A FOURTH AQUABIRNAVIRUS SEROGROUP

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Introduction

There is an extensive world-wide trade in tropical ornamental fish and Singapore is at the forefront of that trade. Many tropical ornamental fish are bred in Singapore, and others are imported for ongrowing there prior to export to markets throughout the world. Any diseases in the fish are investigated by the Agri-Food and Veterinary Authority. Investigations into separate cases of mortality in populations of freshwater angelfish, *Pterophyllum scalare*, dwarf gourami, *Colisa lalia*, and the ram cichlid, *Microgeophagus (=Apistogramma) ramirezi* between 1995 and 1998 demonstrated the presence of viruses, which were identified as aquabirnaviruses. Their serological relationships to the aquabirnavirus Serogroups A, B and C (Hill and Way 1995, John and Richards 1999) were investigated, and showed that the isolates belong to a new aquabirnavirus Serogroup.

Virus isolation

Details of the fish sampled are shown in Table 1; all were taken from populations of cultivated fish undergoing mortalities. Pools of brain and eye, and of liver, spleen and kidney taken from 5 - 10 fish were homogenised, clarified, and inoculated onto bluegill fibroblast (BF-2), *Epithelioma papulosum cyprini* (EPC) and chinook salmon embryo (CHSE-214) cells. The cells were incubated at 25°C (BF-2 and EPC) or 18°C (CHSE-214). If cytopathic effect (CPE) was not observed after 10 days, blind passages were done. In all the diagnostic investigations, CPE was observed in BF-2 cells only, upon primary inoculation with both tissue pools.

Table 1: Details of the fish tested

Case ref	Isolate code	Fish species	Number of fish	Country of origin	Year
A08/12/95	M2[1]	Freshwater angelfish *(Pterophyllum scalare)*	20	Malaysia[2]	1995
A16/09/97	M3	Dwarf gourami *(Colisa lalia)*	10	Malaysia[2,3]	1997
A03/04/97	M4	Ram cichlid *(Microgeophagus ramirezi)*	10	Malaysia[2]	1997
A16/02/98	M5	Ram cichlid	6	Malaysia[2]	1998

[1] The same code as used by Chew-Lim *et al*., (in press)
[2] These are not native to Malaysia
[3] From the same farm site as the freshwater angelfish

Electron microscopy

The viruses were concentrated using 7% polyethylene glycol 6000 as described by Dixon and Hill (1983). Concentrated virus was applied to carbon-coated Formvar grids, stained with 2 % methylamine tungstate and observed with a JEOL 1210 transmission electron microscope. All four isolates were found to be isometric viruses, with a diameter of approximately 60 nm (Figure 1).

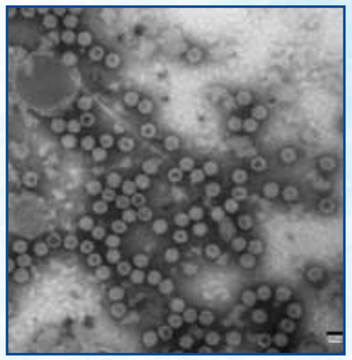

Figure 1: Electron micrograph of the virus (M2) isolated from the freshwater angelfish stained with 2% methylamine tungstate. Bar = 100 nm

Virus properties

The effect of 5-bromo-2'-deoxyuridine (BUDR) on replication of the isolates, and their sensitivity to chloroform were determined as described by Rovozzo and Burke (1973). The isolates were chloroform resistant and replication was not inhibited by BUDR; the latter property indicated that the nucleic acid was RNA. Those properties, and the virion morphology, suggested that the isolates might be members of the family *Birnaviridae*. The isolated viruses were inoculated onto rainbow trout gonad (RTG-2) and fathead minnow (FHM) cells, as well as into EPC and CHSE-214 cells to investigate their cell line host range. Primary inoculation was done at 1:100 and 1:1000 dilutions, the cells were incubated at 25°C for 7 days, then if no CPE was observed, blind passages were done at 1:10 and 1:100 dilutions. Table 2 shows the results, including the cell line specificity of isolates from the three aquabirnavirus Serogroups (serotypes A2, B1 and C1) for comparison.

Table 2: Growth of aquabirnavirus isolates in different cell lines

Isolate	Cell line			
	EPC	RTG	FHM	CHSE-214
A2	Yes	Yes	Yes	Yes
B1	Poor growth	No	Poor growth	No
C1	Yes (variable)*	Yes	Yes	No
M2	No	Yes	No	No
M3	No	Yes	No	No
M4	No	Yes	No	No
M5	No	Yes	No	No

* In some experiments there was CPE, but generally this was when cells were very sub-confluent, and the resulting harvests did not always passage.

RNA extraction and electrophoresis

Each virus was concentrated as above and the nucleic acid was extracted by the SDS-proteinase K-phenol/chloroform/isoamyl alcohol method essentially as described by Ganga *et al*., (1994). Electophoresis of RNA was carried out on 6 % polyacrylamide gels at 200 V for 1.5 h then the gels were silver stained. By comparison with reference aquabirnaviruses, the four isolates contained two strands of double-stranded (ds) RNA (Fig. 2), confirming them to be aquabirnaviruses. The molecular masses of the RNA segments of the four isolates were similar to each other but differed from reference aquabirnaviruses representing serotypes A1, A2, B1 and C1.

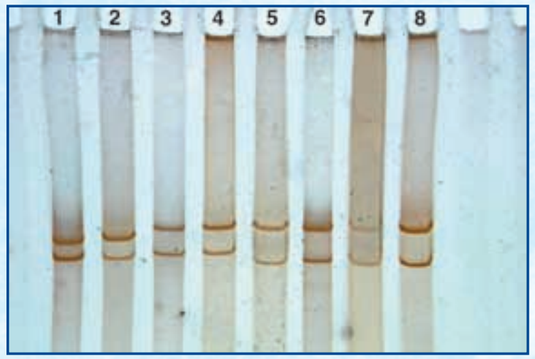

Figure 2: Comparison of the purified RNA segments of isolates M2, M3, M4 and M5 with reference aquabirnaviruses on silver-stained 6% polyacrylamide gels. Lane 1, serotype A1, lane 2, serotype A2, lane 3, serotype B1, lane 4, serotype C1, lane 5, isolate M2, lane 6, isolate M3, lane 7, isolate M4, lane 8, isolate M5

Serological comparisons with reference aquabirnaviruses

The reference aquabirnaviruses (representing serotypes A1, A2, A3, A4, A5, A6, A7, A8, A9, B1) and their homologous antisera were as described by Hill and Way (1995). Blotched snakehead virus (BSNV) representing Serogroup C (John and Richards 1999) was a gift from Dr K.R. John. Details of a second member of Serogroup C (M23/6/91), isolated from the giant snakehead (*Channa micropeltes*) (Subramanian *et al*., 1993) and its homologous antiserum are given in Chew-Lim *et al*., (in press). That virus and its homologous antiserum were used in the serological comparisons, as antiserum against BSNV was not available. Isolates M2 and M3 were purified and antisera against them (numbers 280 and 281, respectively) prepared in rabbits by standard methods. All Serogroup A and B viruses were grown in BF-2 cells at 15°C and the Serogroup C viruses were grown in BF-2 cells at 25°C.

The new isolates were plaque titrated as described by Dixon and Hill (1983) and plaque cloned prior to serological comparisons. Initial plaque neutralisation tests on isolates M2, M3, M4 and M5 using antisera against all reference Serogroup A, B and C viruses were done using 1:100 and 1:1000 (final) dilutions of antisera. The four isolates were not neutralised by 1:100 dilutions of any of the antisera.

Neutralisation tests of all Serogroup A, B and C viruses using antisera 280 (against isolate M2) and 281 (against isolate M3) were done by the constant serum (1:100)/virus dilution (ten-fold step) method (alpha neutralisation, Rovozzo and Burke 1973). The antisera did not neutralise any of the nine Serogroup A serotypes, serotype B1 or serotype C1. Serotype A8 appeared to be neutralised by both antisera in alpha neutralisation tests, and that was investigated further using the 50% plaque reduction method described by Hill and Way (1995). The 50% plaque reduction titres of antisera 280 and 281 against isolate A8 were both <1:200; that level of neutralisation was considered to be non-specific.

The 50% plaque reduction method was used to compare isolates M2, M3, M4 and M5. The serological relationship between isolates M2 and M3 was determined using the formula $r = \sqrt{r1 \times r2}$ (Archetti and Horsfall 1950), where

$$r1 = \frac{\text{reciprocal of heterologous titre obtained with virus 2}}{\text{reciprocal of heterologous titre obtained with virus 1}}$$

and

$$r2 = \frac{\text{reciprocal of heterologous titre obtained with virus 1}}{\text{reciprocal of heterologous titre obtained with virus 2}}$$

The criteria of Hill and Way (1995) that a 1/r value of <10 would indicate that isolates were of the same serotype, and a value significantly >10 would indicate a new serotype, were used. The results are shown Table 3; the 1/r value for isolates M2 and M3 was 555.15.

Table 3: Fifty per cent plaque reduction titres of isolates M2, M3, M4 and M5 with antisera 280 and 281.

Antiserum	Virus			
	M2	M3	M4	M5
280	**646,200**	99	329,750	300
281	2,400	**113,250**	2,300	184,100

Homologous reactions are shown in bold type.

Conclusions

- The morphology of isolates M2, M3, M4 and M5, their insensitivity to chloroform, and their genome comprising two strands of dsRNA showed them to be aquabirnaviruses.
- The serological comparisons showed that the isolates were distinct from the three established aquabirnavirus Serogroups, and that they should be allocated to a fourth aquabirnavirus Serogroup, D.
- The 1/r value of 555.15 for isolates M2 and M3 distinguishes them as different serotypes within Serogroup D. M2 should be reference serotype D1, and M3 should be reference serotype D2.
- Although reciprocal cross neutralisation tests have not been done, the reactions of isolate M4 suggest that it should be provisionally allocated to serotype D1, and those of isolate M5 suggest that it should be provisionally allocated to serotype D2.
- The molecular masses of the two RNA segments of the isolates were different from the other aquabirnaviruses compared, but similar to each other. The size difference between the two genome segments, was greater than that of BSNV, which was reported to have the largest size difference of all the aquabirnaviruses (John and Richards 1999).
- The cell line specificity of the isolates was identical to each other, but distinct from the other serotypes tested.

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