Vol. 93: 1–15, 2010
doi: 10.3354/dao02278

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 7

OPEN ACCESS

Molecular characterisation of Australasian isolates of aquatic birnaviruses

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ABSTRACT: An aquatic birnavirus, first isolated in Australia from farmed Atlantic salmon in Tasmania in 1998, has continued to be re-isolated on an infrequent but regular basis. Due to its low pathogenicity, there has been little urgency to undertake a comprehensive characterisation of this aquatic birnavirus. However, faced with possible incursions of any new aquatic birnaviruses, specific identification and differentiation of this virus from other, pathogenic, aquatic birnaviruses such as infectious pancreatic necrosis virus (IPNV) are becoming increasingly important. The present study determined the nucleic acid sequence of the aquatic birnavirus originally isolated in 1998, as well as a subsequent isolate from 2002. The sequences of the VP2 and VP5 genes were compared to that of other aquatic birnaviruses, including non-pathogenic aquatic birnavirus isolates from New Zealand and pathogenic infectious pancreatic necrosis virus isolates from North America and Europe. The deduced amino acid (aa) sequences indicate that the Australian and New Zealand isolates fall within Genogroup 5 together with IPNV strains Sp, DPL, Fr10 and N1. Thus, Genogroup 5 appears to contain aquatic birnavirus isolates from quite diverse host and geographical ranges. Using the sequence information derived from this study, a simple diagnostic test has been developed that differentiates the current Australian isolates from all other aquatic birnaviruses, including the closely related isolates from New Zealand.

KEY WORDS: Aquatic birnavirus · Infectious pancreatic necrosis virus · Phylogenetic relationships

INTRODUCTION

Viruses in the family *Birnaviridae* belong to 3 genera: *Aquabirnavirus*, *Avibirnavirus* and *Entomobirnavirus* (Delmas et al. 2005). The genus *Aquabirnavirus* is the largest and most diverse of these genera, and aquatic birnaviruses have been isolated from a large number of different aquatic animal species, both marine and freshwater, throughout the world (Hill & Way 1995, Reno 1999). Natural disease caused by or associated with aquatic birnaviruses has been reported mainly in finfish, and these pathogenic aquatic birnaviruses have been responsible for major economic losses within aquaculture industries worldwide (Bernard & Brémont 1995). The type species for the genus *Aquabirnavirus* is infectious pancreatic necrosis virus (IPNV; Wolf et al. 1960), which causes infectious pancreatic necrosis (IPN) in salmonids. Initially, IPN was defined as an acute, contagious disease resulting in high mortality in young salmonid fish (Hill & Way 1995). Since then, however, a number of viruses serologically related to IPNV but causing no apparent disease have been detected in non-salmonid species, including a number of finfish species, molluscs and crustaceans (Reno 1999). Aquatic birnaviruses that do not cause disease in salmonid species are often termed IPNV-like or, preferably, aquatic birnaviruses (Hill & Way 1995).

Aquatic birnaviruses are non-enveloped viruses characterized by a bisegmented double-stranded RNA genome (Dobos 1995). The smaller Segment B encodes VP1, the RNA polymerase of the virus (Dobos et al. 1979). Segment A contains 2 overlapping open reading frames (ORF; Duncan et al. 1987). The small ORF

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encodes VP5, an anti-apoptosis protein (Hong et al. 2002), while the large ORF encodes a polyprotein that, when cleaved, forms 3 viral proteins: pVP2, a non-structural protein (NS) and VP3 (Dobos 1995). NS is a protease that cleaves the polyprotein while the VP3 protein is an internal component of the capsid (Heppell et al. 1992). The VP2, a component of the outer capsid, is formed by cleavage of the pVP2 precursor upon virus maturation (Dobos 1995). VP2 is the major structural and antigenic protein and, as such, is of particular taxonomic importance (Heppell et al. 1992, 1995).

Despite occurring worldwide and in a number of different host species, most aquatic birnaviruses are antigenically related (Hill & Way 1995). Serological studies based on reciprocal neutralization assays with polyclonal antisera have identified 2 serogroups, A and B (Hill & Way 1995). Most aquatic birnaviruses belong to Serogroup A, which contains 9 serotypes (A1 to A9). The type species of Serogroup A viruses is IPNV. A number of serotypes of IPNV have been recorded from finfish in the Asian region (Ahne 1994). In general, these IPNV isolates were most closely related to the Sp, West Buxton and Ab serotypes within Serogroup A. While most serotypes are geographically localized, there appears to be no correlation between serotype and host species (Reno 1999). A number of antigenically unrelated isolates belong to Serogroup B, and, in general, there is far less known about these viruses (Reno 1999). A similar lack of knowledge applies to Serogroups C (John & Richards 1999) and D (Dixon et al. 2008), which have been recognised more recently.

Until recently, few investigations into the relationship between genetic and serological classifications of aquatic birnaviruses existed. Heppell et al. (1992) compared a 310 bp region at the VP2 and NS junction of 17 Serogroup A IPNV isolates, and they proposed that 3 genogroups existed within Serogroup A. More recently, Blake et al. (2001a,b) examined the genetic relationship of 28 IPNV isolates that represented all serotypes of Serogroup A that were drawn from wide geographical and host origins. This investigation focused on the VP2 region and demonstrated that, in fact, 6 genogroups existed within Serogroup A. It was also demonstrated that unique signature aa residues, which distinguish particular serotypes, existed within the VP2 region (Blake et al. 2001a,b). Since then, a 7th genogroup, including isolates mainly from Japan and Korea, has also been proposed (Zhang & Suzuki 2004, Nishizawa et al. 2005).

For the Australasian region, IPNV has remained exotic. While aquatic birnaviruses have been isolated in New Zealand on a regular basis since 1987 (Tisdall & Phipps 1987, Anderson 1995), the first isolation of an aquatic birnavirus in Australia was in 1998 during health surveillance of farmed and wild fish in Tasmania (Crane et al. 2000). At that time, limited immunocytochemical studies in cell culture were undertaken with a panel of monoclonal antibodies in an attempt to compare serological classification of the Australian isolate with that of some known IPNV isolates (Crane et al. 2000). To date, neither Australian nor New Zealand isolates have been associated with clinical signs of disease (Crane et al. 2000). In addition, the DPL isolate was reported from finfish in Thailand (Blake et al. 2001a,b). While the cytochemical studies could not clearly assign the Australian isolate to any of the major serotypes of IPNV, limited sequence analysis suggested that the isolate was most closely related to the N1, Fr-21 and Ab strains in Serogroup A. Since the initial identification, regular surveillance has seen the virus isolated on a regular basis, always from apparently healthy farmed salmon and never outside Macquarie Harbour, Tasmania, Australia.

In order to better understand the relationship between these isolates and others from around the world, this paper describes the complete nucleotide (nt) sequence of Segment A of TAB98, the original Australian isolate, together with the coding region of Segment B. In addition, the VP2 sequences of a subsequent Australian isolate (TAB02) and 2 New Zealand isolates (NZ6 and NZ10) were also obtained to determine the genomic relationship of Australasian isolates with other *Aquabirnavirus* isolates found worldwide, including IPNV and marine aquatic birnaviruses.

MATERIALS AND METHODS

Virus isolates. The isolate designated TAB98 was first detected in 1998 during routine health surveillance of Tasmanian farmed Atlantic salmon *Salmo salar* from Macquarie Harbour. Hence, it was originally called the Macquarie Harbour isolate or Tasmanian aquatic birnavirus (TAB; Crane et al. 2000). The virus was isolated from wild marine fish and poorly growing, 18 mo old, farmed Atlantic salmon. The isolate designated TAB02 was isolated from healthy farmed Atlantic salmon from Macquarie Harbour during routine health surveillance in 2002. Eight additional Australian isolates of TAB, isolated from a variety of hosts and geographical locations, were also used in this study (see Fig. 4). NZ6 was isolated from Quinnat salmon *Oncorhynchus tshawytscha* in New Zealand during export certification testing (Tisdall & Phipps 1987). NZ10 was isolated from wild-caught turbot *Psetta maxima* (M. Hine pers. comm.). The Erwin strain of IPNV was kindly provided by Drs. Barry Hill and Keith Way, CEFAS, Weymouth Laboratory, UK, and has been used in our laboratory as a highly patho-

genic positive control. As there was no published sequence available for the gene encoding the VP2 protein on the Erwin strain, this region was also sequenced for future laboratory reference. Virus identity was confirmed by growth in cell culture, followed by electron microscopy and immunoperoxidase assays using specific antibodies as described previously (Crane et al. 2000).

Virus propagation. Inoculum was prepared by thawing aliquots (10 µl) of frozen stock tissue culture supernatant for each isolate and diluting in 5 ml of Eagle's minimum essential medium containing Earle's salts (Gibco-BRL) supplemented with 10 mM hepes buffer (ICN Biomedical), 2 mM glutamine (ICN Biomedical), 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1} (Sigma-Aldrich) and 2% (v/v) foetal bovine serum (Thermo Trace). For each isolate, the culture medium on 90% confluent cultures of the CHSE-214 cell line, grown in 25 cm^2 flasks (Corning), was replaced by this inoculum, and the cultures were incubated at 15°C with an atmosphere of 5% CO_2/95% air until complete viral cytopathic effect was observed. Cell culture medium was harvested, centrifuged at 1000 × *g* for 10 min at 4°C and supernatant fluid stored at −80°C until use.

RNA extraction and preparation of cDNA. RNA was extracted from supernatants using the QiaAmp Viral RNA Extraction Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised using avian myeloblastosis virus (AMV) reverse transcriptase (Promega). Briefly, 1 µl of RNA was mixed with 0.5 µl deionized formamide, pulse-centrifuged and incubated at 100°C for 40 s, then chilled on ice. The sample was mixed with 19.5 µl master mix containing 1 µl of random hexamer (5 mg ml^{-1}) (Promega), 4 µl of 5× AMV reaction buffer (Promega), 4 µl of 1.25 mM dNTPs, 20 U of AMV reverse transcriptase (Promega), 40 U of RNA inhibitor (RNAsin; Promega) and 7 µl sterile distilled water. Reactions were incubated at 25°C for 10 min followed by 42°C for 30 min with a final incubation at 65°C for 5 min.

Polymerase chain reaction (PCR). PCRs consisted of 1 µl cDNA with PCR master mix containing 2.5 µl 10× reaction buffer (Promega), 1.5 mM magnesium chloride (Promega), 4 µl of 1.25 mM dNTPs, 1.25 U *Taq* polymerase (Promega), 1 µl of 20 µM sense and antisense primers (Table 1) and sterile distilled water to 25 µl. PCRs were undertaken in a Perkin Elmer thermocycler using cycling conditions as follows: 1 cycle at 94°C for 2 min; 35 cycles at 92.5°C for 45 s, 58°C for 45 s and 72°C for 105 s; and finally 1 cycle at 72°C for 7 min. PCR products were visualised on a 2% TAE (Tris-acetate-EDTA) agarose gel containing 0.5 µg ethidium bromide ml^{-1} (BioRad) under UV irradiation. After excision from the agarose gel, PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen).

Sequence analysis. Sequences of Segments A and B were determined for TAB98, while for TAB02, NZ6, NZ10 and Erwin isolates, sequencing was restricted to the VP2 region. The general sequencing strategy was to sequence purified overlapping PCR fragments (as described by Blake et al. 2001a,b) using fluorescence-based dideoxy Big Dye terminator chemistry (PE Applied Biosystems). Each PCR fragment was sequenced in each direction at least twice to ensure that an accurate consensus was obtained.

To sequence the Segment A and B termini of TAB98, a modified single primer amplification technique (SPAT) was used (Attoui et al. 2000, Potgieter et al. 2002). First, viral RNA was isolated from partially purified virus. Briefly, tissue culture supernatant with suspended cells (150 ml) was homogenised in a Dounce homogenizer and clarified by low speed centrifugation at 2000 × *g* for 20 min at 4°C. The supernatant was centrifuged 3 more times, each at 18 000 × *g* for 1 h at 4°C, then ultracentrifuged at 149 000 × *g* for 70 min at 4°C. The resultant pellet was resuspended in a minimal volume of sterile distilled water. RNA was isolated by adding 500 µl of the resuspended viral pellet to 12.5 µl 20 mg ml^{-1} Proteinase K and 5 µl 10% sodium dodecyl sulphate (SDS) and incubating at 37°C for 3 h with occasional vortexing. Total RNA was extracted using phenol and diethylether, ethanol-precipitated and resuspended in 50 µl sterile distilled water. Contaminating chromosomal single-stranded RNA was precipitated overnight from the double-stranded RNA (dsRNA) with 2 M lithium chloride as described by Potgieter et al. (2002). The supernatant was ethanol-precipitated and resuspended in 20 µl distilled water. Purified dsRNA was visualised on a 2% TAE agarose gel containing ethidium bromide (BioRad) under UV irradiation, excised and purified as 2 separate segments using QIAquick Gel Extraction Kit (Qiagen).

Each dsRNA segment was ligated to primer PC3 (5′ PO_4-GGA TCC CGG GAA TTC $GG(A)_{17}$-NH_2 3′) using T4 ligase (Promega) at 16°C overnight as described previously by Vreede et al. (1998) and Lambden et al. (1992). Primer PC3 contained restriction enzyme sequences with both a poly (dA) tail to facilitate oligo dT priming and a 3′ terminal NH_2-group that prevented concatenation. Ligated segments were purified using the QIAquick PCR purification kit (Qiagen), and cDNA was transcribed using Superscript II (Invitrogen) according to the manufacturer's instructions using primer PC2 (5′ PO_4-CCG AAT TCC CGG GAT CC-OH 3′) complementary to the anchor site ligated to either end of the segments. Ligated cDNA was amplified by PCR using primer PC2 and gene specific primers of

Table 1. Primers used in sequencing (A) the full length Segment A of Tasmanian aquatic birnavirus (TAB) and (B) the coding region of Segment B of TAB. Positions of each primer are referenced according to the published sequence of the Jasper strain (Duncan & Dobos 1986). ESAx refer to primers designed for end sequencing. * denotes reverse primer. N/A: not applicable

Primer name	Sequence 5′ to 3′	Position	Amplicon size (bp)	Source
Segment A				
Pb9	GAG AGC TCT TAC GGA GGA G	39	508	Blake et al. (2001a)
Pa8*	GAC ATC AAG CTG TTG TAG G	547		Blake et al. (2001a)
KS3	GAC GTC GCA GGA CCT GAA G	371	623	Present study
KS1R*	ATC TTG GCT GAG ACG CCT CTG	994		Present study
P8	GGA AAT ACG ACA TCC AGA GCT	421	909	Blake et al. (2001a)
P10*	CAC AGG ATC ATC TTG GCA TAG T	1330		Blake et al. (2001a)
P15	GAA CGG AGC AAG GAT GAG GTG	683	1076	Blake et al. (2001a)
P12*	TGC ACC ACA GGA AAG ATG ACT C	1759		Blake et al. (2001a)
KS1	CAG AGG CGT CTC AGC CAA GAT	974	457	Present study
KS2R*	GCA GGT CGC TGG AGA AGT CAG TG	1431		Present study
P14	GTA TCC AAC TAT GAG CTG AT	1224	535	Blake et al. (2001a)
P12*	TGC ACC ACA GGA AAG ATG ACT C	1759		Blake et al. (2001a)
KS2F	TCC GGA AGG TGG CAG CTC CTG TA	1474	316	Present study
KS4*	GAA TGC CTC GCC TGG TGC ACT T	1790		Present study
P17	CCA GTT CAT CGG AGA TCT CAC	1544	616	Blake et al. (2001a)
P1R*	GTT CAT GGG CGG CTA TGG CTT T	2160		Blake et al. (2001a)
P13	GAG TCA TCT TTC CTG GGT GCA	1738	422	Blake et al. (2001a)
P1R*	GTT CAT GGG CGG CTA TGG CTT T	2160		Blake et al. (2001a)
P13	GAG TCA TCT TTC CTG TGG TGC A	1738	334	Blake et al. (2001a)
AK2*	CCT GGT TGG TTG CCA ATG	2072		Present study
P13	GAG TCA TCT TTC CTG TGG TGC A	1738	574	Blake et al. (2001a)
P7R*	TCT CAT CAG CTG GCC CAG GTA C	2313		Blake et al. (2001a)
AK1	TGC CAC TCA TCG GCA ACC	2048	557	Present study
G1R*	GCT TTG ACG GCG TCC AGT GA	2605		Present study
AK1	TGC CAC TCA TCG GCA ACC	2048	731	Present study
G2R*	GCA TTT GGT CTT GGT CGG	2898		Present study
G1	ATA TCA CTG GAC GCC GTC AAA G	2583	315	Present study
G2R*	GCA TTT GGT CTT GGT CGG GTC	2898		Present study
ESA1*	CAA GTA AGT TGC GGT TGC CTT G	33	N/A	Present study
ESA2*	GAT GCT TGC TGG TCC AGT CTC	72	N/A	Present study
ESA3	AGG AGT TCT ACG ACG CAG TTG	2708	N/A	Present study
ESA4	TGC CGA GAA TGG AGG AAG AGG	2857	N/A	Present study
Segment B				
B1F2	ATG TCG GAC ATC TTC AAC TCA C	101	523	Present study
B3R2*	TTC ATT GCC AGT AGC CTG TT	624		Present study
B2F	CAT TCC ACA AGC CAG ACC ATG AC	423	457	Present study
B4R*	CGT CGT GT CTC CTT TGG TTT TG	880		Present study
KSB1	AGC AGA TGG CAA GAC TGA TGG A	684	844	Present study
KSB3*	TTG TGG TGG CAG AAT GGC ACA A	1528		Present study
B4F	CCG GCC TAC CCT ACA TAG GCA AA	840	769	Present study
B7R*	CTC TTT GGT CAT GGG GTT TGG	1609		Present study
KSB2	TGA CTA GGT CCT GCT CCT GAT	1230	844	Present study
KSB4*	GTT GTT CCA GTC GTC CAG GAA T	2074		Present study
B6F	CAG GCC ATG ATG TAC TAC C	1346	1094	Present study
B9R2*	GCT CTT TAG CAG GTT GTT CGC	2440		Present study
B8F2	GAA ACA GCA GCA AAA CAC ATG	1982	458	Present study
B9R2*	GCT CTT TAG CAG GTT GTT CGC	2440		Present study
ESB1*	CCT GCG TGC TCT TCA TGA GTG	170	N/A	Present study
ESB2*	ATC CTT TGC GGG TCT GAA GCG	220	N/A	Present study
ESB3	CAT TGC GAG ACC AAG CAA AGG	2316	N/A	Present study
ESB4	CCG AAG AAC TCG CAG AAC AAC	2382	N/A	Present study

each segment (Table 1) using a reaction mix as described previously with the following cycling conditions: 1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30 s and at 62°C for 2 min. Products were visualized on a 2% gel with ethidium bromide, excised, purified and sequenced as described above. Sequence data were analysed and managed using Chromas (Techelysium) and Sci Ed Central (Scientific and Educational Software). Accession numbers of sequences generated in this study are included in Table 2. Phylogenetic analyses were undertaken with Sci Ed Central Clone Manager 9 (Scientific and Educational Software).

Development of a diagnostic PCR test. The PCR mixture per sample consisted of the following reagents: 9.5 µl of water; 12.5 µl of HotStar *Taq* Master mix; 0.5 µl (20 µM) of the newly designed primer GABF 5′ ACG AAC CCT CAG GAC AA 3′ and of primer GABR 5′ CAC AGG ATC ATC TTG GCA TAG T 3′ (Blake et al. 2001a,b); and 2 µl of cDNA. PCR conditions were as follows: 1 cycle at 94°C for 15 min; 35 cycles at 94°C for 45 s, 45°C for 45 s and 72°C for 2 min; and finally, 1 cycle at 72°C for 7 min. Amplified DNA (775 bp) was detected by agarose gel electrophoresis.

Restriction enzyme digestion of a diagnostic aquatic birnavirus PCR product. If a PCR product of the correct size was amplified, the product was eluted from the agarose gel. To 10 µl of the eluate were added: 2 µl of 10× reaction buffer, 2 µl of BSA (1 mg ml^{-1}), 10 U of the restriction enzyme *Cla*I and water to a final volume of 20 µl. Digestions were performed at 37°C for 1.5 h, followed by incubation at 65°C for 15 min to inactivate the enzyme. Digested and undigested samples were then resolved on an agarose gel containing ethidium bromide and examined under UV illumination. Type strains representing Genogroups 1 to 6 were analysed, including West Buxton, Jasper, VR299, Erwin, He, Can1, Can2, Can3, Te, Ab, Sp and DPL in addition to TAB isolates identified in different host species between 1998 and 2002. Serogroup B viruses TV-1 and an isolate from *Limanda limanda* (Olesen et al. 1988) were also analysed.

RESULTS AND DISCUSSION

Sequence analysis of TAB98

Reverse transcriptase PCR was used to generate 14 overlapping fragments for Segment A and 7 fragments for Segment B of TAB98. Each fragment was then sequenced with both forward and reverse primers, and together, these sequences accounted for 2861 of 3097 bp (93%) for Segment A and 2313 of 2657 bp (87%) for Segment B. SPAT was employed to determine terminal sequences of Segment A. Despite numerous attempts on 2 different preparations of RNA, the complete termini of Segment B could not be determined. However, the coding region for Segment B was determined.

A single large ORF within Segment B was identified which encoded the VP1 protein. The VP1 coding region was 2532 nt (844 aa) in length as was the case with IPNV strain Sp and marine aquatic birnavirus (MAB) strain AY-98 (Zhang & Suzuki 2004). All 3 strains appeared to have one less residue than other IPNV and MAB strains already sequenced. The TAB98 isolate appeared to have a nucleotide deletion at position 2636 (compared with IPNV type strain, Jasper), which resulted in a stop codon. The VP1 region of TAB98 shared the highest identity with Sp (89% nt, 95% aa) and the lowest identity with US strains of West Buxton and Jasper (80% nt, 89% aa).

Within Segment A, 2 separate but partially overlapping ORF were identified. The smaller ORF, 399 nt, encoded the VP5 protein (133 aa). Across the VP5 protein, the TAB98 isolate appeared most similar to the European isolate Fr21 (94% nt, 86% aa) and least similar to North American strains Jasper and VR299 (85% nt, 58% aa).

The second ORF identified in Segment A encoded the polyprotein (VP2-NS-VP3). If the polyprotein cleavage sites were assumed to be $^{508}A\blacktriangledown S^{509}$ (VP2-NS) and $^{734}A\blacktriangledown S^{735}$ (NS-VP3) (Petit et al. 2000), the processed proteins VP2, NS and VP3 would be 508, 225 and 139 aa in length, respectively. Across the entire polyprotein, TAB98 showed the highest identity with IPNV strain Sp (91% nt, 96% aa) and the lowest identity with IPNV strain He (75% nt, 85% aa). However, across the VP2 protein, where more sequence information in GenBank is available, TAB98 showed the highest identity with the 2 NZ isolates (NZ6, NZ10), DPL and Sp (Table 3). Our study supports and extends a previous analysis of TAB98 (Crane et al. 2000), which was based on very limited sequence information (a 459 bp PCR product).

VP2 sequence analyses of other isolates — TAB02, NZ6, NZ10 and Erwin

Further investigation of other Australasian isolates (TAB02, NZ6 and NZ10) was restricted to the sequence of the VP2 region. Being the major capsid protein, the VP2 contains all neutralizing epitopes and cell attachment sites which determine host and cell range (Lipipun et al. 1989). The VP2 has also been shown to contain the central variable domain, which encompasses 2 hypervariable segments (Heppell et al. 1995). Blake et al. (2001a,b) further demonstrated that the VP2 region contains small variable regions, which identify specific serotypes, and they identified highly conserved signature aa residues that distinguish particular serotypes.

Table 2. Infectious pancreatic necrosis virus (IPNV) and marine aquatic birnavirus sequences used in the present study. MAB: marine aquatic birnavirus

Strain	Accession no.		Geographical origin	Host origin	Genogroup	Serotype	Source
	Segment A	Segment B					
West Buxton	AF342727	AF078669	Maine, USA	Trout	1	A1	Blake et al. (2001a,b), Yao & Vakharia (1998)
Dry Mills	AF343571	Not used	Maine, USA	Trout	1	A1	Blake et al. (2001a,b)
VR299	AF343572	Not used	West Virginia, USA	Trout	1	A1	Blake et al. (2001a,b)
Buhl	AF343573	Not used	Idaho, USA	Trout	1	A1	Blake et al. (2001a,b)
Reno	AY026345	Not used	Nevada, USA	Trout	1	A1	Blake et al. (2001a,b)
64-93	AY026346	Not used	Idaho, USA	Trout	1	A1	Blake et al. (2001a,b)
90-11	AY026347	Not used	Idaho, USA	Trout	1	A1	Blake et al. (2001a,b)
91-114	AY026348	Not used	Idaho, USA	Trout	1	A1	Blake et al. (2001a,b)
91-137	AF343570	Not used	Idaho, USA	Trout	1	A1	Blake et al. (2001a,b)
Jasper D	NC_001915	NC_001916	Canada	Trout	1	A1	Duncan & Dobos (1986), Duncan et al. (1991)
DRT	D26526	D26527	Korea	Trout	1	A1	Chung et al. (1994a,b)
Erwin	EU869269	Not used	USA	Trout	1	Not typed	Present study
Can2	AF342733	Not used	Canada	Trout	4	A7	Blake et al. (2001a,b)
Can3	AF342734	Not used	Canada	Artic char	4	A8	Blake et al. (2001a,b)
Ab	AF342729	Not used	Denmark	Trout	2	A3	Blake et al. (2001a,b)
EEV	AY026486	Not used	Japan	Eel	2	A3	Blake et al. (2001a,b)
E1S	AY026487	Not used	Taiwan	Eel	2	A3	Blake et al. (2001a,b)
PV	AY026488	Not used	Taiwan	Perch	2	A3	Blake et al. (2001a,b)
CV-HB1	AY026489	Not used	Taiwan	Clam	2	A3	Blake et al. (2001a,b)
Te	AF342731	Not used	England	Tellina	3	A5	Blake et al. (2001a,b)
Can1	AF342732	Not used	Canada	Trout	3	A6	Blake et al. (2001a,b)
ASV	AY026490	Not used	Canada	Atlantic salmon	3	A6	Blake et al. (2001a,b)
TAB98	EU672429	EU672430	Australia	Atlantic salmon	5	Not typed	Present study
TAB02	EU869272	Not used	Australia	Atlantic salmon	5	Not typed	Present study
NZ6	EU869270	Not used	New Zealand	Pacific salmon	5	Not typed	Present study
NZ10	EU869271	Not used	New Zealand	Turbot	5	Not typed	Present study
Sp	AF342728	M58757	Denmark	Trout	5	A2	Blake et al. (2001a,b), Duncan et al. (1991)
N1	D00701	Not used	Norway	Atlantic salmon	5	A2	Håvarstein et al. (1990)
Fr10	AY026482	Not used	France	Trout	5	A2	Blake et al. (2001a,b)
Fr21	AY026483	Not used	France	Trout	5	A2	Blake et al. (2001a,b)
OV2	AY026484	Not used	England	Oyster	5	A2	Blake et al. (2001a,b)
DPL	AY026485	Not used	Thailand	Snakehead	5	Not typed	Blake et al. (2001a,b)
He	AF342730	Not used	Germany	Pike	6	A4	Blake et al. (2001a,b)
Y-6	NC_004168	NC_004176	Japan	Yellowtail	7	MABV	Suzuki et al. (1998), Zhang & Suzuki (2003, 2004)
YT-01A	AY283782	Not used	Japan	Yellowtail	7	MABV	Zhang & Suzuki (2003, 2004)
H-1	AY283783	Not used	Japan	Japanese flounder	7	MABV	Zhang & Suzuki (2003, 2004)
AY-98	NC_008019	NC_008026	Japan	Ayu	7	MABV	Zhang & Suzuki (2003, 2004)
NC1	AY283784	Not used	Korea	Japanese flounder	7	MABV	Zhang & Suzuki (2003, 2004)

Table 3. Percent nucleotide (lower left) and amino acid (upper right) similarity of the VP2 protein of different strains of aquatic birnavirus

	TAB98	TAB02	NZ10	NZ6	DPL	Sp	Can2	Can3	Ab	Can1	Te	Jasper	WB	VR299	Erwin	He
TAB98		99	99	98	96	96	89	89	91	92	92	86	86	86	88	85
TAB02	99		98	98	96	96	89	89	91	92	92	86	86	86	88	84
NZ10	97	97		99	96	96	89	89	92	92	92	87	87	87	88	85
NZ6	94	94	95		96	96	89	89	91	92	91	87	87	87	88	85
DPL	91	91	92	91		97	89	89	90	90	90	86	86	86	87	86
Sp	91	90	92	91	98		89	89	90	90	90	87	87	87	88	86
Can2	85	84	85	85	84	84		97	87	87	87	86	86	86	86	83
Can3	85	85	85	85	84	84	96		87	88	87	86	86	86	86	83
Ab	86	86	87	86	86	86	82	82		90	90	88	88	88	88	82
Can1	86	86	87	87	86	86	83	84	86		95	96	85	86	86	82
Te	86	86	87	87	87	86	82	83	85	91		85	85	85	86	83
Jasper	80	79	79	79	79	80	78	78	80	79	74		96	99	97	81
WB	79	79	80	80	79	80	79	79	82	79	78	89		96	95	81
VR299	79	79	79	79	79	80	78	78	80	79	78	99	89		97	81
Erwin	83	83	82	81	82	82	79	79	81	80	80	95	87	95		82
He	75	75	76	76	76	76	75	74	74	74	74	73	73	73	74	

The sequence of the TAB02 isolate was found to have a 99% (nt, aa) identity with the TAB98 isolate across the VP2 region (Table 3). Over the 508 aa, alignments revealed 3 aa changes between the 2 isolates at positions 54, 255 and 477, the first being a conserved change and the 2 other changes being significant. These data suggest that the aquatic birnavirus isolates obtained from fish species within Macquarie Harbour on an infrequent but regular basis most likely belong to the same strain, which apparently has not changed significantly over the 4 yr period spanning the isolation of TAB98 and TAB02.

While the TAB found in 2002 was detected during routine health surveillance, this isolate was not recovered from a pinhead fish as was the case with the original isolate in 1998 (Crane et al. 2000). The isolate in 2002 was from healthy Atlantic salmon, showing no overt signs of disease.

Both the 1998 and 2002 TABs were isolated from Atlantic salmon. Following the first isolation in 1998, a number of fish species in Macquarie Harbour were sampled, and TAB was isolated. Limited sequencing was performed on isolates identified from other fish species sampled in 1998, including wild fish. These isolates were found to have 100% identity with TAB98 at the aa sequence level across the 393 bp sequenced within the hypervariable region (data not shown).

A New Zealand isolate from wild-caught turbot, designated NZ10, showed a high level of identity to TAB98 (97% nt, 99% aa), suggesting that the Australian and NZ isolates may have originated from the same source, presumably wild marine species inhabiting the Southern Ocean. A further New Zealand isolate, designated NZ6, was also very similar to the TAB98 isolate (94% nt ,98% aa) (Table 3).

Analysis of the VP2 sequence of the Erwin isolate (used as a virulent positive control in our studies) demonstrated greatest identity with the Jasper and VR299 strains of IPNV (Table 3).

Phylogenetic analysis

To determine how the Australasian isolates were related to other Serogroup A IPNV and MABs, published nucleotide sequences covering the polyprotein ORF for 29 IPNV and 5 MABs were aligned in Clustal X and used to generate a phylogenetic tree using TreeView software (Fig. 1). As demonstrated by Blake et al. (2001a,b), IPNV strains appeared to cluster into 6 genogroups based on geographical and serological similarities. The Australasian isolates fall within Genogroup 5 and appear to be most closely related to the Sp and N1 strains. In contrast to Genogroup 1, which appears to contain viruses isolated solely from trout in North America (USA and Canada), Genogroup 5 consists of isolates from more diverse geographical and host ranges, viz., from trout in Denmark and France, from Atlantic salmon in Norway, from oysters in UK, from various species including Atlantic salmon in Australia, from Quinnat salmon and turbot in New Zealand and from snakehead in Thailand. Interestingly, the aa sequence of VP2 shows no more than 4% divergence between isolates within Genogroup 5 as seen in Table 3. This is in contrast to 9% divergence between the Australasian isolates and Genogroups 3 and 4, and 11, 14 and 16% divergence between the Australasian isolates and Genogroups 2, 1 and 6, respectively.

Full length alignments of the Segment A polyprotein (Fig. 2) revealed that all Australasian isolates investigated in this study had 2 unique aa residues: an argi-

nine at position 81 and an alanine at position 221. Blake et al. (2001a,b) described a unique glycine at position 76 for all Genogroup 5 isolates. Results from our study demonstrate that not all Genogroup 5 isolates shared this unique signature aa residue. Australasian isolates contained a glutamine at position 76, as did isolates of Genogroup 3. However, alignments demonstrated that all Genogroup 5 isolates, including Australasian isolates, shared the unique methionine and leucine residues at positions 263 and 361, as described by Blake et al. (2001a,b).

Several studies (Bruslind & Reno 2000, Santi et al. 2004, Shivappa et al. 2004) have reported the presence of threonine and alanine at positions 217 and 221 of virulent strains, whereas low virulent strains have a proline and alanine at the same residues. As seen in Fig. 2, TAB isolates have a proline and alanine at positions 217 and 221, respectively, indicating low virulence. Bain et al. (2008) recently demonstrated that strains with a proline and alanine at positions 217 and 221, respectively, were in fact highly virulent in field and experimental conditions. These

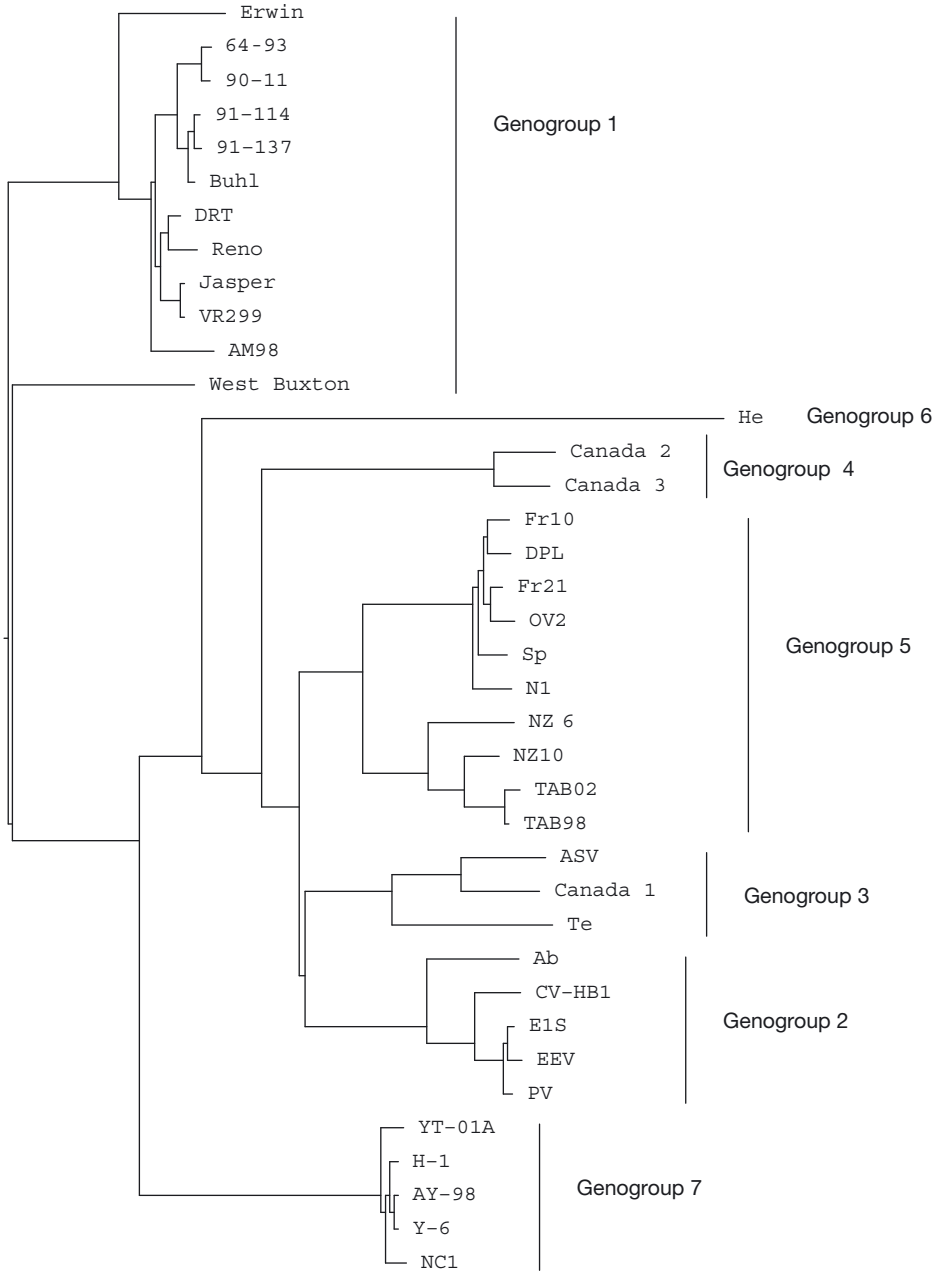

Fig. 1. Phylogenetic analysis of VP2 sequences of Australasian isolates and of published sequences of strains of infectious pancreatic necrosis virus (IPNV) and marine aquabirnaviruses

```
Jasper            1 mstskatatylrsimlpengpasipdditerhilkqetssynlevsesgsgllvcfpgap
VR299             1 ..............................................................
Erwin             1 .n.n.......k......t.....................................i........
WB                1 .n.t.....................................d....................
Ab                1 ...n.......k......t..s.....................d....v........
Can1              1 ...n.......k......t........v.....................v........
Te                1 .n.n.......k......t........v.....................v........
Can2              1 .n.n.......k......t.........................d....................
Can3              1 .n.n.......k....s.t.........................d....................
Sp                1 .n.n.......k......t.....................................i........
DPL               1 .n.n.......k......t.....................................i........
TAB98             1 .n.n.......k......t.....................................i........
TAB02             1 .n.n.......k......t.....................................i.i......
NZ10              1 .n.n.......k......t.....................................i........
NZ6               1 .n.n.......k......s.....................................i........
He                1 .tnn.n.....k......s........v.....................i........

Jasper           61 gsrvgahyrwnlnqtalefdqwletsqdlkkafnygrlisrkydiqsstlpaglyalngt
VR299            61 ..............................................................
Erwin            61 ...........v...e....r.....................................
WB               61 ..............e...........................................
Ab               61 ...........v...e.............................v..............
Can1             61 ...........v...............................................
Te               61 s..........a...e...........................................
Can2             61 ........k..a.........................v.....................
Can3             61 ...........v...............................................
Sp               61 ...i.......a...g...........................................
DPL              61 ...i.......a...g.............................e..............
TAB98            61 ...........v...e....r.....................................
TAB02            61 ...........v...e....r.....................................
NZ10             61 ...........v...e....r.............................f.........
NZ6              61 ...........v...e....r.....................................
He               61 ...i.......a...q.q............r.............................

Jasper          121 lnaatfegslsevesltynslmslttnpqdkvnnqlvtkgitvlnlptgfdkpyvrlede
VR299            121 ..............................................................
Erwin            121 ..............................................................
WB               121 ..............................................................
Ab               121 i.....................................v........................
Can1             121 ................s.....................v........................
Te               121 ................s.....................v........................
Can2             121 ..............................................................
Can3             121 ..............................................................
Sp               121 ..............................a........v........................
DPL              121 .....................................v........................
TAB98            121 .....................................v........................
TAB02            121 .....................................v........................
NZ10             121 .....................................v........................
NZ6              121 .....................................v........................
He               121 i...........pn.s.................i...v...i.....................

Jasper           181 tpqgpqsmngarmrctaaiaprryeidlpserlptvaatgtpttiyegnadivnstavtg
VR299            181 ......................k.................................t...
Erwin            181 ..............................................................t...
WB               181 ...................................g.........d..g......t...
Ab               181 ........................x.....................x.xg......t...
Can1             181 ....lr.....k..................q....ap....l..............t...
Te               181 ....l......k..................q.....p....l..............t...
Can2             181 ....ll.....k.....................paq....l..m...........t...
Can3             181 ....ll.....k.....................pat....l..m.....g.....t...
Sp               181 ....l.....................qs..p.p....l..l...........t...
DPL              181 ....l......k......t...........q...p.pv...l..l...........t...
TAB98            181 ....l......k..................q...p.p...al..l...........t...
TAB02            181 ....l......k..................q...p.p...al..l...........t...
NZ10             181 ....l......k..................q...p.p....l..l...........t...
NZ6              181 ....l......k..................q.m.p.p....l..l...........t...
He               181 ..k.l.....st....gv.s.........n.a..p.p....yv.l...........tgs.

Jasper           241 ditfqleaepvnetrfdfilqflgldndvpvvtvtsstlvtadnyrgasakftqsiptem
VR299            241 ..............................................................
Erwin            241 ...................l.....................p...e.................
WB               241 .v....a.............................s............................l
Ab               241 ..s.s.ann.tadik...q.d..............v..n..........m.m.....n
Can1             241 ..n.s.pra.ttd..y..q.d.i.........s....v.a....fq.v...........d
Te               241 ..n.r.p.a.pad.ky..q.d.v.....i...si...v.a..a.fs.v...........d
Can2             241 ..s.s.ata.aa..t.e.q.d......n......i.ta.a.ke.hl.v...m..a....s
Can3             241 ..s.s.ana.aad.t.k.q.d......n......i.ta.a.te.hl.v...m..a....s
Sp               241 ..n.s.aeq.a.......q.d.m...........v..v.a.n.....v...m.......n
DPL              241 ..n.s.aeh.a...k...q.d.m...........v..v.a.s...s.v...m.......n
TAB98            241 ..n.s.adn.pt..k...q.d.m...........v..v.a.......v...m.......n
```

Fig. 2 (continued overleaf). Amino acid (aa) sequence alignment of Segment A polyproteins. (.) Identical aa residues

```
TAB02          241 ..n.s.ads.pt..q...q.d.m..........v..v.a.......v...m.......n
NZ10           241 ..n.s.adn.pt..k...q.d.m..........v..v.a.......v...m.......n
NZ6            241 ..n.s.adn.pt..k...q.d.m..........v..v.a.......v...m.......n
He             241 ..n.n.qqa.a...k...k.e.......e.....v..v.a.ea....i...m.......n

Jasper         301 itkpitrvklayqlnqqtaianaatlgakgpasvsfssgngnvpgvlrpitlvayekmtp
VR299          301 ............................................................
Erwin          301 ............................................................
WB             301 ..................g.........................................
Ab             301 ..........s.kv........p....tl..............................
Can1           301 ........r.t.kv...e..tt......l.....................v.........
Te             301 ........r.s.kv...a..tt......l.....................v.........
Can2           301 ........r.s.k.....e.g.......l...t..........................
Can3           301 ........r.s.k.....e.........l...t..........................
Sp             301 ..........s.ki......g.v....tm..............................
DPL            301 ..........s.ki......g.v....tm..............................
TAB98          301 ........r.s.ki......g.v....tl..............................
TAB02          301 ........r.s.ki......g.v....tl..............................
NZ10           301 ..........s.ki......g.v....tl..............................
NZ6            301 ..........s.ki......g.v.....l..............................
He             301 ....v.....s.ki....e.g.t....vl.....a........................

Jasper         361 qsiltvagvsnyelipnpdllknmvtkygkydpeglnyakmilshreeldirtvwrteey
VR299          361 ............................................................
Erwin          361 ............................................................
WB             361 ............................................................
Ab             361 ..................e.......r............................k....
Can1           361 ..................e.......r............................k....
Te             361 ..................e.......r.....................d.......k....
Can2           361 ............................................................d.
Can3           361 ............................................................d.
Sp             361 l.................e.......r..............................
DPL            361 l.................e.......r..............................
TAB98          361 l.................e.......r............................k....
TAB02          361 l.................e.......r............................k....
NZ10           361 l.................e.......r............................k....
NZ6            361 l.................e.......r............................k....
He             361 ..v...................r....h.....................d.........d.

Jasper         421 kertrafkeitdftsdlptskawgwrdlvrgirkvaapvlstlfpmaapligaadqfigd
VR299          421 ............................................................
Erwin          421 ............................................................
WB             421 .......n....................................................
Ab             421 .....v.n.....s.............i.........................v...l...
Can1           421 r....v.n.....s.............i.........................v...l...
Te             421 r....v.n.....s.............i.........................v...l...
Can2           421 .....v.n.....s.............i.........................i.......
Can3           421 .....v.n.....s.............i.........................m.......
Sp             421 .....v.n.....s.............i.........................t.......
DPL            421 .....v.n.....s.............i.........................m.......
TAB98          421 r....v.n.....s.............i.........................m.......
TAB02          421 r....v.n.....s.............i.........................m...l...
NZ10           421 r....v.n.....s.............i.........................m.......
NZ6            421 r....v.n.....s.............i.........................m.......
He             421 .....v.n.....s.............i.kq..ri.......m.........m.......

Jasper         481 ltktnsaggrylshaaggryhdvmdswasgseagsyskhlktrlesnnyeevelpkpt
VR299          481 ............................................................
Erwin          481 ..............................r.............................
WB             481 ....................r....t......t..........................
Ab             481 ..n..a.....r.m......k.........gpd.kf.qa..n....a........p.s
Can1           481 ..n..a.....r.m......k..le.....gpd.kf.la..nk...t........p.s
Te             481 ..n..a.....r.m......k..le.....gpd.kf.qa..nk...t........a.s
Can2           481 .....a.....n.m....hhr...e.....kqd.rf.qs..n....a........p.q
Can3           481 .....a.....n.m....hhr..ve.....kqd.rf.qs..n....g........p.q
Sp             481 .....a.....h.m......k..le.....gpd.kf.ra..n....a........p.s
DPL            481 .....a.....h.m......k..le.....gpd.kf.ra..n....a........p.s
TAB98          481 .....a.....h.m......k..le.....gpd.kf.ra..n....a........p.s
TAB02          481 .....a.....h.m......k..le.....gpd.kf.ra..n....a........p.s
NZ10           481 .....a.....h.m......k..le.....gpd.kf.ra..n....a........p.s
NZ6            481 .....a.....h.m......k..le.....gpd.kf.ra..n....a........p.q
He             481 .....as....n........k..let.....nt.rf.ms..k....t........r.e
```

Fig. 2. (continued)

conflicting observations, together with the fact that TAB has not caused disease in Atlantic salmon, support the suggestion by Bain et al. (2008) that viral, host and/or environmental factors in addition to specific aa residues influence pathogenicity. TAB has continued to be isolated from salmon sea cages, but its presence has not been associated with elevated mortality rates. However, TAB has never been isolated from hatcheries in Tasmania, and whether it would be pathogenic to salmonids under hatchery conditions in Tasmania is unknown.

The location of the initiation codon of the VP5 protein may vary (data not shown). Magyar & Dobos (1994) reported that the initiation codon of VP5 is situated at position 68, although Heppell et al. (1995) demonstrated that VP5 expression was strain-dependent and could be initiated from position 68 or 112. Weber et al. (2001) have since demonstrated, by reverse genetics, that the second in-frame methionine codon is responsible for the initiation of VP5 in VR299, which Shivappa et al. (2004) have also demonstrated in Sp strains. The He strain is the only known IPNV to lack an ORF for VP5 (Heppell et al. 1995).

Alignment of VP5 nt sequences (Fig. 3A) demonstrates that all Australasian *Aquabirnavirus* isolates and several IPNV strains have a nucleotide deletion at position 106 that alters the reading frame of these isolates when compared to the IPNV type strain, Jasper. One of the IPNV isolates with this deletion (Strain N1) moves back into the original reading frame due to an

A

```
                                                          ↓
Jasper          68 ATGGCGAAAGCCCTTTCTAACAAACAACCAACAATTCTTATCTACATGAATCA
VR299           68 ..................................................
West Buxton     68 ..........................................-..T.....
Can2            68 ..............................T.....A.C-..A..A...C.AG.
Ab               1 ........................T...A.-......AA....--A.....AG.
E1-S             1 ........................T...A.-......AA....--A.....AG.
N1              79 ............................A...AC.A...ATAT.CA..AC.AG.
Fr21            68 ............................A...AC.A...-..A.CA...C.AG.
NVI-001         68 ............................A...AC.A...-..A.TA...C.AG.
NVI-011         68 ............................A....C.A...-..A.CA...C.AG.
NVI-013         68 ............................A...AC.A...-..A.CA...C.AG.
NVI-015         68 ............................A...AC.A...-..A.CA...C.AG.
NVI-016         68 ............................A...AC.A...-..A.CA...C.AG.
NVI-020         68 ............................A...AC.A...-..A.CA...C.AG.
NVI-023         68 ............................A...AC.A...-..A.CA...C.AG.
TAB98           68 ..............................CA..A...-..A.CA...C.AG.
TAB02           11 ..............................CA..A...-..A.CA...C.AG.
NZ#6            30 ..............................CA..A...-..A.CA...C.AG.
NZ#10           30 ..............................CA..A...-..A.CA...C.AG.
```

B

```
                                        BH4 domain
                                    ******************
Jasper           68 makalsnkqptiliymnhehiqgnrnll
VR299            68 ............................
West Buxton     112 ---------------.....nk......
Can2            112 ---------------.qd..k......f
Ab               1 .......n.-.q..s.kd..k.s.....
E1-S             1 .......n.-.q..s.kd..k.s.....
N1              79 .........tnn.ysiqd..k.......
Fr21            112 ---------------.qd..k.......
NVI-001         112 ---------------.qd..k......p
NVI-011         112 ---------------.qd..k......p
NVI-013         112 ---------------.qd..k......p
NVI-015         112 ---------------.qd..k......p
NVI-016         112 ---------------.qd..k.......
NVI-020         112 ---------------.qd..k......p
NVI-023         112 ---------------.qd..k......p
TAB98           112 ---------------.qd..k.......
TAB02            55 ---------------.qd..k.......
NZ#6             74 ---------------.qd..k.......
NZ#10            74 ---------------.qd..k.......
```

Fig. 3. (A) Nucleotide (nt) sequence alignment of all available VP5 sequences identifying deletion at position number 106 (arrow). (B) Amino acid (aa) sequence alignments of the same VP5 strains. Note strains that have the 106 deletion also have a 15 aa deletion at the N-terminal end (grey shading). (.) Identical bases/aa; (–) deleted nt residue/aa

insertion at nucleotide position 111. A similar insertion was found in the Australian and New Zealand *Aquabirnavirus* isolates. The N1 strain has been demonstrated to produce VP5 (Håvarstein et al. 1990, Heppell et al. 1995), and was also the only IPNV sequence examined that did not contain a second methionine residue.

It is assumed that the initiation codon for any isolate exhibiting a deletion at nucleotide 106 is position 112, and for those with no deletion at 106, the initiation codon is at position 68. Alignments of VP5 aa sequences revealed that Australasian isolates were 133 aa in length, lacking 15 aa at the N-terminal end of full length IPNV VP5 sequences (VR299: Heppell et al. 1993; Jasper: Duncan & Dobos 1986; N1: Håvarstein et al. 1990). Similarly sized VP5 proteins have been identified for Fr21, Can2 (Heppell et al. 1993) and Norwegian Sp strains (Santi et al. 2004), all of which also lack the 15 N-terminal aa.

Hong et al. (2002) demonstrated that the full length VP5 protein, which is composed of domains BH1, BH2, BH3 and BH4, has an anti-apoptotic function. Loss or modification of domains BH1 and BH2 resulted in loss of this function. While these domains were intact for the Australasian isolates, aa sequence alignments (Fig. 3B) indicate that all truncated VP5 proteins lack the first 4 aa of the BH4 domain. The effect of this deletion on the function of VP5 is unclear.

While virulence of IPNV isolates has been linked to Segment A (Sano et al. 1992), no specific sequences or motifs have been identified that correlate with virulence. An attempt to determine specific markers of virulence was recently reported by Santi et al. (2004). They demonstrated that all pathogenic isolates studied encoded a truncated VP5 protein. However, the current study has shown that all Australasian strains also contain a truncated VP5 protein, despite the fact that these isolates were usually detected in healthy fish showing no overt signs of disease (Tisdall & Phipps 1987, Crane et al. 2000). Therefore, the significance of a truncated VP5 protein in determination of virulence in salmon remains unclear. Other studies have shown that the role of VP5 in the expression of virulence is more complex and may be influenced by other viral and/or host factors (e.g. Santi et al. 2005). It is possible that the Australasian isolates may be pathogenic in other hosts or under different environmental or husbandry conditions. TAB has never been detected in any of the Tasmanian salmonid hatcheries and thus it has been assumed to originate in wild, non-salmonid fish populations. It is noteworthy that water temperatures in Tasmania are relatively high for salmonid aquaculture and stocking densities are kept relatively low in comparison to conditions in other salmon producing countries.

While the VP1 sequence was obtained as part of this study, a critical analysis has not been undertaken because VP1 encodes the RNA-dependent RNA polymerase and is likely to be highly conserved. At the aa level, all Australasian isolates were closely related. They showed greatest similarity to Sp (89%) and were least similar to the Jasper isolate (80%). However, there is limited sequence data available in GenBank for the VP1 protein.

Development of a differential diagnostic test

While aquatic birnaviruses have been isolated from both wild and farmed fish in Australasia, pathogenic IPNV remains exotic to this region. Thus, it was important for us to develop a diagnostic test that could differentiate Tasmanian *Aquabirnavirus* from pathogenic strains. To develop a PCR test that was pan-specific for aquatic birnaviruses, a set of generic primers, based on a nucleotide sequence alignment of Segment A from 13 exotic aquatic birnaviruses representing each genogroup and the 2 Australian and New Zealand isolates, was developed in conserved regions located on either side of a known variable region in the gene that encodes VP2. The forward primer was newly designed while the reverse primer was P10 designed by Blake et al. (2001a,b). This 'generic' PCR successfully detected all Serogroup A viruses tested, resulting in a 775 bp product. However, neither of the Serogroup B viruses tested yielded a PCR product (data not shown). While it is recognised that only sequence analysis of the PCR product will provide a definitive diagnosis, the presence of a unique *Cla*1 restriction enzyme site within the PCR product for TAB enabled the development of a rapid test to differentiate the TAB PCR product from the PCR product of other Serogroup A aquatic birnaviruses. The 775 bp *Aquabirnavirus* PCR products from each of the 10 different isolates of TAB were digested by *Cla*1, yielding 2 products (652 and 123 bp). No digestion occurred with the other aquatic birnaviruses that were examined in this study, including the 2 closely related New Zealand isolates (Fig. 4). For regional laboratories that may not have ready access to sequencing facilities, this technique provides a rapid means of tentatively characterising any specific product from the generic aquatic birnavirus PCR. Unfortunately, no MABs were available for examinination with this test, but a review of sequence alignments indicated that, theoretically, this test would also differentiate TAB from any marine birnaviruses as all of the latter lack the *Cla*I restriction site within the generic amplified product.

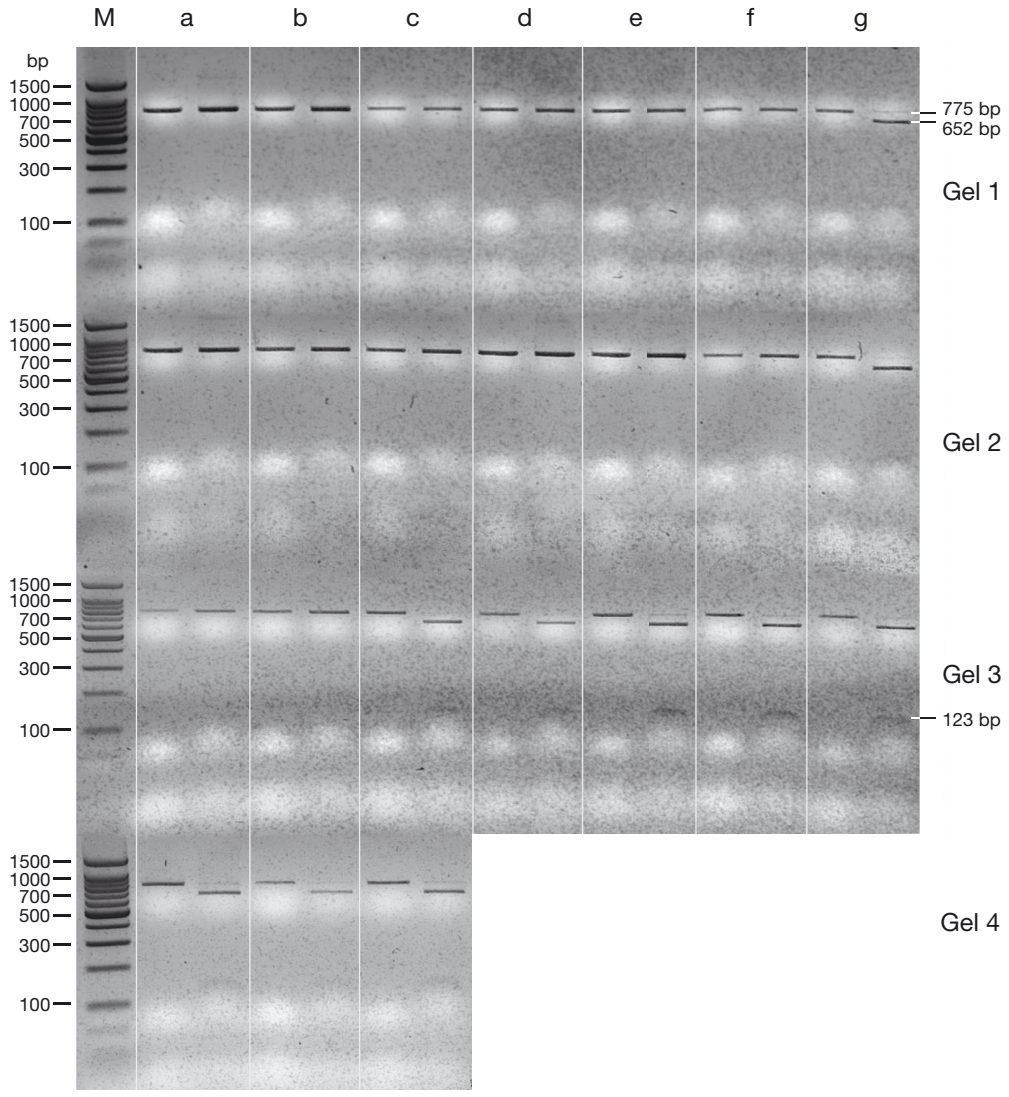

Fig. 4. *Cla*I digestion patterns of generic PCR products. The generic PCR products of various infectious pancreatic necrosis virus (IPNV) and Australasian aquabirnavirus isolates were digested with *Cla*I. For each sample (a to g), the uncut (left) and cut (right) samples were run next to each other to demonstrate fragment size shift. Gel 1: (a) West Buxton; (b) Jasper; (c) VR299; (d) Erwin; (e) He; (f) Canada (Can) 2; (g) TAB98. Gel 2: (a) Can 3; (b) Can 1; (c) Te; (d) Ab; (e) NZ6; (f) NZ10; (g) TAB02. Gel 3: (a) Sp; (b) DPL; (c) TAB Atlantic salmon, Macquarie Harbour (2001 isolate); (d) TAB Atlantic salmon, Hideaway Bay (1998 isolate); (e) TAB Atlantic salmon, Hideaway Bay (1998 isolate); (f) TAB rainbow trout, Macquarie Harbour (1998 isolate); (g) TAB rainbow trout, Macquarie Harbour (2001 isolate). Gel 4: (a) TAB cod, Macquarie Harbour (1998 isolate); (b) TAB flounder, Macquarie Harbour (1998 isolate); (c) TAB baitfish, Macquarie Harbour (1998 isolate). M: 100 bp DNA ladder (Promega). 775 bp is equivalent to the undigested amplicon. The 652 and 123 bp are the sizes of the 2 cut (digested) pieces

Acknowledgements. We gratefully acknowledge the generosity of: M. Hine and R. Morrison (National Centre for Disease Investigation, Upper Hutt, New Zealand) for supply of the New Zealand aquatic birnavirus isolates; B. L. Nicholson (University of Maine, USA) for providing the DPL isolate; B. Hill and K. Way (Weymouth Laboratory, CEFAS, Dorset, UK) for the Erwin isolate of IPNV; and, finally, A. Pye and A. R. Gould (CSIRO AAHL) for sequencing and sharing the partial sequence of the TAB Segment B gene, respectively. The authors also thank F. Filippi (CSIRO AAHL) for his expert assistance with graphic design. This study was supported by funding from the FRDC on behalf of the Australian Government (FRDC Project 2001/620 Aquatic Animal Health Subprogram: Development of improved procedures for the identification of aquatic birnaviruses).

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Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: May 26, 2009; Accepted: August 23, 2010
Proofs received from author(s): November 23, 2010