An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia

R. Speare*, J. R. Smith

Graduate School of Tropical Veterinary Science and Agriculture, James Cook University of North Queensland, Townsville 4811, Australia

ABSTRACT: An enveloped, icosahedral DNA virus of 128 ± 8 nm was isolated from metamorphs of the ornate burrowing frog *Limnodynastes ornatus* (Gray) in Australia. At 25 °C the virus grew readily in 11 mammalian and 4 fish cell lines ($TCID_{50} \text{ ml}^{-1} = 1 \times 10^{5.43}$ to $10^{8.85}$), grew less well in the Atlantic salmon cell line ($TCID_{50} \text{ ml}^{-1} = 1 \times 10^{3.85}$) and failed to grow in 5 insect cell lines. Growth was inhibited at temperatures above 33 °C. Cytopathic effects consisted of basophilic cytoplasmic inclusion bodies, rounding of infected cells, lifting from substrate and extensive destruction of the monolayer usually by 24 to 48 h postinfection. Methylation of DNA occurred during growth. The virus shares characteristics with Iridoviridae of amphibian origin, e.g. Ranavirus, and piscine origin, e.g. epizootic haematopoietic necrosis virus of redfin perch *Perca fluviatilis*.

INTRODUCTION

Interest in the pathogens of amphibians in Australia has been stimulated by the desire to limit the progressive expansion of the range of the cane toad *Bufo marinus* L. *B. marinus* was introduced from South America via Hawaii in 1935 to control beetle pests in sugar cane, but subsequently became a pest species itself (Freeland 1985). Although there are only a few examples of successful biological control of vertebrates (Spratt 1990), a program to investigate the pathogens of *B. marinus* was commenced (Freeland 1985). At the inception of the program no viruses had been reported from amphibians in Australia and viruses had not been found in *B. marinus* elsewhere (Speare 1990). Subsequently an irido-like virus was observed in erythrocytes of *B. marinus* from Costa Rica (Speare et al. 1991).

Viruses with the characteristics of Adenoviridae, Caliciviridae, Herpesviridae, Iridoviridae, Papovaviridae and Togaviridae have been found in amphibians in North and South America and in Europe (Granoff 1983, 1989, Speare et al. 1991).

During a study on the pathogens of the cane toad in Australia a cytopathic viral agent was isolated from metamorphs of the ornate burrowing frog *Limnodynastes ornatus* (Gray). These had been collected as tadpoles from a temporary pond in a backyard at Bohle, a suburb of Thuringowa in north Queensland, Australia (19° 16' S, 146° 47' E), and maintained in a glass aquarium for several weeks. Investigation was prompted by deaths of the frogs during or soon after metamorphosis. This paper describes some of the characteristics of a virus isolated from the metamorphs.

MATERIAL AND METHODS

Isolation of virus. Whole froglets or tissues were macerated, suspended in Dubeco's Minimum Essential Medium with added amphotericin ($2.5 \mu\text{g ml}^{-1}$), benzyl penicillin (200 IU ml^{-1}), gentamycin ($200 \mu\text{g ml}^{-1}$) (DMEM), centrifuged at 10 000 rpm at 15 °C and 0.2 ml of the supernatant placed for 2 h at 25 °C on monolayers of bluegill fry-2 cells (BF-2) (Wolf et al. 1966) grown in glass test tubes with 12×8 mm coverslips. After adsorption 2 ml of DMEM with 5 % foetal bovine serum (FBS) was added to each test tube and the cultures were incubated at 25 °C. The following tests were performed using virus of third to fifth passage.

* Present address: Anton Breinl Centre for Tropical Health and Medicine, James Cook University of North Queensland, Townsville 4811, Australia

Cytopathic effect. Cytopathic effect (CPE) was observed in unstained infected cell cultures by the use of an inverted microscope. Infected cells on glass coverslips (12 × 8 mm) were fixed in Bouin's solution and staining was done with haematoxylin and eosin using standard techniques. Timed infections to determine when inclusion bodies first appeared were done by inoculation of cell cultures on glass coverslips and staining at 0.5, 1, 2, 3, 4, 5, 6, 9, 12 and 24 h post infection.

Electron microscopy. Infectious BF-2 culture medium was centrifuged at 10 000 rpm for 10 min. Virus was examined from both supernatant and lysed pellet using air drying on Formvar-coated copper grids and staining with 2 % phosphotungstic acid (PTA). Thin sections were prepared from BF-2 cells 12 h after inoculation. Cells were suspended with antibiotic-trypsin (0.05 %)-versene solution, pelleted by centrifugation at 10 000 rpm for 10 min, fixed with 2.5 % glutaraldehyde, rinsed twice in 0.1 M sodium cacodylate buffer for 10 min, post-fixed in buffered 1 % osmium tetroxide at room temperature for 1 h, rinsed in 0.1 M sodium cacodylate buffer for 10 min, and dehydrated in a graded series of ethanol to 100 %. Tissue was infiltrated in 1:1 Spurr's resin:ethanol overnight and embedded in Spurr's resin and polymerised at 75 °C overnight. Sections of 500 Å thickness were cut on an LKB ultratome, mounted on copper grids, stained in saturated uranyl acetate in 70 % methanol and in lead citrate. Samples were examined at 80 kV on a Joel 2000 FX transmission electron microscope.

Titration of virus. Samples for titre determination were collected, frozen at –70 °C for periods of 1 wk to 3 mo, thawed by immersion in water at 37 °C and determinations done in batches for each experiment using BF-2 cells, flat-bottomed plastic microtitre trays and 10-fold dilutions. Unstained CPE was determined grossly using an inverted microscope. Titres (TCID₅₀ ml^{–1}) were determined by the technique of Reed & Muench (1938).

Treatment with ether. One ml of diethyl ether was added to 2 ml of BF-2 tissue culture fluid with virus, mixed by vortication and samples collected from test culture fluid with lipid solvent and from the control culture fluid after 18 h at 4 °C (Andrewes & Horstmann 1949). Samples were frozen and processed for titre as indicated above.

Treatment with different pH. The changes in titres with pHs of 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 were studied at 1 h (Kettler et al. 1962). Hydrochloric acid (1 M HCl) and sodium hydroxide (10 M NaOH) were added to DMEM to produce the desired acid and alkaline pHs respectively. Virus in 0.5 ml of tissue culture fluid was added to 5 ml of the test solution, a 0.5 ml aliquot removed at the chosen time, added to 4.5 ml of DMEM,

and final pH adjusted to neutrality using either HCl or NaOH. Samples were frozen and processed for titre as indicated above.

Heat sensitivity. Change in titres at temperatures of 25, 37 and 56 °C were measured over 6 h. Virus in 5 ml of DMEM in glass test tubes (150 mm long × 15 mm wide) was held in waterbaths at 37 and 56 °C and in an incubator at 25 °C and 0.75 ml samples removed at 0.5, 1, 2 and 6 h, care being taken to avoid viral contamination of the upper portions of the test tubes (Kettler et al. 1962). Samples were frozen and processed for titre as indicated above.

Effect of temperature on growth. Cell cultures of the fish cell lines Atlantic salmon (AS), brown bullhead (BB), BF-2, fat head minnow (FHM) and rainbow trout gonad (RTG), and the mammalian cell lines Madin Darby bovine kidney (MDBK), Vero (American Type Culture Collection 1988), Dunnart (CSL235) (Commonwealth Serum Laboratories 1977), porcine standard/equine kidney (PS/EK) (Gorman et al. 1975), feline kidney (FK) (Smith 1986) and bovine trachea (BT) were inoculated with 50 µl of media containing 10⁸ TCID ml^{–1}, absorbed for 1 h and replaced with fresh DMEM with 5 % FBS. Flasks were incubated at 20, 25, 30, 34 and 37 °C, and CPE was noted and samples collected at 7 d post-inoculation. Samples were frozen and processed for titre as indicated above.

Nucleic acid. To determine the type of nucleic acid present in viral inclusions, cell cultures were stained with acridine orange (Thompson 1966). At 6 h after infection, cell cultures of BF-2 were fixed in 50:50 diethyl ether:95 % ethanol for 30 min, hydrated through 80, 70 and 50 % ethanol, rinsed in distilled water, treated with 1 % acetic acid for 6 s, stained in 0.1 % acridine orange for 3 min, washed in McIlvaine's citric acid buffer at pH 3.8 for 1 min, differentiated in 0.1 M calcium chloride for 30 s, mounted in 1 drop of buffer and examined immediately by ultraviolet light (BG 38 filter to give wavelength of about 300 nm) with a Zeiss Photomicroscope II with Zeiss III RS incident light fluorescence attachment.

DNA inhibition was tested by the use of pyrimidine analogues and prevention of this inhibition with thymidine (Hamparian et al. 1960). BF-2 cell cultures and media containing the inhibiting pyrimidine analogues 5-bromo-2'-deoxyuridine (BUdR) and 5-iodo-2'-deoxyuridine (IUdR) at concentrations of 100 and 200 µg ml^{–1} were used. Prevention of this inhibition was tested by the addition of thymidine at 100 and 500 µg ml^{–1} with each concentration of DNA inhibitor. A 2 ml inoculum of virus in DMEM (TCID₅₀ ml^{–1} = 1 × 10⁷) was placed on each cell culture and removed after 1 h. After the cell culture was washed 3 times with DMEM, DMEM containing the DNA inhibitor with or without thymidine was added. Samples were collected after addi-

tion of the new media and at 7 d post-infection. Titres were determined in the usual manner, but with the addition of thymidine at 250 µg ml⁻¹ in titre trays.

Methylation of DNA was evaluated by inhibition with 5-azacytidine (AZC) (Goorha et al. 1984). Virus (TCID₅₀ ml⁻¹ = 1 × 10⁷) was absorbed to BF-2 cell cultures for 1 h and media replaced with DMEM containing 2.5 µg ml⁻¹ of AZC. Samples were collected after media change and at 7 d post-inoculation. Samples were frozen and processed for titre as indicated above.

Statistical analyses. Results for replication in different cell lines at different temperatures were analysed by Statistix 3.1 (Analytic Software, St. Paul, MN, USA) using 1-way analysis of variance (ANOVA) and general analysis of variance with least squares difference (GANOVA). For these analyses results from insect cell lines and AS were omitted since replication did not occur in the former and was minimal in the latter.

RESULTS

Isolation of virus

CPE was detected in the initial isolations at 48 h and was reproduced by inoculation into fresh BF-2 cultures. In subsequent passages monolayers were destroyed within 24 h at 25 °C. CPE developed in all mammalian and piscine cell lines, but in none of the insect cell lines (Table 1). Titres did not increase after inoculation into 3 mosquito cell lines (see Table 4).

The initial change in unstained cell sheets was rounding of cells with increased refractility. Plaques had centres largely devoid of cells and with affected cells aggregated at the circumference. Aggregated cells subsequently lifted and floated into the media. Total destruction of the monolayers usually took 24 to 48 h at 25 °C. Initially in cell cultures of BF-2 there were cytoplasmic inclusion bodies and no other cytopathology (Fig. 1b). Inclusion bodies (IB) were basophilic, roughly oval to spherical, occasionally with irregular margins, surrounded by a narrow halo; single or more commonly multiple with up to 7 IB cell⁻¹. Later in the infection (Fig. 1c) the inclusion bodies were more basophilic, more often spherical, and larger, measuring 3.21 ± 0.86 µm (range: 2.4 to 5.7 µm). There were fewer inclusion bodies per cell and it was uncommon to find more than 2. At this stage the cytoplasm of infected cells was finely granular, strongly eosinophilic, particularly close to the nucleus. Many nuclei were pyknotic, irregular in outline and bean-shaped forms were common. In many cells inclusion bodies were enclosed in the concave hollow of the pyknotic nucleus. The nuclear chromatin had lost its fine granularity and was

Table 1. Cell lines inoculated with the virus isolated from *Limnodynastes ornatus* and appearance of cytopathic effect (CPE) 7 d after infection at 25 °C. (+): CPE present; (-): CPE absent

Cell line	Source of line	CPE
Insect cell lines		
AA.20A	Mosquito	-
AG.55	Mosquito	-
AS.43	Mosquito	-
AM.60	Mosquito	-
C6.36	Mosquito	-
Fish cell lines		
Atlantic salmon (AS)	Fish	+
Brown bullhead (BB)	Fish	+
Bluegill fry 2 (BF-2)	Fish	+
Fathead minnow (FHM)	Fish	+
Rainbow trout gonad (RTG)	Fish	+
Mammalian cell lines		
Baby hamster kidney (BHK21)	Rodent	+
Bovine turbinate (BT)	Cow	+
Dunnart (CSL235)	Marsupial	+
Feline kidney (FK)	Cat	+
G361	Mouse	+
Hela	Human	+
Madin Darby bovine kidney (MDBK)	Cow	+
Ovine lung (OL)	Sheep	+
Porcine standard/equine kidney (PS/EK)	Horse/pig	+
Swine testis (ST)	Pig	+
Vero	Monkey	+
3T3	Mouse	+

aggregated below the nuclear membrane or was clumped. Prior to lifting from the coverslip, the affected cells were shrunken with markedly eosinophilic cytoplasm. Nuclei were irregular in outline and pyknotic with increased basophilia. Inclusion bodies were dense and spherical as before, but some affected cells had no inclusion bodies. The affected cells aggregated into foci of pyknotic nuclei, shrunken eosinophilic cytoplasm and spherical inclusion bodies to form a jumble of structures in which it was difficult to make out individual cell outlines. In timed infections with BF-2 cells inclusion bodies were first detected at 3 h. By 6 h other cytopathology (as described above) was common and by 12 h the monolayers were totally destroyed.

Morphology of virus

Viral particles seen by negative contrast were roughly circular with occasional hexagonal outlines and frequently enveloped (Fig. 2). Particles frequently stained with PTA and consisted of a central spherical core surrounded by a membrane abutting against the capsid with an external envelope. The diameter of the

Fig. 1. Monolayers of BF-2 cells. (a) Normal monolayer. (b) Basophilic inclusion bodies in cytoplasm of BF-2 monolayer 4 h after infection with the virus. Many cells contain multiple inclusions (arrows). The arrowhead marks a contracted artifactual cell common in BF-2 monolayers. (c) Advanced cytopathic effect 6 h after infection with the virus. Cytoplasmic inclusions are aggregated (arrows) and some nuclei are pyknotic. Cell boundaries are less distinct (H&E); 600 $\times$, scale bar = 20 μ m

nucleocapsid was 128 ± 8 nm (range: 117 to 146 nm, $n = 14$) and of the envelope 147 ± 8 nm (range: 137 to 156, $n = 11$).

In thin sections viral particles in cells had cubic symmetry with hexagonal and pentagonal cross sections (Fig. 3). Average diameter from vertex to vertex of the top 25 % of 40 measured particles was 172 ± 5 nm (max: 176 nm). Viruses had a central, spherical, densely staining core surrounded by a capsid and were not enveloped in cells. In the sections examined no

Fig. 2. Negative contrast stain of virus isolated from *Limnodynastes ornatus*. Note the envelope closely adjacent to the nucleocapsid. TEM; phosphotungstic acid; 90 000 $\times$, scale bar = 100 nm

Fig. 3. Unenveloped virus particles in cytoplasm of BF-2 cell. TEM; 60 000 $\times$, scale bar = 100 nm

viral particles were found in the process of escaping from cells.

Biological characteristics of the virus

After treatment with diethyl ether for 18 h, titres were reduced from $10^{7.8}$ ml $^{-1}$ to $10^{4.3}$ ml $^{-1}$, a reduction of about 99 %. Ether sensitivity confirmed the presence of a lipid-soluble envelope essential for infectivity. Viral titres were markedly reduced after 1 h at pHs of 2, 3, 10 and 11 (Table 2). Titre was unchanged after 6 h at 25 and 37 $^{\circ}$ C (Table 3). Titre was reduced by 99.9 % and 99.999 % after 0.5 h and 1 h at 56 $^{\circ}$ C respectively and no viability was detected at 2 and 6 h.

Culture temperature affected replication in all cell lines (Table 4). Growth of virus occurred in Atlantic

Table 2. Infectivity of the virus isolated from *Limnodynastes ornatus* after 1 h in DMEM at different pH

pH	Titre (TCID ₅₀ ml ⁻¹)
Initial	10 ^{5.68}
2	0
3	10 ^{1.09}
4	10 ^{5.11}
5	10 ^{4.11}
6	10 ^{4.18}
7	10 ^{4.85}
8	10 ^{5.11}
9	10 ^{4.68}
10	10 ^{1.85}
11	10 ^{2.35}

Table 3. Stability of the virus isolated from *Limnodynastes ornatus* at different temperatures. Titres are given as TCID₅₀ ml⁻¹. nd: not done

Temp. (°C)	Initial	0.5 h	1 h	2 h	6 h
25	10 ^{7.85}	nd	nd	nd	10 ^{7.52}
37	10 ^{7.85}	nd	nd	nd	10 ^{7.98}
56	10 ^{7.85}	10 ^{3.27}	10 ^{2.23}	0	0

Table 4. Replication of the virus isolated from *Limnodynastes ornatus* 1 wk post-inoculation in different cell cultures at different temperatures. Titres are given as TCID₅₀ ml⁻¹. nd: not done; (-): contaminated

Cell line	Initial titre	20 °C	25 °C	30 °C	34 °C	37 °C
Insect						
AA.A20	10 ²	nd	10 ²	nd	nd	nd
AG.55	10 ²	nd	10 ²	nd	nd	nd
C636	10 ²	nd	10 ²	nd	nd	nd
Fish						
AS	10 ^{2.11}	10 ^{1.85}	10 ^{3.85}	10 ^{2.85}	10 ^{2.11}	0
BB	10 ^{2.5}	10 ^{7.35}	10 ^{7.68}	10 ^{7.35}	10 ^{2.85}	10 ^{2.85}
BF-2 expt 1	10 ^{3.4}	10 ^{8.33}	10 ^{6.5}	10 ^{6.76}	10 ^{1.76}	10 ^{1.5}
BF-2 expt 2	10 ^{3.2}	10 ^{8.67}	10 ^{8.67}	10 ^{8.17}	10 ^{2.67}	10 ^{3.0}
FHM	10 ^{2.35}	10 ^{7.68}	10 ^{8.68}	10 ^{6.85}	10 ^{2.11}	10 ^{2.35}
RTG	10 ^{2.35}	10 ^{6.85}	10 ^{8.85}	10 ^{8.11}	10 ^{2.85}	0
Mammal						
BT	10 ^{2.85}	10 ^{4.65}	10 ^{7.35}	10 ^{6.85}	10 ^{1.11}	10 ^{1.11}
CSL235 expt 1	10 ^{2.5}	10 ^{5.85}	10 ^{7.85}	10 ^{7.85}	10 ^{4.11}	10 ^{3.68}
CSL235 expt 2	10 ^{2.11}	10 ^{4.11}	10 ^{8.85}	10 ^{7.11}	10 ^{2.65}	10 ^{1.85}
FK	10 ^{2.35}	10 ^{4.85}	10 ^{8.35}	—	10 ^{1.11}	10 ^{1.85}
MDBK	10 ^{2.8}	10 ^{4.67}	10 ^{5.43}	10 ^{5.17}	10 ^{4.17}	10 ^{3.0}
PS/EK	10 ^{2.35}	10 ^{5.35}	10 ^{7.85}	10 ^{6.65}	10 ^{4.11}	10 ^{1.85}
Vero	10 ^{2.9}	10 ^{6.11}	10 ^{8.11}	10 ^{5.85}	10 ^{1.85}	10 ^{1.58}

salmon, but only at 25 °C and titre was low compared to other cell lines. Replication did not occur at 37 °C in any cell line or at 34 °C in any fish cell line. Replication did occur at 34 °C in some mammalian cell lines, but titres

were reduced. Maximum titres for all cell lines were attained at 20 or 25 °C. Analysis of titres after omission of AS showed 2 groups, with 20, 25 and 30 °C having titres higher than post-absorption, 34 °C and 37 °C (GANOVA). The vertebrate class of origin of cells was only significant at 20 °C with fish cell lines having higher titres than mammalian cell lines (1-way ANOVA, $p < 0.001$).

Nucleic acid of virus

Inclusion bodies had an apple-green fluorescence when stained with acridine orange and examined with ultraviolet light, indicating that double-stranded DNA was present. BUdR and IUdR in the media prevented CPE development and virus multiplication (Table 5). This inhibition was prevented by the higher concentration (500 µg ml⁻¹) of thymidine while the lower concentration (100 µg ml⁻¹) increased the titre, but not to control levels (Table 5). Inclusion of AZC in the media inhibited growth (Table 5).

Table 5. Effect of inhibitors of DNA synthesis with and without thymidine and the effect of an inhibitor of DNA methylation on infection of BF-2 cell cultures with the virus isolated from *Limnodynastes ornatus*. Titres were measured at 7 d post-inoculation and are given as TCID₅₀ ml⁻¹. AZC: azocytosine; BUdR: 5-bromo-2'-deoxyuridine; IUdR: 5-iodo-2'-deoxyuridine; thym: thymidine

Inhibitor	Conc (µg ml ⁻¹)	CPE	Titre Initial	Final
Inhibitors of DNA synthesis				
BUdR	200	neg	10 ^{2.11}	10 ^{1.85}
BUdR	100	neg	10 ^{2.11}	10 ^{2.11}
IUdR	200	neg	10 ^{2.11}	10 ^{2.11}
IUdR	100	neg	10 ^{2.11}	10 ^{2.35}
Thymidine	500	4 +	10 ^{2.11}	10 ^{6.35}
Thymidine	100	4 +	10 ^{2.11}	10 ^{6.58}
BUdR + thym	100 + 500	4 +	10 ^{2.11}	10 ^{6.11}
BUdR + thym	100 + 100	2 +	10 ^{2.11}	10 ^{3.85}
IUdR + thym	100 + 500	4 +	10 ^{2.11}	10 ^{6.35}
IUdR + thym	100 + 100	2 +	10 ^{2.11}	10 ^{4.85}
Virus alone	0	4 +	10 ^{2.11}	10 ^{7.11}
Inhibitor of DNA methylation				
AZC	2.5	neg	10 ^{1.68}	10 ^{2.85}
Virus alone	0	4 +	10 ^{1.85}	10 ^{7.85}

DISCUSSION

This is the first description of a virus isolated from amphibians in Australia. The size and morphology of this virus, the presence of a lipid envelope, its CPE and the presence of DNA, are consistent with an iridovirus (Francki et al. 1991). The genus Ranavirus of the

Iridoviridae have methylated cytosine residues and AZC inhibits methylation (Essani & Granoff 1989). AZC inhibition of replication of the isolate indicates that DNA methylation is an essential process (Goorha et al. 1984). The isolate is similar in size, biological characteristics and CPE to ranaviruses isolated in North America (Essani & Granoff 1989). The North American ranaviruses 1, 2 and 3 will not replicate above 33 °C (Granoff et al. 1965, Clark et al. 1969, Gravel & Granoff 1970, Essani & Granoff 1989). The replication of the isolate from *Limnodystes ornatus* showed a similar temperature sensitivity. The virus did not grow at 37 °C in all cell lines tested and at 34 °C in fish cell lines used. However, although titres of this isolate were reduced at 34 °C in mammalian cells, replication occurred in 3 of the 7 mammalian cell lines tested.

The Ranaviruses replicated in 9 mammalian cell lines, 21 reptile cell lines, primary chick embryo cell lines, 4 fish cell lines, but did not grow in the piscine cell lines BB and CAR (Darlington et al. 1966, Clark et al. 1968, Essani & Granoff 1989). Frog Virus 3 did not multiply in the mealworm *Tenebrio molitor* (Lunger & Came 1966). Our isolate grew to high titre in 4 fish cell lines and 6 mammalian cell lines. It grew to low titre in the fish cell line AS, and failed to replicate in 4 insect cell lines. Replication of our isolate in BB cells is a biological difference from the ranaviruses. We did not test growth in CAR, another cell line in which ranaviruses 1, 2, and 3 failed to grow (Essani & Granoff 1989).

This virus is the third iridovirus-like agent isolated from poikilothermic vertebrates in Australia. Epizootic haematopoietic necrosis virus (EHNV) was isolated from redfin perch *Perca fluviatilis* (Langdon et al. 1986, Langdon & Humphrey 1987) and an EHNV-like agent was isolated from rainbow trout *Salmo gairdneri* (Langdon et al. 1988), but further study is required to determine the degree of similarity between the 2 isolates (Eaton et al. 1991). The morphologies of EHNV and our isolate are similar and they cause a similar CPE. Like our isolate and the ranaviruses, EHNV also has methylated DNA (Eaton et al. 1991). The only biological difference noted is higher titres at lower temperatures with EHNV, while our isolate replicates equally well at 20 to 30 °C. Although our isolate and EHNV can not be distinguished on published data, there are no reports of attempted infections of mammalian or insect cells with EHNV.

A number of iridoviruses have been found in fish, but only 4, fish lymphocystis disease virus (FLDV), EHNV, goldfish virus (GFV) and sheatfish virus, appear to have been isolated and characterised (Berry et al. 1983, Langdon & Humphrey 1987, Ahne et al. 1989). FLDV is distinct from the ranaviruses, GFV and EHNV (Essani & Granoff 1989, Eaton et al. 1991). EHNV differs in

protein profiles and DNA restriction pattern from GFV and is similar to, but appears to have some differences from, the North American ranaviruses (Eaton et al. 1991). Since GFV is unable to infect mammalian cells, our isolate is different in this respect.

Our isolate is possibly a new strain of iridovirus. A comparative study with the ranaviruses and the iridoviruses of fish, particularly EHNV and sheatfish virus, using monoclonal antibodies and characterisation of DNA and proteins is required for clarification of its status.

Acknowledgements. We thank Tricia O'Shea, Marion Hulme, Sally Bolton and Dick Jones for expert technical assistance, John Humphries of the Australian Fish Health Reference Laboratory for the original provision of BF-2 and Dr Alex Hyatt for advice and review of the manuscript. This study was supported by a James Cook University Prestige Research Grant to R.S. and by funds provided by the Council of Nature Conservation Ministers (CONCOM).

LITERATURE CITED

- Ahne, W., Schlottfeld, H. J., Thomsen, I. (1989). Fish viruses: isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). J. Vet. Med. B36: 333–336
- American Type Culture Collection (1988). American type culture catalogue of cell lines and hybridomas, 6th edn. American Type Culture Collection, Rockville, MD
- Andrewes, C. H., Horstmann, D. M. (1949). The susceptibility of viruses to ethyl ether. J. gen. Microbiol. 3: 290–297
- Berry, E. S., Shea, T. B., Gabliks, J. (1983). Two iridovirus isolates from *Carassius auratus* (L.). J. Fish Dis. 6: 501–510
- Clark, H. F., Brennan, J. C., Zeigel, R. F., Karzon, D. T. (1968). Isolation and characterization of viruses from the kidneys of *Rana pipiens* with renal adenocarcinoma before and after passage in the red eft (*Triturus viridescens*). J. Virol. 2: 629–640
- Clark, H. F., Gray, C., Fabian, F., Zeigel, R., Karzon, D. T. (1969). Comparative studies of amphibian cytoplasmic virus strains isolated from the leopard frog, bullfrog, and newt. In: Mizell, M. (ed.) Biology of amphibian tumours. Springer-Verlag, Berlin, p. 310–326
- Commonwealth Serum Laboratories Cell Culture Handbook (1976). Commonwealth Serum Laboratories, Parkville
- Darlington, R. W., Granoff, A., Breeze, D. C. (1966). Viruses and renal carcinoma of *Rana pipiens*. II. Ultrastructural studies and sequential development of virus isolated from normal and tumor tissue. Virol. 29: 149–156
- Eaton, B. T., Hyatt, A. D., Hengstberger, S. (1991). Epizootic haematopoietic necrosis virus: purification and classification. J. Fish Dis. 14: 157–169
- Essani, K., Granoff, A. (1989). Amphibian and piscine iridoviruses proposal for nomenclature and taxonomy based on molecular and biological properties. Intervirology. 30: 187–193
- Francki, R. I. B., Fauquet, C. M., Knudson, D. L., Brown, F. (1991). Classification and nomenclature of viruses. Springer-Verlag, Vienna
- Freeland, W. J. (1985). The need to control cane toads. Search 16: 211–215

- Goorha, R., Granoff, A., Willis, D. B., Murti, K. G. (1984). The role of DNA methylation in virus replication: inhibition of frog virus 3 replication by 5-azacytidine. *Viol.* 138: 94-102
- Gorman, B. M., Leer, J. R., Filippich, C., Goss, P. D., Doherty, R. L. (1975). Plaquing and neutralization of arboviruses in the PS-EK line of cells. *Aust. J. med. Technol.* 6: 65-70
- Granoff, A. (1983). Amphibian herpesvirus. In: Roizmann, B. (ed.) *The herpesviruses*. Plenum Publ. Corp., p. 367-384
- Granoff, A. (1989). Viruses of Amphibia: an historical perspective. In: Ahne, W., Kurstak, E. (eds.) *Viruses of lower vertebrates*. Springer-Verlag, Berlin, p. 3-12
- Granoff, A., Came, P. E., Rafferty, K. A. (1965). The isolation and properties of viruses from *Rana pipiens*: their possible relationship to the renal adenocarcinoma of the leopard frog. *Ann. N.Y. Acad. Sci.* 126: 237-255
- Gravell, M., Granoff, A. (1970). Viruses and renal carcinoma of *Rana pipiens*. IX. The influence of temperature and host cell on replication of frog polyhedral cytoplasmic deoxy-riboviruses (PCDV). *Viol.* 41: 596-602
- Hamparian, V. V., Ketler, A., Hilleman, M. R. (1961). Recovery of cold viruses (Coryzavirus) from cases of common cold in human adults. *Proc. Soc. exp. Biol. Med.* 108: 444-453
- Ketler, A., Hamparian, V. V., Hilleman, M. R. (1962). Characterization and classification of ECHO 28-Rhinovirus - Coryzavirus agents. *Proc. Soc. exp. Biol. Med.* 110: 821-831
- Langdon, J. S., Humphrey, J. D. (1987). Epizootic haematopoietic necrosis, a new viral disease in redfin perch, *Perca fluviatilis* L., in Australia. *J. Fish Dis.* 10: 289-297
- Langdon, J. S., Humphrey, J. D., Williams, L. M. (1988). Outbreaks of an EHN-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *J. Fish Dis.* 11: 93-96
- Langdon, J. S., Humphrey, J. D., Williams, L. M., Hyatt, A. D., Westbury, H. A. (1986). First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. *J. Fish Dis.* 9: 263-268
- Lunger, P. D., Came, P. E. (1966). Cytoplasmic viruses associated with Lucké tumor cells. *Viol.* 30: 116-126
- Reed, L. J., Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27: 493-542
- Smith, J. R. (1986). Studies on canine parvovirus associated disease and control. Ph.D. thesis, James Cook Univ., Townsville
- Speare, R. (1990). A review of the diseases of the cane toad, *Bufo marinus*, with comments on biological control. *Aust. Wildl. Res.* 17: 387-410
- Speare, R., Freeland, W. J., Bolton, S. J. (1991). A possible iridovirus in erythrocytes of *Bufo marinus* in Costa Rica. *J. Wildl. Dis.* 27: 457-462
- Spratt, D. M. (1990). The role of helminths in the biological control of mammals. *Inter. J. Parasitol.* 20: 543-550
- Thompson, S. W. (1966). Selected histochemical and histopathological methods. Charles C. Thomas Publisher, Springfield
- Wolf, K., Gravell, M., Malsberger, R. G. (1966). Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. *Science* 151: 1004-1005

Responsible Subject Editor: P. Zwart, Utrecht, The Netherlands

Manuscript first received: February 16, 1992

Revised version accepted: June 12, 1992