cells (Fig. 3b). By hours 96–120, a significant part of the monolayer was affected and there were several microscope fields with partial loss of destructed monolayer.

The infectious titres of isolated strains during the primary isolation were between $10^{5..5}$ and $10^{5..6}$ mean tissue culture infective doses ($TCID_{50}$)/cm^3. After 8–10 passages of isolated strains, their infectious titres increased up to $10^{7..6}$ and $10^{8..2}$ $TCID_{50}/cm^3$.

The isolation of IPN virus from pathological specimens upon sensitive cell cultures and the observation of a characteristic CPE as one stage of the diagnostic observations enabled obtaining of comparative data from cultivation of our isolates and the referent IPN viral strains. The results did not show any significant dfifference with respect to the sensitivity of used cell cultures, the CPE features and the obtained infectious titres.

The testing of 8 viral isolates by the NT using a referent anti IPN-Sp serum in a 1:100 dilution revealed that in 6 isolates, the neutralization index varied between lg 3.25 and lg 5.1. The neutralization indexes

Table 1. Neutralization index in IPN viral strains

Viral strains	Hyperimmune sera				
	IPN-Sp (reference)	IPN-Sp (isolate Sm/99)	IPN-Ab (reference)	IPN-Ab (isolate Sm/02)	VHS-F_1 (reference)
IPN-Sp reference	5.10	3.95	0.12	0.65	–
IPN-Sp isolate Sm/93	3.25	2.75	0.12	0.30	–
IPN-Sp Isolate/99	4.50	3.25	0.30	0.60	–
IPN-Ab reference	0.16	0.12	4.80	3.75	–
IPN-Ab isolate Sm/02	0.65	0.60	4.15	3.50	–
VHS-F_1 reference	–	–	–	–	3.35

Table 2. Comparative studies for detection of IPN viral antigen in infected cell cultures by the indirect fluorescent antibody test

Viral strains	Hyperimmune sera					
	IPN-Sp (reference)	IPN-Sp (isolate/ 99)	IPN-Ab (reference)	IPN-Ab (isolate Sm/02)	VHS-F_1 (reference)	Controls of cell cultures
IPN-Sp reference	+	+	–	–	–	–
IPN-Sp isolate /99	+	+	–	–	–	–
IPN-Ab reference	–	–	+	+	–	–
IPN-Ab isolate Sm/02	–	–	+	+	–	–
VHS-F_1 reference	–	–	–	–	+	–

in the other 2 isolates were lower than 1 (0.675 and 0.12 respectively). The same reaction with the referent IPN-Ab serum yielded neutralization indexes of lg 4.15 and lg 4.75 against those 2 isolates and values under 1 for the other 6 isolates (Table 1).

The neutralization titre of our hyperimmune sera was not significantly different from those of referent ones. The studies with homologous viral strains via the NT procedure showed neutralization titres of sera of lg 3.45 and lg 2.85 respectively.

Our investigations confirmed the high sensitivity and specificity of reaction, especially in the cross-over virus neutralization studies. The data supported antigenic identity of the 6 field isolates with the referent IPN-Sp strain whereas the other 2 isolates were antigenically identical with the IPN-Ab serotype.

The appearance of CPE and the immunofluorescence (IF) of infected cell cultures successively followed revealed that preparations treated with referent IPN-Sp and IPN-Ab antisera yielded a specific luminescence only in cell cultures, infected with homologous viral strains, in comparison with the non-infected EPC cell cultures (Table 2). In cell cultures, infected with the reference IPN-Sp and IPN-Ab strains and treated with the respective antisera, specifically luminous perinuclear granules were observed 10–12 hours after the inoculation. They were clearly distinguished from the adjacent red-brown normal cells (Fig. 4). By hour 24, a distinct granular IF was observed in the citoplasm of affected cells, whose extent by hour 48 was about 50–60% of cellular monolayer (Fig. 5 and 6). By hour 72–96, an intense IF of citoplasm was visible as well as formation of clusters of fluorescent cells while the nuclei of infected cells were always negative (Fig. 7). After the 120th hour, the intensity and the number of fluorescent cells decreased considerably as a result of cellular monolayer destruction.

The comparative studies of cell cultures, infected with the heterologous F_1 strain of viral haemorrhagic septicaemia, did not manifest IF after the treatment with either anti IPN-Sp or IPN-Ab sera.

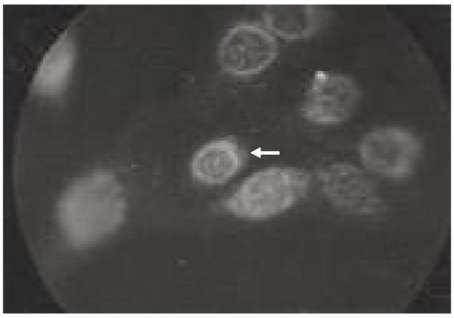

Fig. 4. Stable EPC cell line 10 hours after infection with an IPN viral strain (Sm/02 isolate). A weak perinuclear fluorescence is visible (arrow). Magnification ×400.

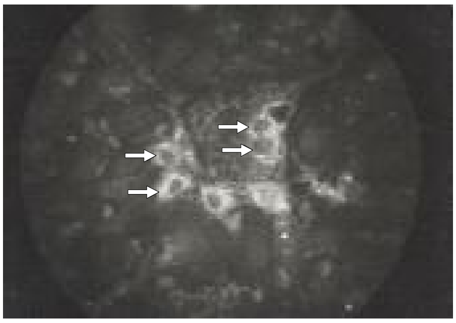

Fig. 5. Stable EPC cell line 24 hours after infection with an IPN viral strain (Sm/02 isolate). A perinuclear fluorescence with single fluorescent granules is present (arrows). Magnification ×400.

Fig. 6. Stable EPC cell line 48 hours after infection with an IPN viral strain (Sm/02 isolate). Cell clusters with granular cytoplasmic fluorescence are visible (arrow). Magnification ×400.

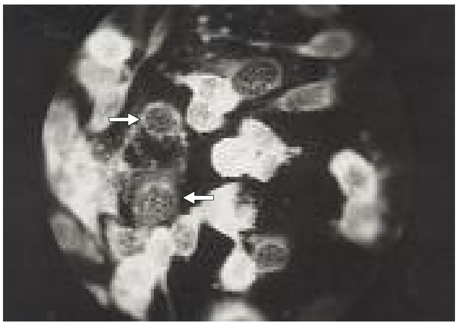

Fig. 7. Stable EPC cell line 72 hours after infection with an IPN viral strain (Sm/02 isolate). A strong cytoplasmic fluorescence in cell clusters is observable (arrows). Magnification ×400.

The control preparations were also negative.

The comparisons between reference and our HIS performed via the IFAT showed an identical sensitivity but lower titres of our HIS. It allowed us to assume that the HIS obtained by us were specific and could therefore be used for diagnostics of IPN in rainbow trouts via the IFAT and NT. At the same time it should be emphasized that obtaining specific HIS with high titres is particularly important for the successful preparation of diagnostic tests.

The data of our studies confirm the opinions of Jorgensen (1974), Swanson and Gillespie (1981) and Ahne (1989) that the NT and the IFAT were appropriate and widely used tests for viral infection diagnostics in fish pathology. At the same time, they underline the dependence of those laboratory diagnostic methods upon the specificity and the titre of used hyperimmune sera and conjugates.

The virological investigations of diagnostic specimens from different trout farms evidenced the distribution of the IPN virus in our country. The number of analysed samples do not permit however a complete evaluation of the epizootological status in our fish farms.

Considering the opportunity that permanently infected with IPN trout farms could occur, there is a need of development and implementation of a system of epizootological control and steps for limitation of IPN infection.

CONCLUSIONS

The virological investigations of diagnostic specimens from various trout farms via the neutralization test and the indirect fluorescent antibody test resulted in identification of 8 field isolates of infectious pancreatic necrosis (IPN) virus in rainbow trouts: 6 IPN-Sp and 2 IPN-Ab isolates.

The reagents prepared by us, showed a high sensitivity and specificity in the NT and the IFAT, similar to that of commercial kits. They could be successfully used for identification of IPN virus isolated in cell cultures when performing diagnostic and screening studies.

REFERENCES

Agius, C., H. Mangunwiryo, R. H. Johnson and D. A. Smail, 1982. A more sensitive technique for isolating infectious pancreatic necrosis virus from asymptomatic carrier rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases*, **5**, 285–292.

Ahne, W., P. E. V. Jorgensen, N. J. Olesen, T. Fischer-Scherl and R. Hoffmann, 1989. Aquatic Birnaviruses: Virus of the serogroup II isolated from an IPN outbreak in brook trout (*Salvelinus fontinalis*). *Bulletin of European Association of Fish Pathology*, **9**, 14–16.

Dobos, P. and T. E. Roberts, 1983. The molecular biology of infectious pancreatic necrosis virus. *Canadian Journal of General Microbiology*, **29**, 377–384.

Dorson, M. and C. Torchy, 1981. The influence of fish age and water temperature on mortalities of rainbow trout, *Salmo gairdneri* Richardson, caused by a European strain of infectious pancreatic necrosis virus. *Journal of Fish Diseases*, **4**, 213–221.

Hill, B. J. and K. Way. 1995. Serological classification of infectious pancreatic necrosis /IPN/ virus and other aquatic birnaviruses. *Annual Review of Fish Diseases*, **5,** 55–77.

Jorgensen, P .E. V., 1974. Indirect fluorescent antibody techniques for demonstration of trout viruses and corresponding antibodies. *Acta Veterinaria Scandinavica*, **15**, 198–205.

Malsberger, R. G. and C. P. Cerini. 1963. Characteristics of infectious pancreatic necrosis virus. *Journal of Bacteriology*, **86**, 1283–1287.

Melby, H. P., P. Caswell-Reno and K.Falk, 1994. Antigenic analysis of Norwegian aquatic birnavirus isolates using monoclonal antibodies with special reference to fish species, age and health status. *Journal of Fish Diseases*, **17**, 85–91.

Swanson, R. N. and J. H. Gillespie, 1981. An indirect fluorescent antibody test for rapid detection of infectious pancreatic necrosis virus in tissues. *Journal of Fish Diseases*, **4**, 309–315.

Wolf, K., S. F. Snieszko, C. E. Dunbar and E. Pyle, 1960. Virus nature of infectious pancreatic necrosis in trout. *Proceedings of the Society for Experimental Biology and Medicine*, **104**, 105–108.

Paper received 15.05.2003; accepted for publication 15.04.2004

Correspondence:
Assoc. Res. Scientist Dr.V. Chikova
Department of Fish Diseases and Aquaculture,
National Diagnostic & Research Veterinary Institute,
15, Pencho Slaveikov Blvd.,
1606 Sofia, Bulgaria