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Biophysical and serological characterization of rhabdovirus 903/87 isolated from European lake trout *Salmo trutta lacustris*

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ABSTRACT: Rhabdovirus 903/87 isolated from European lake trout *Salmo trutta lacustris* (L.) in Finland was compared serologically with 11 previously reported fish rhabdoviruses. By immunofluorescence, the virus was not related to 9 of these rhabdoviruses; however, the lake trout rhabdovirus 903/87 was serologically related to perch rhabdovirus and pike rhabdovirus DK 5533. Electron microscopic examination showed the virus to be bullet-shaped, with dimensions of 156 × 65 nm. SDS-PAGE analyses of the structural proteins indicated that the virus belonged to the *Vesiculovirus* genus of the family Rhabdoviridae, but that the relative mobilities of the glycoprotein and the phosphoprotein (also termed N_s) could be used to differentiate the 903/87 isolate from the perch rhabdovirus and the pike rhabdovirus DK 5533. The rhabdovirus 903/87 appears to be a new pathogen of fish and we suggest that it be renamed the European lake trout rhabdovirus.

KEY WORDS: Virus · Rhabdovirus · *Vesiculovirus* Fish

INTRODUCTION

A rhabdovirus was recently isolated from diseased European lake trout *Salmo trutta lacustris* (L.) in a fish farm in northern Finland. The virus has been preliminarily characterized by Koski et al. (1992) under the designation 'virus 903/87'. In neutralization tests and enzyme-linked immunosorbent assays (ELISA) the virus showed no antigenic relatedness to viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), spring viraemia of carp virus (SVCV), pike fry rhabdovirus (PFR), European eel rhabdovirus (EVEX) or perch rhabdovirus (Koski et al. 1992).

The aim of the present work was to further characterize this new virus by comparing it with the above-mentioned viruses and 5 other previously described fish rhabdoviruses using immunofluorescence tests, and to determine some biophysical properties of the virus.

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MATERIALS AND METHODS

Viruses and antisera. The rhabdoviruses included in this study were VHSV, IHNV, hirame rhabdovirus (HRV), snakehead rhabdovirus (SHRV), carpione brown trout rhabdovirus, SVCV, PFR, EVEX, perch rhabdovirus, ulcerative disease rhabdovirus (UDRV), pike rhabdovirus DK 5533, and lake trout rhabdovirus 903/87 (Table 1). The viruses were propagated in cell cultures as described below and those used for immunization of rabbits were purified by ultracentrifugation on 15 to 45 % linear sucrose gradients for 2.5 h at 27 000 rpm (100 000 × *g*) in a Beckman SW 28 rotor. Rabbit antisera to the viruses were prepared as previously described (Jørgensen et al. 1989, Olesen et al. 1991). Antisera to some of the viruses were obtained from other laboratories (Table 1). Antisera which showed background staining in immunofluorescence were purified using affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) and absorbed with cell culture cells as described by Jørgensen et al. (1993).

Fish cell lines and medium. Monolayer cultures of bluegill fry cells, BF-2 (Wolf et al. 1966) and epithe-

Table 1 Virus isolates and antisera used

Virus name	Virus strain	Strain isolated from	Supplier of virus	Supplier of serum	Reference
Viral haemorrhagic septicaemia virus	VHSV, F1	Rainbow trout *Oncorhynchus mykiss*	NVL[a]	NVL	Jensen (1965)
Infectious haematopoietic necrosis virus	IHNV, Coleman	Chinook salmon *Oncorhynchus tshawytscha*	Wolf, K.	NVL	Amend et al. (1969)
Hirame rhabdovirus	HRV, 8401 H	Japanese flounder *Paralichthys olivaceus*	Kimura, T.	Kimura, T	Kimura et al. (1986)
Snakehead rhabdovirus	SHRV	Snakehead *Ophiocephalus striatus*	Wattanavijarn, W.	NVL	Ahne et al. (1988)
Carpione brown trout rhabdovirus	583	Carpione brown trout *Salmo trutta carpione*	Bovo, G.	Bovo, G.	Bovo et al. (unpubl.)
Spring viraemia of carp virus	SVCV, 56/80	Common carp *Cyprinus carpio*	Fijan, N.	NVL	Fijan et al. (1971)
Pike fry rhabdovirus	PFR	Northern pike *Esox lucius*	de Kinkelin, P.	NVL	de Kinkelin et al. (1973)
Eel rhabdovirus	EVEX	European eel *Anguilla anguilla*	Castric, J.	NVL	Castric et al. (1984)
Perch rhabdovirus	No strain designation	Perch *Perca fluviatilis*	de Kinkelin, P.	NVL	Dorson et al. (1984)
Ulcerative disease rhabdovirus	UDRV 19	Snakehead *Ophiocephalus striatus*	Hill, B.	Hill, B.	Frerichs et al. (1986)
Pike rhabdovirus	DK 5533	Northern pike *Esox lucius*	NVL	NVL	Jørgensen et al. (1993)
Lake trout rhabdovirus	903/87	European lake trout *Salmo trutta lacustris*	Neuvonen, E.	NVL	Koski et al. (1992)

[a] NVL = National Veterinary Laboratory

lioma papulosum cyprini cells, EPC (Fijan et al. 1983) were grown at 15°C in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin (100 IU ml^{-1}) and dihydrostreptomycin (125 µg ml^{-1}). Medium for cells grown in open systems (24- or 96-well plates and petri dishes) was buffered with Tris-HCl buffer while that used for cells grown in tissue culture flasks was buffered with sodium bicarbonate.

Electron microscopy. Monolayer cultures of BF-2 cells were infected with trout rhabdovirus 903/87 at a low multiplicity of infection (MOI 0.1) and incubated at 15°C. After 24 h and 48 h incubation, cells were fixed with 2.5% glutaraldehyde in 0.07 M phosphate-buffered saline (PBS) (pH 7.6). The cells were postfixed with 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, dehydrated in acetone series and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL JEM-100SX electron microscope. A culture of noninfected BF-2 cells was included as a control.

Immunofluorescence tests. Monolayer cultures of EPC cells were grown on 9 × 22 mm coverglasses and infected with each of the 12 rhabdoviruses. At 24 h after infection (or 96 h after infection in the case of UDRV), the cultures were rinsed twice in MEM without fetal bovine serum and fixed for 10 min in 80% aqueous acetone. After air-drying, the coverglasses were overlaid with 30 µl of homologous or heterologous rabbit antiserum at predetermined dilutions (1:25 to 1:800) in MEM containing 10% fetal bovine serum. The coverglasses were incubated for 30 min at 37°C in a humid chamber, rinsed twice for 10 min in 0.9% NaCl with 0.05% Tween 20, rinsed once for 10 min in deionized water with 0.05% Tween 20, air-dried, and overlaid with 30 µl of a 1:100 dilution of tetramethyl rhodamine isothiocyanate (TRITC) labelled swine antibody to rabbit Ig (Dakopatts, Copenhagen, Denmark). After incubation for 30 min at 37°C, the rinse cycles were repeated and the coverglasses were mounted on microscope slides with cell side down in a drop of buffered glycerol, pH 7.6, and examined with an Olympus Vanox epifluorescence microscope equipped with a 200 W mercury lamp and 40× and 100× fluorescence oil objectives.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method described by Laemmli (1970) using 0.75 mm slab gels in a vertical elec-

trophoresis system. Purified viruses were mixed with tracking dye (bromphenol blue) and sample buffer (0.01 M Tris-HCl, pH 6.8; 0.001 M EDTA; 10% SDS; 5% 2-mercaptoethanol) and heated to 100°C for 3 min. Electrophoresis of virus proteins was performed using 13% acrylamide separating gels overlaid with 3.5% acrylamide stacking gels. After electrophoretic separation the gels were stained with silver nitrate as described by Morrissey (1981). The molecular weights of the viral structural proteins were estimated by comparing their relative mobilities with those of molecular weight protein standards (Pharmacia, Uppsala, Sweden).

RESULTS

Cytopathic effect

Cytopathic effects, consisting of granular, rounded and eventually lysed cells, were typically observed 2 to 3 d after infection of BF-2 cells with the new rhabdovirus 903/87. The cytopathic effects progressed over a 3 to 4 d period until the monolayer was destroyed. The virus 903/87 did not induce cytopathic effects in EPC cells, although multiplication occurred, as evidenced by the immunofluorescence results.

Electron microscopy

Bullet-shaped virus particles were seen in electron micrographs of BF-2 cultures infected with the lake trout rhabdovirus 903/87 (Fig. 1). No virus replication was seen in cells fixed 24 h after infection, whereas numerous virus-releasing cells were seen in samples fixed 48 h after infection. Based on measurements of 20 particles, the mean length of the virion was 156 ± 18 nm and the mean diameter was 65 ± 4 nm. The minimum and maximum lengths observed were 140 and 210 nm, and the minimum and maximum diameters were 60 and 70 nm.

Immunofluorescence tests

Using homologous antiserum, intense granular fluorescence was seen in the cytoplasm of EPC cells infected with the lake trout rhabdovirus 903/87 (Fig. 2). The same antiserum produced only a weak fluorescence in the cytoplasm of EPC cells infected with perch rhabdovirus (Fig. 3). No fluorescence was seen in EPC cells infected with pike isolate DK 5533 or with any of the other viruses. Cells infected with virus 903/87 were weakly fluorescent following incubation with antisera to perch rhabdovirus or to the pike isolate DK 5533

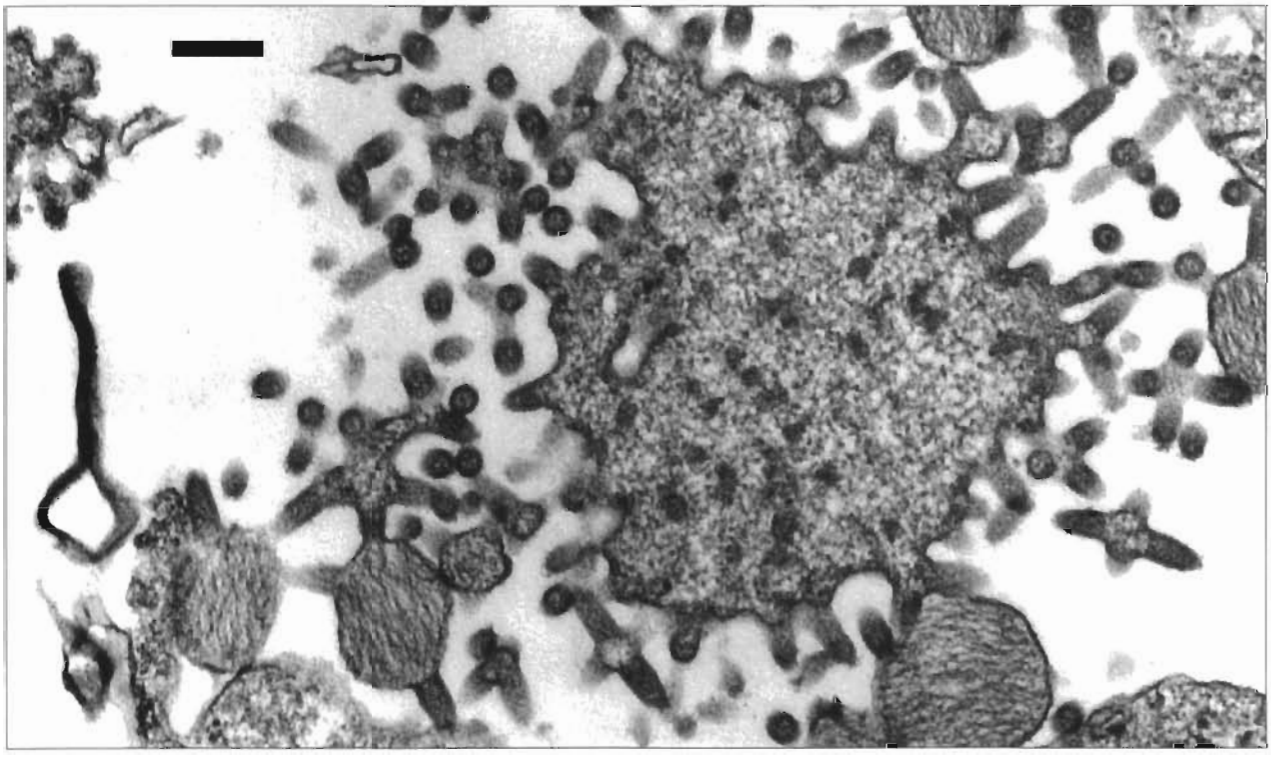

Fig. 1. Electron micrograph showing bullet-shaped virus particles in BF-2 cells infected with lake trout rhabdovirus 903/87. Stained with uranyl acetate and lead citrate (scale bar = 200 nm)

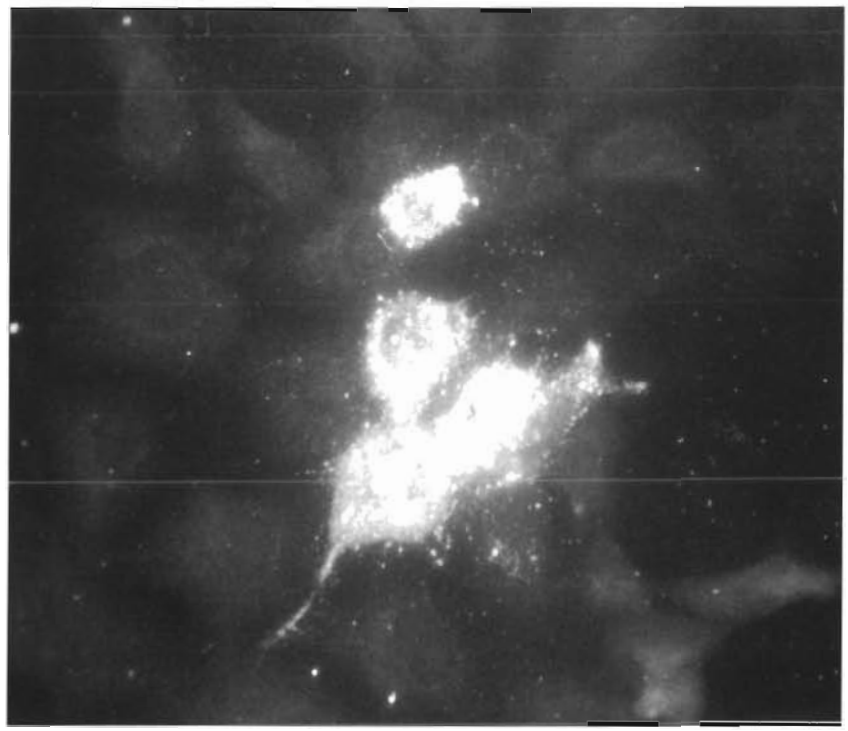

Fig. 2. Immunofluorescence micrograph showing the staining of lake trout rhabdovirus 903/87-infected EPC cells with rabbit antiserum to virus 903/87 as the primary antibody and TRITC-labelled swine antibody to rabbit Ig as the secondary antibody (×370)

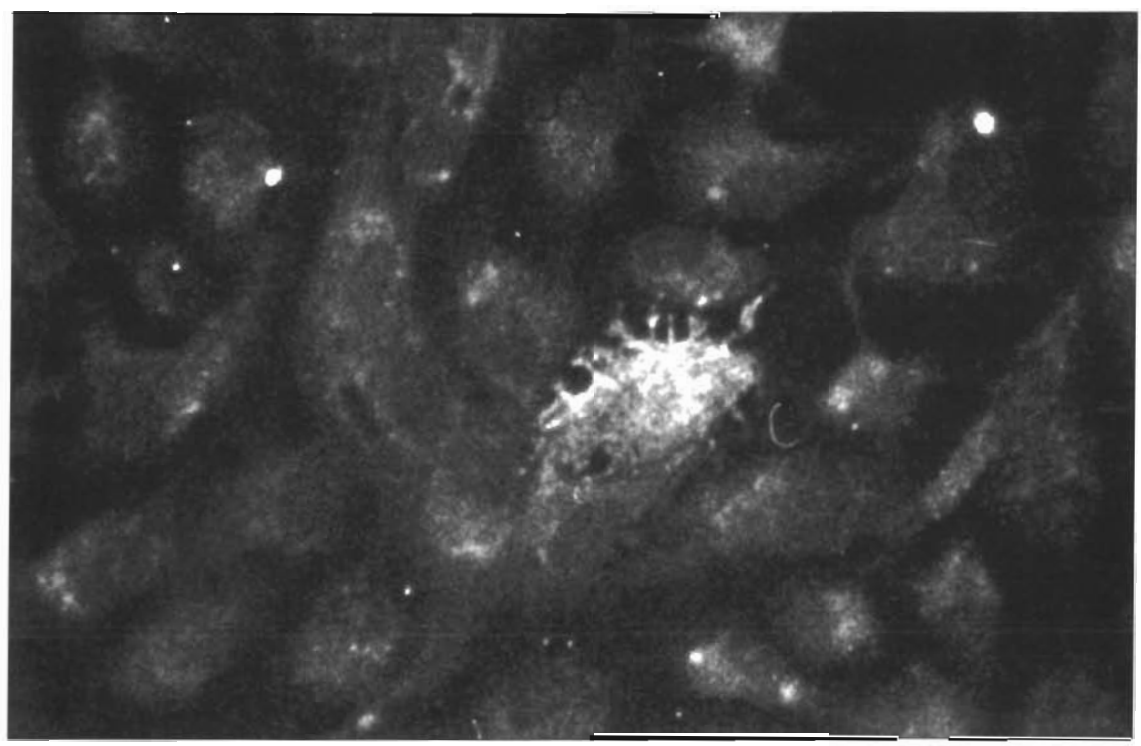

Fig. 3. Immunofluorescence micrograph showing the cross-reaction staining of a perch rhabdovirus-infected EPC cell with rabbit antiserum to virus 903/87 as the primary antibody and TRITC-labelled swine antibody to rabbit Ig as the secondary antibody (×370)

Table 2. Immunofluorescence results with rhabdovirus 903/87 and 2 other fish rhabdoviruses using homologous and heterologous antisera. Reactions are 0: no reaction; +: weak; ++: moderate; +++: strong

Virus	Antiserum		
	903/87	DK 5533	Perch rhabdovirus
Rhabdovirus 903/87	+++	+	+
Pike rhabdovirus DK 5533	0	+++	+++
Perch rhabdovirus	+	+++	+++
Uninfected EPC cells	0	0	0

(Table 2). These reactions rated '+++' or '+' in Table 2 were the only positive reactions observed in immunofluorescence tests using antiserum to virus 903/87 against the 12 fish rhabdoviruses, or in tests using antisera to the same 12 rhabdoviruses against virus 903/87.

SDS-PAGE

Comparative analysis of virus isolate 903/87, pike isolate DK 5533 and perch rhabdovirus by SDS-PAGE indicated that they all belong to the *Vesiculovirus* genus of the family Rhabdoviridae (McAllister & Wagner 1975) (Fig. 4). Small differences were evident among the relative mobilities of the glycoprotein (G) and the phosphoprotein (labeled N_s) of the 3 isolates, whereas the polymerase (L), the nucleoprotein (N) and the matrix protein (M) of the 3 viruses showed identical mobilities (Table 3).

Table 3. Estimated molecular weights (in kilodaltons) of structural proteins of rhabdovirus 903/87 and 2 other fish rhabdoviruses belonging to the genus *Vesiculovirus*. Molecular weights estimated from the electropherogram shown in Fig. 4

Virus	Viral proteins				
	L	G	N_s	N	M
Rhabdovirus 903/87	200	61	51	44	28
Pike rhabdovirus DK-5533	200	60	49	44	28
Perch rhabdovirus	200	62	47	44	28

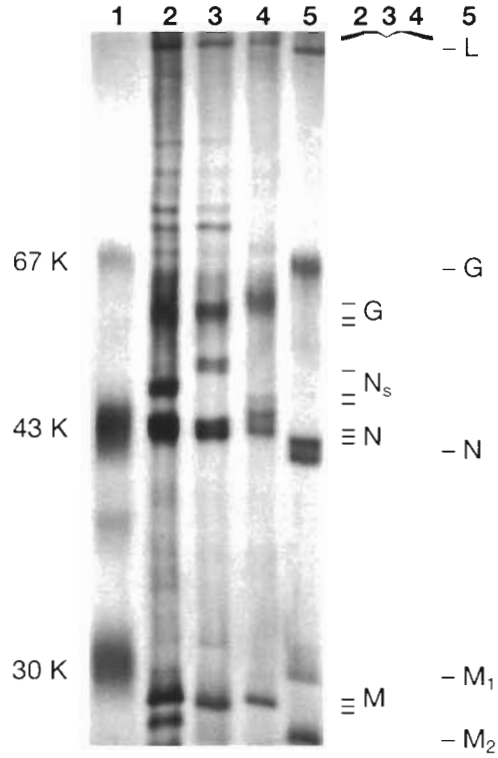

Fig. 4. Electropherogram of purified virus preparations in SDS-PAGE. Lane 1: molecular weight markers (molecular weights given in kilodaltons); Lane 2: pike rhabdovirus DK 5533; Lane 3: lake trout rhabdovirus 903/87; Lane 4: perch rhabdovirus; Lane 5: VHS virus. The localization of the structural proteins G, N_s, N and M (Lanes 2, 3 and 4) and G, N, M_1 and M_2 (Lane 5), respectively, is indicated. L: polymerase. The virus isolates in Lanes 2, 3 and 4 belong to the genus *Vesiculovirus*, while the virus isolate in Lane 5 belongs to the genus *Lyssavirus* of rhabdoviruses

DISCUSSION

The cytopathic effects and the morphology of virus 903/87 were typical of those of a rhabdovirus. The dimensions of the virus were measured to 156 × 65 nm. Based on an average of 15 negatively stained virus particles, Koski et al. (1992) reported the size of the rhabdovirus 903/87 as approximately 170 × 102 nm. However, because different methods were used for harvesting and purification of the virus and fixation for electron microscopy, the sizes of the virus 903/87 from the 2 studies are not directly comparable.

Koski et al. (1992) reported that in ELISA and serum neutralization tests virus 903/87 appeared to be distinct from VHSV, IHNV, PFR, SVCV, EVEX and perch rhabdovirus, and they concluded that virus 903/87 was a new fish rhabdovirus. Our results confirmed that virus 903/87 was not serologically related to VHSV, IHNV, PFR, SVCV and EVEX nor to an additional 4 rhabdoviruses, i.e. HRV, SHRV, UDRV and carpione

brown trout rhabdovirus. However, in contrast to the ELISA results obtained by Koski et al. (1992), our data show that the virus 903/87 shares a limited number of antigenic determinants with perch rhabdovirus and also with the recently isolated pike rhabdovirus DK 5533, not examined by Koski et al. (1992). The discrepancy between the results may be due to a higher sensitivity of our immunofluorescence technique compared to the ELISA used by Koski et al. (1992), or due to differences in the cross-reactivity of the antisera. It has been shown that different immunization protocols and different animals may give sera with very different cross-reactivities (Jørgensen 1972, Jørgensen et al. 1989).

The relative mobilities of the virus 903/87, pike rhabdovirus DK 5533 and perch rhabdovirus M and N proteins were very similar, whereas those of G and N_s were somewhat different (Fig. 4 and Table 3). The relative mobilities of the proteins G and N_s could be used to differentiate the virus 903/87 from the perch rhabdovirus and the pike rhabdovirus DK 5533.

The immunofluorescence test data shown in Table 2 indicate that pike rhabdovirus DK 5533 and perch rhabdovirus are more related to each other than to rhabdovirus 903/87. The strong immunofluorescence cross-reactions between pike rhabdovirus DK 5533 and perch rhabdovirus are in agreement with previous findings of Jørgensen et al. (1993). However, more detailed studies of the virus genomes, as well as additional serological examination using techniques such as Western blotting, will be required to further characterize the relationship of rhabdovirus 903/87, pike rhabdovirus DK 5533 and perch rhabdovirus, and to determine to what extent the 3 rhabdoviruses share antigens on their structural proteins.

Since number designations are not overly informative, we suggest that virus 903/87 be renamed the European lake trout rhabdovirus.

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LITERATURE CITED

Ahne, W., Jørgensen, P. E. V., Olesen, N. J., Wattanavijarn, W. (1988). Serological examination of a rhabdovirus isolated from snakehead (*Ophiocephalus striatus*) in Thailand with ulcerative syndrome. J. appl. Ichthyol. 4: 194–196

Amend, D. F., Yasutake, W. T., Mead, R. W. (1969). A hematopoietic virus disease of rainbow trout and sockeye salmon. Trans. Am. Fish. Soc. 98: 796–804

Castric, J., Rasschaert, D., Bernard, J. (1984). Evidence of lyssaviruses among rhabdovirus isolates from European eel (*Anguilla anguilla*). Annls Virol. (Inst. Pasteur) 135E: 35–55

de Kinkelin, P., Galimard, B., Bootsma, P. (1973). Isolation and identification of the causative agent of 'red disease' of pike (*Esox lucius* L. 1766). Nature 241: 465–467

Dorson, M., Torchy, S., Chilmonczyk, S., de Kinkelin, P., Michel, C. (1984). A new rhabdovirus pathogenic for perch, *Perca fluviatilis* L.: isolation and preliminary study. J. Fish Dis. 7: 241–245

Fijan, N., Petrinec, Z., Sulimanovic, D., Zwillenberg, L. O. (1971). Isolation of the viral causative agent from the acute form of infectious dropsy of carp. Vet. Arhiv Zagreb 41: 125–135

Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinic, D., Zwillenberg, L. O., Chilmonczyk, S., Vautherot, J. F., de Kinkelin, P. (1983). Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp (*Cyprinus carpio*). Annls Virol. (Inst. Pasteur) 134: 207–220

Frerichs, G. N., Millar, S. D., Roberts, R. J. (1986). Ulcerative rhabdovirus in fish in Southeast Asia. Nature 322: 216

Jensen, M. H. (1965). Research on the virus of Egtved disease. Ann. N.Y. Acad. Sci. 126: 422–426

Jørgensen, P. E. V. (1972). Freund's adjuvants: their influence on the specificity of viral antisera. Acta pathol. microbiol. scand., Sect. B 80: 931–933

Jørgensen, P. E. V., Olesen, N. J., Ahne, W., Lorenzen, N. (1989). SVCV and PFR viruses: serological examination of 22 isolates indicates close relationship between the two fish rhabdoviruses. In: Ahne, W., Kurstak, E. (eds.) Viruses of lower vertebrates. Springer, Berlin, p. 349–366

Jørgensen, P. E. V., Olesen, N. J., Ahne, W., Wahli, T., Meier, W. (1993). Isolation of a previously undescribed rhabdovirus from pike (*Esox lucius*). Dis. aquat. Org. 16: 171–179

Kimura, T., Yoshimitzu, M., Gorie, S. (1986). A new rhabdovirus isolated in Japan from cultured hirame (Japanese flounder) (*Paralichthys olivaceus*) and ayu (*Plecoglossus altivelis*). Dis. aquat. Org. 1: 209–217

Koski, P., Hill, B. J., Way, K., Neuvonen, E., Rintamäki, P. (1992). A rhabdovirus isolated from brown trout (*Salmo trutta* m. *lacustris* [L]) with lesions in parenchymatous organs. Bull. Eur. Ass. Fish Pathol. 12(5): 177–180

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685

McAllister, P. E., Wagner, R. R. (1975). Structural proteins of two salmonid rhabdoviruses. J. Virol. 15: 733–738

Morrissey, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Analyt. Biochem. 117: 307–310

Olesen, N. J., Lorenzen, N., Jørgensen, P. E. V. (1991). Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). Dis. aquat. Org. 10: 31–38

Wolf, K., Gravell, M., Malsberger, R. G. (1966). Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. Science 151. 1004–1005

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