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Preliminary characterization of a reovirus isolated from golden ide *Leuciscus idus melanotus*

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ABSTRACT: Some characteristics of a reovirus recently isolated from golden ide *Leuciscus idus melanotus* and tentatively designated as golden ide reovirus (GIRV) were determined. Spherical non-enveloped particles with an outer capsid of about 70 nm and an inner capsid of about 50 nm were observed by electron microscopy. The density of the virus determined in CsCl gradients was 1.36 g ml^{-1} The genome contained 11 segments of dsRNA. GIRV differed from other aquareoviruses by a slight reduction of infectivity after treatment with chloroform and by the absence of forming syncytia in cell monolayers.

KEY WORDS: Golden ide · Reovirus · Characterization

INTRODUCTION

Golden ide *Leuciscus idus melanotus* are often kept as ornamental fish in garden ponds. Furthermore, they are frequently used as test fish for toxicological monitoring. Therefore, golden ide were included in a survey program on fish diseases. During this program some fish developed epidermal hyperplasia. The skin alterations were associated with the presence of herpesvirus-like particles (Steinhagen et al. 1992). Attempts to isolate this virus using the epithelioma papulosum cyprini (EPC; Fijan et al. 1983) and fathead minnow (FHM; Gravell & Malsberger 1965) cell lines failed. However, evidence of cytopathic effects (CPE) was observed in both EPC and FHM cell cultures inoculated with homogenized internal organs from a single tumor-bearing golden ide. The CPE were characterized by the formation of focal aggregates of rounded cells (Neukirch & Kruse 1993). Formations of syncytia were not observed. In infected cells virus-like spherical particles different from herpesviruses and similar to reoviruses or birnaviruses were demonstrated in the cytoplasma by electron microscopical observations (Neukirch & Kruse 1993). However, the isolate could not be neutralized by polyvalent antisera against infectious pancreatic necrosis virus (IPNV strains Ab, Sp and VR 299), a birnavirus which has been isolated from numerous species of fish. The following contribution presents some biochemical and physical characteristics of this isolate.

MATERIALS AND METHODS

Cell culture. EPC cells were used for virus propagation and quantification of virus infectivity. The cells were grown as monolayer cultures using Eagle's minimal essential medium (Cytogen) with Eagle's salts (EMEM) supplemented with 10% fetal bovine serum ($EMEM_{10}$).

Virus propagation and assay. Virus which had been plaque purified in 3 consecutive cycles according to the method described by Mayr et al. (1974) was propagated in cell cultures grown in 75 cm^2 flasks (Nunc) using Dulbecco's modification of EMEM. The medium was supplemented with either 5% fetal bovine serum ($EDulb_5$) or 10 µg trypsin (Sigma; type XIII, TPCK [tosylamide-2-phenylethyl chloromethyl ketone] treated; 12 200 BAEE [Na-benzoyl-L-arginine ethyl ester] U mg^{-1}) per ml medium ($Edulb/T_{10}$) which enhanced virus infectivity (Neukirch & Kruse 1993). IPNV and CSV (chum salmon virus) were used as ref-

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erence viruses and were propagated with the same media in CHSE (chinook salmon embryo) cells. Virus infectivity was quantified by end-point dilution tests in 96-well microplates, the titres were calculated by the method of Kärber (1931). Virus propagation and assays were carried out at 20°C.

Treatment with chloroform, various pH values and IUDR. The sensitivity of the isolate to lipid solvents (10% chloroform), various pH values and IUDR (5-iodo-2-deoxyuridine, 100 µg ml^{-1}) was investigated according to the methods described by Mayr et al. (1974).

Hemagglutination. The hemagglutinating activity was examined in microplates (Severe 1962) using both infectious clarified culture fluid ($EDulb_5$ and $EDulb/T_{10}$) and virus which was concentrated 1:150 by ultracentrifugation. The virus suspensions were diluted 2-fold and incubated with human type 0, carp and chicken erythrocytes at 20°C for 2 h.

Neutralization. Neutralization tests were carried out in microtiter plates according to the method described by Mayr et al. (1974) using anti-CSV serum. (Antiserum against CSV and the corresponding virus were kindly provided by Dr S. LaPatra.) About 50 $CCID_{50}$ of golden ide reovirus (GIRV) and CSV were present in each test well.

Buoyant density of the virus. The density of the virus was determined by isopycnic centrifugation in CsCl gradients. Clarified infectious supernatant was mixed with CsCl (resulting density: 1.31 g ml^{-1}) and centrifuged at equilibrium for 40 h at 39 000 rpm (185 000 × *g*) in a SW41 rotor (Beckman). One ml fractions were collected by piercing the bottom of the centrifuge tube. The density of each fraction was calculated from the refractive index, and the viral infectivity was quantified by the end-point dilution test after the fractions had been dialysed against phosphate-buffered saline overnight.

Virus purification. Virus used for polyacrylamide gel electrophoresis to determine RNA segments and to analyse viral proteins was purified according to the method of Winton et al. (1987).

Electron microscopy. Virus used for electron microscopical studies was purified according to Winton et al. (1987) but without CsCl gradient centrifugation. Purified virus was resuspended in 0.1 ml of 0.1 M Tris buffer. Preparations placed on formvar-coated copper grids were negatively stained with neutralized 2% phosphotungstic acid and examined with a Zeiss EM 10A electron microscope. Particle dimensions were measured on photographic prints and by comparison with spherical latex particles of known size (mean diameter: 83 ± 19 nm; W. Plannet, Wetzlar, Germany).

Polyacrylamide gel electrophoresis. Five percent polyacrylamide slab gels (Laemmli 1970) were used to determine the number of RNA segments. The nucleic acid was extracted from the isolate as described by Winton et al. (1987). Equal volumes of purified virus and phenol saturated with buffer were mixed for 5 min. After centrifugation at 2000 × *g* for 10 min the aqueous phase was removed. The RNA was precipitated with cold ethanol, collected by centrifugation at 10 000 × *g* for 15 min, suspended in sample buffer and applied to the gel. After electrophoresis for 3 h at 10 mA and 1 h at 15 mA, the RNA segments were stained with silver (Merril et al. 1981). A second run was accomplished to better resolve the large segments using a 10% polyacrylamide gel for 50 h at 10 mA. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous system devised by Laemmli (1970) with 10% separating gel and 5% stacking gel. Purified virus was heated (100°C, 2 min) in sample buffer and applied to the gel. After electrophoresis for 16 h at 4 mA, the gels were fixed and stained with Coomassie Brilliant Blue.

RESULTS

Influence of chloroform, IUDR and pH on virus infectivity

After treatment of infectious supernatant ($EDulb_5$) with 10% chloroform for 2 h the virus infectivity decreased slightly by 1 to 2 log_{10} steps (Table 1). Treatment of virus grown in $EDulb/T_{10}$ resulted in a strong reduction of virus infectivity of more than 5 log_{10} steps. The stability of IPNV and CSV against chloroform treatment was not influenced by the medium used for virus propagation. Inhibition of virus synthesis by IUDR was not observed (Fig. 1). The infectivity was stable between pH 3 and 9 and at pH 2 and 10.5 only a slight reduction of virus infectivity was measured (Table 2).

Table 1 Influence of chloroform (10%; 2 h treatment) on golden ide reovirus (GIRV) infectivity. CSV (chum salmon virus) and IPNV (infectious pancreatic necrosis virus) were used as reference viruses. Infectivity titers in log_{10} $CCID_{50}$ ml^{-1}

Virus	Growth medium	Untreated	Chloroform treated	Difference in log_{10}
GIRV	$EDulb_5$	5.0	3.0	+2.0
	$EDulb/T_{10}$	7.25	2.0	+5.25
IPNV	$EDulb_5$	7.75	8.0	−0.25
	$EDulb/T_{10}$	7.5	7.5	0.0
CSV	$EDulb_5$	5.6	6.0	−0.4
	$EDulb/T_{10}$	8.4	8.5	−0.1

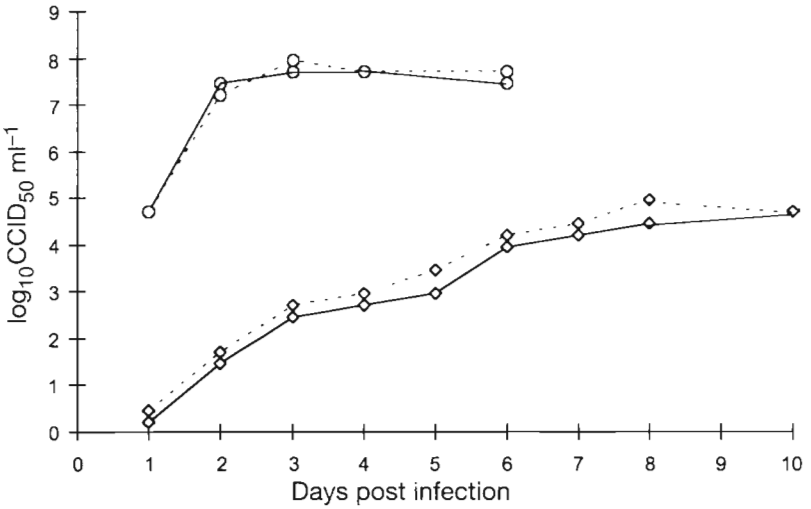

Fig. 1. Influence of IUDR (100 µg ml^{-1}) on the replication of the golden ide virus isolate. VHSV (viral hemorrhagic septicemia virus) was used as RNA reference virus. (—◇—) golden ide isolate; (—○—) VHSV; (---◇- --) golden ide isolate with IUDR; (---○---) VHSV with IUDR

Hemagglutination

Clarified infectious tissue culture fluid ($EDulb_5$ and $EDulb/T_{10}$) did not hemagglutinate human type 0, chicken or carp erythrocytes. With concentrated virus weak hemagglutinating activity (maximum titer: 1:32) was observed only when using chicken red blood cells.

Neutralization

The golden ide virus isolate was not neutralized by anti-CSV serum (Table 3).

Buoyant density of the virus

The maximum infectivity of the isolate was observed at a density of 1.36 g ml^{-1} (Fig. 2). A second infectivity peak occurred at 1.18–1.20 g ml^{-1}. IPNV, used as reference virus, showed maximum infectivity at a density of 1.34 g ml^{-1} (Fig. 2).

Table 2. Influence of pH on virus infectivity (in $\log_{10}$ $CCID_{50}$ ml^{-1}). Initial titer: $10^{4.5}$ $CCID_{50}$ ml^{-1}. nd: not done

pH	Virus infectivity after			
	30 min	60 min	120 min	180 min
2	nd	3.5	3.5	3.0
3	4.0	4.0	4.25	nd
5	4.25	4.0	4.0	nd
7	nd	nd	nd	4.5
9	4.25	4.0	4.0	nd
10.5	nd	4.0	3.75	3.5

Electron microscopy

In negatively stained preparations (Fig. 3a–e) non-enveloped spherical particles approximately 50 to 70 nm in diameter could be observed. The majority of the particles were of small size, about 50 nm, showing a single capsid shell probably due to the growth of virus in medium containing trypsin ($EDulb/T_{10}$). Only a few particles about 70 nm in diameter exhibited a double capsid shell (arrows in Fig. 3a,d) or an incomplete (coreless) structure (Fig. 3d,e). Propagation of

Table 3. Neutralization of the golden ide virus isolate (GIRV) and chum salmon virus (CSV) with anti-CSV serum. Values are given as reciprocal of serum dilution at which 50% of the test cultures were protected

Virus	Anti-CSV serum	Control serum
CSV	6090	<20
GIRV	<20	<20

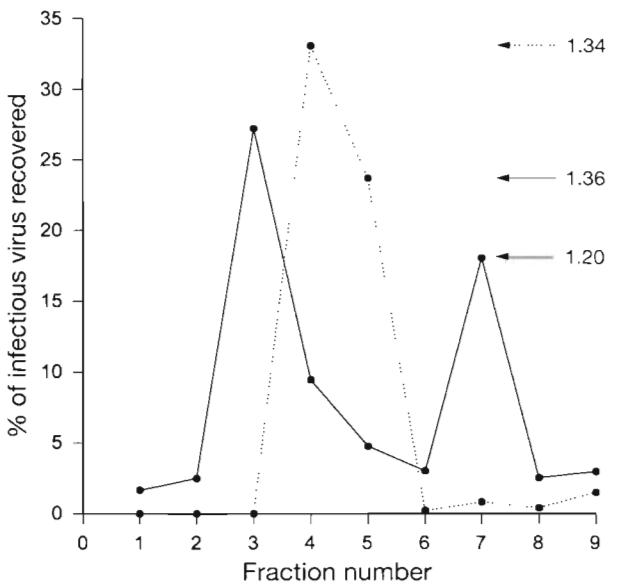

Fig. 2. Densities of golden ide isolate (—●—) and IPNV (···●···) after isopycnic centrifugation in a CsCl density gradient. Arrows indicate densities (g ml^{-1}) of the corresponding peaks

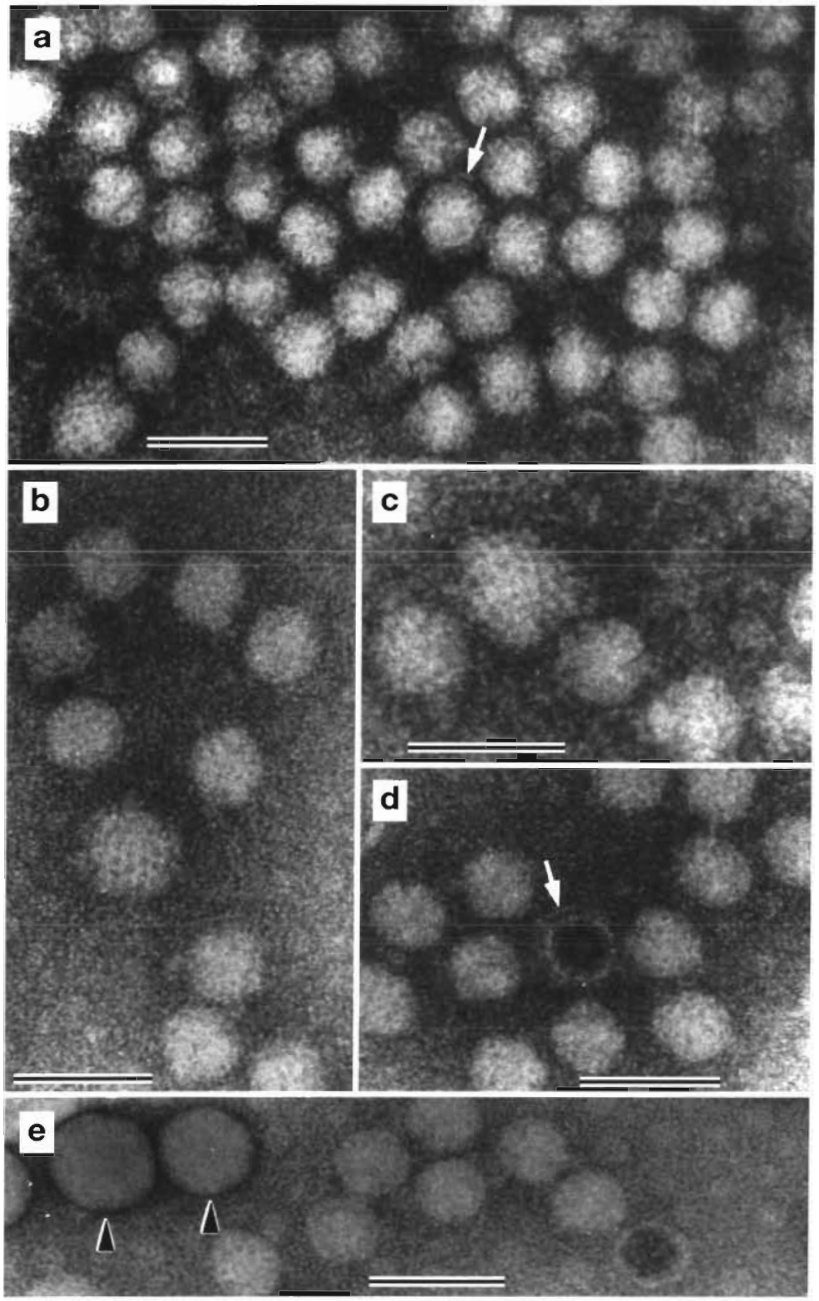

Fig. 3. Electron micrographs of negatively stained virus particles of the golden ide isolate. Most virions are approximately 50–55 nm in diameter (a). (b–d) Particles of different sizes (50–70 nm); some exhibiting a double capsid shell (arrows in a and d) or an incomplete (coreless) structure (d and e). (e) Latex particles (black arrowheads) of known size (83 ± 19 nm). Scale bars = 100 nm

virus with EDulb$_5$ resulted mostly in particle sizes of about 70 to 75 nm (Fig. 3c).

Polyacrylamide gel electrophoresis of RNA segments and polypeptides

Electrophoresis of viral nucleic acid using SDS-polyacrylamide vertical slab gels revealed that the genome of the virus was composed of 11 discernible segments of double-stranded RNA (Fig. 4a,b), divided in groups of small (5), medium (3) and large (3) segments.

SDS-PAGE of the proteins showed 2 major polypeptides with molecular weights of approximately 135 000 and 120 000 and 1 polypeptide with a molecular weight of nearly 70 000 (Fig. 4c).

DISCUSSION

Due to particle structure, host range, sensitivity to chemical and physical treatments and number of genome segments the family Reoviridae has been subdivided into 9 genera (Francki et al. 1991). Ten to twelve segments of dsRNA were found to be characteristic for the genome of Reoviridae (Matthews 1982). Reoviruses isolated from numerous fish species have been classified in the genus *Aquareovirus* according to the work of Winton et al. (1987). Viruses belonging to this genus are characterized by an efficient replication in fish cell monolayers forming syncytia, resistance of virus infectivity to lipid solvents, a size of 70 to 75 nm, bouyant density in CsCl of 1.34 to 1.36 g ml^{-1} and a genome containing 11 segments of dsRNA. Electrophoretic patterns of the genomic segments showed a 3-3-5 distribution for aquareoviruses (Winton et al. 1987). Within the genus *Rotavirus*, the other genus of the Reoviridae family with 11 genomic segments, the electropherotype of group A rotaviruses was characterized as a 4-2-3-2 pattern and group B rotaviruses exhibited different electrophero-types, including 4-3-1-1-1-1, 4-2-2-1-1-1 and 4-2-1-1-1-1-1 patterns (Eiden & Allen 1992).

The virus isolated from golden ide revealed characteristics of the genus *Aquareovirus*. The particle structure was found to be spherical, non-enveloped and about 70 nm in diameter with 2 capsid shells. The bouyant density was 1.36 g ml^{-1} and the genome was

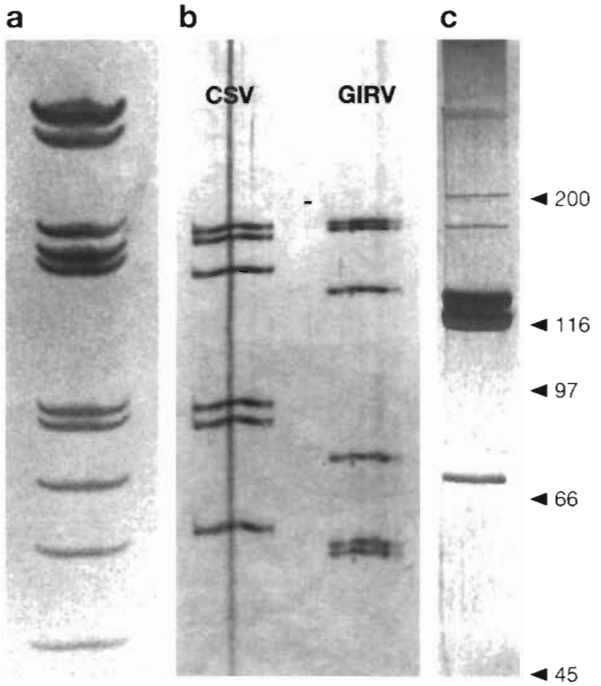

Fig. 4. Analysis of (a, b) virion RNA and (c) structural proteins in polyacrylamide slab gels. (b) PAGE using a 10% polyacrylamide gel for 50 h at 10 mA. Only the large and medium segments of CSV, used as reference virus, and GIRV are shown

composed of 11 segments of dsRNA distributed as 3 large, 3 medium and 5 small segments. With polyacrylamide gels of 5% only 2 large segments could be detected (Fig. 4a). Extended running times did not resolve an additional segment eventually comigrating in the first band. However, electrophoresis for 50 h using a 10% polyacrylamide gel revealed 3 segments (Fig. 4b).

The molecular weight of the 3 polypeptides demonstrated by SDS-PAGE is in accordance with those of members of the genus *Aquareovirus* (Winton et al. 1987). Two other major polypeptides of lower molecular weight usually demonstrated in aquareoviruses (Hedrick et al. 1984, Winton et al. 1987) could not be detected. This may be attributed to the fact that the virus had been propagated in medium supplemented with trypsin, resulting in a lack of the outer capsid shell.

Neutralization tests with antiserum against CSV showed no neutralizing capacity against GIRV indicating that GIRV is not related to CSV.

The properties of GIRV differed somewhat from other aquareoviruses in 2 characteristic ways. GIRV replicated in FHM and EPC cells, forming focal aggregates of rounded cells followed by lysis, but did not initiate the formation of syncytia in contrast to other reoviruses isolated from fish and shellfish (Plumb et al. 1979, Hedrick et al. 1984, Ahne & Kölbl 1987, Winton 1989, Varner & Lewis 1991). The other point of interest concerns the sensitivity of the golden ide isolate against chloroform treatment. The observed reduction of infectivity titers by 1 to 2 $\log_{10}$ steps does not agree with the resistance of aquareoviruses against treatment with lipid solvents (Winton et al. 1987, Winton 1989). Furthermore, GIRV became much more sensitive against chloroform when the virus had been propagated in medium containing trypsin. This phenomenon could not be observed with IPNV and CSV (Table 1). The treatment with chloroform did not alter the infectivity of IPNV no matter which medium was used for virus multiplication. The growth of CSV in EDulb/T_{10} resulted in enhanced virus infectivity but did not influence the stability against chloroform.

On the basis of morphological features and a genome with 11 dsRNA segments, characterized as a 3-3-5 pattern, the described isolate is proposed to be a member of the genus *Aquareovirus*. However, GIRV differs from other strains because of a variable sensitivity against lipid solvents and the absence of syncytia formations in cell cultures.

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