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Characterization of a rhabdovirus isolated from carpione *Salmo trutta carpio* in Italy

G. Bovo[1], N. J. Olesen[2], P. E. V. Jørgensen[2], W. Ahne[3], J. R. Winton[4]

[1] Istituto Zooprofilattico Sperimentale delle Venezie, Via G. Orus 2, I-35129 Padova, Italy
[2] National Veterinary Laboratory, Hangøvej 2, DK-8200 Aarhus N, Denmark
[3] Institute of Zoology, Fishery Biology and Fish Diseases, Kaulbachstrasse 37, D-80539 Munich, Germany
[4] National Fisheries Research Center, Bldg 204 Naval Station, Seattle, Washington 98115, USA

ABSTRACT: A virus, strain 583, was isolated from carpione *Salmo trutta carpio* fry exhibiting high mortality. The virus was not neutralized by rabbit antisera against the fish rhabdoviruses viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus, eel rhabdovirus European X, spring viraemia of carp virus or pike fry rhabdovirus, or against the birnavirus infectious pancreatic necrosis virus. The virus replicated in several fish cell lines incubated at 20 to 25°C and grew optimally in the bluegill fry (BF-2) and fathead minnow (FHM) cell lines. Electron microscopy of infected BF-2 cell cultures revealed the presence of typical rhabdovirus particles, and immunofluorescent staining was observed using various polyclonal and monoclonal antibodies (MAbs) against Egtved virus, the causative agent of viral haemorrhagic septicaemia. The staining by a MAb against the nucleoprotein (N) of VHSV was particularly strong, a MAb against the glycoprotein (G) gave a moderate reaction, whereas a second MAb against the G protein and MAbs against the matrix proteins, M_1 and M_2, of VHSV did not react. Fluorescence titres using 3 rabbit antisera against whole Egtved virus varied between negative and moderately positive. Western blotting using polyclonal and monoclonal sera confirmed that both the N and G proteins of the carpione virus shared some epitopes with those of VHSV, but the M_1 and M_2 proteins did not. SDS-PAGE showed the structural proteins of the carpione virus produced a pattern typical of members of the *Lyssavirus* genus of the Rhabdoviridae and the molecular weights were very similar to those of VHSV, except for the M_2 protein which was somewhat smaller. Infection trials showed the carpione virus induced high mortalities in carpione fry but not in rainbow trout *Oncorhynchus mykiss* fry. The carpione virus was clearly distinguishable from Egtved virus despite limited serological cross reaction. Since it was also easily distinguishable by immunofluorescence from the other fish rhabdoviruses included in the present study as well as in studies published elsewhere, it is concluded that the virus is a previously undescribed one. It is proposed that the virus be given the preliminary designation 'carpione rhabdovirus'

KEY WORDS: Rhabdovirus · Carpione *Salmo trutta carpio* · Fish virus

INTRODUCTION

According to Melotto & Oppi (1987), carpione *Salmo trutta carpio* is a salmonid species with an uncertain taxonomic classification. Apparently, carpione are present only in Garda Lake, Italy, where they have become highly adapted, spawning twice a year in reproduction areas at depths of 70 to 300 m. The species is mainly planktophagous. In spring 1988, attempts were initiated by the Ente Sviluppo Agricolo del Veneto (ESAV) to propagate carpione artificially with the aim of increasing the population, which in recent years has suffered a significant reduction (Melotto & Oppi 1987). Fertilized eggs, obtained from captured wild spawners, were transferred to the ESAV experimental lake station in Bardolino, Italy, and hatched at 10 to 12°C. Fry were fed with phytoplankton. After 2 wk, the fry were transferred to a trout farm supplied with river water. At the end of June, 3 mo after hatching, high mortality occurred in the fry. This paper describes the isolation of a novel virus from the diseased fish.

MATERIALS AND METHODS

Virological examination. Diseased, 3 mo old carpione fry were homogenized and examined by cell culture inoculation according to standard diagnostic procedures using the rainbow trout gonad (RTG-2) (Wolf & Quimby 1962) and the *epithelioma papulosum cyprini* (EPC) (Fijan et al. 1983) cell lines incubated at 15°C. Virus identification was attempted by neutralization tests using rabbit antisera to: viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), eel rhabdovirus European X (EVEX), spring viraemia of carp virus (SVCV), pike fry rhabdovirus (PFR) and infectious pancreatic necrosis virus (IPNV).

Bacteriological examination. Kidney material was tested for bacteria by inoculation of blood agar and TSA agar plates which were incubated at room temperature for 5 d.

Cell cultivation. All the cell lines used were cultivated using Eagle's minimum essential medium to which had been added 10% (or occasionally 2%) foetal bovine serum and antibiotics and Tris buffer in standard concentration, using commercially available plastic flasks and trays. Incubation temperatures, unless otherwise stated, were 20°C during cell outgrowth and 15°C after inoculation of virus.

Virus isolates. IHNV strain 32/87, isolated in France, was obtained from Dr P. de Kinkelin, INRA, Paris, France. The VHSV isolates used were reference strain F1 (Jensen 1965) and strains DK-3592-B and Saone, isolated by the authors in Denmark and Italy, respectively.

Production of antiserum in rabbits. Antiserum to the virus isolate from carpione fry was produced by immunizing rabbits with sucrose-gradient-purified virus according to a previously described purification and immunization schedule (Olesen et al. 1991).

Immunofluorescence (IF). Coverglass (9 × 22 mm) cultures of EPC cells were infected by immersion in 1:50 or 1:500 dilutions of growth medium from cell cultures showing complete cytopathic effect (CPE). The cultures were incubated at 15°C for 24 h. Coverslips were rinsed twice in cell culture medium without serum, fixed in acetone for 15 min, air dried, and stored at –20°C until used. Polyclonal rabbit antisera to VHSV (K05, K59 and 'Pool b'), IHNV (K2702), EVEX (K13), PFR (K45), SVCV (K42) and to carpione isolate 583 as well as monoclonal antibodies (MAbs) to the G (IP1D11 and IP1H3), N (IP5B11), M_1 (IP1C6), and M_2 (IP1C3) proteins of VHSV (Lorenzen et al. 1988) were applied as the first antibody layer and the coverglasses were incubated in a humid chamber, either for 30 min at 37°C (rabbit sera) or overnight at 4°C (MAbs). Following 3 rinsing cycles as described by Lorenzen et al. (1988), staining was performed for 30 min at 37°C using fluorescein isothyocyanate (FITC) conjugated swine anti-rabbit Ig or rabbit anti-mouse Ig, respectively, (Dakopatts, Copenhagen) at a dilution of 1.100. Rinsing was then repeated before mounting in glycerol-PBS (phosphate-buffered saline) mixture (pH 8.0). Examination was carried out using an epifluorescence microscope (Olympus Vanox) supplied with a 200 W mercury lamp and fluorescence oil objectives (40× and 100×).

During experimental infection trials with the carpione virus, tissue from fry that showed signs of disease (e.g. muscle haemorrhages) were processed for IF. Specimens were frozen on metal dishes inside a cryostat chamber at –20°C. After 30 min, 5 to 10 µm sagittal sections were cut. After air drying, sections were fixed in acetone at –20°C for 10 min. Immunofluorescent staining was conducted using the indirect technique, where rabbit antisera to IHNV, VHSV or the carpione virus were applied as the first antibody and incubated in a humid chamber at 37°C for 30 min. Following 3 rinsing cycles, staining was performed at 37°C for 30 min using FITC conjugated goat antibody against rabbit Ig (Pasteur Institute, Paris, France) at a dilution of 1:100. Rinsing was repeated before mounting in glycerol-PBS mixture buffered at pH 9 with carbonate-bicarbonate solution. Observation was carried out at 400× using an epifluorescence microscope (Zeiss, Axioskop) supplied with an HBO 100 W mercury lamp.

Electron microscopy. BF-2 cells infected with the carpione virus at a multiplicity of infection (MOI) of 0.1 were incubated at 20°C for 48 h. Afterwards, cells were fixed in 2.5% glutaraldehyde in 0.067 M Sorensen's phosphate buffer (pH 7.4) for 24 h, washed 3 times with 0.2 M saccharose-Sorensen-phosphate buffer and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. After dehydration in a graded acetone series, the specimens were embedded in Epon 812. Semi-thin sections were stained with 1% toluidine blue in 1% borax. Ultrathin sections stained with uranyl acetate and lead citrate were examined using a transmission electron microscope (Zeiss EM 109).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (1970) in 1 mm thick slab gels in a vertical slab gel system. The stacking gel contained 3.5% acrylamide and 0.11% bisacrylamide, the separating gel 10% and 0.31%. The gels were loaded with gradient-purified carpione virus, IHNV (strain 32/87) and VHSV (strain F1). After electrophoresis, the gels were stained by the silver staining method described by Morrissey (1981) or were used for immunoblotting.

Immunoblotting. The protein bands were transferred from polyacrylamide gels to polyvinylidene difluoride membranes (Immobilon, Millipore) according to procedures described by Kyhse-Andersen (1984). Free binding sites were blocked by incubation in PBS with 5% BSA (bovine serum albumin) for 1 h at 37°C. After washing in PBS with 0.05% Tween-20 (PBS-T), the membranes were cut into 30 mm wide strips, each containing the polypeptides of the 3 viruses. The strips were then incubated overnight with 1:5000 dilutions of protein-A purified rabbit Ig to IHNV or VHSV previously absorbed on EPC and BF-2 cells, or with a pool of the MAbs IP1D11, IP1H3, IP5B11, IP1C6, and IP1C3 against the VHSV polypeptides G (2 MAbs), N, M_1 and M_2, respectively (Lorenzen et al. 1988). Following three 5 min rinses in PBS-T, horseradish-peroxidase-conjugated swine antibodies to rabbit Ig or rabbit antibodies to mouse Ig (Dakopatts) were added at a dilution of 1:1000. Visualization of bound conjugate and total protein was performed as described by Lorenzen et al. (1988).

Virus replication in different cell lines. Replication of the carpione virus in different cell lines was examined by inoculation of a stock culture (6th passage on EPC cells) into 24 h old cultures of the following cell lines: RTG-2, EPC, BF-2 (Wolf & Quimby 1966), fathead minnow (FHM) (Gravell & Malsberger 1965), chinook salmon embryo (CHSE-214) (Lannan et al. 1984), pike gonad (PG) (Ahne 1979), ovary fluid cells (OFC) (G. Bovo unpubl.) and sea bass line (SBL) (Castric 1984). The cells were grown in 75 cm^2 flasks to about 90% confluence. Cells were inoculated with virus at an MOI ranging from 0.6 to 1.2. After virus adsorption for 1 h at 15°C, the inoculum was removed and the cell sheet washed twice with 5 ml of medium. Afterwards, 20 ml of new medium containing 2% serum was added to each flask, which was incubated at 20 or 25°C as shown in Table 1.

After 7 d, the virus titre in each flask was determined by plaque assay using 24-well plates of EPC cells. Serial 10-fold dilutions were prepared and triplicate inocula (0.1 ml $well^{-1}$) of each dilution were plated and allowed to adsorb for 60 min at 15°C. Afterwards, the inoculum was removed, 1 ml medium with 0.8% methylcellulose was added to each well, and plates were incubated at 15°C for 7 d. The titres were expressed as the average number of plaque forming units per ml (PFU ml^{-1}).

Virus replication at different temperatures. Virus replication in EPC cells incubated at selected temperatures was examined using 75 cm^2 flasks infected at an MOI of 0.1. Virus was inoculated onto drained cell monolayers for 60 min at 15°C followed by 2 washes with 5 ml cell culture medium. The cultures were incubated at 15, 20, 25 and 30°C for 7 d and the virus concentrations were determined by plaque titration on EPC cells as described.

Virus stability at selected temperatures. Aliquots of carpione virus in cell culture medium were exposed for 15 min to each of the following temperatures: 4, 30, 37, 42 and 56°C. Subsequently the residual infectivity was titrated on EPC cells and expressed as PFU ml^{-1}

***In vivo* passage of carpione virus in rainbow trout fry.** Twenty-five rainbow trout *Oncorhynchus mykiss* fry (1.0 g) were given an intraperitoneal injection of carpione virus (cell culture passage 8). The virus was reisolated from a pool of 2 fish which developed signs of disease such as exophthalmia, petechial haemorrhages in muscles and gills, presence of ascitic fluid in the coelom, cerebral disturbances and severe kidney haemorrhages approximately 5 d post-infection. The first cell culture passage of this reisolated virus was used in bath infection experiments with rainbow trout fry (1.25 g).

Experimental infection. Virus titres in the experimental infections were determined by tissue culture infective doses per ml ($TCID_{50}$ ml^{-1}) as described by Reed & Muench (1938). During infection experiments, experimental fish were kept in 8 l aquaria supplied with tap water at 10 or 15°C (see Table 3). The trout fry were fed commercially available trout dry food whereas the carpione fry were not fed because phytoplankton was not available.

Groups of 50 carpione fry (0.1 g) were exposed by bath infection to 2×10^6 $TCID_{50}$ ml^{-1} of the carpione virus, 8th cell culture passage, or to 4×10^3 $TCID_{50}$ ml^{-1} of VHSV (strain Saone), 2nd cell culture passage. Groups of 50 rainbow trout fry (2 g) were exposed to carpione virus and Saone isolates of VHSV at a titre of 1×10^5 $TCID_{50}$ ml^{-1}. In an additional experiment, replicate groups of 50 rainbow trout fry (1.25 g) were exposed to bath infection with 1×10^5 $TCID_{50}$ ml^{-1} of carpione virus (1 cell culture passage after 8 *in vitro* subcultivations and 1 *in vivo* passage in trout fry) or with VHSV, strain 3592B, cell culture passage 3 (see Table 3) In both experiments, control fish were exposed to water containing cell culture medium without virus. Moribund fry were examined visually for the presence of disease signs and selected samples were processed for virus isolation and/or indirect IF tests.

RESULTS

Isolation of virus

Cytopathic effect appeared 48 to 72 h after inoculation of tissue suspensions from diseased carpione fry into RTG-2 and EPC cell cultures. Bacteriological examination of the affected fry was negative. The virus was not neutralized by rabbit antisera to VHSV, IHNV, EVEX, SVCV, PFR, or IPNV

Immunofluorescence

By IF, a positive reaction was observed with 2 of 3 reference quality rabbit antisera made against reference-strain F1 of VHSV. One of the sera (Pool b) gave a weak response, the other (K59) a moderate one compared to the reactions obtained with homologous virus.

These 3 rabbit antisera have routinely given strong reactions with a large number of field isolates of VHSV representing all known serotypes (data not shown).

MAb IP5B11, directed against the N protein of VHSV, and MAb IP1D11, directed against the G protein of VHSV, gave a very strong and a moderate reaction (Fig. 1), respectively. No reactions were observed with MAbs recognizing the M_1 or M_2 proteins of VHSV nor with MAb IP1H3, which is directed against a G protein epitope different from that reacting with IP1D11 (not shown). Rabbit antisera to IHNV, SVCV, PFR, EVEX and IPNV all gave negative results. The antiserum to the carpione virus induced a brilliant cytoplasmic staining of carpione virus in infected cell cultures and in cryostat sections of infected fish. In contrast the antiserum did not stain cultures infected with VHSV or IHNV.

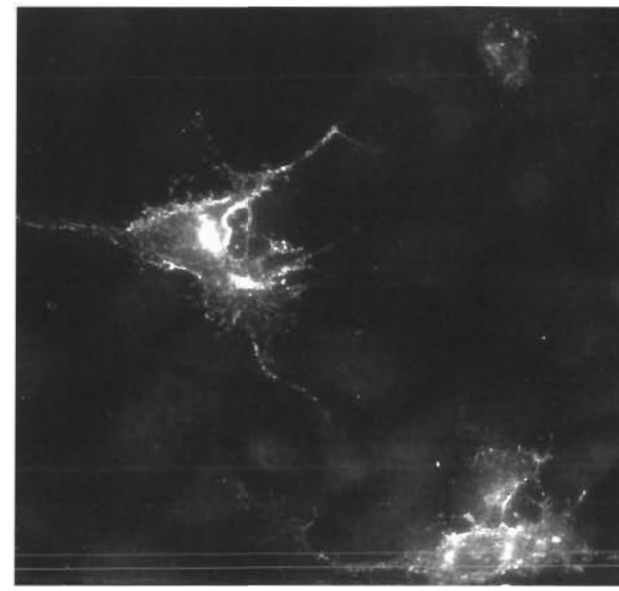

Fig. 1. Immunofluorescence micrograph showing the staining of a carpione-rhabdovirus-infected EPC cell culture with monoclonal antibodies to VHSV-G protein as the primary antibody and FITC-labelled rabbit antibody to mouse Ig as the secondary antibody. ×370

Electron microscopy

Thin sections of infected BF-2 cells revealed bullet-shaped virus particles (Fig. 2). Based on measurements of 10 particles the mean length of the virion was 175 ± 16.4 nm and the mean diameter was 52 ± 3 nm. Budding nucleocapsids were observed and virions were present in cytoplasmic vesicles. Areas of the cytoplasm associated with budding contained numerous ribosomes.

SDS-PAGE

Gradient-purified carpione virus gave rise to 5 major bands in silver-stained gels (Fig. 3). Based on the nomenclature of rhabdovirus polypeptides proposed by Wagner et al. (1972) these bands are called L, G, N, M_1 and M_2. The relative mobilities of the respective 5 polypeptides suggested that they had molecular weights of about 190, 70, 41, 28 and 23 kDa (data not

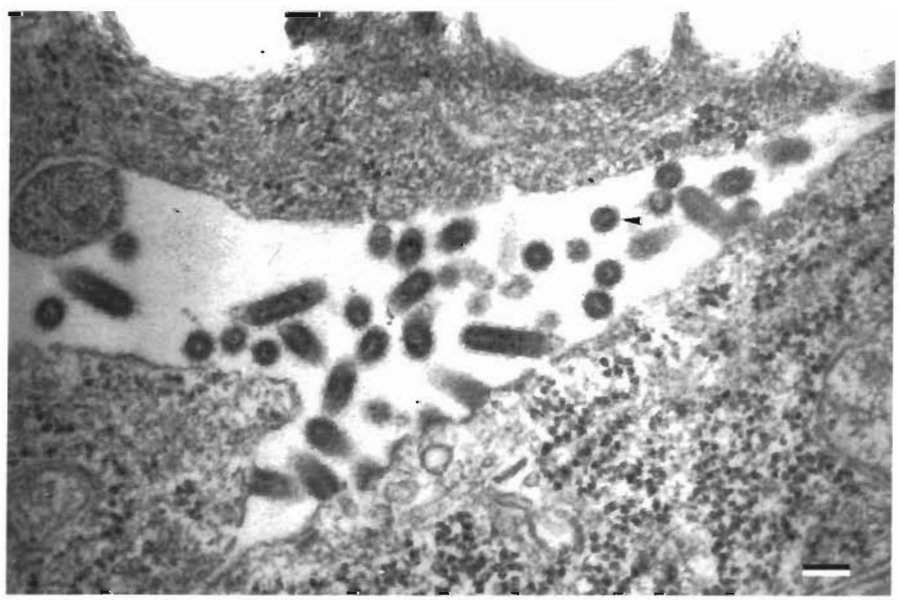

Fig. 2. Electron micrograph showing a thin section of a BF-2 cell infected with the carpione virus. Bullet-shaped particles within a cytoplasmic vesicle and budding nucleocapsids are seen. In the vesicle, cross sections of particles are visible (arrowhead). Virions measure about 175 × 52 nm. Note the presence of ribosomes in the vicinity of the budding site. Scale bar = 100 nm

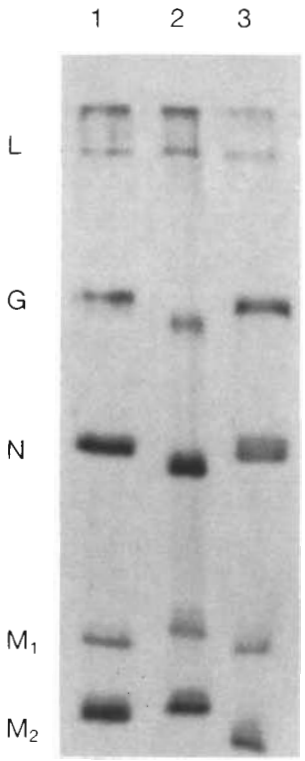

Fig. 3. SDS-PAGE gel stained with silver to reveal the 5 structural proteins (L, G, N, M_1, and M_2) of: (1) VHSV, (2) IHNV, and (3) carpione virus

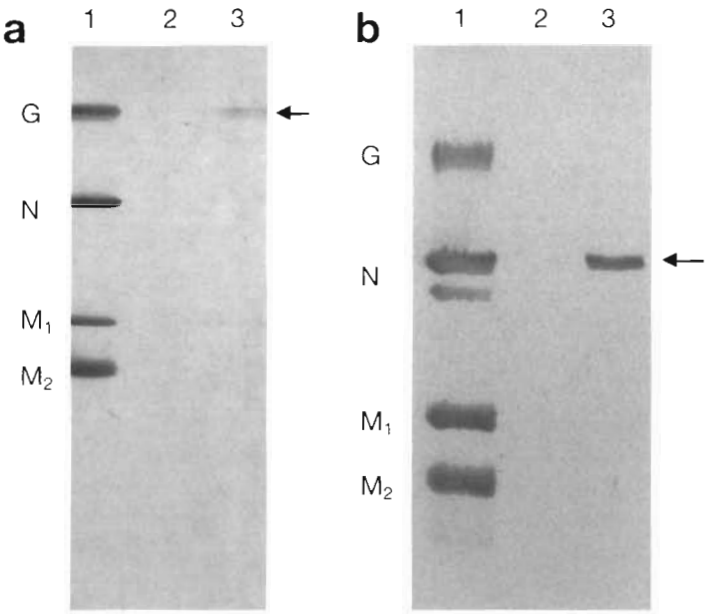

Fig. 4. Western blotting with (a) K59 (rabbit Ig to VHSV) and (b) a pool of MAbs to VHSV, on the structural proteins of: (1) VHSV, (2) IHNV, and (3) carpione virus. Note reactions between K59 and carpione virus G protein (arrow, a) and between the MAb pool and carpione N protein (arrow, b)

shown) and were thus very similar to those of VHSV (Lorenzen et al. 1988). Only the M_2 protein of the carpione virus appeared to differ slightly in that it was about 1 kDa smaller than the M_2 protein of VHSV

Immunoblotting

An immunoblot developed using antiserum (K59) to VHSV is shown in Fig 4a. The rabbit anti-VHSV serum reacted with the G, N, M_1 and M_2 proteins of VHSV as expected and weakly with the G protein of the carpione virus. The same antiserum also gave a weak reaction with the M_2 polypeptide of IHNV. Polyclonal antiserum to IHNV did not react with the carpione virus polypeptides (not shown). Strong staining of the carpione N protein was observed with MAb IP5B11, which recognizes the VHSV N protein. No staining was observed with MAbs recognizing the G, M_1 and M_2 polypeptides of VHSV (Fig. 4b).

Growth and stability of the virus

Replication of the carpione virus in different fish cell lines is summarized in Table 1. The highest titres of virus were produced using BF-2 and FHM cells. The temperature optimum for virus replication in EPC cells

Table 1 Replication of the carpione virus in selected fish cell lines.

Cell line	Growth temp. (°C)	Virus titre[a] (PFU ml^{-1})
BF-2	25	2×10^7
CHSE-214	20	3×10^4
EPC	25	8×10^6
FHM	20	2×10^7
OFC	20	3×10^5
PG	20	< detection limit
RTG-2	20	4×10^6
SBL	20	2×10^6

[a]Titre determined on samples taken after 7 d incubation

was 20°C. The virus was considered to be heat sensitive as it was completely inactivated following a 15 min exposure to 56°C (Table 2), but the virus appeared to be relatively stable at temperatures of 37°C and below.

Infection trials

All groups of carpione fry exposed to carpione virus or to VHSV developed typical signs of rhabdoviral infection, i.e. exophthalmia, dark pigmentation and petechial haemorrhages in muscles and viscera

Table 2. Titres in EPC cells of the carpione virus following exposure for 15 min at 5 selected temperatures

Temperature (°C)	Virus titre (PFU ml^{-1})
4	1.3×10^6
30	1.3×10^6
37	1.1×10^6
42	4.1×10^5
56	< detection limit

(Fig. 5). High mortality occurred among groups of infected carpione beginning at Day 5 (carpione virus) and Day 7 (VHSV) post-infection. The experiment was stopped after 18 d. Most of the fish from this experiment, with the exception of a few taken for histological examination and for IF, were submitted to virological examination using EPC cells. Unfortunately, mortalities were also observed in the carpione control groups (Table 3), but virus was isolated only from experimentally infected fish. The cause of the mortality in the control groups was not determined but it is suspected that problems with nutrition and management were involved. In bath experiments, rainbow trout fry were not susceptible to carpione virus at passage level 8, nor to cell culture passage 1 of virus reisolated after passage in fish (Table 3). In contrast, i.p. injection of carpione virus into rainbow trout fry at a concentration of 10^7 $TCID_{50}$ per fish induced a 30% mortality while injection of 10^5 $TCID_{50}$ per fish produced only a 12% mortality (results not shown). Specimens of symptomatic fish from carpione virus challenge experiments that were processed for indirect IF using antiserum to carpione virus were positive for carpione virus antigen, whereas control fish and fish infected with VHSV were negative.

DISCUSSION

The rhabdovirus isolated from carpione fry in Italy appears to be a member of the *Lyssavirus* genus of the Rhabdoviridae. The estimated molecular weights of the structural polypeptides of the virus were very similar to those reported for VHSV.

The carpione virus was antigenically related to VHSV as shown by IF and immunoblotting. MAbs to both the N and G proteins of VHSV reacted with the carpione virus in IF, whereas only the MAb to the N protein reacted positively in immunoblotting. Together, the findings clearly show that the carpione and VHS viruses share antigenic determinants on the N and G proteins. The moderate, weak, or negative reaction observed with 3 rabbit polyclonal antisera to VHSV was somewhat surprising, considering the reactions observed with the MAbs, because these sera reacted strongly with the 3 isolates of VHSV in both IF and immunoblotting. It has previously been shown that both polyclonal antibodies and pools of monoclonal antibodies made against VHSV reference strain F1 react strongly in IF and immunoblotting with the polypeptides of virus isolates representing all the currently known serological groups of VHSV (Olesen et al. 1993, Olesen & Jørgensen unpubl. data). One possi-

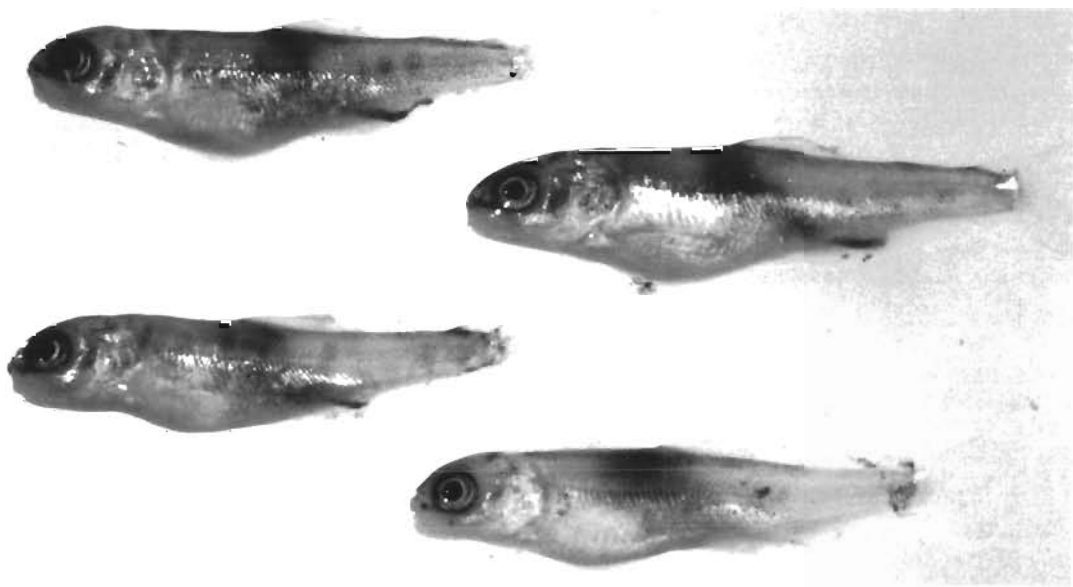

Fig. 5. *Salmo trutta carpio*. Darkening of the body surface and presence of haemorrhagic lesions in carpione fry experimentally infected with the carpione virus

Table 3. Results of bath infection of rainbow trout *Oncorhynchus mykiss* and carpione *Salmo trutta carpio* fry with VHSV and carpione virus

Species	n	Size (g)	Virus	Challenge* ($TCID_{50}$ ml^{-1})	Mortality %
Carpione	50	0.1	Carpione 583	2×10^6 (a)	86
	50	0.1	Carpione 583	2×10^6 (a)	92
	50	0.1	Carpione 583	2×10^6 (a)	74
	50	0.1	VHSV (Saone)	4×10^3 (a)	66
	50	0.1	VHSV (Saone)	4×10^3 (a)	72
	50	0.1	Control	– (a)	52
	50	0.1	Control	– (a)	26
Rainbow trout	50	2.0	Carpione 583	1×10^5 (a)	0
	50	2.0	Carpione 583	1×10^5 (a)	0
	50	2.0	VHSV (Saone)	1×10^5 (a)	100
	50	1.25	Carpione 583	1×10^5 (b)	0
	50	1.25	Carpione 583	1×10^5 (b)	0
	50	1.25	VHSV (3592B)	1×10^5 i(b)	98
	50	1.25	VHSV (3592B)	1×10^5 (b)	98
	50	1.25	Control	– (b)	0
	50	1.25	Control	– (b)	0

* a = 10°C, b = 15°C

ble reason for the relatively weak staining of carpione antigens by rabbit antisera against VHSV is a lower average affinity of the polyclonal antibodies than of the MAbs. The difference in affinity might have been particularly obvious when the antibodies were reacted with antigens which were related but not completely identical with the homologous ones.

In the immunoblotting, the occurrence of 2 stained protein bands at 41 kDa (the N protein) with the MAbs (Fig. 4b) most likely was due to proteolysis of the N protein (Lorenzen 1992). The slight reaction observed between rabbit anti-VHSV and the M_2 protein of IHNV may indicate that IHNV and VHSV possess related epitopes on the M_2 proteins, as has also been suggested by studies using certain MAbs (Dr J.-P. Enzmann, Tübingen, Germany, pers. comm.; author's unpubl. results).

Despite the fact that VHSV and the carpione virus contained an unknown number of related antigenic sites, it was concluded that the carpione virus was clearly distinct from VHSV and the other fish rhabdoviruses tested. The relatively weak reaction with polyclonal antibodies to VHSV suggested that the serological relationship between the 2 viruses is somewhat distant. In another study (Jørgensen et al. 1993), the carpione virus was found to be serologically related only with VHSV among the 12 currently available fish rhabdoviruses, including the recent isolates from European lake trout in Finland (Koski et al. 1992) and pike in Denmark (Jørgensen et al. 1993).

On the basis of the compiled serological data we conclude that the virus from carpione brown trout is a distinct, previously undescribed fish rhabdovirus. As a preliminary name we propose 'carpione rhabdovirus'.

Based upon the results of the infection trials, it seemed likely that the carpione virus was responsible for the losses recorded in the carpione fry. Unfortunately, only very small carpione fry (0.1 g) were available for experimental infection, and thus it cannot be excluded that the pathogenicity of the virus might be restricted to fry of that size. The virus did not infect rainbow trout fry by bath infection and only injection of a large number of infective particles induced mortality in fish of this species. The lack of pathogenicity of the virus for rainbow trout apparently was not due to cell culture adaptation, since carpione virus isolated from moribund experimental rainbow trout and passed only once in cell cultures was also nonpathogenic.

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