Vol. 89: 191–198, 2010
doi: 10.3354/dao02198

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published April 9

Evaluation of a polyclonal antibody for the detection and identification of ranaviruses from freshwater fish and amphibians

Katerina Cinkova, Stanislava Reschova, Pavel Kulich, Tomas Vesely*

Department of Bacteriology & Virology, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic

ABSTRACT: A rabbit polyclonal antibody (PAb) raised against European catfish virus (ECV; isolated from black bullhead *Ameiurus melas* in France) was produced and then evaluated using a panel of 9 ranavirus isolates collected from different lower vertebrate species originating from Australia, North and South America, Southeast Asia, and Europe. Using ranavirus-infected epithelioma papillosum cyprini (EPC) cell cultures, the specificity of the PAb was determined by Western blot, immunogold electron microscopy, and direct enzyme-linked immunosorbent assay (ELISA). Western blot analysis demonstrated that the PAb reacted strongly with a protein with a molecular weight corresponding to approximately 49 kDa. Immunogold electron microscopy provided direct evidence that the epitopes recognized by this PAb were located on the outer surface of virions. The PAb was used for the preparation of a peroxidase-labeled conjugate for the direct ELISA detection of ranaviruses in infected EPC cell cultures. The specificity of the conjugated PAb was tested using ranaviruses, some representative fish viruses of the genera *Rhabdovirus* and *Birnavirus*, and samples from various non-infected fish species. The PAb detected all tested ranaviruses except for 2 Santee-Cooper ranaviruses. The direct ELISA enabled the detection of ranavirus from a concentration of $10^{3.5}$ to $10^{3.8}$ $TCID_{50}$ ml^{-1} cell culture. The results of this study revealed that the rabbit PAb raised against ECV could be useful for the development of specific and standardized diagnostic assays for the detection of ranaviruses from freshwater fish and amphibians.

KEY WORDS: *Iridoviridae* · *Ranavirus* · Polyclonal antibody · ELISA · Freshwater fish · Ornamental fish · Amphibians

INTRODUCTION

Ranaviruses belonging to the genus *Ranavirus* in the family *Iridoviridae* contain a linear double-stranded DNA molecule (105 to 140 kbp in length) (Chinchar et al. 2009) within an icosahedral capsid surrounded by a lipid membrane. The virus particles range in size from 152 to 175 nm (Hyatt et al. 2000). Ranaviruses are well known as causative agents of systemic diseases in lower vertebrates including farmed food fish, ornamental fish, and wild fish (Langdon et al. 1986, Ahne et al. 1989, Pozet et al. 1992, Hedrick & McDowell 1995, Bigarre et al. 2008) as well as amphibians (Granoff et al. 1965, Speare & Smith 1992) and reptiles (Mao et al. 1997, Hyatt et al. 2002).

Many ranaviruses have been identified in association with considerable morbidity and mortality observed in wild and cultured fish as well as amphibian species (Chinchar 2002). Ranaviruses can be extremely virulent, and the World Organisation for Animal Health (OIE) recently added to its list 'Diseases notifiable to the OIE' epizootic haematopoietic necrosis (EHN) — a systemic iridoviral infection of finfish already infected with epizootic haematopoietic necrosis virus (EHNV) — and infection of amphibians with ranavirus — an infection of amphibians with any species of the genus *Ranavirus*, with the exception of EHNV and European catfish virus (ECV) (OIE 2008).

Several methods have been developed for the detection and diagnosis of these pathogenic viruses, including the isolation of virus in cell culture, detection of virions by electron microscopy, immunological methods such as enzyme-linked immunosorbent assay (ELISA),

*Corresponding author. Email: vesely@vri.cz

immunofluorescence assay, and immunohistochemistry (Hyatt et al. 1991, Steiner et al. 1991, Hedrick et al. 1992, Hengstberger et al. 1993, Whittington & Steiner 1993). These tests are not able to distinguish one particular virus from other fish and amphibian ranaviruses because they share conserved group-specific antigens. Polymerase chain reaction (PCR), combined with either restriction endonuclease analysis (Marsh et al. 2002) or sequence analysis, can differentiate ranaviruses from each other, except European sheatfish virus (ESV) from ECV, both of which belong to the group of ECV. ESV and ECV have been classified as separate ranavirus isolates on the basis of their different host species, sheatfish and catfish. However, on the basis of genetic studies (Mao et al. 1997, Ahne et al. 1998, Hyatt et al. 2000, Pallister et al. 2007), it has been suggested that ESV and ECV are likely to be 2 isolates of the same virus but that this is a different virus from EHNV.

In connection with a rapidly developing aquaculture industry, rapid, accurate, and simple methods for the detection and identification of viral pathogens are important tools that assist in reducing losses from disease outbreaks and in preventing the spread of viral diseases by trade in live aquatic animals/products. Thanks to its sensitivity and specificity, rapid processing of large numbers of samples, low cost, reagent stability, and ease of procedure, serology could be very helpful in screening some fish populations but it has not yet been validated for routine diagnosis. The aim of this study was to prepare polyclonal antibody (PAb) for the identification of ranaviruses from a variety of poikilothermic vertebrate hosts, isolated in cell cultures. The methods applied in the characterization of the antibody were: ELISA, Western blot, and immunogold electron microscopy.

MATERIALS AND METHODS

Viruses and cells. The panel of viral isolates used in the present study and their origin are listed in Table 1. Fish rhabdovirus isolates (infectious haematopoietic necrosis virus [IHNV], spring viraemia of carp virus [SVCV], and viral hemorrhagic septicemia virus

Table 1. Host and geographic origin of virus isolates used in the present study

Virus isolate	Abbreviation	Original host	Country of isolation	Source
Epizootic haematopoietic necrosis virus	EHNV[a]	Redfin perch *Perca fluviatilis*	Australia	Langdon et al. (1986)
European sheatfish virus	ESV[a]	Wels catfish *Silurus glanis*	Germany	Ahne et al. (1989)
European catfish virus	ECV[a]	Black bullhead *Ameiurus melas*	France	Pozet et al. (1992)
Bohle iridovirus	BIV[a]	Ornate burrowing frog *Limnodynastes ornatus*	Australia	Speare & Smith (1992)
Frog virus 3	FV3[a]	Leopard frog *Rana pipiens*	USA	Granoff et al. (1965)
Rana esculenta iridovirus[b]	REV[a]	Edible frog *Rana esculenta*	Italy	G. Bovo (pers. comm.)
Guppy virus 6	GV6[a]	Guppy *Poecilia reticulata*	USA[c]	Hedrick & McDowell (1995)
Doctor fish virus	DFV[a]	Bluestreak cleaner wrasse *Labroides dimidiatus*	USA[c]	Hedrick & McDowell (1995)
Pike-perch iridovirus	PPIV[a]	Pike-perch *Sander lucioperca*	Finland	Tapiovaara et al. (1998)
Infectious haematopoietic necrosis virus	IHNV	Rainbow trout *Oncorhynchus mykiss*	France	Hattenberger-Baudouy et al. (1989)
Spring viraemia of carp virus	SVCV	Common carp *Cyprinus carpio*	Czech Republic	Koutna et al. (2003)
Viral hemorrhagic septicemia virus	VHSV	Rainbow trout *Oncorhynchus mykiss*	Czech Republic	Reschova et al. (2008)
Infectious pancreatic necrosis virus	IPNV	Rainbow trout *Oncorhynchus mykiss*	Czech Republic	Reschova et al. (2008)

[a]Viruses were kindly provided by E. Ariel of the European Community Reference Laboratory for Fish Diseases, Århus, Denmark. [b]Isolate REV, named REV-Italy 282/I02, was obtained from moribund *Rana esculenta* toads (Bonferraro, Verona province, Italy) (G. Bovo pers. comm.). [c]Viruses were isolated from imported ornamental fish from Southeast Asia

[VHSV]) and a fish birnavirus isolate (infectious pancreatic necrosis virus [IPNV]) were used as negative controls. Epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983) grown in minimal essential medium with Earle's salts and L-glutamine (MEM; GIBCO) were used for virus propagation and titration. The infected cell cultures were incubated at 24°C for ranavirus isolates, and at 15°C for other isolates. After 3 to 7 d, when advanced cytopathic effect (CPE) was observed, the cell cultures were harvested and stored at –20°C until further examination.

Concentration of viruses. Virus-infected cell cultures with fully developed CPE were harvested, and cell debris was removed by centrifugation at 3000 × *g* for 15 min at 4°C (JOUAN CR 3i, T40 rotor). Viral suspension was concentrated by ultracentrifugation at 76 000 × *g* for 2 h at 10°C (Beckman Optima™ LE-80K, SW 55 Ti rotor). The virus pellet was resuspended in phosphate buffered saline (PBS) to obtain a 100× concentrate of the original volume. The concentrated virus was used as antigen for immunization, in immunogold electron microscopy, and in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The non-infected EPC cells were processed in the same way to obtain a negative antigen for SDS-PAGE.

Preparation of polyclonal antibody to ECV. The concentrated ECV suspension, containing 80 µg ml^{-1} protein, was emulsified in an equal volume of Freund's complete adjuvant (FCA; for 1st inoculation) or Freund's incomplete adjuvant (FIA; for 2nd and 3rd inoculation). A New Zealand white rabbit aged 4 mo was injected subcutaneously with 40 µg of virus protein. Booster immunizations were given to the rabbit with 40 µg of virus protein in FIA 2 times intramuscularly at 21 d intervals. A final boost of 40 µg of virus protein in PBS (4th inoculation) was given intravenously to the rabbit after 21 d. Five d after the 4th inoculation, the rabbit was bled. Immunoglobin (IgG) fraction was separated from the immune serum by threefold precipitation with ammonium sulphate to 33% saturation and then centrifuged at 3000 × *g* for 20 min at 15°C (Jouan CR 3i, T40 rotor). To remove the non-specific antibodies, which cross-react with the antigens from fish cells, the IgG was purified by affinity chromatography using immunosorbents (CNBr Sepharose 4b; Pharmacia) with bound proteins from homogenized non-infected EPC cell cultures and fetal bovine serum (FBS) according to Rodak et al. (1993).

SDS-PAGE and Western blot analysis. Protein analysis of the concentrated ranavirus isolates was carried out by SDS-PAGE under conditions similar to those of Laemmli (1970) and Vesely et al. (2004). After electrophoretic separation, the proteins were stained with silver nitrate (Moeremans et al. 1985) or transferred onto a nitrocellulose membrane (pore size 0.45 µm; Bio-Rad) for Western blot analysis (Towbin et al. 1979). The blotted membrane was rinsed with distilled water and then incubated at room temperature (RT) for 1 h in PBS containing 0.1% Tween-20 and 1% lactalbumin hydrolysate (PBST-LAH), pH 7.2, to block unoccupied sites. Thereafter, the membrane was incubated for 1 h at RT with PAb raised against ECV (RAECV; 7.4 mg ml^{-1}) diluted 1:1000 in PBST-LAH and washed 3 times with PBST for 3 min. Secondary incubation was carried out with swine anti-rabbit IgG-horseradish peroxidase conjugate (SwARIgG-HRP; DAKO) diluted 1:1000 in PBST-LAH for 1 h at RT, and the membrane was washed again 3 times with PBST. The immunoreactive bands were visualized using a substrate 3-amino-9-ethylcarbazole (AEC; Sigma). After electrophoretic separation and blotting, the lane with standards of relative molecular weight (MW) was cut out from the nitrocellulose replica and then subsequently stained with colloid gold (Moeremans et al. 1985).

Electron microscopy. ***Negative staining:*** The concentrated viruses were adsorbed onto Formvar-coated, carbon-stabilized grids and then negatively stained with 2% NH_4MoO_4 and examined using a Philips 208 electron microscope Morgagni (FEI) at 18000× magnification and an accelerating voltage of 90 kV.

Immunogold staining: Colloidal gold with an average diameter of 20 nm was prepared from 0.02% gold trichloride (Sigma) with 1% sodium citrate as described by Gupta et al. (1992). The pH of the colloidal gold solution was adjusted to 6.5 with 1% potassium carbonate. To 1 ml of colloidal gold solution, 10 µl of PAb RAECV (1 mg ml^{-1}) or 10 µl of polyclonal rabbit antibody specific for IHNV (PAb RAIHNV; 1 mg ml^{-1}) as a negative control was added. The mixture was stirred for 20 min at RT and then centrifuged twice for 20 min at 4000 × *g* (Jouan CR 3i, T40 rotor). The pellet was resuspended in the remaining 50 µl of supernatant. The optimal amount (2 to 5 µl) of the antibody-colloidal gold conjugate was mixed with concentrated ranavirus particles so that the separate virions were abundantly coated with colloidal gold particles. The samples were adsorbed onto Formvar-coated, carbon-stabilized grids and then negatively stained and examined using an electron microscope. The antibody-colloidal gold conjugate was mixed with concentrated SVCV particles in the same way to obtain another negative control.

Preparation of peroxidase conjugate. The purified PAb RAECV was labelled with horseradish peroxidase (HRP; Sigma) using the oxidation method described by Farr & Nakane (1981). The produced conjugate (RAECV-HRP) was tested by a direct antigen-capture ELISA method.

Antigen-capture ELISA. Microtitre plates (GAMA) were coated with the PAb RAECV (7.4 mg ml^{-1}) dilu-

ted 1:2000 in carbonate-bicarbonate coating buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed 3 times in PBST. The culture medium of virus-infected cells for each isolate was then added to the wells and the plate incubated for 1 h at 37°C. After washing, the plates were incubated with the RAECV-HRP conjugate diluted 1:4000 for 1 h at 37°C. The enzymatic reaction was visualized by 3,3´,5,5´-tetramethylbenzidine substrate solution (TMB; TEST-LINE) and stopped by the addition of 1M H_2SO_4. The optical densities (OD) were measured at a wavelength of 450 nm using a spectrophotometer (SLT Spektra; Schoeller). Samples were examined either in the basic dilution (1:2) or in a series of 2-fold dilutions (1:2 to 1:256). PBST supplemented with 2% FBS was used as diluent for samples, polyclonal antibody, and conjugate. The culture media from non-infected cell cultures and from cultures inoculated with non-infected fish tissue (see following section) were used as negative control antigens.

Processing of fish tissue homogenates. The (non-specific) reactivity of the polyclonal antisera to normal fish tissues was evaluated using various species of non-infected farmed and ornamental fish (Table 2). Processed fish tissues were also inoculated onto EPC cell cultures to confirm the absence of viral infection. Preparation of fish tissues for ELISA was carried out according to standard virus isolation procedures as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE 2006). Fish less than 40 mm body length were processed whole after removal of the head and tail fin. Internal organs (heart, spleen, and head kidney), aseptically removed, were used from fish over 40 mm body length. All samples were homogenized in Tris MEM with Earle's salts and L-glutamine (pH 7.4 to 7.8), supplemented with 10% FBS, 100 IU ml^{-1} of penicillin, 100 µg ml^{-1} of streptomycin, and 100 µg ml^{-1} of gentamycin, and then centrifuged (Jouan CR 3i, T40 rotor) at 3000 × *g* for 15 min at 4°C. The resulting supernatants were passed through a 0.45 µm filter membrane, mixed with antibiotics (100 IU ml^{-1} of penicillin, 100 µg ml^{-1} of streptomycin) and incubated overnight at 4°C. Filtrates were inoculated onto EPC cells in 24 well plates (NUNC) in dilutions of 1:10, 1:100, and 1:1000. The inoculated cell cultures were incubated at 15 or 24°C and checked periodically during 7 d for CPE. On Day 7, the plates were freeze-thawed and subcultured onto a new culture of EPC cells. After 2 to 3 passages, if no CPE was observed, the samples were considered negative.

Table 2. Check for cross-reactivity of the polyclonal antibody raised against European catfish virus (PAb RAECV) with a panel of non-infected fish samples. Farmed and ornamental fish were processed individually or in pools of a maximum of 10 fish, homogenized, and assayed by cell culture technique. The ELISA was performed in the sub-cultivated samples from the second cell culture pass. OD: optical densities (mean ± SD); N: no. of samples

Latin designation	Family	Material	N	OD 450 nm (mean ± SD)
Arius jordanii	*Ariidae*	Whole fish	6	0.143 ± 0.017
Danio rerio	*Cyprinidae*	Whole fish	3	0.192 ± 0.013
Carassius auratus	*Cyprinidae*	Kidney, spleen	14	0.181 ± 0.036
Carnegiella marthae	*Gasteropelecidae*	Whole fish	15	0.170 ± 0.036
Corydoras hastatus	*Callichtyidae*	Whole fish	10	0.134 ± 0.019
Corydoras julii	*Callichtyidae*	Whole fish	15	0.147 ± 0.013
Oncorhynchus mykiss	*Salmonidae*	Ovarian fluid	3	0.172 ± 0.023
	Salmonidae	Kidney, liver, spleen	40	0.176 ± 0.027
Otocinclus affinis	*Loricariidae*	Whole fish	15	0.190 ± 0.035
Poecilia reticulata	*Poeciliidae*	Whole fish	12	0.190 ± 0.034
Potamotrygon reticulatus	*Dasyatidae*	Kidney, liver, spleen	4	0.152 ± 0.042
Pterophyllum scalare	*Cichlidae*	Whole fish	7	0.182 ± 0.049
Xiphophorus maculatus	*Poeciliidae*	Whole fish	13	0.176 ± 0.034

RESULTS

The analysis of the ranavirus polypeptides is shown in Fig. 1. Each virus isolate possessed a minimum of 20 distinguishable structural proteins, with estimated molecular masses ranging from approximately 8 to 121 kDa. All ranaviruses investigated possessed a major polypeptide of approximately 49 kDa, with the exception of EHNV, where the polypeptide was approximately 50 to 51 kDa. In addition, the major capsid proteins (MCPs) of guppy virus 6 (GV6) and doctor fish virus (DFV) migrated slightly more slowly than the corresponding proteins from other ranaviruses. Examination of the protein profiles indicated that no specific signature polypeptides exist for the differentiation of one virus from another. Whilst the protein profiles from ranaviruses were generally similar, minor differences could be seen between most isolates, such as EHNV, GV6, and DFV. The protein profiles further revealed that ranaviruses from specific geographic regions appeared identical (e.g. ESV and ECV; GV6 and DFV). The last lane represents the polypeptide profile from non-infected EPC cells, which were concentrated and processed in the same way as virus isolates.

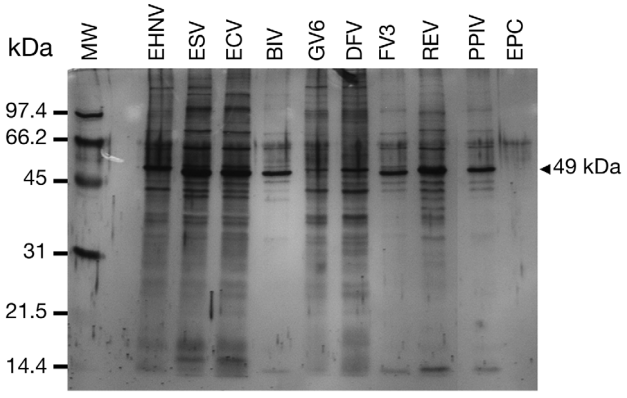

Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of a panel of ranaviruses (stained with silver nitrate). MW: molecular weight standards. The 49 kDa band indicates the position of the major capsid protein. See Table 1 for virus abbreviations

Western blot was carried out to identify the reactivity of the PAb to ranaviral proteins. The PAb showed a strong reaction to the homologous protein of the ECV isolate of MW 49 kDa (Fig. 2). A few other less immunoreactive bands of ECV were also revealed by this PAb. As shown in Fig. 2, the antibody also reacted strongly with the 49 to 51 kDa MCP of most ranaviruses studied. In the case of GV6 and DFV, no bands of MCP protein were observed, although some reactions to other proteins were observed. No bands were found in the non-infected EPC cells.

The localization of the antibody binding sites on the ranavirus virions was identified using immunogold electron microscopy (Fig. 3). The presence of gold-labelled virions suggested that the epitopes recognized by the PAb RAECV are exposed on the surface of

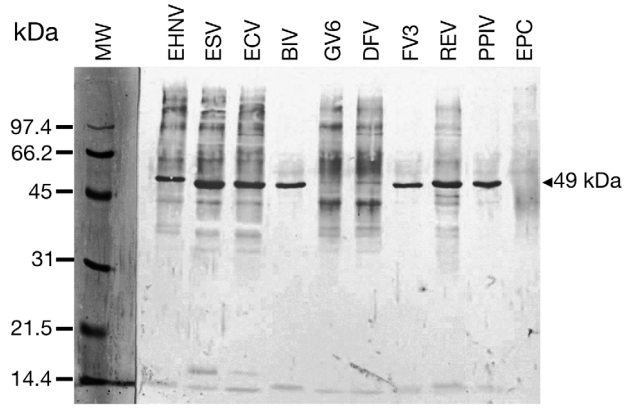

Fig. 2. Western blot analysis of viral proteins in concentrated ranaviruses reacted with the polyclonal antibody raised against European catfish virus (PAb RAECV). MW: molecular weight standards stained with colloidal gold. The 49 kDa band indicates the position of the major capsid protein recognized by the PAb RAECV. See Table 1 for virus abbreviations

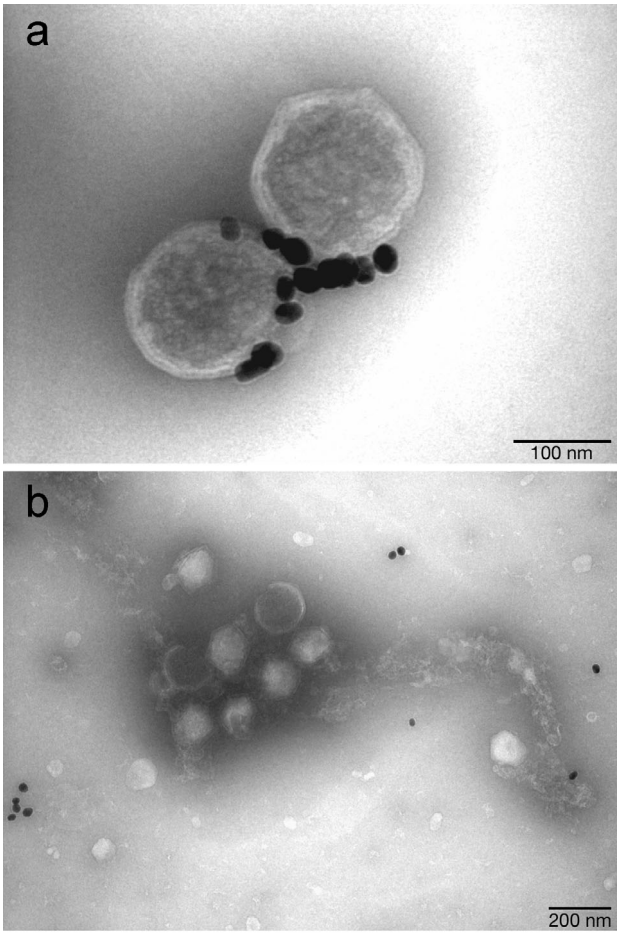

Fig. 3. Immunogold electron micrograph of ranaviruses. Virus particles were incubated with the polyclonal antibody raised against European catfish virus (PAb RAECV) conjugated with colloidal gold (20 nm particles, black coloured). Virions were then negatively stained and examined by electron microscopy. (a) European catfish virus (ECV) virions; colloidal gold particles were located on the outer surface of virions. (b) Doctor fish virus (DFV) virions; no colloidal gold particles were located on the outer surface of virions

the ECV virus (Fig. 3a). The same results were obtained in all other ranaviruses studied (data not shown), except for the isolates GV6 and DFV. In the case of both isolates GV6 and DFV (Fig. 3b), no gold particles were bound to the surface of the virion. The control experiments carried out with rhabdoviruses showed no presence of gold-labelled virions. In the control experiments, no gold-labelling and ranavirus particles in the presence of PAb RAIHNV and no gold SVCV particles in the presence of PAb RAECV could be seen (data not shown).

The PAb RAECV was conjugated using the periodate technique, and subsequently tested for sensitivity and specificity in a direct ELISA test. The analytical sensitivity of the ELISA format was estimated by a

series of 2-fold dilutions of ECV isolates in cell cultures. The virus antigen titre was defined as the last dilution at which the OD value of antigen was higher than the background OD of EPC cells (diluted 1:2). End-point dilution was obtained when 3 samples in duplicate with an identical virus concentration (10^5 $TCID_{50}$ ml^{-1}) were examined. As shown in Fig. 4, the OD dropped to a level approaching background when viruses were diluted to a concentration below $10^{3.2}$ $TCID_{50}$ ml^{-1}.

The OD cut-off was determined by analysis of frequency distributions of the OD data. For the specific determination of OD cut-off, the sensitivity of the ELISA format was defined as the minimum virus concentration that produced an OD value higher than the mean OD value of negative samples (EPC cells) (0.146) plus 3-fold standard deviation (0.117). OD cut-off was calculated and rounded to 0.3. OD values greater than 0.3 were considered as positive results, and the sensitivity of the ELISA developed in this study was therefore $10^{3.5}$ to $10^{3.8}$ $TCID_{50}$ ml^{-1} (Fig. 4).

The specificity of the ELISA and the quality of the PAb RAECV produced was confirmed using the panel of virus isolates. As shown in Fig. 5, all ranaviruses except for GV6 and DFV produced OD values above the cut-off value. The highest intensity of the reactions was observed in ECV and ESV viruses. The low OD values for GV6 and DFV were not a consequence of low inoculum titre. Original $TCID_{50}$ values for all isolates were in the range 4.0×10^4 ml^{-1} to 7.1×10^9 ml^{-1}. No positive reactions were detected even when concentrated viruses GV6 and DFV were examined (data not shown). The antibody also did not react with the antigens prepared from 4 other unrelated fish viruses of the genera *Rhabdovirus* and *Birnavirus*.

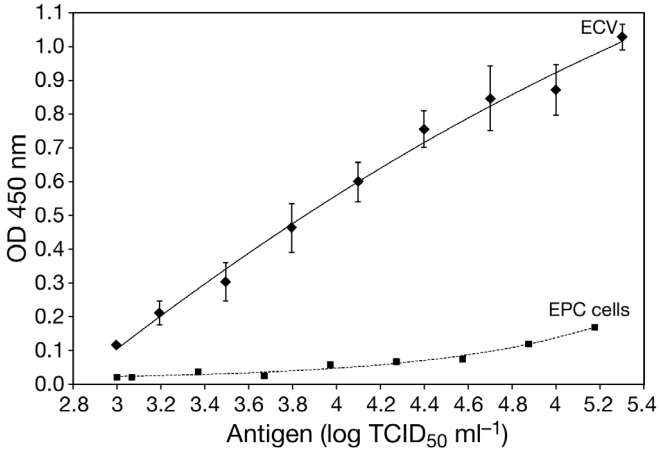

Fig. 4. Sensitivity of direct ELISA by titration of European catfish virus (ECV) isolates (◆—◆) from virus-infected epithelioma papillosum cyprini (EPC) cells with identical $TCID_{50}$ (4×10^5 ml^{-1}). Titration was carried out on 3 samples in duplicate. (■---■): non-infected EPC cells. Error bars are ±SD

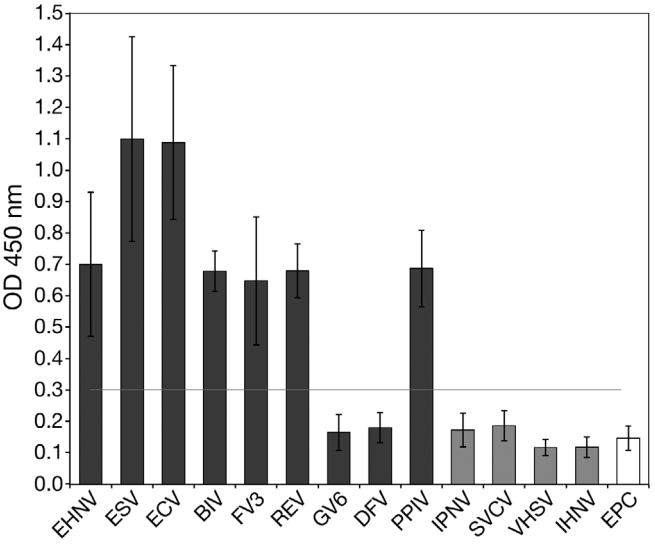

Fig. 5. Antigen-capture ELISA data from examination of virus-infected EPC cells (diluted 1:2). Results are expressed as optical densities read at 450 nm. Each scale bar represents an average and a standard deviation (SD) of multiple replicates. Black bar: ranavirus; grey bar: fish virus unrelated to ranavirus; white bar: negative control, non-infected EPC cells; horizontal line: optical densities positive-negative cut-off. See Table 1 for virus abbreviations

Reactivity of the PAb was tested further in a total of 157 sub-cultivated samples from various non-infected farmed fish and ornamental fish species (Table 2). The pooled samples were diluted 1:2. All samples from non-infected fish produced low OD values below the cut-off value, indicating that there was no non-specific antibody reaction with cell culture or medium in any of the non-infected fish samples.

DISCUSSION

Interest in viruses belonging to the genus *Ranavirus* has increased due to the findings that some of its members can be highly virulent and may be responsible for serious diseases in a broad range of poikilothermic vertebrate hosts including native fish, amphibians, and reptiles (Mao et al. 1997). Experimental studies have shown that some individual ranaviruses have the potential for cross-species transmission (Moody & Owens 1994, Hedrick & McDowell 1995) and that one class of poikilothermic vertebrates (fish, amphibians, and reptiles) can become a reservoir or a carrier of viruses infecting another class of low vertebrates (Mao et al. 1997). The possible spread of ranaviral diseases is a serious threat to national and international trade. This has led to the need for the development of accurate, rapid, and sensitive diagnostic tests for the identification of the causative agents of the disease.

In the present study, a rabbit polyclonal antibody against ECV was produced for the development of immunoassays for the detection of ranaviral antigens. Ranaviruses were confirmed using negative staining electron microscopy and SDS-PAGE, and subsequently used to characterize the PAb. Specificity of the PAb was analyzed by Western blot and immunogold electron microscopy. Western blot analysis revealed that the PAb reacted strongly with the 49 kDa protein corresponding to the size of the major capsid protein, which is the main structural component of iridoviruses, comprising 40 to 45% of all viral proteins (Williams et al. 2005). Similar results were found in EHNV and BIV (Hengstberger et al. 1993), and in ESV and ECV (Hedrick et al. 1992) with antisera raised against EHNV. Immunogold electron microscopy provided visual evidence that the epitopes recognized by this PAb were located on the outer surface of virions. The results of Western blot and immunogold electron microscopy illustrated that the PAb is specific for all ranaviruses except for 2 (GV6 and DFV), designated as Santee-Cooper ranaviruses, and that the 49 kDa viral protein is a major antigen responsible for PAb binding. The absence of cross-reactivity of GV6 and DFV with the PAb indicates that these viruses contain different epitopes.

The PAb was further used for the production of peroxidase-labeled conjugate for direct ELISA detection of ranavirus antigens in infected EPC cell cultures. ELISA was capable of detecting $10^{3.5}$ to $10^{3.8}$ $TCID_{50}$ ml^{-1} of the virus-infected cell cultures when an optical density value >0.3 was judged as positive. Similar sensitivity (10^3 to 10^4 $TCID_{50}$ ml^{-1}) was found in antigen-capture ELISA validated to detect EHNV in cell cultures in the OIE Reference Laboratory for EHNV (OIE 2006).

The specificity of the conjugated PAb was evaluated against 9 ranaviruses, 4 representative fish viruses of the genera *Rhabdovirus* and *Birnavirus*, and in a total of 157 samples from various non-infected fish species. The ELISA results showed that all ranaviruses described in this paper with the exception of GV6 and DFV were successfully detected with the PAb in cell cultures. This observed cross-reactivity implies that ranaviruses possess similar antigenic epitopes. The poor cross-reactivity observed with the Santee-Cooper ranaviruses in ELISA indicates that these species are significantly different from other ranaviruses. The techniques used in this study confirm results described by Hyatt et al. (2000) that these viruses imported from Southeast Asia are not members of the genus *Ranavirus*.

The data presented show that the antibody can be useful as a diagnostic reagent for the development of a rapid, precise and sensitive method for identification of viruses belonging to the genus *Ranavirus*, with the exception of Santee-Cooper ranavirus isolates, which currently also belong to this genus.

Acknowledgements. This work was supported by the RANA project 'Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems' (SSPE-CT-2005-006459) under the sixth framework programme. We thank E. Ariel (European Community Reference Laboratory for Fish Diseases, Denmark) for providing ranavirus isolates. We also thank I. Halikova, L. Leharova, and J. Martinu for technical assistance, and A. Bayley and L. Faldikova for proofreading the translated manuscript.

LITERATURE CITED

Ahne W, Schlotfeldt HJ, Thomsen I (1989) Fish viruses: isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). J Vet Med B Infect Dis Vet Public Health 36:333–336

Ahne W, Bearzotti M, Bremont M, Essbauer S (1998) Comparison of European systemic piscine and amphibian iridoviruses with epizootic haematopoietic necrosis virus and frog virus 3. J Vet Med B 45:373–383

Bigarre L, Cabon J, Baud M, Pozet F, Castric J (2008) Ranaviruses associated with high mortalities in catfish in France. Bull Eur Assoc Fish Pathol 28:163–168

Bovo G, Comuzi M, DeMas S, Ceschia G, Giorgetti G, Giacometti P, Cappellozza E (1993) Isolamento di un agente virale irido-like da pesce gatto (*Ictalurus melas*) dàllevamento. Boll Soc It Patol Ittica 11:3–10

Chinchar VG (2002) Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers — brief review. Arch Virol 147: 447–470

Chinchar VG, Hyatt A, Miyazaki T, Williams T (2009) Family *Iridoviridae*: poor viral relations no longer. In: Van Etten JL (ed) Lesser known large dsDNA viruses. Springer, Berlin, 328:123–170

Farr AG, Nakane PK (1981) Immunohistochemistry with enzyme labeled antibodies: a brief review. J Immunol Methods 47:129–144

Fijan N, Sulimanovic D, Bearzotti M, Muzinic D and others (1983) Some properties of the *Epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio.* Ann Virol 134:207–220

Granoff A, Came PE, Rafferty KA (1965) Isolation and properties of viruses from *Rana pipiens*: their possible relationship to renal adenocarcinoma of leopard frog. Ann NY Acad Sci 126:237–255

Gupta R, Talwar GP, Gupta SK (1992) Rapid antibody capture assay for detection of group-a streptococci using monoclonal-antibody and colloidal gold-monospecific polyvalent antibody conjugate. J Immunoassay 13:441–455

Hattenberger-Baudouy AM, Danton M, Merle G, Torchy C, de Kinkelin P (1989) Serological evidence of infectious haematopoietic necrosis in rainbow trout from a French outbreak of disease. J Aquat Anim Health 1:126–134

Hedrick RP, McDowell TS (1995) Properties of iridoviruses from ornamental fish. Vet Res 26:423–427

Hedrick RP, McDowell TS, Ahne W, Torhy C, de Kinkelin P (1992) Properties of three iridovirus-like agents associated with systemic infections of fish. Dis Aquat Org 13:203–209

Hengstberger SG, Hyatt AD, Speare R, Coupar BEH (1993) Comparison of epizootic hematopoietic necrosis and Bohle iridoviruses, recently isolated Australian iridoviruses. Dis Aquat Org 15:93–107

Hyatt AD, Eaton BT, Hengstberger S, Russel G (1991) Epizootic hematopoietic necrosis virus: detection by ELISA, immunohistochemistry and immunoelectronmicroscopy. J Fish Dis 14:605–617

Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA and others (2000) Comparative studies of piscine and amphibian iridoviruses. Arch Virol 145:301–331

Hyatt AD, Williamson M, Coupar BEH, Middleton D and others (2002) First identification of a ranavirus from green pythons (*Chondropython viridis*). J Wildl Dis 38:239–252

Koutná M, Veselý T, Pšikal I, Hulová J (2003) Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. Dis Aquat Org 55:229–235

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

Langdon JS, Humphrey JD, Williams LM, Hyatt AD, Westbury HA (1986) First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. J Fish Dis 9:263–268

Mao J, Hedrick RP, Chinchar VG (1997) Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229:212–220

Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD, Chisholm O (2002) Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Mol Cell Probes 16:137–151

Moeremans M, Daneels G, Demey J (1985) Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. Anal Biochem 145:315–321

Moody NJG, Owens L (1994) Experimental demonstration of the pathogenicity of a frog virus, Bohle iridovirus, for a fish species, barramundi *Lates calcarifer.* Dis Aquat Org 18: 95–102

OIE (World Organisation for Animal Health) (2006) Epizootic haematopoietic necrosis. In: Manual of diagnostic tests for aquatic animals, 5th edn. OIE, Paris, p 82–103

OIE (World Organisation for Animal Health) (2008) Epizootic haematopoietic necrosis. In: Aquatic animal health code, 11th edn. OIE, Paris

Pallister J, Gould A, Harrison D, Hyatt A, Jancovich J, Heine H (2007) Development of real-time PCR assays for the detection and differentiation of Australian and European ranaviruses. J Fish Dis 30:427–438

Pozet F, Morand M, Moussa A, Torhy C, de Kinkelin P (1992) Isolation and preliminary characterization of a pathogenic icosahedral deoxyribovirus from the catfish *Ictalurus melas.* Dis Aquat Org 14:35–42

Reschova S, Pokorova D, Hulova J, Kulich P, Vesely T (2008) Surveillance of viral fish diseases in the Czech Republic over the period January 1999–December 2006. Vet Med 53:86–92

Rodak L, Pospisil Z, Tomanek J, Vesely T, Obr T, Valicek L (1993) Enzyme-linked immunosorbent assay (ELISA) for the detection of spring viremia of carp virus (SVCV) in tissue homogenates of the carp, *Cyprinus carpio* L. J Fish Dis 16:101–111

Speare R, Smith JR (1992) An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. Dis Aquat Org 14:51–57

Steiner KA, Whittington RJ, Petersen RK, Hornitzky C, Garnett H (1991) Purification of epizootic haematopoietic necrosis virus and its detection using ELISA. J Virol Methods 33:199–209

Tapiovaara H, Olesen NJ, Lindén J, Rimaila-Pärnänen E, von Bonsdorff CH (1998) Isolation of an iridovirus from pike-perch *Stizostedion lucioperca.* Dis Aquat Org 32:185–193

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Vesely T, Nevorankova Z, Hulova J, Reschova S, Pokorova D (2004) Monoclonal antibodies to nucleoprotein and glycoprotein of the virus of infectious haematopoietic necrosis of salmonids (IHNV) and their use in immunoperoxidase test. Bull Eur Assoc Fish Pathol 24:218–230

Whittington RJ, Steiner KA (1993) Epizootic haematopoietic necrosis virus (EHNV): improved ELISA for detection in fish tissues and cell cultures and an efficient method for release of antigen from tissues. J Virol Methods 43: 205–220

Williams T, Barbosa-Solomieu V, Chinchar VG (2005) A decade of advances in iridovirus research. Adv Virus Res 65:173–248

Editorial responsibility: Alex Hyatt, Geelong, Victoria, Australia

Submitted: May 22, 2009; Accepted: January 11, 2010
Proofs received from author(s): March 16, 2010