

Identification of grass carp haemorrhage virus as a new genogroup of aquareovirus

Aymara A. C. Rangel,¹ Daniel D. Rockemann,² Frank M. Hetrick³ and Siba K. Samal²

¹ Universidade Federal Rural do Rio de Janeiro, Brazil

² Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD 20742, USA

³ Maryland Institute for Agriculture and Natural Resources, College Park, MD 20742, USA

Three aquareovirus strains isolated from grass carp (*Ctenopharyngodon idellus*), geoduck clams (*Panope abrupta*) and herring (*Clupea harengus*) in North America and Asia were examined by RNA–RNA blot hybridization to determine their genogroup. The isolates from clams and herring were identified as members of genogroup A, but the isolate from grass carp did not hybridize to any of the known genogroups, suggesting that this virus probably represents a new, seventh genogroup.

Aquareoviruses have been isolated from a wide variety of aquatic animals, including finfish and crustacea, throughout the world (Lupiani *et al.*, 1995). These viruses replicate in cell cultures of piscine and mammalian origins, at temperatures between 15 °C and 25 °C (Samal *et al.*, 1998). They provide a characteristic cytopathic effect consisting of large syncytia (Samal *et al.*, 1990; Winton *et al.*, 1989). While their role in pathogenesis is not well understood, it is clear that some members of this group are important fish pathogens, capable of causing haemorrhagic disease, hepatitis and pancreatitis (Amend *et al.*, 1984; Chen & Jiang, 1984; Marshall *et al.*, 1990). These diseases pose a significant threat to aquacultural industries throughout the world.

Aquareoviruses are placed in the genus *Aquareovirus* in the family *Reoviridae* (Francki *et al.*, 1991). The diameter of aquareovirus in negative stain is approximately 75 nm and the particle exhibits multilayered protein capsid morphology (Shaw *et al.*, 1996). Aquareovirus is stable at pH 3 and ether-resistant. The genome of aquareovirus is composed of 11 segments of double-stranded (ds)RNA, a similar composition to the members of the genus *Rotavirus* within the family *Reoviridae*. However, aquareoviruses do not show any genetic or antigenic relatedness to the members of the genus *Rotavirus* (Samal *et al.*, 1990).

Author for correspondence: Siba Samal.

Fax +1 301 935 6079. e-mail ss5@umail.umd.edu

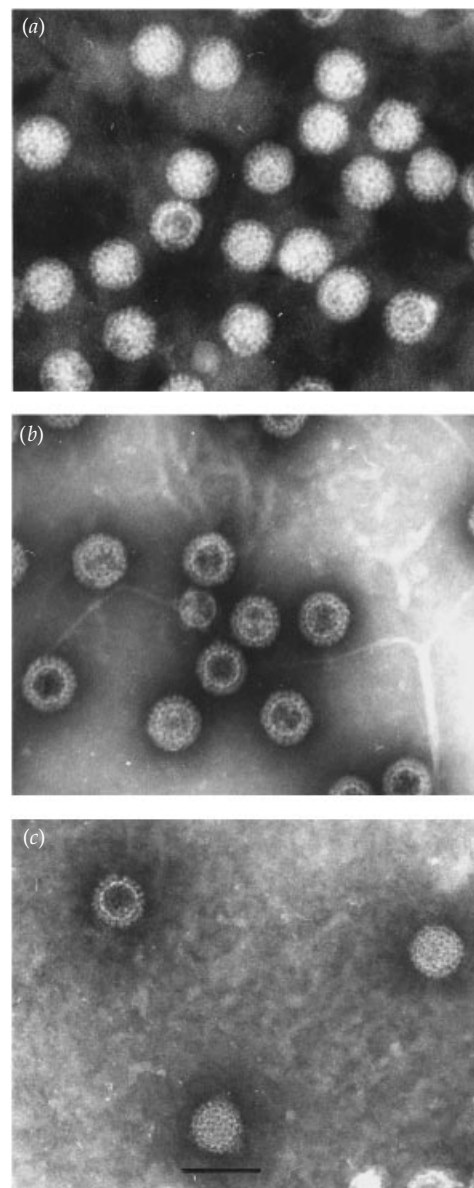


Fig. 1. Electron micrograph of purified aquareovirus isolates negatively stained with 2% phosphotungstic acid. Note the double capsid structure. The scale bar represents 100 nm. Grass carp haemorrhage virus (a), geoduck clams aquareovirus (b) and herring aquareovirus (c).

Using reciprocal RNA–RNA blot hybridization, six different genetic groups (genogroups) have been established (designated A to F) among the 42 aquareovirus isolates (Lupiani *et al.*, 1993, 1994; Subramanian *et al.*, 1997). Genogroup A was represented by 18 isolates, genogroup B by 19 isolates, genogroups C, D and E by one isolate each and genogroup F by two isolates. Genogroup A appeared to be the most heterogeneous, containing members from both cold and warm water fish and from fish on several continents. RNA–RNA hybridization between genogroups A and B showed that segment 10, the genome segment that encodes the major outer capsid protein, was the most variable gene (Lupiani *et al.*, 1993). The genome segment migration pattern (electrophenotype), as analysed by electrophoresis in 1% agarose gel, is consistent within a single genogroup but shows significant variation between viruses from different genogroups (Samal *et al.*, 1991). Viruses within a single genogroup do show variations in electrophenotype when their dsRNA genome segments are analysed by electrophoresis in high percentage (> 6%) polyacrylamide gels (Lupiani *et al.*, 1993; Subramanian *et al.*, 1997).

In this study, we have analysed the electrophoretic pattern of the genomic RNA segments of three additional aquareovirus isolates obtained from different geographical areas of the world and have studied their genetic relatedness by reciprocal RNA–RNA blot hybridization. This study includes for the first time grass carp haemorrhage virus (GCV), the most pathogenic aquareovirus. Our results identified GCV as a new, seventh genetic group of aquareovirus (genogroup G). These data will be useful in studying the epidemiology, taxonomy and classification of aquareoviruses in the family *Reoviridae*.

The GCV strain was isolated from a grass carp (*Ctenopharyngodon idellus*) in the People's Republic of China (Chen & Jiang, 1984). This virus causes severe haemorrhagic disease in grass carp, affecting about 85% of fingerling and yearling populations (Jiang & Ahne, 1989). GCV was previously characterized as a member of the family *Reoviridae* (Ke *et al.*, 1990), but its genetic relatedness with other members of the genus *Aquareovirus* was never determined. The other two aquareoviruses used in this study were isolated from apparently healthy finfish and shellfish during routine examinations. The geoduck clams aquareovirus (CLV) strain was isolated from a geoduck clam (*Panope abrupta*) from Vallenar Bay, Alaska in 1977. The herring aquareovirus (HRV) strain was isolated from an Atlantic herring (*Clupea harengus*) in Massachusetts in 1997.

The Chinook salmon embryo cell line (CHSE-214) was used to propagate the GCV, CLV and HRV strains. The CHSE-214 cells were also used to propagate striped bass reovirus (SBR, genogroup A), Chinook salmon reovirus (LBS, genogroup B), golden shiner virus (GSV, genogroup C), channel catfish reovirus (CRV, genogroup D), turbot reovirus (TRV, genogroup E) and salmon reovirus (SCR, genogroup F) strains. The simian embryonic kidney cell line MA104 was used to

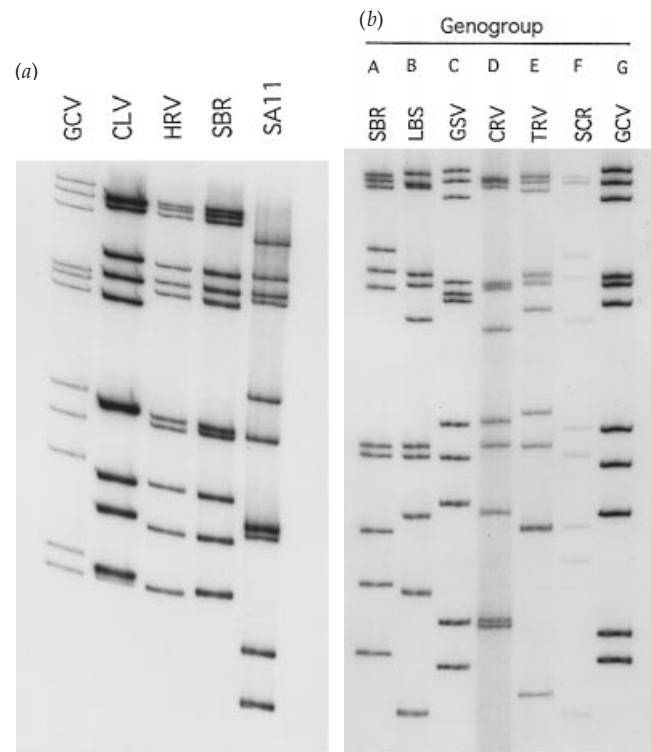


Fig. 2. Comparative genome profiles of (a) aquareovirus strains and simian rotavirus strain SA11, and (b) SBR, LBS, GSV, CRV, TRV, SCR and GCV representing genogroups A to G. Genomic dsRNAs were extracted from purified viruses, 3'-end labelled with [³²P]pCp and subjected to electrophoresis in a 7.5% polyacrylamide gel, 1–2 mm thick and 4–5 cm long. Electrophoresis was carried out for 18 h at 200 V. The genomic dsRNA segments were detected by autoradiography.

propagate simian rotavirus SA11. CHSE-214 and MA104 cells were grown at 16 °C and 37 °C, respectively. Aquareovirus, as well as SA11 virus, was purified as described elsewhere (Subramanian *et al.*, 1994). Small drops of purified GCV, CLV and HRV were negatively stained with 2% phosphotungstic acid and viewed with a JEOL 1200EX electron microscope. Electron micrographs of negatively stained viruses (Fig. 1) showed spherical particles 70–80 nm in diameter with a double-layered capsid. The ultrastructural features of GCV, CLV and HRV strains were similar to those of aquareovirus, suggesting that these viruses belong to the genus *Aquareovirus*.

The virus genomic dsRNAs were extracted from purified virus by the method of McCrae & Joklik (1978) and the 3' ends were labelled with cytidine-3',5'-bisphosphate using RNA ligase as described by England & Uhlenbeck (1978) and England *et al.* (1980). Samples of radiolabelled virus genomic RNAs were analysed in 7.5% polyacrylamide gels using a Tris–glycine buffer system (Laemmli, 1970). Radiolabelled dsRNAs of SBR strain (genogroup A aquareovirus) and rotavirus strain SA11 were also included in the electrophoretic analysis for the purpose of comparison. The migration pattern of the genomic dsRNAs of the three aquareovirus isolates in polyacrylamide gels is shown in Fig. 2(a). Each aquareovirus

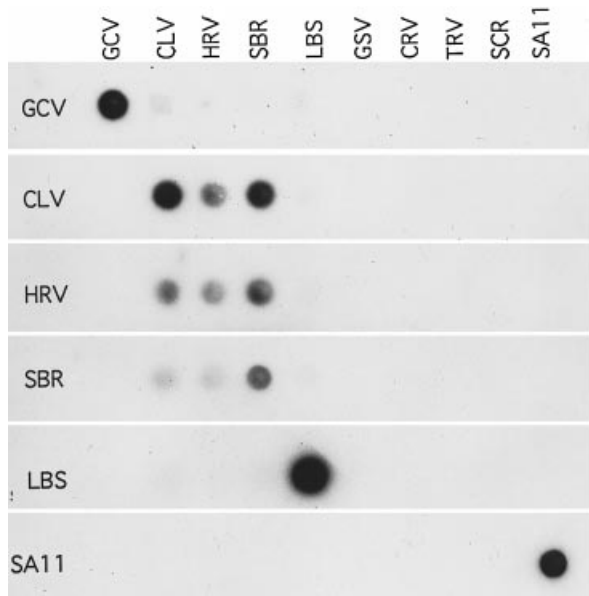


Fig. 3. Reciprocal RNA-RNA dot blot hybridization between GCV, CLV and HRV with genogroup A (SBR), genogroup B (LBS), genogroup C (GSV), genogroup D (CRV), genogroup E (TRV), genogroup F (SCR) and rotavirus SA11. Genomic dsRNAs were extracted from purified viruses, denatured by heating at 100 °C for 2 min and applied to a nylon membrane. Each probe was made by 3'-end labelling of total genomic dsRNAs and hybridized to a blot containing RNAs from different viruses. Hybridization was at 52 °C in 50% formamide and blots were washed at 72 °C.

isolate exhibited a distinctive profile of 11 dsRNA segments in polyacrylamide gels. The electrophoretic patterns of GCV, CLV and HRV were similar to other aquareoviruses in having three large (segments 1–3), three medium (segments 4–6) and five small segments (segments 7–11). Two large dsRNA segments (segments 1 and 2) and two small dsRNA segments (segments 7 and 8) of the CLV strain were difficult to resolve in most gels but were resolved in longer gels (results not shown). The dsRNA profiles of the three virus isolates were unlike that of rotavirus SA11. The presence of 11 dsRNA segments in the genome of these three virus isolates confirms them as being aquareoviruses. Furthermore, the electrophoretic pattern of the genomic dsRNAs of these aquareovirus isolates can be used for molecular epidemiological studies to monitor virus outbreaks and transmission.

The genetic relatedness of GCV, CLV and HRV strains among themselves and with the six known genogroups (A to F) was studied by reciprocal RNA-RNA dot blot hybridization (Fig. 3). Total genomic dsRNA probes were prepared following standard protocols. Briefly, the viral genomic dsRNA was extracted from purified viruses by the method of McCrae & Joklik (1978) and cytidine-3',5'-bisphosphate (England & Uhlenbeck, 1978). For dot blot hybridization, purified total genomic RNAs were denatured by boiling and applied to a Gene Screen Plus membrane (Dupont). The conditions used for the prehybridization and hybridization of membranes were

similar to those of Bodkin & Knudson (1985*a*). Blots were prehybridized for 2 h at 52 °C using hybridization buffer containing 50% formamide, 5 × SSC, 50 mM sodium phosphate pH 6.5%, sonicated salmon testes DNA (100 µg/ml), 0.2% SDS and 0.1% each of BSA, ficoll and polyvinylpyrrolidone. After overnight hybridization at 52 °C, membranes were washed at room temperature twice for 30 min in 1 × SSC–0.1% SDS at 72 °C. The membranes were then dried, sealed in plastic bags and exposed to X-ray films. When dsRNA from GCV was used as a probe, hybridization signals were observed with itself, but not with any of the known genogroups (A to F). This result indicates that GCV probably represents a new, seventh genogroup (genogroup G) in the genus *Aquareovirus*. When dsRNA from CLV was used as a probe, hybridization signals were observed with itself, HRV and SBR. Similarly, when dsRNA from HRV was used as a probe, hybridization signals were observed with itself, CLV and SBR. These results suggested that CLV and HRV belong to genogroup A. Reciprocal hybridization with SBR (genogroup A) probe confirmed that CLV and HRV are members of genogroup A. Simian rotavirus strain SA11 did not hybridize with any of the aquareovirus isolates, indicating that SA11 is not genetically related to the aquareovirus (Fig. 3).

Reciprocal RNA-RNA blot hybridization has been used to genetically group reoviruses (Bodkin & Knudson, 1985*b*), rotaviruses (Flores *et al.*, 1982; Nakagomi & Nakagomi, 1989, 1991; Nakagomi *et al.*, 1989) and orbiviruses (Bodkin & Knudson, 1986; Kowalik & Li, 1987). Using this approach, we had previously identified six genogroups (A to E) among 42 strains of aquareovirus (Lupiani *et al.*, 1993; Subramanian *et al.*, 1997). In this study, we have extended this approach to determine the genogroup of three additional aquareovirus strains. Based on our hybridization results, the GCV strain was found to represent a new, seventh genogroup, designated G. The electrophoretic pattern of the six established genogroups, A to F (SBR, LBS, GSV, CRV, TRV and SCR) and the new genogroup G (GCV) are shown in Fig. 2(*b*). Since GCV is a highly pathogenic aquareovirus, it remains to be seen if future members of this genogroup are also pathogenic. Grouping of aquareovirus strain CLV was also interesting since it was isolated from a shellfish. Surprisingly, the CLV strain was found to be a member of genogroup A. Our results suggest that viruses belonging to genogroup A can infect both finfish and shellfish. This is in contrast to rotaviruses, where viruses belonging to one genogroup are usually derived from the same animal species (Nakagomi & Nakagomi, 1991). Although CLV and HRV belong to genogroup A, they were isolated from Alaska and Massachusetts, respectively. The location of CLV and HRV isolation reinforces our previous observation that viruses belonging to genogroup A are present in a wide range of geographical areas (Lupiani *et al.*, 1993; Subramanian *et al.*, 1997).

The results of this study confirm and extend the results of our previous studies (Lupiani *et al.*, 1993; Subramanian *et al.*,

1997), which demonstrated that there is a wide genetic diversity in the genus *Aquareovirus* and provide an experimental method for grouping aquareoviruses on the basis of genetic relatedness. Reassortment of aquareovirus among genogroups does not seem to occur (unpublished data). However, the possibility of reassortment among strains of a genogroup in nature cannot be ruled out at present. The genogroups determined in this study could be used for comparison with new aquareoviruses as they are isolated. Further biological and serological studies on the isolates in each genogroup are required before the divisions made here can be fully substantiated.

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