Vol. 26: 159–162, 1996 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published August 15

NOTE

Antigenic differences among aquareoviruses correlate with previously established genogroups

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ABSTRACT: Five aquareoviruses, selected to include 1 from each of the 5 genogroups established by Lupiani et al. (1993; Virology 197:475–479), were compared by cross-neutralization and immunodot test procedures. Serological ratios by cross-neutralization were extremely high, demonstrating that the 5 selected strains correspond to 5 different serogroups. On the other hand, the ratios obtained from cross-immunodot test were much lower, and in some cases under 20. Results demonstrate that the serogroups determined by cross-neutralization correlate with the previously established genogroups of aquareoviruses.

KEY WORDS: Serogroups · Neutralization · Aquareovirus

Over the last 15 yr, aquareoviruses have been increasingly isolated from different aquatic animals in different geographic areas. These isolates share several biochemical and biophysical characteristics, but are different in other respects. Few attempts have been undertaken to clarify the taxonomic status of the strains included in the genus *Aquareovirus*. Serological heterogeneity among several reoviruses isolated from aquatic animals was observed by some authors employing neutralization tests (Hedrick et al. 1984, Ahne & Kölbl 1987, Brady & Plumb 1988). Dopazo et al. (1992), studying serological relationships among 5 aquareovirus strains by means of cross-neutralization, immunodot and ELISAs (enzyme-linked immunosorbent assays), established the existence of at least 2 different serogroups among aquareoviruses which correlated with differences in genome electropherotypes. These studies, however, were based on the comparison of few viral strains which limited the significance of the results. Recently, Lupiani et al. (1993) reported the comparison of most of the aquareovirus isolates known at that time (19 strains) on the basis of their RNA profiles and cross RNA-RNA hybridizations. Based on their results, 5 different genogroups (named A, B, C, D, and E) have been established within the genus *Aquareovirus*.

In the present study, viral strains belonging to each of the 5 genogroups were compared by seroneutralization and immunodot techniques, in order to determine if genogroups and serogroups are correlated.

Materials and methods. The aquareovirus strains used in the present study are listed in Table 1. Chinook salmon embryo cells (CHSE-214) grown at 15°C were used for the replication of all the isolates, except the strain from catfish (CRV) which was grown in channel catfish ovary cells (CCO) at 23°C. The cells were cultured in Eagle's minimum essential medium (EMEM), supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 50 µg ml^{-1} gentamicin).

Viral isolates were inoculated at an MOI (multiplicity of infection) of 0.1 and when cytopathic effects (CPE) were extensive cells were scraped into the medium and the cell debris pelleted at 3000 × *g* for 20 min. The supernatant was transferred to a sterile bottle and polyethylene glycol (PEG, molecular weight 8000) added to a final concentration of 10%. The pellet was resuspended in 10 ml of SSC buffer (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.2), vigorously shaken for 5 min, centrifuged at 3000 × *g* for 10 min and the supernatant transferred to the former PEG suspension. After incubating overnight at 4°C on an orbital shaker, the fluid was centrifuged at 10000 × g for 30 min and the pellet resuspended in 10 ml of SSC. An equal volume of Freon was added to the suspension and the mixture was vigorously shaken for 5 min. The resultant emulsion was separated into the Freon and aqueous phase by centrifugation at 8000 × *g* for 30 min, and the aqueous phase was then centrifuged at 85000

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Table 1. Aquareovirus strains employed

Genogroup[a]	Viral strain	Species	Location
A	Striped bass reovirus (SBR)	*Morone saxatilis*	USA
B	Simpson coho salmon (SCS)	*Oncorhynchus kisutch*	USA
C	Golden shiner virus (GSV)	*Notemigonus crisoleucas*	USA
D	Catfish reovirus (CRV)	*Ictalurus punctatus*	USA
E	Turbot reovirus (TRV)	*Scophthalmus maximus*	Spain

[a]Genogroups as established by Lupiani et al. (1993)

$\times g$ for 90 min. The pellet was resuspended in 0.5 ml of SSC, sonicated for 30 s, layered on a 10 to 50% sucrose gradient and centrifuged at 150000 $\times g$ for 90 min. The visible virus band was collected, concentrated by centrifugation (85000 $\times g$ for 90 min), resuspended in 500 µl of buffer and centrifuged for a second time on a sucrose gradient as above indicated. The visible band was finally concentrated at 85000 $\times g$ for 90 min, and the virus resuspended in SSC and stored at 4°C until used.

The 5 antisera were obtained by inoculating young New Zealand white rabbits with purified virus according the following schedule: 100 µl of purified virus (approximately 10^{10} $TCID_{50}$ ml^{-1}) was diluted (1/5) in phosphate buffered saline (PBS), mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly into each hind leg of the rabbit, and subcutaneously (2 aliquots). One month later, 1 ml of purified virus diluted in a solution of $Al(OH)_2$ was inoculated by intramuscular injection. Two months after the first injection, a last injection was made intramuscularly with purified virus diluted in PBS, and 1 wk later the rabbits were bled. The sera were inactivated (56°C, 30 min) and stored at −20°C until used. Prior to the first immunization, sera from the rabbits were tested and found to be negative for neutralizing antibodies against the 5 aquareoviruses.

The seroneutralization test was performed following the constant virus-varying antiserum dilution method (Okamoto et al. 1983). The viruses were diluted in EMEM without FBS to obtain a suspension containing approximately 10^3 $TCID_{50}$ ml^{-1} (initial viral titers ranging from $10^{6.5}$ to 10^8 $TCID_{50}$ ml^{-1}). The antisera were diluted from 1/10 to 1/100000 in EMEM, and viruses and antisera dilutions were mixed (1:1). After 1 h incubation at room temperature, 0.1 ml of each mixture were inoculated in triplicate wells of confluent cells in 48-well plates. The plates were sealed and incubated at 15°C for the CHSE-214 cells, or 23°C for the CCO cells, for a maximum period of 3 wk. The neutralizing antibody titers were calculated according to the Reed & Müench (1938) method and expressed as the reciprocal of the highest dilution of the antiserum protecting 50% of the inoculated cells.

The immunodot assay was performed as previously described (Dopazo et al. 1992). Briefly, pieces of nitrocellulose membrane (HA, 0.45 µm, Millipore) were washed with PBS and dried under warm air before use. Then, 10 µl of crude virus (non-purified tissue culture supernatants, with an approximate titer of 10^7 $TCID_{50}$ ml^{-1}) was added to the membranes and allowed to dry for 15 min at room temperature. Infectious pancreatic necrosis (IPN) virus, EMEM with 5 and 10% FBS and EMEM without serum were used as controls. To block non-specific reactive sites, the membranes were immersed in a solution containing 5% powdered milk

Table 2. Antigenic relatedness among 5 aquareoviruses by cross-neutralization tests. Numbers in bold correspond to the homologous titers

Geno-group[a]	Viral strain	Antiserum SBR	SCS	GSV	CRV	TRV
A	SBR	**7943**	<10	<10	<10	2512
B	SCS	<10	**7080**	84	<10	<10
C	GSV	<10	<10	**19953**	<10	<10
D	CRV	199.5	<10	94.5	**5000**	94.5
E	TRV	<10	<10	80.2	<10	**15850**

[a]Genogroups as established by Lupiani et al. (1993)
[b]Neutralization titers expressed as reciprocal of the 50% neutralization endpoint

Table 3. Antigenic relatedness among 5 aquareoviruses as determined by cross-immunodot tests. Numbers in bold correspond to the homologous titers

Geno-group[a]	Viral strain	Antiserum SBR	SCS	GSV	CRV	TRV
A	SBR	**10000**[b]	500	1000	1000	2500
B	SCS	500	**10000**	5000	1000	1000
C	GSV	1000	<10	**25000**	1000	1000
D	CRV	500	<10	500	**10000**	1000
E	TRV	10	<10	1000	100	**25000**

[a]Genogroups as established by Lupiani et al. (1993)
[b]Immunodot titers, expressed as the reciprocal of the highest dilution of antisera giving a positive test

in PBS for 30 min at room temperature and then washed 3 times (10 min each) with PBS containing 0.02% Tween 20. The antisera against the 5 aquareoviruses were diluted as described for the former experiment and the membrane was immersed in the respective dilution of each antiserum and incubated at room temperature for 1 h. After washing as above, goat antirabbit IgG conjugated with peroxidase (diluted 1/1000 in PBS) was added and the mixture incubated for 1 h at room temperature. The membranes were washed and immersed in the color-developing solution (30 mg of 1-Cl-α-naphthol dissolved in 1 ml of cold methanol, plus 50 ml of PBS and 30 µl of 30% H_2O_2). After the appearance of a blue color, which indicates a specific positive reaction, membranes were washed, air dried, and conserved in vacuum sealed plastic bags.

The serological relationships ($1/r$) between the aquareoviruses were calculated as described by Archetti & Horsfall (1950) from the formula $r = \sqrt{r_1 \times r_2}$, where r_1 and r_2 are the heterologous titers divided by the homologous titers for the corresponding antisera. A value of $1/r = 1$ between 2 viruses indicates that they are serologically identical, and a value of 2 will indicate that there is a relatedness of 50%. The higher the value, the lower the relatedness.

Table 4. Cross-neutralization ratios among selected strains of 5 *Aquareovirus* genogroups

Genogroup[a]	Virus	Virus: SBR	SCS	GSV	CRV	TRV
A	SBR	**1**	750	1259	141	71
B	SCS	–	**1**	410	595	1059
C	GSV	–	–	**1**	325	628
D	CRV	–	–	–	**1**	290
E	TRV	–	–	–	–	**1**

[a]Genogroups as established by Lupiani et al. (1993); data are given as $1/r$, with $r = \sqrt{r_1 \times r_2}$, and r_1 and r_2 are the titer ratios calculated as the ratio between the heterologous and the homologous titers

Table 5. Cross-immunodot ratios among selected strains of 5 *Aquareovirus* genogroups

Genogroup[a]	Virus	Virus: SBR	SCS	GSV	CRV	TRV
A	SBR	**1**	20	16	14	100
B	SCS	–	**1**	71	100	158
C	GSV	–	–	**1**	22	25
D	CRV	–	–	–	**1**	50
E	TRV	–	–	–	–	**1**

[a]Genogroups as established by Lupiani et al. (1993); data are given as $1/r$, with $r = \sqrt{r_1 \times r_2}$, and r_1 and r_2 are the titer ratios calculated as the ratio between the heterologous and the homologous titers

Results and discussion. Cross-neutralization has been reported by Dopazo et al. (1992) as the most useful procedure to study serological relatedness among aquareoviruses. This assay was therefore chosen for an immunological comparison of viral strains from the 5 genogroups of the genus *Aquareovirus*. The immunodot assay was employed for comparative purposes.

Titers of antisera against homologous and heterologous viruses obtained by the neutralization and immunodot assays are shown in Tables 2 & 3, respectively. Homologous titers were high ranging from 1/5000 (for CRV) to 1/19953 (GSV) by neutralization and from 1/10000 (SBR, SCS and CRV) to 1/25000 (GSV and TRV) by immunodot. In a previous report (Dopazo et al. 1992), the aquareoviruses were thought to be poorly immunogenic in rabbits based on the titers of antisera obtained by neutralization (maximum value: 1/7080). The antiserum titers obtained in the present study are remarkably high, which rule out that conclusion. Since in the protocol to obtain antisera, viral purification was modified with respect to previous reports (Dopazo et al. 1992) to avoid use of sonication, mercaptoethanol and deoxicholate, integrity of the virus may have been better maintained, which could explain those higher antisera titers.

Some authors have employed cross-neutralization titers to determine serological relatedness among aquareoviruses (Brady & Plumb 1988). However, use of cross-neutralization ratios is a better approximation to serological comparisons among viral strains, and these have been employed by many authors (Okamoto et al. 1983, Hedrick et al. 1984, Ishiguru et al. 1984, Dopazo et al. 1992). In the present report, the serological relationship ratios were calculated according to Okamoto et al. (1983) from the antisera titers obtained by cross-neutralization and cross-immunodot, and the results are shown in Tables 4 & 5.

The ratio value determining that 2 viral strains correspond to 2 different genogroups was established at >20 by Jørgensen (1972) based on a decision by the Committee on Enteroviruses in 1962. As shown in Table 3, ratios by cross-neutralization were much higher than 20, clearly demonstrating that the 5 selected aquareovirus strains employed correspond to 5 separate serogroups. Serological ratios calculated from cross-immunodot tests yielded different results. The

values were not only lower than by neutralization, but also under 20 in 2 cases (serological relatedness of SBR with GSV and CRV). This disparity between results from the 2 techniques could be due to involvement of different epitopes in the antibody-antigen reaction.

In a previous study Dopazo et al. (1992) found some relationship between neutralization ratios and RNA profiles of several aquareovirus strains, and established that cross-neutralization is the best tool for studying serological relatedness among aquareoviruses. The results presented here agree with that conclusion. On the other hand, since the $^1/_r$ values are usually lower by immunodot than by neutralization tests, as shown in the present study and in previous reports (Dopazo et al. 1992), perhaps the criterion for determining serogroups by neutralization ($^1/_r$ > 20) should be changed for immunodot data. Finally, it appears that the serogroups determined by cross-neutralization correlate with the genogroups established by Lupiani et al. (1993); however, further studies including a larger number of aquareovirus strains must be conducted to confirm that conclusion.

Acknowledgements. This work was supported by Grants XUGA 20002A94 from Xunta de Galicia and MAR 89-0270 from the Comisión Interministerial de Ciencia y Tecnología (CICYT), Spain.

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Responsible Subject Editor: F. M. Hetrick, College Park, Maryland, USA

Manuscript first received: January 16, 1996
Revised version accepted: May 21, 1996

Erratum

Re: L. Margolis, M. L. Kent, P. Bustos
"Diseases of salmonids resembling myxosporean whirling disease, and the absence of *Myxobolus cerebralis*, in South America"
Dis Aquat Org 25: 33–37 (1996)

- An error occurred in the first author's initials. The correct form is given above