In vitro host range of aquatic birnaviruses

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Abstract
One hundred and three isolates of aquatic birnaviruses (ABV) belonging to all 9 serotypes of serogroup A were tested for their ability to grow in 4 continuous cell lines derived from salmonids and non-salmonids. All 103 isolates replicated and produced rapid and extensive cytopathogenic effects (CPE) in the salmonid cell lines derived from chinook salmon (CHSE-214) and rainbow trout (RTG-2), whereas only half produced visible CPE in two non-salmonid cell lines tested (from carp (EPC) and minnows (FHM)) in 7 days. There was a significant positive correlation between the level of CPE and the virus titer produced in the infected cells. In addition, it was found that 19/46 isolates that failed to produce CPE in EPC and FHM cells at 7 days post-infection, had replicated to titers up to $10^5$ TCID$_{50}$/mL, and that 27/46 isolates produced no evidence of virus replication. The ability to produce CPE in non-salmonid cells was strongly correlated with the virus serotype. Isolates belonging to serotypes A$_1$ (subtypes WB and VR-299), A$_2$ (Sp), A$_4$ (He), and A$_5$ ( Jasper), produced high levels of CPE in both EPC and FHM cells. By contrast, isolates belonging to serotype A$_1$ (Buhl subtype only), A$_3$ (Ab), A$_5$ (Tellina), A$_6$ (Canada 1), A$_7$ (Canada 2), and A$_8$ (Canada 3) serotypes did not produce CPE on EPC or FHM cells. This ability to cause CPE in non-salmonid cells was highly correlated with the presence of the W4 epitope on the virus, with those isolates possessing the epitope 50-100 times more likely to produce CPE in these cells than those isolates lacking this epitope ($P<0.001$).

Introduction
Aquatic birnaviruses (ABV), members of the family Birnaviridae, have been isolated from more than 80 aquatic vertebrates and invertebrates worldwide (Reno, 1999). They have frequently been cited as infectious pancreatic necrosis virus (IPNV), biochemically and serologically identical to the aquatic birnaviruses, if they caused disease in salmonids (Reno, 1999; Kent and Poppe, 1998). Aquatic birnaviruses are routinely replicated in a variety of teleost cell lines including AS, BF-2, CHSE-214 (Nims et al., 1970; Wolf and Mann, 1980); EPC (Novoa et al., 1993), and FHM and RTG-2 (Wolf and Quimby, 1962; Kelly et al., 1978; Novoa et al., 1993). Comparative susceptibility to ABV and virus yield in various cell lines have been studied often (Nims et al., 1970; Scherrer and Cohen, 1975; Kelly et al., 1978; Wolf and Mann, 1980; Novoa et al., 1993; Rodriguez and Perez, 1993). However, in all cases, a single or only a few isolates of virus, or those belonging to one or two serotypes were tested. All IPNV isolates tested thus far grow on CHSE-214 cells but not all grow in non-salmonid cells (Kelly et al., 1978).

This study was designed to determine the ability of aquatic birnaviruses from all 9

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serotypes of serogroup A (Hill and Way, 1995) to replicate and/or cause cytopathogenic effect (CPE) in two salmonid and two non-salmonid cell lines commonly utilized in detection of virus from field samples. In addition, the relationship between the presence or absence of certain virus epitopes and in vitro host range was evaluated.

Materials and methods

Aquatic Birnaviruses

The isolates used in this study were a sub-sample of more than 300 isolates held in liquid nitrogen at the Hatfield Marine Science Center. The various aquatic birnavirus isolates were obtained from a variety of aquatic species, sources and investigators over a period of more than 20 years. The isolates were stored at -80°C, or in liquid nitrogen. Viruses were grown in CHSE-214 cells (Lannan et al., 1984) according to the methods of Caswell-Reno et al. (1989). The viruses were tested for their epitope composition with a suite of 11 monoclonal antibodies as described by Caswell-Reno et al. (1989).

Cell lines and their maintenance

The following cell lines were used in this study: chinook salmon embryo cells, (CHSE-214, Lannan et al. (1984), epithelioma papulosum cyprini, (EPC) Fijan et al. (1983), fathead minnow, (FHM) Gravell and Malsberger (1965), rainbow trout gonad, (RTG-2) Wolf and Quimby (1962). Cells were grown in Eagle’s Minimum Essential Medium (MEM) (HyClone Laboratories, Inc.) supplemented with 10% fetal bovine serum (MEM-10). No antibiotics were used in the routine culture of cell lines. The procedures for the passage of these cell lines were carried out as described in Lannan (1994). Cell cultures were incubated in CO2 incubators at 18°C. Serotyping of the isolates by Enzyme Immunodot Assay was done as described in Caswell-Reno et al. (1989).

Virus inoculation

All ABV isolates were titrated prior to use in this experiment and adjusted to a concentration of 10^6 TCID₅₀/ml. One tenth ml of virus was added into each of 24 wells of a 24 well microtitration plate, for a multiplicity of infection (MOI) of approximately 0.02. A single ABV isolate was used on each 24 well plate to avoid cross contamination. After inoculation with virus, the 24 well plates were incubated for 1-2 hours to allow time for virus adsorption. They were then washed three times with 1-2 mL of MEM without fetal bovine serum, and 1.0 ml of MEM-10 was added into each well. Assays of the final wash volume failed to detect virus. Daily from the second to the 7th day after inoculation, all wells were examined and the level of CPE was evaluated microscopically and scored on an ordinal basis from 0-4 (0= no visible CPE; 1-4 for additional quartiles of the monolayer affected). After 7 days incubation at 18°C, culture medium from four random replicates was pooled, aliquots were taken and assayed for virus within 48 h. Titrations were carried out as described in Caswell-Reno et al. (1986) on the CHSE-214 cell line, and amount of virus was quantified using 50% tissue culture infectious dose (TCID₅₀/ml) method by Sperman-Kärber method (Payment and Tudel, 1993).
Results

Of the 103 aquatic birnavirus isolates tested in four continuous teleost cell lines, all produced extensive CPE in two salmonid cell lines, CHSE-214 and RTG-2 by 7 days post-exposure (dpe). All 103 isolates produced maximum CPE (4) on CHSE-214 cells, and 102 of 103 isolates produced maximum CPE on RTG-2 also; a single isolate (Tellina virus, serotype A5) produced a minimally detectable level (1) of CPE on the RTG-2 (Table 1 and 2). However, only half of the isolates tested produced CPE in two non-salmonid cell lines, EPC and FHM (Table 1). The levels of CPE produced both in FHM (mean CPE = 1.91) and in EPC cells (mean CPE = 1.71) were not statistically different from each other.

Table 1. Cytopathic effects (CPE) of aquatic birnaviruses on four teleost cell lines. Serotype refers to the classification scheme of Hill and Way (1995). (Parenthesis indicates the archetypes of serotype, and n: the number of isolates tested).

<table>
<thead>
<tr>
<th>Serotype</th>
<th>n</th>
<th>EPC</th>
<th>CHSE-214</th>
<th>FH M</th>
<th>RTG-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (WB)</td>
<td>63</td>
<td>29</td>
<td>63</td>
<td>29</td>
<td>63</td>
</tr>
<tr>
<td>A2 (Sp)</td>
<td>14</td>
<td>12</td>
<td>14</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>A3 (AB)</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>A4 (He)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A5 (Te)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>A6 (CAN-1)</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>A7 (CAN-2)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A8 (CAN-3)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>A9 (Jasper)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>54</td>
<td>103</td>
<td>55</td>
<td>103</td>
</tr>
</tbody>
</table>

Table 2. Propagation of IPNV isolates belonging to various serotype and subtypes in four fish cell lines: two salmonid and two non-salmonid cell lines.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Subtypes</th>
<th>CHSE-214</th>
<th>RTG-2</th>
<th>EPC</th>
<th>FHM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>WB</td>
<td>4.00</td>
<td>8.88</td>
<td>4.00</td>
<td>8.13</td>
</tr>
<tr>
<td>A1</td>
<td>VR-299</td>
<td>4.00</td>
<td>8.63</td>
<td>4.00</td>
<td>7.32</td>
</tr>
<tr>
<td>A1</td>
<td>BUHL</td>
<td>4.00</td>
<td>8.62</td>
<td>3.96</td>
<td>8.21</td>
</tr>
<tr>
<td>A2</td>
<td>SP</td>
<td>4.00</td>
<td>8.12</td>
<td>4.00</td>
<td>6.71</td>
</tr>
<tr>
<td>A3</td>
<td>AB</td>
<td>4.00</td>
<td>8.56</td>
<td>4.00</td>
<td>7.69</td>
</tr>
<tr>
<td>A3</td>
<td>EVE</td>
<td>4.00</td>
<td>9.25</td>
<td>4.00</td>
<td>9.00</td>
</tr>
<tr>
<td>A4</td>
<td>HE</td>
<td>4.00</td>
<td>8.37</td>
<td>4.00</td>
<td>7.63</td>
</tr>
<tr>
<td>A5</td>
<td>TE</td>
<td>4.00</td>
<td>8.75</td>
<td>1.00</td>
<td>4.00</td>
</tr>
<tr>
<td>A6</td>
<td>CAN-1</td>
<td>4.00</td>
<td>8.75</td>
<td>3.80</td>
<td>7.90</td>
</tr>
<tr>
<td>A7</td>
<td>CAN-2</td>
<td>4.00</td>
<td>10.25</td>
<td>4.00</td>
<td>8.00</td>
</tr>
<tr>
<td>A8</td>
<td>CAN-3</td>
<td>4.00</td>
<td>7.50</td>
<td>4.00</td>
<td>7.50</td>
</tr>
<tr>
<td>A9</td>
<td>JASPER</td>
<td>4.00</td>
<td>9.19</td>
<td>4.00</td>
<td>7.94</td>
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<tr>
<td>A9</td>
<td>BC</td>
<td>4.00</td>
<td>7.69</td>
<td>4.00</td>
<td>7.38</td>
</tr>
</tbody>
</table>

*mean CPE scored on ordinal scale: 0= no evidence of CPE; 1=25% of monolayer affected; 2=25-50% of monolayer affected; 3=50-75% of monolayer affected; 4=75% of monolayer affected.

** titer = geometric mean titer (log_{10} TCID_{50}/mL).
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(Wald-Wotvitz Runs Test (W-WRT); $z = 0.7502$, $P = 0.45$), but were significantly lower than that produced by CHSE-214 cells ($P = 0.0001$) and RTG-2 cells ($P < 0.0001$).

**Figure 1a.** The box and whisker plot of mean cytopathic effects (MC) produced by 103 aquatic birnaviruses isolates in EPC cells ($0$ = no CPE; $1$ = 25% or less; $2$ = 25-50%; $3$ = 50-75%; $4$ = >75%) (Values indicate mean (dot), standard error of the mean (box) and 1.96 standard errors of mean (whisker)).

**Figure 1b.** The geometric mean titers (GMT) of 103 aquatic birnaviruses in EPC cells tested.
The virus yields produced by various isolates in the cell lines were also compared. Virus yields were the highest in CHSE-214 cells (geometric mean titer (GMT) = 10^{8.43} TCID_{50}/ml),
and RTG-2 cells (GMT=10^{7.56} TCID_{50}/ml). The yields of the isolates which produced CPE in EPC (GMT =10^{7.35} TCID_{50}/ml) and FHM (GMT=10^{6.88} TCID_{50}/ml) were not significantly different from each other (W-WRT; z = 0.068, P = 0.95) or from RTG-2 cells, but were significantly lower than the GMTs of CHSE-214 cells (Dunnett’s Test.; F= 90.82, P = 0.0001) (Figure 1 and 2). A regression analysis indicated that level of virus produced was significantly associated with CPE readings in EPC cells (r^2 = 0.78, Figure 3).

Unexpectedly, in many cases large amount of virus (more than inoculated level, 10^5 TCID_{50}/ml) was obtained in the absence of cell monolayer destruction. Moreover, this phenomenon occurred with the same isolates belonging to all serotypes except A1 and A9 on both EPC and FHM cell lines. That is, if occurred in EPC cell lines, it also always occurred in FHM cells (See Ogut, 2001 for full list of responses of the isolates).

Serotypes and In Vitro Host Range
Although all viruses were capable of replicating in the two salmonid cell lines, there was a highly significant relationship between aquatic birnavirus serotype and the ability to replicate and cause CPE in the two non-salmonid cell lines. As indicated in Table 1, aquatic birnaviruses belonging to the A1 (WB), A2 (Sp), A4 (He) and A9 (Ja) serotypes were generally capable of causing CPE in the non-salmonid cells, whereas those belonging to serotypes A3, A5, A6, A7, and A8, were not. Within the A1 serotype, 2 of the 3 subtypes of virus (WB (n=13) and VR-299 (n=19)) as assessed with a panel of monoclonal antibodies, were capable of replicating in the non-salmonid cell lines while the isolates belonging to the Buhl subtype (n=31) did not
replicate in either of these cell lines. The single isolate tested of the A7 serotype (Canada 2) was capable of replicating and producing CPE in the FHM cell line but not in the EPC cell line.

**Epitope composition and in vitro host range**

Utilizing a panel of monoclonal antibodies, all of the isolates were tested for the presence of 11 epitopes on the primary capsid proteins (VP2 and VP3) of the aquatic birnaviruses. When the ability to replicate in non-salmonid cell lines was related to the presence or absence of various epitopes, it was determined that the presence of the W-4 epitope on the VP2 protein was strongly correlated with the ability to grow on the non-salmonid cell lines: 40/41 isolates tested which possessed the W4 epitope were capable of growing on the 2 non-salmonid cell lines (c2 1 d.f. = 53.4; P<0.0001). The calculated odds ratio for the presence of W4 and the ability to replicate in the non-salmonid cell lines indicated that those viruses which have the W4 epitope are about 50 times more likely to replicate in these cells than viruses lacking the epitope (EPC odds ratio = 50.5; FHM odds ratio = 44.9).

**Discussion**

**Ability of aquatic birnaviruses to produce CPE in four teleost cell lines**

The in vitro host range of a virus is variable and dependent on a variety of factors, both inherent to the virus, and to the cells in which the virus may replicate. Aquatic birnaviruses have been shown to replicate in a wide variety of teleost cell lines, as well as some non-teleost cell lines (Wolf and Mann, 1980). The virus has been found to replicate better in certain cell lines than others. For example, Kelly et al. (1978) found that the Buhl isolate (Serotype A1, Buhl subtype) replicated in CHSE-214 cells but not in FHM cells, whereas VR-299 (also A1 serotype) replicated well in that cell line. The purpose of this study was systematically to determine the in vitro host range of a large number of ABV isolates and to determine if there was a correlation between the ability of viruses to replicate in certain cell lines and virus antigenic composition.

In this study, all 103 aquatic birnaviruses examined caused complete (level=4) cytopathogenic effect (CPE) in CHSE-214 cells, which are derived from chinook salmon Lannan et al. (1984). Complete CPE usually occurred within 3 days post-exposure. All cells were exposed to 10^4 TCID<sub>50</sub> of virus and yields of virus in this cell line were consistently > 10^8 TCID<sub>50</sub>/mL, significantly higher than in any of the other 3 cell lines tested (Table 1). The results reported here support and significantly extend the results of Kelly et al. (1978), Novoa et al. (1993) and Thoesen, (1994) indicating that the CHSE-214 cell line is uniformly susceptible to a wide range of ABV from all known members of serogroup A, producing readily discernible CPE rapidly (within 3 dpe), and producing high yields of virus. The use of CHSE-214 cells as a standard salmonid cell line for the isolation of ABV is confirmed by the results obtained in this study.

Similarly, all the other aquatic birnaviruses replicated to high levels and caused extensive cytopathology in RTG-2 cells, (derived from rainbow trout), except the A5 (Te) serotype of ABV which produced =25% CPE at 7 d post-
exposure. This would suggest that the RTG-2 cell line could be somewhat less reliable than CHSE-214 when used to detect ABV in a variety of aquatic animals.

The most significant finding in this study was the clearly demarcated separation of ABV into 2 groups based on their ability to produce CPE in the 2 non-salmonid cell lines, EPC & FHM. Six groups of viruses corresponding to serologic subtypes of serogroup A did not produce CPE in these cells (Hill and Way, 1995). These were members of serotype A1, subtype Buhl; serotype A3 (Ab); serotype A5 (Te); serotype A6 (Canada 1); serotype A7 (Canada 2); serotype A8 (Canada 3). The remainder of the serotypes (A1, subtypes WB & VR-299; A2 (Sp); A5 (He) and A9 (Jasper) were capable of consistently causing CPE in these two cell lines. Rodriguez and Perez (1993) reported that all IPNV isolates tested replicated (all of which were members of the A2 or A3 serotypes) on CHSE-214 and RTG-2 cell lines (n=93). Of the 93 isolates tested, 81.9% (77) were positive on FHM cell line, and in EPC cells, 86.17% (81) replicated. In our study, 10/12 A2 isolates and 1/6 A3 isolates replicated in the two cell lines.

The most interesting group of viruses examined is serotype A1, subtype Buhl, that have been isolated mostly from salmonids in the western United States and from non-salmonids in Korea (Reno, 1999; Reno, unpublished results). While the other subtypes within this serotype, VR-299 (n=16) and WB (n=14), replicate well in EPC & FHM cells, members of the Buhl subtype (n=31) uniformly failed to produce CPE in the EPC & FHM cells. Kelly et al. (1978) compared the ability of VR-299 and Buhl isolates to replicate in FHM cells and reported that the Buhl isolate failed to produce virus and cytopathology, whereas the VR-299 isolate caused significant amounts of CPE. In another study carried out by Nicholson and Dexter (1975), no CPE was detected in FHM cells inoculated with the Buhl isolate. Darragh and MacDonald (1982) also reported the failure to isolate Buhl isolate on FHM cells. These studies strongly indicated that the Buhl isolate did not replicate efficiently in FHM cells. The present study, however, used 31 isolates of the Buhl subtype rather than one as in previous studies, to examine the ability of this subtype of virus to replicate in both FHM and EPC cells. This inability of viruses belonging to the Buhl subtype to replicate in EPC or FHM cells can be used as a characteristic of this subtype of aquatic birnavirus in clinical isolates. If the unknown virus shows growth in FHM or EPC cells as well as in CHSE-214 cells, it is unlikely that the isolate belongs to the Buhl subtype.

As noted in table 2, all of the subtypes that failed to replicate in EPC and FHM (A1/Buhl, A3, A5, A6, A7, A8) lacked the W4 epitope (Caswell-Reno et al., 1989). This is a neutralizing epitope present on the capsid protein VP2 (Caswell-Reno et al., 1986), and is therefore essential to the process of infecting cells. These data indicate that the W4 epitope may be an important factor in the determination of host range in vitro. For example, both the 93-321 and ST isolates were the closest to the WB subtype in their epitope structure (10/11 epitopes identical), differing only in the lack the W4 epitope, and in our tests did not replicate in EPC or FHM cells.
The W4 epitope is not present on the Buhl subtype of A1 ABV, but is present on viruses of the A1, VR-299 and West Buxton subtypes. Of the isolates that lacked the W4 epitope and replicated in EPC, all were from the European serotypes A2 and A4; none of the North American subtypes lacking W4 (A1, Buhl) replicated in EPC or FHM cells.

The reason that some isolates of aquatic birnaviruses produce cytopathology in certain cells and not in the others is not known, but may be due to a lack of EPC & FHM receptor-ligands on the virus. Darragh and MacDonald (1982) suggested that cellular receptors for IPNV subtypes are different on FHM and CHSE-214 cells, based the failure of the OV-7 isolate (a member of A2 serotype) to be adsorbed by FHM cells. This may have resulted in reduced CPE in EPC and FHM cells and low virus yields. The majority of serotype A2 (Sp) isolates tested in this study (11/12) replicated in both EPC and FHM, although they did not react with monoclonal antibody to W4. This ability to replicate may have been due to an alternate epitope conformation, which enabled the virus to successfully infect these cells, but not bind the Mab.

While Buhl subtype isolates did not produce CPE in EPC and FHM cells, this was not necessarily the case for virus replication. We found that in nearly half of the cases (21 of 48 isolates in both EPC and FHM cells) detectable amounts of virus were recovered from the cells even though no CPE was observed by 7dpe. At levels of virus production greater than $10^{5.5}$ TCID$_{50}$/ml, CPE was produced in infected cells within 7dpe, whereas at the levels lower than this, CPE was not evident. Holding cells for a further 7 days resulted in the production of CPE for those isolates that had evidence of replication at 7dpe, but those isolates showing no evidence of virus replication after 7 dpe did not produce virus even after 14 dpe (Reno, unpublished results). These results confirm the utility of holding cells for 14 dpe after inoculation with samples for diagnosis and for blind passaging of cells to enhance the potential for producing CPE. This should be encouraged for diagnostic work since the absence of CPE may not mean the absence of replicating virus.

In conclusion, our results supported the results of other studies suggesting that the best cell line for detecting aquatic birnaviruses in clinical samples is CHSE-214 cells, with RTG-2 cells nearly as efficient in terms of the range of isolates/serotypes that replicated in the cells and the yield of virus. Clinical sample testing should not be carried out on either EPC or FHM cells since they are not susceptible to some subtypes of IPNV. Since CPE formation is the target response of clinical sample testing, false negative results may be obtained. If a clinical sample produces CPE in CHSE-214 or RTG-2 cell, but not in EPC or FHM cells, it is likely that if the sample is from North America, it will belong to the Buhl subtype of the A1 serotype of aquatic birnaviruses, and if from Europe or Asia, it will belong to the A3, or A5 serotype. Additionally, there was a strong correlation between the presence of the W4 epitope on VP2 and the ability to replicate in EPC and FHM cells.
Acknowledgement
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References


