FINAL REPORT

Aquatic Animal Health Subprogram: development of improved procedures for the identification of aquatic birnaviruses

Kenneth A. McColl, Kelly R. Steeper and Mark St. J. Crane

April 2004

FRDC Project No. 2001/620

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Non-technical Summary

2001/620	Development of improved procedures for the identification of aquatic birnaviruses

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OBJECTIVES:

1. Import a range of aquatic birnaviruses which are representative of the most important strains.
2. Evaluate the range of birnavirus-specific PCR primers which are currently available for their ability to (a) act as generic (or pan-specific) primers, capable of detecting any aquatic birnavirus; (b) differentiate pathogenic from non-pathogenic isolates and (c) specifically identify the MH aquatic birnavirus.
3. Following evaluation (objective 2), select or develop a standard set of PCR primers which are generic for aquatic birnaviruses. Validate immunodiagnostic procedures (immunocytochemical and immunohistochemical tests) for the detection and identification of aquatic birnaviruses in cell cultures and in histological sections.
4. Develop a set of PCR primers which are specific for the MH aquatic birnavirus isolate.
5. Evaluate the ability of newly developed PCR primers to distinguish between pathogenic and non-pathogenic aquatic birnaviruses.
6. Evaluate the ability of newly developed PCR primers to identify, not only cell culture isolates of viruses but also, birnaviruses in infected fish tissues.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

A Standard Diagnostic Technique for the detection and identification of IPNV and related pathogens, in particular Serogroup A viruses, has been drafted, and is now available for peer review. The techniques encompass virus isolation, immunohistochemistry and also molecular techniques, and they will allow the detection of putative aquabirnaviruses by regional laboratories around Australia. Information on the reagents and procedures involved in this SDT are now available to State and Commonwealth agencies, as required. As an integral part of this process, we have also developed a two-step, PCR-based technique that allows differentiation

of the MH isolate (also known as TAB) from other potentially pathogenic aquabirnaviruses.

Together, these procedures and reagents will be useful to Australian regional laboratories in health certification programs, and also in managing any future disease outbreaks where there is a suspicion of involvement by aquabirnaviruses. The ability to rapidly characterize the virus involved in any such outbreak would certainly assist those decision-makers faced with developing a disease management plan.

Aquabirnaviruses are the most widespread pathogenic organisms in aquatic animal species. Those that are pathogenic for salmonid fish are known as infectious pancreatic necrosis virus (IPNV), and they may cause up to 100% mortality in fry, fingerlings and smolt. However, there are also many non- (or weakly) pathogenic aquabirnaviruses, known as IPNV-like viruses.

The determination of the pathogenicity of any new aquabirnavirus relies on experimental infection of young fish (usually brook trout). Apart from the fact that fish of the required age are not always available (depending on the season), there is also no standard procedure recommended for such pathogenicity trials, and the results of such trials are notoriously variable. The isolation of an aquabirnavirus from farmed salmonids and wild fish in Macquarie Harbour, Tasmania in 1998 (the MH isolate) highlighted the need for: (1) a generic procedure that will allow recognition of all aquabirnaviruses; (2) a test that is specific for the MH isolate (also known as Tasmanian aquabirnavirus, TAB); and, (3) an alternative rapid diagnostic procedure that can distinguish between pathogenic and non-pathogenic viruses.

The results of this project are best summarized by reference to the original objectives:

Objective 1

The AFDL has now gathered a representative range of aquabirnaviruses from all parts of the world, including Australasia. This puts us in a very good position to be able to fully characterize any future aquabirnaviruses isolated from this region of the world.

Objective 2

While many diagnostic tests for aquabirnaviruses were already available, it quickly became apparent that a more rational approach to developing a general test for any aquabirnavirus (known as a generic test), a specific test for TAB, and a procedure for distinguishing between pathogenic and non-pathogenic viruses required a complete understanding of the genetic structure of TAB. As a result of this project, this has now been achieved.

Objective 3

A molecular diagnostic test that is capable of detecting all of the most common aquabirnaviruses (a generic aquabirnavirus test) has now been developed. Furthermore, other diagnostic tests, used by the AFDL and based on older technologies, were also shown to be effective in the detection of a wide range of aquabirnaviruses, including TAB.

Objective 4

A molecular test has also been developed that is capable of tentatively differentiating a TAB isolate from other aquabirnaviruses. Thus, we now have a procedure that, firstly, detects the presence of any of the common aquabirnaviruses, and, secondly, provides a tentative identification of TAB isolates. These tests may be very useful for regional Australian laboratories, allowing them to undertake the preliminary characterization of any aquabirnaviruses that they might isolate.

Objective 5

The new generic test for aquabirnaviruses allows some preliminary molecular characterization of any new virus isolate. However, the lack of a standard virulence test for aquabirnaviruses made it impossible to correlate molecular information about a virus with its pathogenicity. It was therefore not possible to develop a simple test that could differentiate pathogenic from apathogenic aquabirnaviruses. We did, however, obtain comparative data on the virulence of a TAB isolate and a known pathogenic IPNV isolate, the Erwin strain, and these data suggested the relative innocuity of the Tasmanian aquabirnavirus.

Objective 6

The newly-developed generic test for aquabirnaviruses was tested on a number of samples of TAB-infected fish. While the test worked, the overall sensitivity was low. Therefore, while the test is perfectly suitable for characterizing suspect aquabirnaviruses identified in tissue culture, the sensitivity of the test needs to be improved for direct use on fish samples.

In conclusion, a Standard Diagnostic Technique for the detection and identification of IPNV and related pathogens has been drafted, and is now available for peer review. The techniques encompass virus isolation, immunohistochemistry and also molecular techniques, and they will allow the detection of putative aquabirnaviruses by regional laboratories around Australia. An integral part of this process is the development of a two-step, molecular technique that allows differentiation of the MH isolate (also known as TAB) from other potentially pathogenic aquabirnaviruses.

Together, these procedures and reagents will be useful to Australian regional laboratories in health certification programs, and also in managing any future disease outbreaks where there is a suspicion of involvement by aquabirnaviruses. The ability to rapidly characterize the virus involved in any such outbreak would certainly assist those decision-makers faced with developing a disease management plan.

While the new generic aquabirnavirus test is very useful for the characterization of viruses that have been isolated from infected fish, it is much less sensitive for the direct detection of virus in fish samples. There are two possible molecular approaches that could be used to increase the sensitivity of the test, and the AFDL is currently pursuing one of these options.

KEYWORDS: **aquabirnaviruses, Tasmanian aquabirnavirus, diagnosis, sequence.**

Acknowledgements

Many of the viruses used in this study were kindly provided by overseas laboratories: The Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, Weymouth, Dorset, UK provided a number of viruses during the early years of the AFDL; Dr Mike Hine and Ruairidh Morrison, National Centre for Disease Investigation, Upper Hutt, New Zealand, generously provided the New Zealand aquabirnaviruses that were used in this study; and, Professor Bruce L. Nicholson, Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, Maine, USA kindly provided the DPL isolate.

A number of people at AAHL, although not directly involved in this project, made valuable contributions. Dr Linfa Wang was the main source of sage advice on molecular issues, although Meng Yu was also very helpful; Dr Allan Gould provided some unpublished sequence data on TAB that we were able to compare with our newly-acquired data; Lynette Williams and Tamasine Chamberlain gave valuable advice and assistance with aspects of tissue culture and immunoperoxidase tests, respectively.

Background

Aquatic birnaviruses (family: Birnaviridae) are the most widespread pathogenic organisms in aquatic animal species. Aquatic birnaviruses which are pathogenic for salmonid fish species cause infectious pancreatic necrosis (IPN), an acute, contagious disease of mainly fry and fingerlings which can result in upto 100% mortality (Silim et al. 1982). The aetiological agent, IPN virus, was the first virus to be isolated from fish (Wolf et al. 1960) and since then a range of biochemically and serologically similar viruses (the aquatic birnaviruses) have been isolated from a wide variety of finfish (e.g. Ahne 1977, 1982; Hedrick et al. 1983; Wolf 1988), crustacea (Hill 1982) and molluscs (Underwood et al. 1977).

The classification of aquatic birnaviruses is complex and unclear and a range of serological and molecular tools has been used in attempts to elucidate the relationships between IPN and IPN-like viruses (see Reno 1999, for review). Currently, AAHL's ability to detect aquatic birnaviruses is well-developed (Crane et al. 2000) but our ability to differentiate between IPNV (pathogenic viruses) and IPN-like virus (non-pathogenic viruses) is limited.

The recent, first isolation of an aquatic birnavirus from farmed salmonids and wild fish from Macquarie Habour, Tasmania, has highlighted the need for development of improved procedures for the identification of IPN and IPN-like viruses (Crane et al. 2000). While the Macquarie Harbour (MH) isolate (later known as Tasmanian aquabirnavirus, TAB) proved to be of low pathogenicity, there is no guarantee that aquatic birnaviruses isolated in the future would be of similar pathogenicity. More pathogenic variants may be found. Currently, determination of pathogenicity requires experimental infection of young (1-4 month old) fish (brook trout or other relevant salmonid species) which, depending on the season, are not always available. Other techniques for the rapid identification of pathogenic isolates are required.

Need

Although the birnavirus isolated from several fish species in Macquarie Harbour proved to be non-pathogenic, the incident did highlight deficiencies in our ability to identify pathogenic birnaviruses. Rapid diagnostic tests which can distinguish between pathogenic (IPNV) and non-pathogenic (IPN-like viruses) aquatic birnaviruses are not available. The inability to determine quickly whether an aquatic birnavirus is pathogenic, or not, is a serious deficiency in our capability to interpret the significance of any future birnavirus isolations. Pathogenicity trials are complex and can take several weeks to complete (even when fish of the appropriate age are available), making implementation of appropriate on-farm management strategies difficult.

It is likely that the MH isolate (or TAB), or similar viruses, will be isolated during future surveillance. Currently, it could not be determined quickly whether a birnavirus which is different from TAB would, or would not, be pathogenic. Thus it is essential to develop, firstly, a generic procedure that will allow recognition of all aquatic birnaviruses, pathogenic and non-pathogenic. A polymerase chain reaction (PCR) offers one means of achieving this aim.

Such a procedure could be used in regional laboratories as a means of identifying unknown viruses, isolated during routine surveillance, as birnaviruses. For further characterization of these isolates, a more discerning procedure would also be required, one that would quickly allow differentiation of pathogenic from non-pathogenic birnavirus variants. The latter test could be used at AAHL (where pathogenic positive controls would be held) for the further, multi-pronged investigation of any aquatic birnavirus isolates detected by a regional laboratory. Any PCR tests that are developed will also be trialled on infected fish tissues.

Objectives

1. Import a range of aquatic birnaviruses which are representative of the most important strains.
2. Evaluate the range of birnavirus-specific PCR primers which are currently available for their ability to (a) act as generic (or pan-specific) primers, capable of detecting any aquatic birnavirus; (b) differentiate pathogenic from non-pathogenic isolates and (c) specifically identify the MH aquatic birnavirus.
3. Following evaluation (objective 2) select or develop a standard set of PCR primers which are generic for aquatic birnaviruses. Validate immunodiagnostic procedures (immunocytochemical and immunohistochemical tests) for the detection and identification of aquatic birnaviruses in cell cultures and in histological sections.
4. Develop a set of PCR primers which are specific for the MH aquatic birnavirus isolate.
5. Evaluate the ability of newly developed PCR primers to distinguish between pathogenic and non-pathogenic aquatic birnaviruses.
6. Evaluate the ability of newly developed PCR primers to identify, not only cell culture isolates of viruses but also, birnaviruses in infected fish tissues.

Methods

Importation of viruses

A range of aquatic birnaviruses was imported from the OIE Reference Laboratory (CEFAS, Weymouth, UK), and from other overseas laboratories. The identity and purity of imported isolates was established according to procedures outlined in the AAHL Microsecurity Manual prior to development work being initiated. Fish cell lines currently used at AAHL are known to be susceptible to infection by IPNV and other aquatic birnaviruses.

Virus isolation

Media

Eagle's minimum essential medium containing Earle's salts, 2% (v/v) foetal bovine serum, and 100 IU per mL penicillin and 100 ug per mL streptomycin for CHSE-214 cells, and Leibovitz's L-15 medium containing 2% foetal bovine serum and 100 IU per mL penicillin and 100 ug per mL streptomycin for EPC cells.

Procedure

i) Inoculation and monitoring cultures

1. Prepared tissue homogenate(s), and applied the appropriate volume to a 24-hour cell monolayer.
2. Allowed to adsorb for 30 min to 1 hour at 10-15°C, and, without removing the inoculum, added the appropriate cell culture medium. Incubated at 15°C.
3. Microscopic examination of the cultures was undertaken daily for 7 days using an inverted light microscope with 4X, 10X and 40X objectives. The cultures were examined for viral CPE or abnormalities such as sample cytotoxicity or contamination by reference to positive and negative control cultures.
4. If CPE appeared in cell cultures inoculated with the tissue homogenate, aquabirnavirus identification procedures were undertaken immediately (see below).
5. If no CPE occurred after 7 days of incubation (except in the positive control cultures), the inoculated cultures were passaged.

ii) Passaging cultures

1. Subjected cell culture monolayers to one freeze-thaw cycle. Pooled aliquots of the supernatants from all cell monolayers inoculated with dilutions of organ homogenates.
2. Diluted 1/20 and 1/100, and inoculated new 24-hour cell monolayers as described previously.
3. Incubated at 15°C, and monitored as already described.

Immunoperoxidase test

Reagents

Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)

80% (v/v) acetone in water

0.05% (v/v) Tween 20 in PBSA (PBST)

0.1% (w/v) skim milk powder solution in PBSA

Primary antibody: polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)

Normal sheep serum

Secondary antibody: biotinylated donkey anti-sheep Ig

Streptavidin-horseradish peroxidase conjugate

AEC (3 amino-9-ethyl carboxyzole)

DMF

0.05 M acetate buffer, pH 5.0

H_2O_2

Deionised water

Mayer's haematoxylin (Lillie's modification)

Scott's tap water

Procedure

1. The optimal dilutions (in 0.1% skim milk) of each reagent (antibodies, conjugates, substrates) were determined prior to use and checked on a regular basis.
2. Removed supernatant fluid from the wells of the cell culture, and washed the wells twice with 0.05% PBST.
3. Added 50-150 µL of primary antibody to each well (for a 96- or 24-well plate, respectively). Incubated at 37°C for 1 hour on a plate shaker set at low speed.
4. Removed the primary antibody, and washed the wells 3 times with PBST. Added the secondary antibody to all wells. Incubated at 37°C for 1 hour on a plate shaker set at low speed.
5. Removed the secondary antibody, and wash the wells 3 times with PBST. Added conjugate to each well. Incubated at 37°C for 1 hour on a plate shaker set at low speed.
6. Removed the conjugate from the wells, and washed the wells 3 times with PBST. Added freshly prepared substrate (AEC) solution (2 mg AEC, 200 µL dimethlyformamide, 10 mL 0.05 M acetate buffer pH 5.0, 5 µL 30% [v/v] hydrogen peroxide) for 20 min at room temperature.
7. Stopped the reaction with water, and counterstained with 50 µL per well of Mayer's haematoxylin. Incubated for 90 seconds at room temperature, rinsed twice with water, and blued the stain by adding Scott's tap water for 90 seconds.
8. Rinsed with water and allowed to air dry.
9. Examined processed wells by inverted light microscopy.

Interpretation

Positive reaction: grainy, focal, brick-red staining of cells indicated the presence of virus identified by the diagnostic antibody.

Negative reaction: no red staining apparent. All cells should be stained pale blue due to the counterstain.

Background staining: non-grainy, non-focal, pale pinkish staining may occur and could be due to any of a number of factors.

PCR-based tests

Reagents

Reagents stored at –20°C

Deionised formamide
Bovine serum albumin (BSA; 1mg/mL)
dNTPs (1.25mM)
100% Ethanol AR grade
70% Ethanol
Proteinase K (2mg/ml)
Primers (20 µM)
TaqMan Reverse Transcriptase Reagent Kit (AB Cat# N808-0234)
Random hexamers (MAB Cat# N808-0127)
PCR Mastermix (QIAGEN Cat# 201443)
100 bp DNA ladder & loading dye (Promega Cat# G2101)

Reagents stored at 4°C

Deionised formamide
Buffer AVL

Reagents stored at room temperature

QIAamp viral RNA Mini Kit
QIAGEN Buffer AW2
QIAGEN Buffer AW1
QIAGEN Buffer AVE
TNET
Agarose (BIORAD Cat # 162-0134)
Ethidium bromide (BIORAD Cat #161-0430)
40 x TBE Buffer (Promega Cat # 428A)

Procedure

Sample preparation

Inactivation was carried out by the following procedures

1. Cell-free samples, e.g., tissue culture supernatants at room temperature, were added to an appropriate commercially prepared buffer (e.g., Qiagen AVL buffer) containing guanidinium isothiocyanate.
2. Tissue samples, blood and other specimens containing cells were homogenised at approximately 10% w/v in phosphate buffered saline or

similar buffer, and then frozen and thawed. Samples were then microfuged (approximately 13000 x g for 20-30 sec), and the supernatant fluid collected. An appropriate volume of this was then mixed with an appropriate volume of a commercially prepared buffer (e.g., Qiagen AVL buffer).

Nucleic acids were extracted from submitted samples in the Biological Safety Cabinet Class II in the PCR suite.

Nucleic acid extraction and cDNA preparation

Nucleic acid (including aquabirnavirus dsRNA) was obtained from cell-free samples using the QIAamp Viral RNA extraction kit (QIAGEN cat no. 52904) or from tissues using the RNeasy Viral RNA Extraction kit (QIAGEN cat no. 74904). cDNA was then prepared from the viral RNA using a standard protocol that has been adopted for all RNA agents.

The TaqMan Reverse Transcription kit (Applied Biosystems cat no. N808-0234) was used for production of cDNA. A single cDNA reaction mixture consisted of the following reagents: 5.7 µL of water; 2 µL of 10x reaction buffer; 4.4 µL of 25 mM $MgCl_2$; 4 µL of 1.25 mM dNTPs; 1 µL of 50 mM random hexamers; 0.4 µL of RNAsin (20-40U); 0.5 µL of Multiscribe reverse transcriptase (50 U/µL); and 2 µL of extracted nucleic acid from the sample. The mixture was incubated at room temperature for 10 mins, followed by 48°C for 30 mins, and 95°C for 5 mins. For multiple samples, the volumes were multiplied appropriately.

Generic aquabirnavirus PCR

Following production of cDNA, a PCR was then conducted. The PCR mixture for a single sample consists of the following reagents: 9.5 µL of water; 12.5 µL of HotStar Taq Master mix; 0.5 µL of the forward primer (20 µM); 0.5 µL of the reverse primer (20 µM); and 2 µL of cDNA. For multiple samples, the volumes were multiplied appropriately. The mixture was incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that was programmed for: one cycle at 94°C for 15 minutes; 35 cycles at 94°C for 45 sec, 45°C for 45 sec and 72°C for 2 minute; and, finally, one cycle at 72°C for 7 minutes. Amplified DNA (775 bp) was detected by agarose gel electrophoresis.

The two primers that were used in the PCR were:

Generic forward primer: 5’-acgaaccctcaggacaa-3’

Generic reverse primer: 5’-cacaggatcatcttggcatagt-3’

Restriction enzyme digestion of a specific aquabirnavirus PCR product

If a PCR product of the correct size was amplified with the aquabirnavirus primers, the product was purified from the agarose gel (e.g., Qiagen Gel Extraction kit, Cat No. 28704). To 10 µL of the eluate was added: 2 uL of 10X reaction buffer, 2 µL of BSA (1 mg/mL), 10 IU of the restriction enzyme Cla I and water upto a final volume of 20 µL. Digestions were performed at 37°C for 1-2 hr, followed by incubation at 65°C for 15 min to inactivate the enzyme. Digested and undigested samples were then examined on an agarose gel containing ethidium bromide, and examined under UV illumination.

Immunohistochemistry

Reagents

10% formalin

Trypsin solution (1 mg per mL trypsin; 0.1 M Tris/HCl; 1 mg per mL $CaCl_2$; pH 8.0)

Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)

0.1% (w/v) skim milk powder solution in PBSA

Primary antibody: polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)

Normal sheep serum

Secondary antibody: biotinylated donkey anti-sheep Ig

3% (v/v) peroxide solution in methanol

Streptavidin-horseradish peroxidase conjugate

AEC (3 amino-9-ethyl carboxyzole) substrate (2 mg AEC, 200 µL dimethylformamide, 10 mL 0.05 M acetate buffer, pH 5.0, 5 uL 30% (v/v) H_2O_2)

Deionised water

Mayer's haematoxylin (Lillie's modification)

Scott's tap water

Equipment

Poly-L-lysine coated glass slides

Humid chamber, e.g., a plastic sandwich box with an air-tight lid

Light microscope fitted with 4X, 10X and 40X objectives

Procedure

1. The tissues for examination were fixed in aldehyde fixatives, e.g., 10% formaldehyde, for 48 hours or in absolute alcohol or 96% alcohol. Presumably frozen sections could also be used, but these have not been tried at AFDL.
2. After fixation, the sample was processed according to normal histological procedures, and then embedded in liquid paraffin.
3. Histological sections were mounted on silanised slides.
4. Sections were then deparaffinised, using standard procedures, followed by incubation with a trypsin solution for 20 min at $37^{O}C$. The reaction was stopped using cold ($4^{O}C$) PBSA.
5. Sections were then incubated for 1 hour at $37^{O}C$ with polyclonal sheep antiserum raised against IPN virus or with normal sheep serum diluted to its optimal concentration in 0.1% (w/v) skim milk in PBSA.
6. The sections were then washed in PBSA, and incubated for 1 hour at $37^{O}C$ with biotinylated donkey anti-sheep Ig diluted to optimal concentration in 0.1% (w/v) skim milk in PBSA.

7. After a further wash in PBSA, endogenous peroxidase activity was blocked by immersion of the sections in 3% (v/v) peroxide solution in methanol for 20 min at room temperature.
8. Following a wash in PBSA, the sections were incubated for 1 hour at 37°C with streptavidin-horseradish peroxidase diluted to optimal concentration in 0.1% (w/v) skim milk in PBSA.
9. After another wash in PBSA, the sections were incubated with freshly prepared AEC substrate solution for 20 min at room temperature.
10. The sections were rinsed in tap-water, counterstained with Mayer's haematoxylin and mounted in mounting medium (Quickmount, Daido Sangyo Co., Ltd, Japan) for microscopic examination.

Results and Discussion

Objective 1

Viruses imported into AAHL include some belonging to Serotype A: IPN-West Buxton and –VR299 (serotype A1); -Sp (A2); -Ab (A3); -He (A4); -Te (A5); -Canada 1 (A6); -Canada 2 (A7); -Canada 3 (A8); and –Jasper (A9). Additional serotype A viruses that were also imported included: two New Zealand aquabirnaviruses, NZ6 and NZ10 (kindly supplied by Dr Mike Hine, National Centre for Disease Investigation, Upper Hutt, NZ); the DPL strain, originally from Thailand (supplied by Dr Bruce Nicholson, University of Maine, USA); and the Erwin strain (A1). Originally, only one Serotype B virus was available for examination, TV-1. However, during the course of the project another, uncharacterized, isolate also became available (as it was part of the European Union Ring Test in 2002, an international diagnostic proficiency test that the AFDL participates in annually).

In addition, two isolates of the Tasmanian aquabirnavirus (TAB) were examined, the original isolate (TAB98), and an isolate from 2002 (TAB02).

Objective 2

There are numerous publications on PCR primers designed for the detection of aquabirnaviruses. For this project, the initial aim was to examine the efficacy of those published recently by Crane et al (2000) and Blake et al (2001) to act as generic primers, capable of detecting any aquabirnavirus. The two primers described by Crane et al (2000) were TAB 1 and TAB 2, the latter being originally designed by Heppell et al (1992). TAB 2 was used to prepare cDNA from RNA of the following viruses: IPN-Ab, -Sp, -VR299 and –Erwin; TAB98 and TAB02. PCRs were then conducted with TAB 1 and TAB 2 at different annealing temperatures. It was found that, at an annealing temperature of 45°C, a specific PCR product (~422 bp) was found for all viruses, but not for water (the negative control). When the annealing temperature was increased to 60°C, only the IPN isolates were amplified; no specific product was found for the TAB isolates (nor for the negative control) at this higher temperature. Therefore, it appears that TAB 1 and TAB 2, when used under the less stringent conditions (45°C), may potentially be useful as generic (or pan-specific) primers for the detection of aquabirnaviruses.

Blake et al (2001) used 14 different primers to sequence the large open reading frame in segment A of a number of aquabirnaviruses. Potentially, various combinations of these primers may also have been useful in developing a generic aquabirnavirus PCR. They may also have been useful for developing PCRs that were capable of differentiating pathogenic from non-pathogenic isolates, and for specifically identifying Australian aquabirnaviruses. However, it became apparent that, rather than simply testing these primers randomly on a variety of aquabirnaviruses (including TAB), it would be more sensible to actually sequence the original TAB isolate. Alignment of the subsequent TAB sequence with those of other aquabirnaviruses (obtained from sequence databases), would then allow the rational design of not only generic primers, but also TAB-specific primers. The value of the process could be increased by sequencing a more recent isolate of TAB (to gain some understanding of the variation in the genome over time) and, also, two New Zealand aquabirnavirus isolates. In addition, such an approach might allow the

development of a PCR for differentiation of pathogenic and non-pathogenic aquabirnavirus isolates.

Objective 3

TAB, like other aquabirnaviruses, has a bisegmented dsRNA genome. Segment A is approximately 3.1 kb in length, and segment B about 2.8 kb. The initial general strategy that was used to sequence segment A of TAB 98 was to amplify, by PCR, overlapping fragments of the segment using the primers of Blake et al (2001), and then to sequence the individual fragments. Where the established primers failed to amplify regions of segment A (for example, around the known variable region, and at the C-terminal end), new primers, based on nucleotide alignments of 24 different aquabirnaviruses, were designed. For segment B, very few primers had been published, and so new primers were designed based on alignments of the few sequences that were available for other aquabirnaviruses. The termini of both segments A and B were determined by using anchored PCRs (Attoui et al, 2000). A similar strategy was employed to sequence VP2 of TAB 2002, and of two New Zealand aquabirnavirus isolates (NZ6 and NZ10).

The full sequence of TAB98 is shown in Figure 1, and a phylogenetic comparison between Australasian aquabirnaviruses and others (using Genebank sequences) is shown in Figure 2. This analysis demonstrates six distinctly clustered groups of viruses, with each group generally corresponding to the geographical origin and original serological classification of isolates, as first noted by Blake et al (2001). The TAB and NZ isolates fall within Genogroup 5, which also includes five European isolates (Sp, N1, Fr10, and OV2) and one Asian isolate (DPL). Table 1 emphasizes the similarities among Australasian isolates by showing the percentage similarities (at nucleotide and amino acid levels) between Australian and New Zealand isolates. Close examination of all sequences has revealed a signature amino acid for TAB at position 221 (an alanine residue compared with a threonine for all other aquabirnaviruses that we have examined).

Based on a nucleotide alignment of segment A from 13 exotic aquabirnaviruses, two TAB isolates, and two New Zealand isolates, a set of generic primers was developed in conserved regions located on either side of a known variable region in a gene that encodes VP2 (Figures 3, 4). One of these primers (the forward primer) was newly-designed, as part of this project, while the other had been designed previously (P10 designed by Blake et al, 2001). The primers were then tested on representatives from each serotype of Serogroup A aquabirnaviruses. The generic PCR successfully detected all Serogroup A viruses held at AAHL, resulting in a 775 bp specific product. However, neither of the Serogroup B viruses yielded a specific PCR product (see later).

While it is recognised that it is only sequencing of the specific PCR product that will provide the ultimate definitive diagnosis, the presence of a unique Cla 1 restriction enzyme site within the PCR product for TAB allowed the development of a rapid test to differentiate the TAB PCR product from the PCR product of other aquabirnaviruses. The specific 775 bp aquabirnavirus PCR product from TAB isolates alone is digested by Cla 1, yielding two products (652 and 123 bp). No digestion occurred with the other aquabirnaviruses that were examined in this study, including the two closely-related New Zealand isolates (Figure 5). For regional laboratories that may not have ready access to sequencing facilities, this technique

provides a rapid means of tentatively characterising any specific product produced from the generic aquabirnavirus PCR.

The generic primers were tested on suspect TAB isolates from a variety of fish. A specific PCR product was detected in Atlantic salmon, rainbow trout, cod, flounder and baitfish collected between 1998 and 2001 from a variety of locations around Macquarie Harbour. Three isolates collected in 1998 from cod, doggy shark and ling, and also suspected of being TAB, were negative with this PCR. It is possible that the stock virus for these isolates was of very low titre, and attempts to reculture these viruses proved unsuccessful.

In summary, the alignment of nucleotide sequences from different aquabirnaviruses, including two TAB isolates and two New Zealand aquabirnaviruses, has allowed the development of a set of primers that are generic, or pan-specific, for all Serogroup A aquabirnaviruses that have been examined. At the completion of the PCR, specific PCR fragments of the correct size are identified by agarose gel electrophoresis. For interpretation, the following must be considered:

The negative control sample must have no evidence of specific amplified products.

A positive control sample must yield a specific aquabirnavirus fragment (775 bp).

Amplified fragments of the correct size are then eluted from the gel, and a Cla I restriction enzyme digest is performed. If the PCR product has been amplified from TAB, two digestion products will be seen (652 and 123 bp). No digestion will occur with other aquabirnaviruses.

The remainder of the eluate is used to determine the DNA sequence (by using the PCR primers as sequencing primers). Note that, an apparently specific PCR product, and the reactivity of the restriction enzyme with the PCR product, only provide a tentative diagnosis. The PCR product must be sequenced to make a definitive diagnosis.

Sequence identity and genotype are determined by a Blast search of the Genbank database.

Unfortunately this PCR test will not detect Serogroup B aquabirnaviruses. There is very little sequence data available for serogroup B aquabirnaviruses, and, for this reason, we did not feel it was justified to attempt to modify the current generic PCR in an attempt to produce one that would recognize aquabirnaviruses from both serogroups. We did attempt to design an all-encompassing PCR, one that would detect serogroup A and B viruses, based on the VP1 gene of segment B. This gene encodes the viral polymerase, and it is accepted that RNA-dependent RNA polymerase genes are among the most conserved of all viral genes (Shwed et al, 2002). This gene would be expected to be quite conserved across both serogroup A and B viruses. It was anticipated that possibly the more important issue with a PCR test based on this gene would have been avoiding false positive results, i.e., amplifying a specific PCR product from an RNA virus that, despite sequence similarities, belonged to a different taxonomic group. As it turns out, no matter which aquabirnavirus gene segment we used, we were unsuccessful in designing a genuinely generic PCR. Given that the most important aquabirnaviruses appear to belong to Serogroup A, this deficiency is probably of no great importance.

It could be asked, if segment B sequence offered so much, why did we not base our initial attempts to develop a generic PCR on the VP1 gene? We preferred to

concentrate our efforts on VP2 in segment A because: (1) there is much more sequence information available for this region, and (2) by designing primers that are located in conserved regions of the VP2 gene, but which amplify a PCR product that spans the variable region of the gene, we obtain a product that, when sequenced, may be very valuable in understanding the molecular epidemiology of that particular isolate. Such information might be very valuable in disease control programs.

Indirect immunoperoxidase (IPX) tests were developed using a sheep anti-IPN polyclonal antibody (Microtek, Saanichton, Canada) as the primary antibody for detection of virus in tissue sections and in tissue culture. A number of aquabirnaviruses were also characterised using a panel of commercial anti-IPN monoclonal antibodies (DiagXotics, Wilton, CT, USA) that detected virus in tissue culture.

Tissue sections from a brook trout experimentally infected with the Erwin strain of IPN (SAN 98-1825/12A) or from an uninfected fish (SAN 98-1823/1A) were reacted with the sheep anti-IPN antibody. Sheep serum containing anti-corynetoxin antibodies was also used as a negative control on these sections. Whereas negative control slides were uniformly devoid of specific staining, in the Erwin-infected fish there was specific, multifocal, red granular staining throughout the pancreas and kidney, and in the muscle layers of the intestinal wall. The IPX test also worked well on TAB-infected fish (SAN 98/1224/3, 8 and SAN 02/3293/3, /23), the tissue distribution of viral antigen being similar to that seen in Erwin-infected fish.

TAB was also cultured in CHSE monolayers in 96-well plates, and, when cytopathic effect was noted, the monolayers were fixed in formalin, and an IPX test performed (as described in the standard diagnostic technique, Appendix 1). Repeated testing has shown that the sheep anti-IPN antibody works well on TAB in tissue culture.

Objective 4

In an attempt to develop an MH (also known as TAB)-specific PCR, the complete sequence of segments A and B of the original isolate of TAB (TAB98) was determined (Figure 1). Partial sequences of the VP2 gene in segment A of TAB were aligned with the numerous sequences available in the public databases, and primers were designed that were considered to be specific for TAB. Random hexamers were used to produce cDNA from a variety of aquabirnavirus isolates (including 10 TAB isolates), and a PCR was then run (94°C for 2 min followed by 35 cycles of 94°C for 30 sec and 64°C for 2 min).

Initial results were encouraging with a specific PCR product being obtained for all 10 TAB isolates, but not for a variety of other aquabirnaviruses. However, further examination of the specificity of the PCR eventually resulted in it breaking down (in that it also detected some non-TAB aquabirnaviruses). We were unable to modify the primers, or the PCR conditions, such that complete specificity for only TAB could be obtained. For this reason, we explored other options, and eventually discovered the specificity of the Cla 1 restriction enzyme site in the PCR product of TAB isolates (see previous section). As already mentioned, this provides a rapid test for tentatively differentiating the PCR product of a TAB isolate from those of other aquabirnaviruses. Ultimately, of course, determining the sequence of the generic PCR product is the best way of characterising any aquabirnavirus.

Objective 5

An attempt to develop a molecular test that would distinguish between pathogenic and non-pathogenic aquabirnaviruses was always regarded as a challenging exercise. The anticipated difficulty was confirmed almost as soon as efforts got underway. The underlying problem is a paucity of information on the virulence of individual aquabirnavirus isolates. While there is now a substantial amount of sequence data available for many IPN viruses, the corresponding data on the virulence of many of these isolates is simply not available. In large part, this is due to the lack of a standardized protocol for measuring virulence. There have been many attempts to develop such a protocol (for example: Bowden et al, 2002; Isshiki et al, 2001; McAllister and Owens, 1986; Silim et al, 1982; Taksdal et al, 1997), but none of these has been universally accepted. This makes it very difficult to compare mortality data from different studies.

Because we wished to gain some understanding of the comparative pathogenicity of TAB, we conducted a virulence trial with TAB, comparing it with the Erwin strain of IPN virus (an isolate that is considered to be highly virulent). Three-month old Atlantic salmon and brook trout were infected with TAB or the Erwin isolate by either the intraperitoneal route (~10^5 $TCID_{50}$ per fish) or by immersion (~10^5 $TCID_{50}$ per mL for 3 hr). The experimental procedure is summarized in Table 2, and the results of the trial are shown in Table 3.

In an earlier unpublished trial, the Erwin virus was associated with 90% mortality in brook trout compared with <10% in the TAB-infected fish when each of the viruses were administered intraperitoneally. Results from the current trial followed the same pattern, although the differences between Erwin and TAB were not as marked (27% and 13%, respectively, following IP inoculation of brook trout). Only 1% mortality was noted in Atlantic salmon with either virus in the current trial. Clearly there is a difference between the two viruses in brook trout, and the variation in this difference between trials probably reflects the normal trial-to-trial variation that is a hallmark of infection experiments with IPN viruses. The much lower mortality in Atlantic salmon, with either virus, is consistent with previous observations on the variation in the susceptibility of different species of fish to aquabirnaviruses (reviewed by Sadasiv, 1995).

The variation in the mortality rate between trials (not to mention between studies by different groups) highlights the problem of interpretation of mortality data. If one virus causes 5% mortality in challenge experiments, and another causes 90% mortality, are they both regarded as pathogenic? What if the same virus causes 27% mortality in one trial, and 90% in another trial? What is the cut-off point for what is regarded as “pathogenic”? These are unresolved issues that currently make it very difficult to identify virulence markers in aquabirnaviruses. Julin et al (2003) have made an attempt to correlate virulence with a small number of amino acid residues in the VP2 protein, but their own data appear to contain inconsistencies. There is another issue that is not addressed in their work, but which also needs to be considered when trying to correlate sequence changes with virulence, i.e., how to differentiate a putative marker of virulence for a virus from a marker of the geographical origin of the virus. As an example, our work has demonstrated a lysine residue at amino acid position # 245 in VP2 of the (at best) weakly virulent TAB, New Zealand aquabirnaviruses, DPL and Ab strains of IPN, whereas the more virulent VR299, Sp

and Erwin strains of IPN have an arginine residue at that location. However, while this lysine/arginine residue MAY be a potential marker of virulence, it could also simply be a marker of Australasian viruses. Ultimately, it will probably be a reverse genetics approach that allows identification of virulence markers, an approach that is already being used successfully on another birnavirus, infectious bursal disease virus (Brandt et al, 2001).

Objective 6

The newly-developed generic aquabirnavirus PCR primers were tested on TAB-infected fish. The test samples were fish tissues that had been submitted to the AFDL as part of an ongoing virus surveillance program, and TAB was isolated from the samples using standard tissue-culture techniques (SAN 03-3516). The titre of virus in these samples was not determined. However, after tissues from the same infected fish had been prepared for PCR, the generic PCR failed to detect the presence of TAB nucleic acid in the samples.

Tissues from uninfected fish were then spiked with dilutions of tissue culture-derived TAB (10^8 $TCID_{50}$ per 50 µL), and samples of the "infected" tissue were prepared for PCR. These experiments demonstrated that the generic PCR required at least 10^7 $TCID_{50}$ of virus per 50 µL of supernatant fluid from infected fish tissue for a positive result. However, when 1 µL of the final PCR sample was subjected to a further round of the same PCR (using the same primers), very strong responses were observed for both 10^6 and 10^7 $TCID_{50}$ per 50 µL. This finding suggested that, by converting the generic aquabirnavirus PCR to a conventional nested PCR, there would be a marked increase in the sensitivity of the former.

Thus, while the generic PCR was shown to work in fish spiked with TAB, the overall sensitivity of the test was low (as also demonstrated by the inability to detect TAB in naturally-infected fish from which virus was cultured). Therefore, while the test is perfectly suitable for characterizing suspect aquabirnaviruses identified in tissue culture, the sensitivity of the test needs to be improved for direct use on fish samples. This could be done by developing either a nested PCR or a real-time PCR. The AFDL is currently pursuing the latter option.

Conclusions

Overall, the project has been very successful as judged by the level to which each of the Objectives has been satisfied:

Objective 1

The AFDL now has one or more viruses representing each serotype of the aquabirnaviruses. Importantly, we now also have representative isolates of many of the aquabirnaviruses isolated from fish in the Australasian region (e.g., from Australia, New Zealand and Thailand). This puts us in a very good position to be able to fully characterize any future aquabirnaviruses isolated from this region of the world.

Objective 2

Initially, a number of birnavirus-specific PCR primers, that were already available when this project began, were characterized for their potential as diagnostic reagents. While one set of such primers showed promise as generic reagents (i.e., capable of detecting any aquabirnavirus), it became apparent that a more rational approach to satisfying the diagnostic aims of this project required the complete sequence of TAB.

Objective 3

After acquiring the full nucleotide sequence of TAB, and aligning it with other known aquabirnavirus sequences, a generic PCR capable of detecting all serotype A aquabirnaviruses that were available (including a number of TAB isolates), was developed. Despite numerous attempts, based on a variety of genes, we were unsuccessful in developing a generic PCR that could detect both Serogroup A and B viruses. Given that the most important aquabirnaviruses appear to belong to Serogroup A, this deficiency is probably of little importance. Immunodiagnostic tests using a commercially-available sheep anti-IPN polyclonal antibody (Microtek, Saanichton, Canada) were demonstrated to be effective in the detection of aquabirnaviruses, including TAB, in tissue sections and in cell culture.

Objective 4

Although we were unable to design PCR primers that were specific for TAB (also known as the MH aquabirnavirus isolate), we did develop a test, based on restriction enzyme analysis of the generic PCR product, that is capable of tentatively differentiating a TAB isolate from other aquabirnaviruses. Thus, we have produced a two-step test that, firstly, detects the presence of any Serogroup A aquabirnavirus, and, secondly, provides a tentative identification of TAB isolates. While this test may be useful for regional laboratories that lack a nucleic acid sequencing facility, it must be recognized that the definitive characterization of any aquabirnavirus isolate can only come from sequencing of the generic PCR product.

Objective 5

The aim of developing a PCR that could differentiate pathogenic from apathogenic aquabirnaviruses was based on the substantial amount of sequence data that were available before the current project began. Unfortunately, the paucity of data on the virulence of many of these isolates was not anticipated, and the absence of these data made it impossible to fulfil this objective. We did, however, obtain comparative

data on the virulence of a TAB isolate and a known pathogenic IPN isolate, the Erwin strain, and these data suggested the relative innocuity of the Tasmanian aquabirnavirus.

Objective 6

The newly-developed generic aquabirnavirus PCR primers were tested on a number of samples of TAB-infected fish. While the PCR was shown to work in such situations, the overall sensitivity of the test was low. Therefore, while the test is perfectly suitable for characterizing suspect aquabirnaviruses identified in tissue culture, the sensitivity of the test needs to be improved for direct use on fish samples. This could be done by developing either a nested PCR or a real-time PCR. The AFDL is currently pursuing the latter option.

In the process of satisfying each of these Objectives, there have been a number of outputs that have allowed us to address the planned outcomes of the overall project. Firstly, a Standard Diagnostic Technique for the detection and identification of IPNV and related pathogens, in particular Serogroup A viruses, has been drafted, and this is now available for peer review (see Appendix 3). The techniques encompass virus isolation, immunohistochemistry and also molecular techniques, and they will allow the detection of putative aquabirnaviruses by regional laboratories around Australia. Information on the reagents and procedures involved in this SDT are now available to State and Commonwealth agencies, as required. An integral part of this process, and one that allows satisfaction of the second of the planned outcomes, is that we have also developed a two-step, PCR-based technique that allows differentiation of the MH isolate (also known as TAB) from other potentially pathogenic aquabirnaviruses.

Together, these procedures and reagents will be useful to Australian regional laboratories in health certification programs, and also in managing any future disease outbreaks where there is a suspicion of involvement by aquabirnaviruses. The ability to rapidly characterize the virus involved in any such outbreak would certainly assist those decision-makers faced with developing a disease management plan.

Benefits

The identification of aquatic birnaviruses, including pathogenic infectious pancreatic necrosis virus (IPNV) has been problematical, partly due to the vast host range of this group of viruses as well as its geographical range which is most likely to be worldwide. Until relatively recently (Crane *et al.*, 2000), aquatic birnaviruses were exotic to Australia. Since 1999, when the first birnavirus was isolated from farmed Atlantic salmon in Tasmania, birnaviruses have been isolated on an infrequent, but regular, basis.

While the original isolate appears to be of low pathogenicity, there has been no simple assay to determine whether subsequent aquabirnavirus isolates are the same or different to the original isolate or whether they are pathogenic or not. In fact, the relationship between most aquabirnaviruses is not fully understood. The aims of this project were to develop research and diagnostic tools which would assist with the identification of aquabirnavirus isolates detected in Australia and, in particular, in the Atlantic salmon farming regions of Tasmania.

Thus it is clear that the major beneficiary is the salmonid aquaculture sector, the vast majority of which operates out of Tasmania. It has been shown that the aquabirnavirus isolates from Atlantic salmon farmed in SE Tasmania are closely related to each other and can be differentiated from other, exotic viruses. The diagnostic procedures established here permit the rapid detection and identification of the Tasmanian aquabirnavirus (TAB) and other aquatic birnaviruses, including IPNV, which, in turn, assists the regulatory authorities and industry to make timely decisions on disease management.

State laboratories with a requirement for the detection and identification of aquabirnaviruses will also benefit. The immunohistochemical and PCR procedures can be transferred to all diagnostic laboratories with PCR and histology capabilities. Commercial antibodies are readily available and their diagnostic application under Australian conditions has been demonstrated. The PCR primers and other molecular reagents are also readily available and the procedures are straightforward.

Further Development

While the diagnostic procedures established here provide additional and improved tools for the detection and identification of aquatic birnaviruses, the relationship between pathogenic and non-pathogenic viruses remains unclear. The development of a reliable and reproducible experimental model that reflects acute and chronic infection of salmonid fish species with pathogenic aquatic birnaviruses, such as IPNV, is required. There are very few examples, in the literature, of experimental infections of salmonids with IPNV.

Without improved, biosecure aquarium facilities, it is highly unlikely that this capability will be attained in Australia. As the aquaculture industries expand and grow, the need for such a facility will undoubtedly increase, not only for pathogenicity trials in a range of aquatic animals but also for undertaking basic and applied research in aquatic animal diseases.

One other useful area of development relates to the generic PCR test for aquabirnaviruses. While the test that has been developed is perfectly suitable for characterizing suspect aquabirnaviruses identified in tissue culture, the sensitivity of the test needs to be improved for direct use on fish samples. This could be done by developing either a nested PCR or a real-time PCR. The AFDL is currently pursuing the latter option.

Planned outcomes

Infectious pancreatic necrosis (IPN), caused by IPN virus, is a serious disease of salmonid fish species which can cause significant losses in farmed salmonids. The validation of internationally recognised Standard Diagnostic Techniques for the detection and identification of IPNV and related pathogens and the development of a PCR-based technique to differentiate TAB (and possibly other non-pathogenic aquatic birnaviruses) from pathogenic isolates will permit State/Commonwealth agencies to establish accurate information on the presence or absence of these pathogens in fish populations. Thus the use of these procedures will play an important role not only in health certification programs but also in the management of any disease outbreak. Early detection of a pathogenic isolate prior to accidental introduction to a hatchery would avert significant losses. In addition, reagents and procedures which can identify TAB will provide a means by which severe control measures may be avoided.

Thus the industry sectors which will benefit from these outcomes include salmonid aquaculture in Tasmania and other states. Identification of infected and uninfected stock will allow industry and state officials to implement a disease management plan based on accurate information. Isolation of infected and uninfected fish populations will be possible thus enhancing our capability to control and/or eradicate the disease. In addition, since aquatic birnaviruses have a very broad host range, including non-farmed and ornamental fish species, other industries may benefit from project outcomes.

State diagnostic laboratories will benefit from being provided with sensitive and specific reagents and procedures. Based on accurate diagnoses, State officials responsible for conducting activities of the Local Disease Control Centre (LDCC) will be able to make informed decisions during implementation of disease management procedures.

Sequence data comparing TAB with other IPN and IPN-like isolates will assist in determining the relationship between TAB and these exotic and pathogenic strains. Such data will be useful in identifying any new variants which may occur in the future as well as identifying any incursion of potentially highly pathogenic viruses. Again, this type of information will allow appropriate management procedures to be put in place, as required.

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Appendix 1 - Intellectual Property

All information arising from this project has been used for the development and/or establishment of Standard Diagnostic Procedures. No intellectual property has been identified.

Appendix 2 – Staff

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Appendix 3

Australian and New Zealand Standard Diagnostic Procedure - Identification of aquatic birnaviruses draft

for endorsement by

National Aquatic Animal Health Technical Working Group (NAAH-TWG)

and

Sub-committee on Animal Health Laboratory Standards (SCAHLS)

Identification of aquatic birnaviruses

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SUMMARY

Aquatic birnaviruses (aquabirnaviruses), which are double-stranded bisegmented RNA viruses belonging to the Family Birnaviridae, include both virulent and avirulent viruses of aquatic animal species. Isolates that are pathogenic for species within the Family Salmonidae are known as infectious pancreatic necrosis (IPN) viruses, and they are of particular economic importance to the salmonid industries of Norway and Scotland. Tasmanian aquabirnavirus (TAB), which is the only known aquabirnavirus in Australia, has been found in wild and farmed fish in Macquarie Harbour, Tasmania. It has never been associated with mortalities in freshwater hatcheries.

IPN viruses may cause an acute, contagious disease with upto 100% mortality in young first-feeding fry, and, more recently, mortalities have also been recorded in older Atlantic salmon smolt in some Nordic and Scottish farms. The main histological lesions are in the pancreas and intestine. Survivors may become life-long carriers of the virus.

***Identification of the agent:** Diagnosis of aquabirnaviruses is based on a range of procedures. A presumptive diagnosis of a virulent aquabirnavirus may be made on clinical and gross pathological signs, but there may be no gross evidence of infection with avirulent strains. A definitive diagnosis of any aquabirnavirus depends upon detection of virus, viral antigen or viral genome in affected tissues or in cell culture.*

***Status of Australia and New Zealand:** Aquatic birnaviruses have been described in Australia and New Zealand. However, in both cases, the viruses are restricted in their distribution to localized marine environments, and there is no evidence of disease in freshwater sites. Consequently, both countries are still regarded as being free of IPN.*

Introduction

Aquatic birnaviruses (aquabirnaviruses) are the most widespread pathogenic organisms in aquatic animal species having been identified in most parts of the world, and in 32 families of fish, 11 species of mollusc and 4 spp of crustacean.[1] Aquabirnaviruses are of particular economic importance to the salmonid industries of Norway and Scotland.[2,3]

Within Australasia, aquabirnaviruses have been isolated in Thailand, the People's Republic of China, Taiwan, Korea, Japan, and New Zealand.[4,5] None had been recognized in Australia until 1998 when Tasmanian aquabirnavirus (TAB) was isolated from wild and farmed fish in Macquarie Harbour, Tasmania.[6] In subsequent years, there have been a few further isolations in Tasmania, but always restricted to Macquarie Harbour, and never in the freshwater hatcheries.

Aquabirnaviruses have been most recently reviewed by Reno.[7]

Aetiology

The genus Aquabirnavirus includes double-stranded (ds), bisegmented RNA viruses belonging to the Family Birnaviridae. Viruses within this genus are assigned to one of two serogroups: A, within which there are nine serotypes, and B, containing just one serotype.[1] TAB is included in serogroup A.[6]

The genus includes both virulent and avirulent viruses with the term infectious pancreatic necrosis (IPN) virus being reserved for those isolates that are pathogenic for species within the Family Salmonidae. The term "marine aquabirnaviruses", on the other hand, refers to a group of viruses that have been largely isolated from around Japan.[8] The taxonomic status of these viruses is not clear.

Epidemiology

IPN viruses have long been known to cause an acute, contagious disease resulting in up to 100% mortality in very young first-feeding fry (summarized by Reno[7]). The susceptibility of young fish declines as they age, and, generally, when fish are moved to the marine environment they are no longer susceptible to disease, although subclinical infection may still occur. However, recently, mortality due to IPN virus has also become important in Atlantic salmon smolt on some Nordic and Scottish farms.[2,3]

There is variation in the sensitivity of salmonids to infection with IPN virus. Sadasiv[9] noted that generally brook trout (*Salmo fontinalis*) were the most sensitive to the lethal effects of the virus followed by rainbow trout (*O. mykiss*) and, lastly, Atlantic salmon (*S. salar*).

Survivors of infection with IPN virus may become life-long latent carriers of the virus.[10] They, along with more acutely-infected fish, may transmit the virus both horizontally or vertically.[11,12,13] It is also postulated that aquabirnaviruses may be transmitted to fish, directly or indirectly, by fish-eating birds and by many invertebrates.[14,15]

IPN virus is a relatively stable virus under environmental conditions, e.g., there was little effect on virus titre after storage for 4 weeks at approximately 18° C.[5,16] The virus also appears to be resistant to changes in pH.[5]

Pathogenesis

Wolf et al[17] postulated that fish become infected with IPN virus by ingestion or via the gills. In acute infections, virus may be isolated from many tissues including kidney, spleen, pancreas, pyloric caecae, liver and gonads.[18,19] By comparison, in chronic infections virus is generally restricted to pancreas, pyloric caecae, intestine, anterior kidney and gonads of most fish.[19]

It has been postulated that compromised function of the pancreas and intestine in infected fish may account for a poorer food conversion rate in these fish compared with uninfected fish.[20] It has also been postulated that infection with IPN virus may predispose fish to other infectious diseases.[20]

Pathogenesis trials have not been reported for TAB.

Clinical signs

IPN virus may cause an acute disease in young salmonids with clinical signs typical of a systemic infection.[21] These include: darkening of the skin; exophthalmia; abdominal swelling (especially in Atlantic salmon); and, cutaneous petechiae, especially at the base of the fins. Cast-like pseudofaeces and abnormal behavioural signs may also be seen, the latter ranging from lethargy to short bursts of erratic swimming (e.g., "corkscrewing"). Acute infections may culminate in high mortality (up to 10-90%).

No abnormal clinical signs have been reported in fish infected with TAB.[6]

Pathology

Gross lesions associated with IPN include those seen clinically (darkening of the skin, exophthalmia, abdominal swelling, cutaneous petechiae). In addition, petechiae may be present throughout the viscera, and the stomach and anterior intestine may contain a clear-milky mucus which is said to be pathognomonic.[20]

Histologically, the most severe lesions occur in the pancreas and the intestine. Pancreatic necrosis may range from multifocal to extensive, and may affect both acinar and islet cells. Surrounding adipose tissue may also be affected, possibly due to release of pancreatic enzymes. An acute catarrhal enteritis is characterized by necrosis and sloughing of the intestinal mucosa. McKnight and Roberts[20] consider the gut lesion to be the most likely cause of mortality in acute cases. When fish survive an outbreak of IPN, there may residual chronic inflammatory lesions in the pancreas.

Pancreatic lesions consistent with IPN virus infection have been noted in some fish naturally infected with TAB (Figure 1).[6] However, virus was also isolated from a number of fish that had no histological lesions.

Lesions associated with one virus belonging to the marine aquabirnaviruses (AY-98) included pancreatic necrosis and body deformities.[22]

Control

In those countries where IPN is a problem, control relies on management practices that aim to prevent exposure of young fish to IPN virus, e.g., the use of fertilized eggs from broodstock certified to be free of IPN virus, and the use of an IPN virus-free water supply. No effective vaccines are currently available. In the face of an outbreak, there are no effective treatments, although reducing the population density may contribute to an overall reduction in mortality.[23]

Diagnosis

Limitation statement

Because aquabirnaviruses are among the most common pathogens of aquatic organisms, claims about the validity of the following tests must necessarily be restricted to those groups of viruses represented by the isolates that have been tested at the AFDL. These include the six genogroups of serogroup A. However, there is only limited access to serogroup B viruses, and no marine aquabirnaviruses have been examined.

General comments

This standard diagnostic technique paper documents the methods currently used at the AFDL for the isolation and identification of aquabirnaviruses. The methods are based on those recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals for IPN virus.[23] Virus isolation is still considered to be the most sensitive technique for the detection of viral infections of fish, and its use is of fundamental importance in the management of disease outbreaks and the control of disease spread.[24] The procedures used for sampling fish have been documented in other SDTs and will not be discussed here.[25,26] Processing of samples for virus isolation has also been documented previously.[27]

Storage of samples

Samples should not be frozen prior to processing but should be maintained between 4-10°C (shipping on wet ice in a styrofoam shipping container is appropriate). To maximise sensitivity, samples for virus

isolation and RT-PCR should be processed and assayed within 24 hr of sampling, but, when this is not possible, they must be processed within 72 hr of sampling. Samples for immunoperoxidase (IPX) tests should be processed within 2 hr, and samples for histopathological or electron microscopic examination should be placed in the appropriate fixative immediately after euthanasia of fish.

Tests available

The clinical signs associated with infection by aquabirnaviruses are non-specific. While histological lesions may be suggestive of infection, a definitive diagnosis depends upon detection of virus, viral antigen or viral genome in affected tissues or in cell culture.

When an aquabirnavirus infection is suspected, virus isolation should be attempted from the whole viscera, including kidney, or, for larger fish, liver, kidney and spleen (Appendix 1). Virus isolation attempts should be by inoculation of fish cell lines, e.g., CHSE-214 (chinook salmon embryo; ATCC CRL 1681), or EPC (*epithelioma papulosum cyprini*[28]) cell lines. The BF-2 (bluegill fry) and RTG-2 (rainbow trout gonad; ATCC CCL 55) cell lines are also recommended.[23]

Cultures are incubated at 15°C. At 7 days post-inoculation (pi), material from cultures displaying cytopathic effect (CPE) may be passaged on to fresh cell cultures. When CPE is evident in the second passage at about 2-3 days pi, cultures can be used for a virus neutralization test (Appendix 2), or an IPX test (Appendix 3). Tissue culture supernatant fluid from the second passage may also be used for electron microscopic examination.

Where a tentative diagnosis of aquabirnavirus infection is made on the basis of CPE in cell culture, a PCR-based procedure has been developed, firstly, to confirm the diagnosis (generic aquabirnavirus PCR), and then to differentiate, tentatively, a TAB infection from other aquabirnaviruses (Appendix 4).

Depending on the size of the affected fish, either a range of tissues, or the entire fish, should be fixed in 10% buffered formalin for histopathological examination. An immunohistochemical test may also be conducted on unstained sections of formalin-fixed tissues (Appendix 5).

Acknowledgements

Dr Mike Hine, National Centre for Disease Investigation, Upper Hutt, New Zealand, and Dr Bruce Nicholson, University of Maine, Maine, USA, generously provided the New Zealand aquabirnavirus isolates, and the DPL isolate, respectively.

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Appendices

Appendix 1
Virus isolation

Equipment

Equipment required to establish and maintain a fish cell culture laboratory has been discussed previously.[27]

Reagents

Media: Eagle's minimum essential medium containing Earle's salts, 2% (v/v) foetal bovine serum, and 100 IU per mL penicillin and 100 ug per mL streptomycin for CHSE-214 cells, and Leibovitz's L-15 medium containing 2% foetal bovine serum and 100 IU per mL penicillin and 100 ug per mL streptomycin for EPC cells.

Procedure

i) Inoculation and monitoring cultures

a) Prepare tissue homogenate(s),[27] and apply the appropriate volume to a 24-hour cell monolayer.[29]
b) Allow to adsorb for 30 min to 1 hour at 10-15°C, and, without removing the inoculum, add the appropriate cell culture medium. Incubate at 15°C.
c) Microscopic examination of the cultures is undertaken daily for 7 days using an inverted light microscope with 4X, 10X and 40X objectives. The cultures are examined for viral CPE or abnormalities such as sample cytotoxicity or contamination by reference to positive and negative control cultures.
d) If CPE appears in cell cultures inoculated with the tissue homogenate, aquabirnavirus identification procedures must be undertaken immediately (see below).
e) If no CPE occurs after 7 days of incubation (except in the positive control cultures), the inoculated cultures may be passaged.

ii) Passaging cultures

a) Subject cell culture monolayers to one freeze-thaw cycle. Pool aliquots of the supernatants from all cell monolayers inoculated with dilutions of organ homogenates.
b) Dilute 1/20 and 1/100, and inoculate new 24-hour cell monolayers as described previously.
c) Incubate at 15°C, and monitor as already described.

Interpretation

The test is valid if the negative control cell cultures retain normal cellular morphology for the full period of incubation.

The test sample is negative if the inoculated cell cultures retain normal cellular morphology similar to the negative control cultures, i.e., do not demonstrate viral CPE.

If any of the cell cultures inoculated with test samples demonstrate CPE, further investigations are required such as: a virus neutralisation test (Appendix 2); an immunoperoxidase test (Appendix 3); PCR (Appendix 4) or examination by electron microscopy.

Appendix 2
Virus neutralisation test

Reagents

Polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)
Pan-specific anti-aquabirnavirus monoclonal antibody AS1 (DiagXotics Inc., USA #93-AS1UC, if still commercially available)

Procedure

a) Dilute the virus-containing medium from 10^{-2} to 10^{-4}.
b) Mix aliquots of each dilution with equal volumes of the optimal dilution

of the neutralising polyclonal antibody solution or the monoclonal antibody. In addition, negative controls include aliquots of each virus dilution treated with either cell culture medium, normal sheep and/or mouse serum, or a pan-specific non-neutralising anti-aquabirnavirus monoclonal antibody (e.g., E5 from DiagXotics Inc., USA, #93-E5UC, if still commercially available).

c) Parallel neutralisation tests must be performed against a homologous virus (positive control), and a heterologous virus (negative control).
d) Incubate all the mixtures at 15°C for 1 hour.
e) Transfer aliquots of each of the above mixtures on to 24-hour cell monolayers (inoculate two cell cultures per dilution). Incubate at 15°C.
f) Check the cultures for the onset of CPE, and read the results as soon as CPE occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination, or after discarding the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

Interpretation

The tested virus is identified as an aquabirnavirus when CPE is prevented, or noticeably delayed, in the cell cultures that received the virus suspension treated with an anti-aquabirnavirus antibody, whereas CPE is evident in all other cell cultures.

Appendix 3
Immunoperoxidase test

Equipment

Humid chamber, e.g., a plastic sandwich box with an air-tight lid
Acetone (80% v/v)-fixed cell cultures in multi-well plates
Plate shaker
Refrigerator
Inverted light microscope fitted with 4X and 10X objectives

Reagents

Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)
80% (v/v) acetone in water
0.05% (v/v) Tween 20 in PBSA (PBST)
0.1% (w/v) skim milk powder solution in PBSA
Primary antibody: polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)
Normal sheep serum
Secondary antibody: biotinylated donkey anti-sheep Ig
Streptavidin-horseradish peroxidase conjugate
AEC (3 amino-9-ethyl carboxyzole)
DMF
0.05 M acetate buffer, pH 5.0
H_2O_2
Deionised water
Mayer's haematoxylin (Lillie's modification)
Scott's tap water

Procedure

a) The optimal dilutions (in 0.1% skim milk) of each reagent (antibodies, conjugates, substrates) need to be determined prior to use and checked on a regular basis.
b) Remove supernatant fluid from the wells of the cell culture, and wash the wells twice with 0.05% PBST.
c) Add 50-150 uL of primary antibody to each well (for a 96- or 24-well plate, respectively). Incubate at 37°C for 1 hour on a plate shaker set at low speed.
d) Remove the primary antibody, and wash the wells 3 times with PBST. Add the secondary antibody to all wells. Incubate at 37°C for 1 hour on a plate shaker set at low speed.
e) Remove the secondary antibody, and wash the wells 3 times with PBST. Add conjugate to each well. Incubate at 37°C for 1 hour on a plate shaker set at low speed.
f) Remove the conjugate from the wells, and wash the wells 3 times with PBST. Add freshly prepared substrate (AEC) solution (2 mg AEC, 200 uL

dimethlyformamide, 10 mL 0.05 M acetate buffer pH 5.0, 5 uL 30% [v/v] hydrogen peroxide) for 20 min at room temperature. Use of a plate shaker is optional.

g) Stop the reaction with water and counterstain with 50 uL per well of Mayer's haematoxylin. Incubate for 90 seconds at room temperature, rinse twice with water, and blue the stain by adding Scott's tap water for 90 seconds.
h) Rinse with water and allow to air dry.
i) Examine processed wells by inverted light microscopy.

Interpretation

Positive reaction: grainy, focal, brick-red staining of cells indicates the presence of virus identified by the diagnostic antibody.

Negative reaction: no red staining apparent. All cells should be stained pale blue due to the counterstain.

Background staining: non-grainy, non-focal, pale pinkish staining may occur and could be due to any of a number of factors.

Appendix 4
PCR-based tests

Reagents

Reagents stored at –20°C

Deionised formamide
Bovine serum albumin (BSA; 1mg/mL)
dNTPs (1.25mM)
100% Ethanol AR grade
70% Ethanol
Proteinase K (2mg/ml)
Primers (20 µM)
TaqMan Reverse Transcriptase Reagent Kit (AB Cat# N808-0234)
Random hexamers (MAB Cat# N808-0127)
PCR Mastermix (QIAGEN Cat# 201443)
100 bp DNA ladder & loading dye (Promega Cat# G2101)

Reagents stored at 4°C

Deionised formamide
Buffer AVL

Reagents stored at room temperature

QIAamp viral RNA Mini Kit
QIAGEN Buffer AW2
QIAGEN Buffer AW1
QIAGEN Buffer AVE
TNET
Agarose (BIORAD Cat # 162-0134)
Ethidium bromide (BIORAD Cat #161-0430)
40 x TBE Buffer (Promega Cat # 428A)

Equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (e.g., refrigerators, freezers, vortex mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and sequencer.

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay run and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be conducted as specified in the protocol. As a norm, formalin fixed controls would be conducted with formalin fixed test samples, and appropriate unfixed controls would be conducted with fresh tissue or tissue culture supernatant samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a check-testing regimen and as controls for the conduct of disease investigations.

Procedure

Sample preparation

Due to the sensitivity of PCR tests, care at every step of sample preparation must be taken to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated, i.e., not pre-exposed to aquatic pathogens. Wherever possible it is recommended that disposable reagents and plasticsware are used. At AAHL, samples would be handled and processed using sterile disposable single use containers, instruments and reagents to minimise the risks of contamination of the samples.
As a general principle, samples to be used in the PCR suite at AAHL for molecular diagnosis will be inactivated by an approved method prior to movement to the PCR suite.

Inactivation will be carried out by the following procedures by staff approved to work with the categories of agents

1. Cell-free samples, e.g., tissue culture supernatants at room temperature are added to an appropriate commercially prepared buffer (e.g., Qiagen AVL buffer) containing guanidinium isothiocyanate.

2. Tissue samples, blood and other specimens containing cells are homogenised at approximately 10% w/v in phosphate buffered saline or similar buffer, and then frozen and thawed. Samples are then microfuged (approximately 13000 x g for 20-30 sec), and the supernatant fluid collected. An appropriate volume of this is then mixed with an appropriate volume of a commercially prepared buffer (e.g., Qiagen AVL buffer).

Nucleic acids are extracted from submitted samples in the Biological Safety Cabinet Class II in the PCR suite. All samples handled in the PCR suite will be less than 5mL and considered to be of low or intermediate titre (or inactivated if required as above).

Nucleic acid extraction and cDNA preparation

Nucleic acid (including aquabirnavirus dsRNA) is obtained from cell-free samples using the QIAamp Viral RNA extraction kit (QIAGEN cat no. 52904) or from tissues using the RNeasy Viral RNA Extraction kit (QIAGEN cat no. 74904). cDNA is then prepared from the viral RNA using a standard protocol that has been adopted for all RNA agents.

The TaqMan Reverse Transcription kit (Applied Biosystems cat no. N808-0234) is used for production of cDNA. A single cDNA reaction mixture consists of the following reagents: 5.7 µL of water; 2 µL of 10x reaction buffer; 4.4 µL of 25 mM $MgCl_2$; 4 µL of 1.25 mM dNTPs; 1 µL of 50 mM random hexamers; 0.4 µL of RNAsin (20-40U); 0.5 µL of Multiscribe reverse transcriptase (50 U/µL); and 2 µL of extracted nucleic acid from the sample. The mixture is incubated at room temperature for 10 mins, followed by 48°C for 30 mins, and 95°C for 5 mins. For multiple samples, the volumes are multiplied appropriately.

Generic aquabirnavirus PCR

Following production of cDNA, a PCR is then conducted. The PCR mixture for a single sample consists of the following reagents: 9.5 µL of water; 12.5 µL of HotStar Taq Master mix; 0.5 µL of the forward primer (20 µM); 0.5 µL of the reverse primer (20 µM); and 2 µL of cDNA. For multiple samples, the volumes are multiplied appropriately. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is programmed for: one cycle at 94°C for 15 minutes; 35 cycles at 94°C for 45 sec, 45°C for 45 sec and 72°C for 2 minute; and, finally, one cycle at 72°C for 7 minutes. Amplified DNA (775 bp) is detected by agarose gel electrophoresis.

The two primers that are used in the PCR are:

Generic forward primer:
5'-acgaaccctcaggacaa-3'
Generic reverse primer[30]:
5'-cacaggatcatcttggcatagt-3'

Restriction enzyme digestion of a specific aquabirnavirus PCR product

If a PCR product of the correct size is amplified with the aquabirnavirus primers, the product is purifed from the agarose gel (e.g., Qiagen Gel Extraction kit, Cat No. 28704). To 10 uL of the eluate is added: 2 uL of 10X reaction buffer, 2 uL of BSA (1 mg/mL), 10 IU of the restriction enzyme Cla I and water upto a final volume of 20 uL. Digestions are performed at $37^{O}C$ for 1-2 hr, followed by incubation at $65^{O}C$ for 15 min to inactivate the enzyme. Digested and undigested samples are then examined on an agarose gel containing ethidium bromide, and examined under UV illumination.

Interpretation

At the completion of the PCR, specific PCR fragments of the correct size are identified by agarose gel electrophoresis:

- The negative control sample must have no evidence of specific amplified products.
- A positive control sample must yield a specific aquabirnavirus fragment (775 bp) (Figure 2).
- Amplified fragments of the correct size are then eluted from the gel, and a Cla I restriction enzyme digest is performed. If the PCR product has been amplified from TAB, two digestion products will be seen (652 and 123 bp). No digestion will occur with other aquabirnaviruses (Figure 2).
- The remainder of the eluate is used to determine the DNA sequence (by using the PCR primers as sequencing primers). Note that an apparently specific PCR product, and the reactivity of the restriction enzyme with the PCR product, only provide a tentative diagnosis. ***The PCR product must be sequenced to make a definitive diagnosis.***
- Sequence identity and genotype are determined by a Blast search of the Genbank database.

An assay is valid only when all controls yield the expected results.

Appendix 5
Immunohistochemistry

Reagents

10% formalin
Trypsin solution (1 mg per mL trypsin; 0.1 M Tris/HCl; 1 mg per mL $CaCl_2$; pH 8.0)
Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)
0.1% (w/v) skim milk powder solution in PBSA
Primary antibody: polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)
Normal sheep serum
Secondary antibody: biotinylated donkey anti-sheep Ig
3% (v/v) peroxide solution in methanol
Streptavidin-horseradish peroxidase conjugate
AEC (3 amino-9-ethyl carboxyzole) substrate (2 mg AEC, 200 uL dimethylformamide, 10 mL 0.05 M acetate buffer, pH 5.0, 5 uL 30% (v/v) H_2O_2)
Deionised water
Mayer's haematoxylin (Lillie's modification)
Scott's tap water

Equipment

Poly-L-lysine coated glass slides
Humid chamber, e.g., a plastic sandwich box with an air-tight lid
Light microscope fitted with 4X, 10X and 40X objectives

Procedure

a) The tissues for examination have to be fixed in aldehyde fixatives, e.g., 10% formaldehyde, for 48 hours or in absolute alcohol or 96% alcohol. Presumably frozen sections could also be used, but these have not been tried at AFDL.
b) After fixation, the sample is processed according to normal histological procedures, and then embedded in liquid paraffin.
c) Histological sections are mounted on silanised slides.
d) Sections are then deparaffinised, using standard procedures, followed by incubation with a trypsin solution for

20 min at 37°C. The reaction is stopped using cold (4°C) PBSA.

e) Sections are then incubated for 1 hour at 37°C with polyclonal sheep antiserum raised against IPN virus or with normal sheep serum diluted to its optimal concentration in 0.1% (w/v) skim milk in PBSA.

f) The sections are then washed in PBSA, and incubated for 1 hour at 37°C with biotinylated donkey anti-sheep Ig diluted to optimal concentration in 0.1% (w/v) skim milk in PBSA.

g) After a further wash in PBSA, endogenous peroxidase activity can be blocked by immersion of the sections in 3% (v/v) peroxide solution in methanol for 20 min at room temperature.

h) Following a wash in PBSA, the sections are incubated for 1 hour at 37°C with streptavidin-horseradish peroxidase diluted to optimal concentration in 0.1% (w/v) skim milk in PBSA.

i) After another wash in PBSA, the sections are incubated with freshly prepared AEC substrate solution for 20 min at room temperature.

j) The sections are rinsed in tap-water, counterstained with Mayer's haematoxylin and mounted in mounting medium (Quickmount, Daido Sangyo Co., Ltd, Japan) for microscopic examination.

Figure Legends

Figure 1. Pancreas from a TAB infected brook trout. There is extensive acute necrosis of acinar cells in the pancreas.

Figure 2. Generic aquabirnavirus PCR, and Cla I digestion of the PCR products from selected aquabirnaviruses. Viral nucleic acid was extracted, and cDNA prepared. The generic aquabirnavirus PCR was conducted, and specific products from each virus were purified, and digested with the restriction enzyme Cla I. (A) Lanes 1,2: IPN (Canada 3); 3,4: IPN (Canada 1); 5,6: IPN (Te); 7,8: IPN (Ab); 9,10: NZ isolate (NZ6); 11,12: NZ isolate (NZ10); 13,14: TAB02 isolate (2002 AS). (B) Lanes 1,2: IPN (Sp); 3,4: IPN (DPL); 5,6: TAB12 (2001 RT); 7,8: TAB11 (1998 AS); 9,10: TAB6 (1998 AS); 11,12: TAB 23 (1998 RT); 13,14: TAB16 (2001 RT). Even-numbered lanes: undigested PCR product; odd-numbered lanes: Cla I digestion of PCR product.

Figure 1

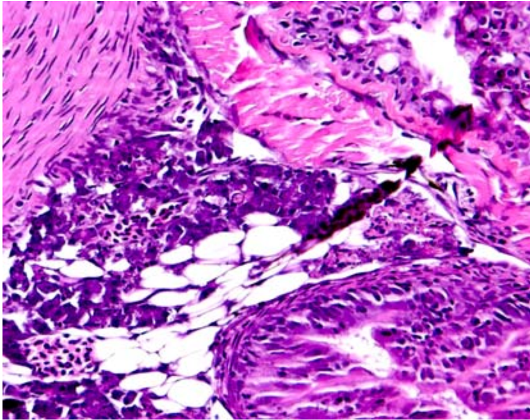
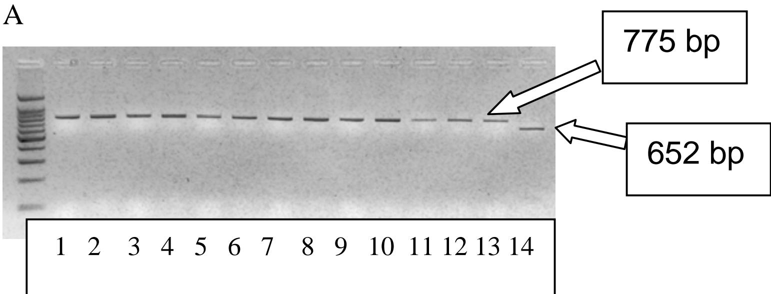
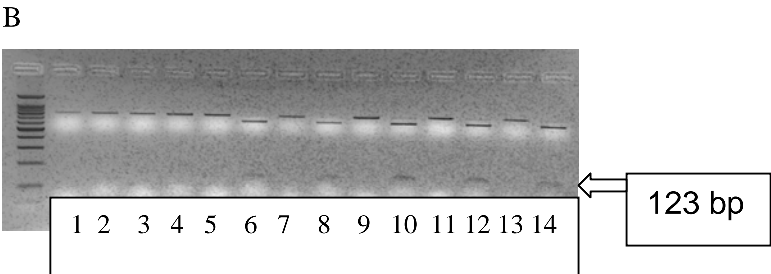


Figure 2

A



B



Appendix 4 – Figures and Tables

Figure 1. The full nucleotide sequence of Segments A and B of Tasmanian aquabirnavirus 98 (TAB98)

Segment A:
GGAAAGAGAGTTTCAACGTTAGTGGTAACCCACGAGCGGAGAGCTCTTACGGA
GGAGCTCTCCGTCGATGGCGAAAGCCCTTTCTAACAAACAACCACAAAATCTAT
ATCAATGCAAGATGAACACAAACAAGGCAACCGCAACTTACTTGAAATCCATTAT
GCTTCCCGAGACTGGACCAGCAAGCATCCCGGACGACATAACGGAGAGACACA
TCTTAAAACAAGAGACCTCGTCATACAACTTAGAGGTCTCAGAATCAGGAAGTG
GCATTCTTGTTTGTTTCCCTGGAGCGCCAGGATCAAGGGTCGGTGCTCACTACA
GATGGAATGTGAACCAGACGGAGCTGGAGTTCGACCGGTGGCTGGAGACGTC
GCAGGACCTGAAGAAGGCCTTCAACTATGGGAGGCTGATTTCACGGAAGTACG
ACATCCAAAGCTCCACACTGCCGGCTGGCCTCTATGCCCTGAACGGAACGCTC
AACGCTGCCACATTCGAAGGCAGTCTGTCTGAGGTCGAGAGCCTGACCTACAA
CAGCCTGATGTCTCTAACAACGAACCCTCAGGACAAGGTCAACAACCAGCTGGT
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CAGCCGACAACTACAGAGGAGTCTCAGCCAAGATGACCCAGTCCATCCCGACC
GAGAACATCACCAAGCCGATCACAAGAGTCAGGCTGTCATACAAGATCAACCAG
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CACCAGAGGAGTTCTACGACGCAGTTGCAGCTGTGTTTGCCGAGAATGGAGGA
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GAAACGACGACCCCGGAACGCCGAGACACCACGGAGAACTAGAGCACCAGCG
GAACCGGCACCGCCCAGGAGCTCGAGGTTCACCCCCCACGGAGATAACGCGG
AGGTGTAACGGCTACTCTCTTTCCTGACCGATCCCCTGGCCAAAATCCCCGGCC
CCCCAGGGGGCCCC

Segment B:
GGAAACAGGGGGTCAACGTTGGTGGCACCCGACATACCACGACTGTTTATGTAT
GCACGCAAGTGCCCCCTTTAAAATCTCTACAATATACAACTTATGATATGTCGGA
CATCTTCAACTCACCTCAGAACAAGGCTTCTATCTTGAATGCACTCATGAAGAGC
ACGCAGGGAGACGTGGAGGATGTTCTAATACCTAAGCGCTTCAGACCCGCAAA
GGATCCGTTAGACAGCCCACAAGCAGCAGCCGCGTTCCTAAAAGAACACAAGT
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CAAACCCGACCATGATGACGTAGGAACGTTTGGGGCTCCAGACATTACACTACT
CAAACAGCTAACCTTCTTCCTGCTGGAGAATGACTTTCCAACCGGACCAGAGAC
CCTACGGCAAGTTCGAGAAGCAATAGCAACCCTGCAATATGGGTCAGGCAGCT
ACTCCGGACAGCTGAACAGGCTACTGGCAATGAAGGCCGTAGCAACGGGCCG
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CCCTTCCATGGGGGAATGGACAGAATCATGACCATCATCAGAGAGCACCTGGAT
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GGGGCGACCTCGAAGGAATCTCACTGACAGAGCCACTGACCAACCAGACACTC
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AACTGCTGATGAAAGCGAACAACCTACTAAAGAGTAGCCTCACGGAAGCAAGG
GAAGCCCTCGAGACCGTGCAGTCAGACAAAATAATCTCAAGCAAAACCTCTCCA
GAGAAGAACCCCGGGACTGCCGCAAACCCCGTGGTTGGCTATGGAGAGTTCAG
CGAAAAGATTCCTCTCACTCCCACGCAAAAGAAGAACGCCAAGCGATGGGAGA
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Figure 2. A phylogenetic comparison of Australasian and other international aquabirnaviruses

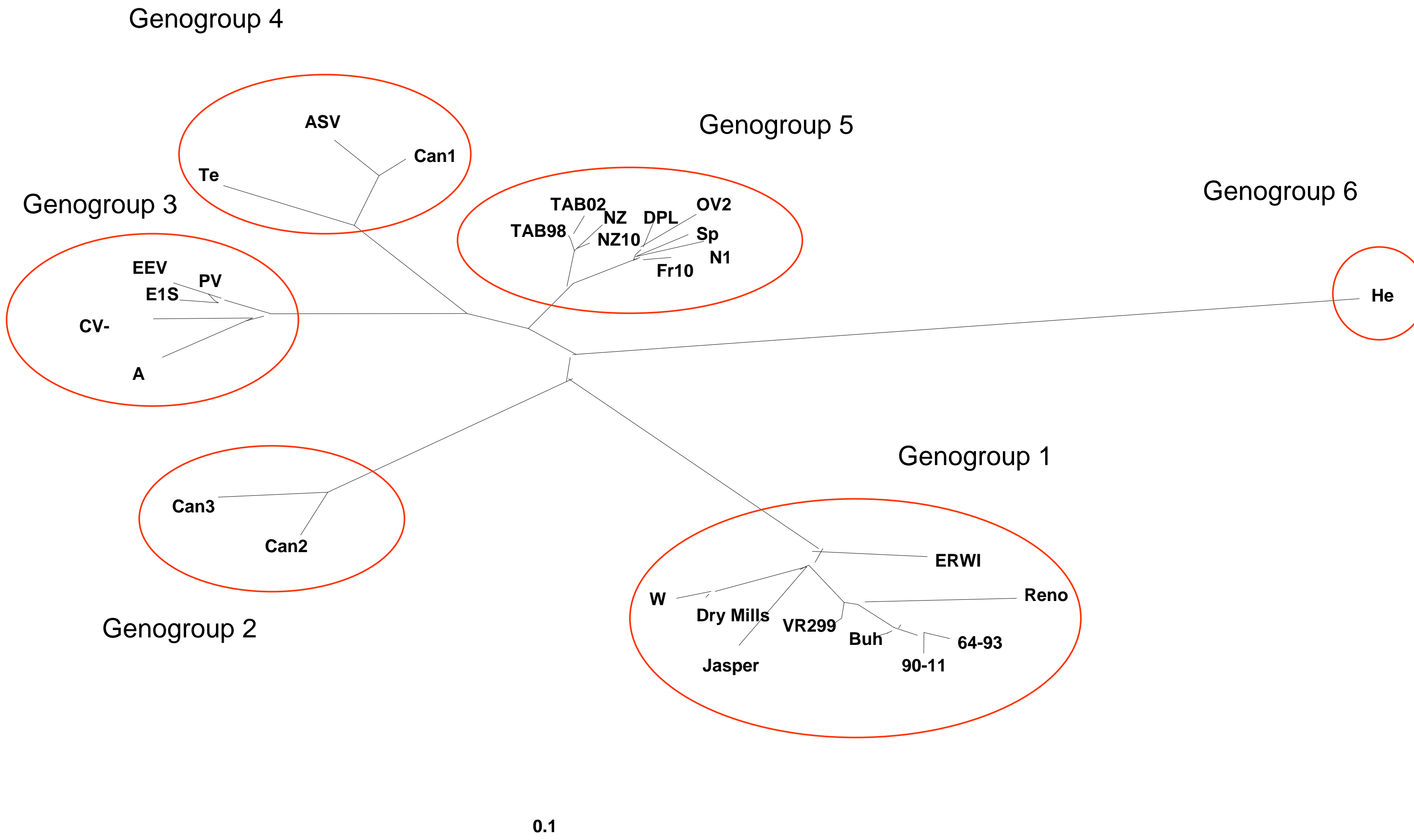

Figure 3. Multiple sequence alignment (Clustal W 1.7) over the variable region of VP2 in segment A of 15 Serogroup A aquabirnaviruses. The forward and reverse generic aquabirnavirus primers are boxed.

Forward primer 5'-ACGAACCCTCAGGACAA

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West Buxton      ACAAACCCCCAGGACAAGGTCAACAACCAACTAGTGACCAAAGGAATAACCGTCCTGAAC
VR299            ACAAACCCACAGGACAAGGTCAACAATCAACTAGTGACCAAAGGAATTACCGTCCTGAAT
Sp Seg A         ACGAACCCCCAGGACAAAGCCAACAACCAGCTGGTGACCAAAGGAGTCACCGTCCTGAAT
Ab               ACAAACCCCCAGGACAAGGTCAACAACCAGCTCGTGACCAAAGGAGTAACCGTCCTGAAC
He               ACAAACCCGCAGGACAAAGTCAACAACCAACTCATAACAAAAGGGGTGACAGTCCTAATT
Te               ACGAACCCCCAGGACAAAGTCAACAACCAGCTCGTGACCAAAGGCGTCACGGTCCTGAAT
Canada 1         ACGAACCCCCAGGACAAGGTCAACAACCAGCTCGTGACCAAAGGAGTCACGGTCCTGAAC
Canada 2         ACGAACCCACAGGACAAGGTCAACAACCAACTGGTGACAAAAGGGATCACCGTCCTGAAC
Canada 3         ACGAACCCACAGGACAAGGTCAACAACCAACTGGTGACCAAAGGGATCACCGTCCTGAAC
Jasper           ACAAACCCACAGGACAAGGTCAACAACCAACTGGTGACCAAAGGAATAACCGTCCTGAAC
DPL              ACGAACCCCCAGGACAAAGTCAACAACCAGCTGGTGACCAAAGGAGTCACCGTCCTGAAT
TAB1998 SegA     ACGAACCCTCAGGACAAGGTCAACAACCAGCTGGTGACCAAAGGAGTCACAGTCCTGAAC
TAB2002 Seg A    ACGAACCCTCAGGACAAGGTCAACAACCAGCTGGTGACCAAAGGAGTCACCGTCCTGAAC
NZ#6             ACGAACCCCCAGGACAAGGTCAACAACCAGCTCGTGACCAAAGGAGTCACCGTCCTGAAC
NZ#10 Seg A      ACGAACCCTCAGGACAAGGTCAACAACCAGCTGGTGACCAAAGGAGTCACCGTCCTGAAC

West Buxton      CTTCCAACTGGGTTTGACAAGCCATACGTCCGCCTTGAGGACGAGACACCGCAGGGTCCC
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VR299	CTACCAACTGGGTTTGACAAGCCATACGTCCGCCTAGAGGACGAGACGCCACAGGGCCCC
Sp Seg A	CTACCAACAGGGTTCGACAAACCATACGTCCGCCTAGAGGACGAGACACCCCAGGGTCTC
Ab	CTTCCAACTGGGTTCGACAAGCCATACGTCCGCCTTGAGGACGAGACACCGCAGGGTCCC
He	CTACCCACCGGGTTTGACAAACCATACGTCCGACTTGAGGACGAGACACCAAAAGGTCTC
Te	CTGCCAACAGGGTTCGACAAACCATACGTCCGACTTGAGGACGAGACACCCCAAGGGCTC
Canada 1	CTACCAACCGGGTTCGACAAACCGTACGTCCGACTTGAGGACGAGACGCCCCAAGGGCTC
Canada 2	CTGCCAACAGGGTTCGACAAGCCATACGTCCGGCTAGAGGACGAGACCCCCCAGGGTCTC
Canada 3	CTACCAACAGGGTTCGACAAACCATACGTCCGACTAGAGGACGAGACACCCCAGGGTCTC
Jasper	CTACCAACCGGGTTTGACAAGCCATACGTCCGCCTTGAGGACGAGACACCACAGGGTCCC
DPL	CTACCAACAGGGTTCGACAAGCCATACGTCCGCCTAGAGGACGAGACACCCCAGGGTCTC
TAB1998 SegA	CTACCAACCGGGTTCGACAAGCCATACGTCCGACTAGAGGACGAGACACCCCAGGGTCTC
TAB2002 Seg A	CTACCAACCGGGTTCGACAAGCCATACGTCCGACTAGAGGACGAGACACCCCAGGGTCTC
NZ#6	CTACCAACAGGGTTCGACAAGCCGTACGTCCGACTAGAGGACGAGACACCCCAGGGTCTC
NZ#10 Seg A	CTACCAACCGGGTTCGACAAACCATACGTCCGACTAGAGGACGAGACACCCCAGGGTCTC

West Buxton	CAGTCCATGAACGGAGCCAGGATGAGGTGCACCGCTGCAATCGCACCAAGGAGGTACGAA
VR299	CAGTCCATGAACGGAGCAAGGATGAGGTGCACAGCTGCCATCGCACCAAGGAAGTATGAA
Sp Seg A	CAGTCAATGAACGGGGCCAGGATGAGGTGCACAGCTGCAATTGCACCACGGAGGTACGAG
Ab	CAGTCCATGAACGGAGCCAGGATGAGGTGCACCGCTGCAATCGCACCAAGGAGGTACGAA
He	CAGTCGATGAATGGGTCAACGATGAGATGCACGGGTGTGATTTCACCACGAAGGTATGAA
Te	CAGTCAATGAACGGTGCGAAGATGAGGTGCACCGCTGCAATTGCGCCGCGAAGGTACGAG
Canada 1	CGGTCAATGAACGGTGCCAAGATGAGGTGCACCGCTGCAATCGCACCGCGGAGGTATGAG
Canada 2	CTGTCGATGAACGGGGCGAAGATGAGGTGCACAGCTGCAATTGCACCACGGAGGTATGAG

Canada 3	CTGTCAATGAACGGGGCGAAGATGAGGTGCACAGCTGCAATTGCACCACGGAGGTACGAG
Jasper	CAATCCATGAACGGAGCCAGGATGAGGTGCACCGCTGCCATTGCACCAAGGCGGTATGAA
DPL	CAGTCAATGAACGGGGCCAAGATGAGGTGCACAGCTGCAACTGCACCGCGGAGGTACGAG
TAB1998 SegA	CAATCGATGAACGGGGCGAAGATGAGGTGCACAGCTGCGATTGCACCGAGGAGGTACGAG
TAB2002 Seg A	CAATCGATGAACGGGGCGAAGATGAGGTGCACAGCTGCGATTGCACCGAGGAGGTACGAG
NZ#6	CAGTCAATGAACGGGGCAAAGATGAGGTGCACAGCTGCAATTGCACCGAGGAGGTACGAG
NZ#10 Seg A	CAGTCGATGAACGGGGCGAAGATGAGGTGCACAGCTGCGATTGCACCGAGGAGGTACGAG

West Buxton	ATAGACCTCCCATCTGAGCGTCTACCAACCGTGGGAGCAACTGGGACCCCAACAACAATC
VR299	ATCGACCTCCCATCCGAACGACTGCCGACCGTGGCCGCGACTGGGACCCCAACAACAATT
Sp Seg A	ATCGACCTCCCATCCCAAAGCCTACCCCCCGTTCCTGCGACAGGAACCCTCACCACTCTC
Ab	ATNGACCTCCCATCTGAGCGACTACCAACCGTGGCAGCAACTGGGACCCCAACAACAATC
He	ATCGACCTGCCCAACGAAGCCCTTCCTCCAGTTCCAGCGACAGGGACCTATGTTACACTA
Te	ATCGACCTTCCATCCCAACGACTACCCACCGTACCAGCCACTGGAACCCTCACCACGATC
Canada 1	ATCGACCTCCCATCCCAGAGACTACCCACCGCACCAGCCACCGGAACCCTCACCACAATC
Canada 2	ATCGACCTCCCATCAGAGCGACTCCCCCCCGCACAAGCGACAGGAACCCTCACCACCATG
Canada 3	ATCGACCTCCCATCAGAGCGACTCCCCCCCGCAACAGCGACAGGAACTCTCACCACCATG
Jasper	ATCGACCTCCCATCCGAACGGGTGCCGACCGTGATGGCAACCGGGACCCCAACAACAATC
DPL	ATCGACCTCCCATCCCAACGGCTACCCCCCGTTCCTGTGACAGGAACCCTCACCACTCTC
TAB1998 SegA	ATCGACCTCCCATCCCAGCGCCTGCCCCCCGTTCCAGCGACAGGGGCCCTCACCACCCTC
TAB2002 Seg A	ATCGACCTCCCATCCCAGCGCCTGCCCCCCGTTCCAGCGACAGGGGCCCTCACCACCCTC
NZ#6	ATCGACCTCCCATCTCAGCGCATGCCCCCCGTTCCAGCGACAGGAACCCTCACCACTCTC
NZ#10 Seg A	ATCGACCTCCCATCCCAGCGCCTGCCCCCCGTCCCAGCGACAGGAACCCTCACCACCCTC

West Buxton	TATGATGGGAACGGCGACATTGTGAACTCAACCACAGTGACAGGAGACGTAACCTTCCAA
VR299	TATGAGGGGAATGCTGACATCGTGAACTCCACAACAGTCACCGGGGACATAACATTCCAG
Sp Seg A	TACGAGGGAAACGCCGACATCGTCAACTCCACAACAGTGACGGGAGACATAAACTTCAGT
Ab	TATGANGGGAANGGCGACATTGTGAACTCAACCACAGTGACAGGAGACATTAGCTTCAGC
He	TACGAAGGCAATGCAGACATAGTTAACTCCACCACAGGTTCGGGTGACATCAACTTCAAT
Te	TACGAGGGGAACGCCGACATTGTCAATTCGACAACTGTGACCGGAGACATCAACTTCCGT
Canada 1	TATGAGGGGAACGCCGACATTGTCAATTCGACAACAGTCACCGGAGACATCAATTTCAGT
Canada 2	TACGAGGGCAATGCAGACATCGTCAACTCAACAACAGTGACGGGAGACATCAGCTTCAGT
Canada 3	TACGAGGGCAACGCAGGCATCGTCAACTCAACAACAGTGACGGGAGACATCAGTTTCAGT
Jasper	TATGAGGGGAACGCTGACATAGTGAACTCAACCACAGTGACCGGGGACATAACCTTCCAG
DPL	TACGAGGGAAATGCCGACATCGTCAACTCTACAACAGTGACGGGAGACATAAACTTCAGT
TAB1998 SegA	TACGAGGGGAACGCAGACATCGTCAACTCAACAACAGTGACGGGAGACATCAACTTCAGT
TAB2002 Seg A	TACGAGGGGAACGCAGACATCGTCAACTCAACAACAGTGACGGGAGATATCAACTTCAGT
NZ#6	TATGAGGGGAATGCAGACATCGTCAACTCAACAACAGTGACGGGAGACATCAACTTCAGT
NZ#10 Seg A	TACGAGGGGAACGCAGACATCGTCAACTCAACAACAGTGACGGGAGACATCAACTTCAGT

West Buxton	CTAGCAGCCGAACCCGTCAAT-GAGACGCGGTTCGACTTCATCCTACAATTCCTTGGGCT
VR299	CTCGAGGCCGAACCCGTCAAT-GAGACACGGTTCGACTTCATTCTACAGTTCCTGGGGCT
Sp Seg A	CTGGCAGAACAACCCGCAAAC-GAGACCAGGTTCGACTTCCAGCTGGACTTCATGGGCCT
Ab	CTCGCAAACAACCCCACCGCA-GACATCAAGTTCGACTTCCAGCTGGACTTCCTCGGTCT
He	CT-GCAGCAGGCCCCAGCAAACGAAACCAAGTTTGACTTCAAGCTTGAGTTCCTCGGGCT
Te	CTACCGGCAGCCCCCCCCGCA-GACACAAAGTATGACTTCCAGCTGGACTTCGTCGGGCT

Canada 1 CTACCAAGAGCTCCCACCACA-GACACCAGGTATGACTTCCAGCTGGACTTCATCGGGCT
Canada 2 CTTGCAACCGCCCCCGCAGCA-GAAACCACGTTTGAATTCCAGCTGGACTTCCTTGGACT
Canada 3 CTTGCAAACGCCCCCGCAGCA-GACACCACGTTCAAATTCCAGCTGGACTTCCTTGGACT
Jasper CTAGAAGCCAAACCCGCCAAC-GAGACGAGGTTCGACTTCATCCTGCAGTTCCTGGGGCT
DPL CTGGCAGAACATCCCGCAAAC-GAGACCAAGTTCGACTTCCAGCTGGACTTCATGGGCCT
TAB1998 SegA CTGGCGGACAACCCCCCAACG-GAGACCAAGTTCGACTTCCAACTGGACTTCATGGGACT
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NZ#6 CTAGCAGACAACCCCCCCACG-GAGACCAAGTTCGACTTCCAGCTGGACTTCATGGGACT
NZ#10 Seg A CTGGCAGACAACCCCCCCACG-GAGACCAAGTTCGACTTCCAACTGGACTTCATGGGACT

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VR299 GGACAACGACGTCCCCGTGGTTACCGTGACAAGCTCCACGCTAGTCACAGCGGACAACTA
Sp Seg A TGACAATGACGTCCCAGTGGTCACAGTGGTCAGCTCCGTGCTGGCCACAAACGACAACTA
Ab CGACAACGACGTCCCGGTTGTCACGGTGACCAGCTCCGTGCTGGTAAACGCAGACAACTA
He TGACAATGATGAACCAGTTGTCACAGTCGTGAGCTCAGTGCTCGCAACTGAGGCAAACTA
Te CGACAATGACATTCCCGTTGTCTCAATAACCAGCTCTGTGCTGGCGACTGCAGCCAACTT
Canada 1 AGACAACGACGTGCCCGTTGTCTCGGTCACCAGCTCTGTGCTGGCAACCGCAGACAACTT
Canada 2 AGACAACAACGTACCCGTCGTCACCGTCATCAGCACAGCGCTGGCCACGAAAGAAAACCA
Canada 3 AGACAACAACGTACCCGTCGTCACCGTCATCAGCACAGCGCTGGCCACGACAGAGAACCA
Jasper GGACAACGACATCCCCGTGGTCTCCGTGACAAGCTCAGCGCTGGTCACAGCCGACAACCA
DPL TGACAATGACGTCCCAGTGGTCACAGTGGTCAGCTCCGTGCTGGCCACAAGCGACAACTA
TAB1998 SegA TGACAACGACGTCCCTGTGGTAACGGTGGTCAGCTCAGTGCTGGCAACAGCCGACAACTA
TAB2002 Seg A TGACAACGACGTCCCTGTGGTAACGGTGGTCAGCTCAGTGCTGGCAACAGCCGACAACTA

NZ#6	TGACAATGACGTCCCTGTGGTAACGGTGGTCAGCTCAGTGCTGGCAACAGCCGACAACTA
NZ#10 Seg A	TGACAATGACGTCCCTGTGGTAACGGTGGTCAGCTCAGTGCTGGCAACAGCCGACAACTA

West Buxton	CAGGGGTGCCTCCGCCAAGTTTACGCAGTCAATCCCAACGGAACTAATAACTAAGCCCAT
VR299	CAGGGGGGCGTCAGCCAAGTTCACCCAGTCAATCCCAACAGAAATGATTACCAAACCAAT
Sp Seg A	CAGAGGAGTCTCAGCCAAGATGACCCAGTCCATCCCGACCGAGAACATTACCAAGCCGAT
Ab	CAGAGGCGCGTCAGCCAAGATGACGATGTCCATACCCACCGAGAACATCACGAAGCCGAT
He	CCGAGGGATCTCAGCGAAAATGACACAGTCGATACCAACTGAAAACATCACAAAACCGGT
Te	CAGCGGAGTGTCAGCCAAGTTCACCCAGTCCATCCCAACAGAAGACATCACCAAGCCCAT
Canada 1	CCAAGGCGTGTCCGCCAAGTTCACGCAGTCCATACCAACCGAAGACATAACCAAACCAAT
Canada 2	CCTAGGAGTCTCGGCCAAGATGACCCAAGCAATACCAACGGAGAGCATAACAAAGCCAAT
Canada 3	CCTAGGAGTCTCGGCCAAGATGACCCAAGCAATACCAACGGAGAGCATAACAAAGCCAAT
Jasper	CAGAGGCGCCTCGGCCAAGTTCACGCAGTCAATCCCAACAGAGATGATCACCAAACCAAT
DPL	CAGCGGAGTCTCAGCCAAGATGACCCAGTCCATCCCGACCGAGAACATCACCAAGCCGAT
TAB1998 SegA	CAGAGGAGTCTCAGCCAAGATGACCCAGTCCATCCCGACCGAGAACATCACCAAGCCGAT
TAB2002 Seg A	CAGAGGAGTCTCAGCCAAGATGACCCAGTCCATCCCGACCGAGAACATTACCAAGCCGAT
NZ#6	CAGAGGAGTCTCAGCCAAGATGACCCAGTCCATACCGACCGAGAACATCACCAAGCCGAT
NZ#10 Seg A	CAGAGGAGTCTCAGCCAAGATGACCCAGTCCATTCCGACCGAGAACATCACCAAGCCGAT

West Buxton	TACAAGGGTCAAGCTGGCTTACCAGCTCAACCAGCAGACCGCAATCGGAAACGCCGCAAC
VR299	CACACGGGTCAAGCTGGCCTACCAGCTCAACCAGCAGACCGCAATTGCAAACGCAGCAAC
Sp Seg A	CACCAGGGTCAAGCTGTCATACAAGATCAACCAGCAGACAGCAATCGGCAATGTCGCCAC
Ab	CACAAGAGTCAAGCTGTCCTACAAAGTCAACCAGCAGACAGCGATAGCCAACCCAGCCAC

He	GACGAGGGTCAAGCTATCCTACAAAATCAATCAGCAAACTGAGATCGGAAACACCGCCAC
Te	CACCAGGGTCAGACTTTCCTACAAAGTCAACCAGCAGGCTGCAATCACCACCGCAGCCAC
Canada 1	CACCAGGGTCAGGCTAACCTACAAAGTCAACCAACAGGAAGCCATCACCACCGCCGCCAC
Canada 2	CACCAGAGTCAGGCTGTCCTACAAACTCAACCAACAGACAGAAATCGGAAACGCAGCTAC
Canada 3	CACCAGAGTCAGGCTGTCCTACAAACTCAACCAACAGACAGAAATCGCAAACGCAGCTAC
Jasper	CACAAGGGTCAAGCTGGCCTACCAACTCAACCAGCAGACCACAATTGGAAATGCGGCAAC
DPL	CACCAGGGTCAAGCTGTCATACAAGATCAACCAGCAGACAGCAATCGGCAATGTCGCCAC
TAB1998 SegA	CACAAGAGTCAGGCTGTCATACAAGATCAACCAGCAGACAGCCATCGGCAACGTTGCCAC
TAB2002 Seg A	CACAAGAGTCAGGCTGTCATACAAGATCAACCAGCAGACAGCCATCGGCAACGTTGCCAC
NZ#6	CACAAGAGTCAAGCTGTCATACAAGATCAACCAGCAGACAGCCATCGGCAACGTCGCCAC
NZ#10 Seg A	CACAAGAGTCAAGCTGTCATACAAGATCAACCAGCAGACAGCCATCGGCAACGTCGCCAC

West Buxton	ACTCGGGGCCAAAGGACCCGCGTCAGTCTCATTCTCATCAGGGAATGGCAATGTGCCGGG
VR299	GCTCGGAGCCAAGGGGCCGGCATCAGTCTCATTCTCATCCGGGAACGGCAATGTGCCGGG
Sp Seg A	CCTGGGCACAATGGGTCCAGCATCCGTCTCCTTTTCATCGGGGAACGGAAATGTCCCCGG
Ab	CCTGGGGACACTAGGTCCAGCGTCCGTCTCCTTTTCTTCAGGAAACGGCAATGTCCCCGG
He	CCTTGGGGTGCTTGGACCAGCCTCAGTGGCCTTCTCGTCAGGCAACGGCAACGTTCCTGG
Te	CCTAGGAGCACTAGGCCCCGCATCCGTTTCATTCTCCTCTGGAAATGGAAATGTGCCGGG
Canada 1	TCTGGGGGCCCTAGGACCGGCATCCGTCTCCTTCTCCTCTGGAAACGGGAACGTGCCTGG
Canada 2	ACTGGGGGCCCTTGGGCCCGCAACTGTCTCCTTCTCATCAGGGAATGGAAACGTACCTGG
Canada 3	ACTGGGGGCCCTTGGGCCCGCAACTGTCTCCTTCTCATCAGGGAATGGAAACGTACCTGG
Jasper	ACTCGGAGCCATGGACCGGCATCAGTCTCATTCTCATCAGGAAACGGCAACGTGCCTGG
DPL	CCTGGGCACAATGGGTCCAGCTTCCGTCTCCTTCTCATCGGGGAACGGAAATGTCCCAGG

TAB1998 SegA	CCTGGGAACCCTGGGGCCCGCATCCGTGTCCTTTTCATCAGGAAACGGGAACGTCCCAGG
TAB2002 Seg A	CCTGGGAACCCTGGGGCCCGCATCCGTGTCCTTTTCATCAGGAAACGGGAACGTCCCAGG
NZ#6	CCTAGGCGCACTGGGACCTGCATCCGTATCCTTCTCATCAGGAAACGGGAATGTCCCAGG
NZ#10 Seg A	CCTGGGCACCCTGGGACCCGCATCCGTGTCCTTTTCATCAGGAAACGGGAACGTCCCAGG

West Buxton	GGTTCTAAGACCCATAACCTTGGTGGCATACGAGAAGATGACCCCCCAGTCAATTCTGAC
VR299	GGTCCTAAGACCCATAACCCTAGTGGCGTACGAGAAGATGACCCCCCAGTCAATCCTGAC
Sp Seg A	CGTGCTCAGACCAATCACACTGGTGGCATATGAGAAGATGACACCGCTGTCCATCCTGAC
Ab	TGTCCTGAGACCCATCACACTGGTGGCCTATGAGAAAATGACACCCCAGTCCATCCTAAC
He	CGTGCTCAGACCCATAACCCTCGTCGCGTATGAAAAGATGACGCCACAGTCAGTTCTGAC
Te	AGTACTCCGACCCATCACCCTGGTGGCCTATGAGAAGATGACACCACAGTCCATCCTGAC
Canada 1	GGTACTCAGACCAGTAACCCTGGTGGCCTACGAGAAAATGACACCACAGTCAATCCTGAC
Canada 2	AGTGCTGCGGCCAATCACACTGGTGGCCTATGAGAAAATGACACCCCAGTCAATCCTAAC
Canada 3	AGTGCTGCGGCCAATCACACTGGTGGCCTATGAGAAAATGACACCCCAGTCAATCCTAAC
Jasper	GGTCCTAAGACCCATAACCCTAGTGGCATATGAGAAGATGACCCCTCAGTCAATTCTGAC
DPL	CGTGCTCAGACCAATCACACTGGTGGCCTATGAGAAGATGACACCGCTGTCCATCCTGAC
TAB1998 SegA	GGTGCTCAGGCCAATCACCCTGGTGGCCTATGAGAAGATGACACCACTGTCAATCCTGAC
TAB2002 Seg A	GGTGCTCAGGCCAATCACCCTGGTGGCCTATGAGAAGATGACACCACTGTCAATCCTGAC
NZ#6	AGTGCTCAGACCAATCACACTGGTGGCCTATGAGAAGATGACACCACTGTCGATCCTGAC
NZ#10 Seg A	AGTGCTCAGACCAATTACACTGGTGGCCTATGAGAAGATGACACCACTGTCAATCCTGAC

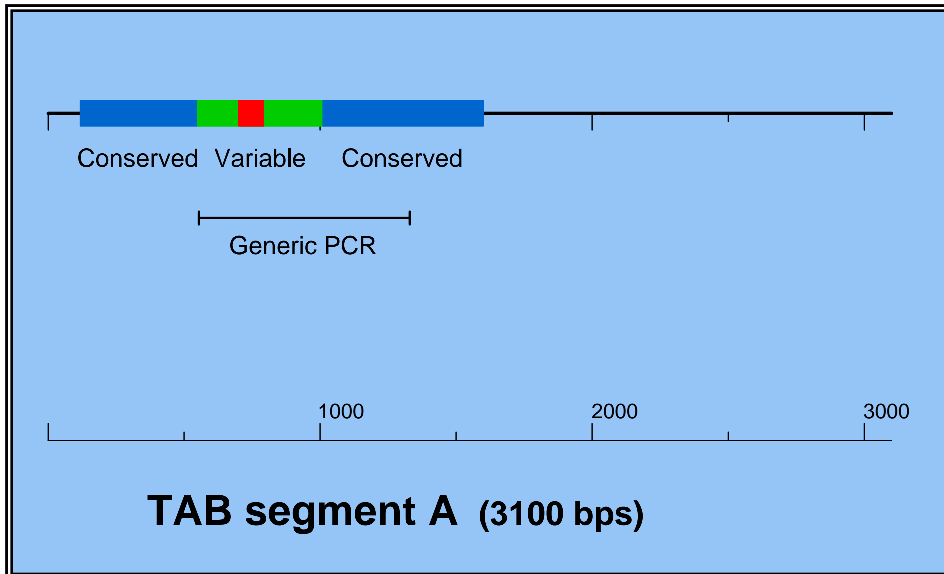
West Buxton	CGTGGCCGGCGTATCCAACTATGAGCTGATCCCCAACCCAGACCTCCTGAAGAACATGGT
VR299	CGTGGCTGGCGTATCCAACTATGAGCTGATCCCAAACCCAGACCTACTGAAGAACATGGT

Sp Seg A	CGTAGCTGGAGTGTCCAACTACGAGCTGATCCCAAACCCAGAACTCCTCAAGAACATGGT
Ab	TGTAGCTGGAGTGTCCAACTACGAGCTGATCCCCAACCCAGAACTCTTGAAGAACATGGT
He	AGTCGCCGGAGTGTCAAACTATGAACTAATCCCAAACCCTGACCTCCTGAGGAACATGGT
Te	CGTAGCAGGAGTGTCCAACTACGAGCTGATCCCCAACCCAGAACTCCTGAAGAACATGGT
Canada 1	CGTAGCAGGAGTGTCCAACTACGAGCTGATCCCAAATCCAGAACTCCTGAAGAACATGGT
Canada 2	CGTGGCAGGGGTTTCCAACTACGAACTGATCCCCAACCCAGACCTCCTGAAGAACATGGT
Canada 3	CGTAGCAGGGGTTTCCAACTACGAACTGATCCCCAACCCAGACCTCCTGAAGAACATGGT
Jasper	CGTGGCCGGCGTATCCAACTACGAGCTGATTCCAAACCCAGACCTCCTGAAGAACATGGT
DPL	CGTAGCTGGAGTGTCCAACTACGAGCTGATCCCGAACCCAGAACTCCTCAAGAACATGGT
TAB1998 SegA	CGTAGCAGGAGTGTCCAACTACGAGCTGATCCCAAATCCAGAGCTCCTGAAGAACATGGT
TAB2002 Seg A	CGTAGCAGGAGTGTCCAACTACGAGCTGATCCCAAATCCAGAGCTCCTGAAGAACATGGT
NZ#6	CGTAGCAGGAGTGTCCAACTACGAGCTGATCCCGAACCCAGAACTCCTGAAGAACATGGT
NZ#10 Seg A	CGTAGCAGGAGTGTCCAACTACGAGCTGATCCCAAATCCAGAGCTCCTGAAGAACATGGT

West Buxton	CACCAAGTATGGCAAATATGACCCTGAGGGCCTCAACTATGCCAAGATGATCCTGTC
VR299	CACCAAGTATGGAAAGTATGACCCTGAGGGCCTCAACTATGCCAAGATGATCCTGTC
Sp Seg A	GACACGCTATGGCAAGTACGACCCCGAAGGTCTCAACTATGCCAAGATGATCCTGTC
Ab	GACACGCTATGGCAAGTATGACCCCGAAGGGCTCAACTATGCCAAGATGATCCTGTC
He	AACCCACTACGGGAAGTATGACCCCGAAGGTCTAAACTATGCCAAGATGATTCTTTC
Te	GACACGCTATGGCAAGTATGACCCCGAAGGTCTGAACTATGCCAAGATGATCCTGTC
Canada 1	GACACGCTATGGCAAGTACGACCCCGAGGGCCTCAACTATGCCAAGATGATCCTGTC
Canada 2	CACAAAGTACGGCAAATATGACCCGGAAGGTCTGAACTATGCCAAGATGATCCTATC
Canada 3	CACAAAGTATGGCAAATACGACCCGGAGGGTCTGAACTACGCCAAGATGATCCTATC
Jasper	CACCAAATATGGAAAGTATGACCCTGAGGGCCTAAACTACGCCAAGATGATCCTGTC
DPL	GACACGCTATGGCAAGTACGACCCCGAAGGTCTCAACTATGCCAAGATGATCCTGTC
TAB1998 SegA	GACACGCTATGGCAAGTACGACCCAGAGGGCCTGAACTACGCCAAGATGATCCTGTC
TAB2002 Seg A	GACACGCTATGGCAAGTACGACCCAGAGGGCCTGAACTACGCCAAGATGATCCTGTC
NZ#6	GACACGCTATGGCAAGTATGACCCAGAGGGCCTGAACTATGCCAAGATGATCCTGTC
NZ#10 Seg A	GACACGCTATGGCAAGTACGACCCAGAGGGCCTGAACTATGCCAAGATGATCCTGTC

TGATACGGTTCTACTAGGACAC-5'

Reverse primer



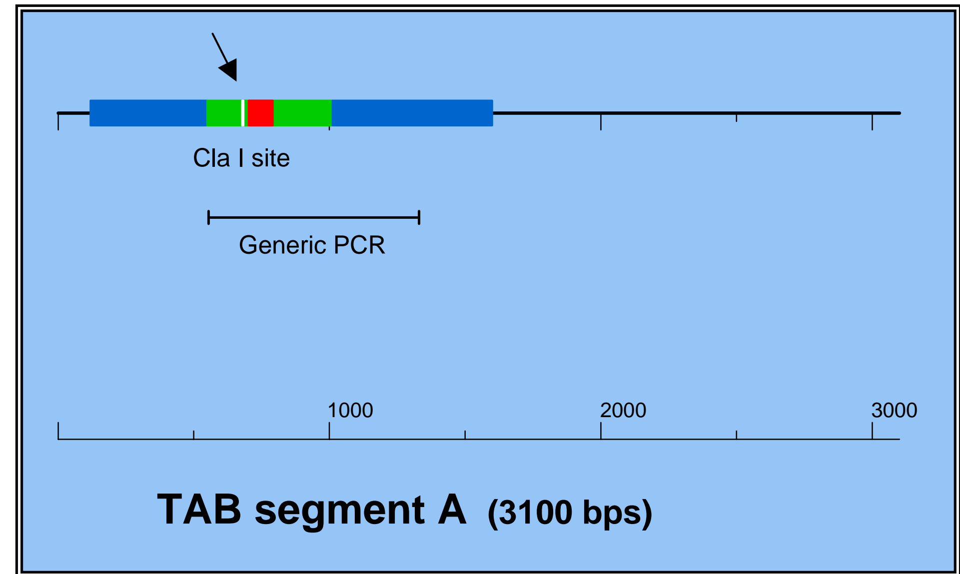


Figure 4. Diagrammatic representation of an aquabirnavirus segment A showing conserved and variable regions of the VP2 gene (left). The location of the generic (pan-specific) aquabirnavirus PCR is shown, as is the location of the Cla1 restriction enzyme site (right).

Figure 5. Cla 1 digestion of purified generic aquabirnavirus PCR products.

Lanes 1, 3: Undigested PCR products from IPN isolate; lanes 2, 4: Cla 1 digested IPNv PCR products; lanes 5, 7, 9, 11, 13: undigested PCR products from various TAB isolates; lanes 6, 8, 10, 12, 14: Cla 1 digested PCR products from TAB isolates. Undigested products: 775 bp; Cla 1 digested products: 652, 123 bp

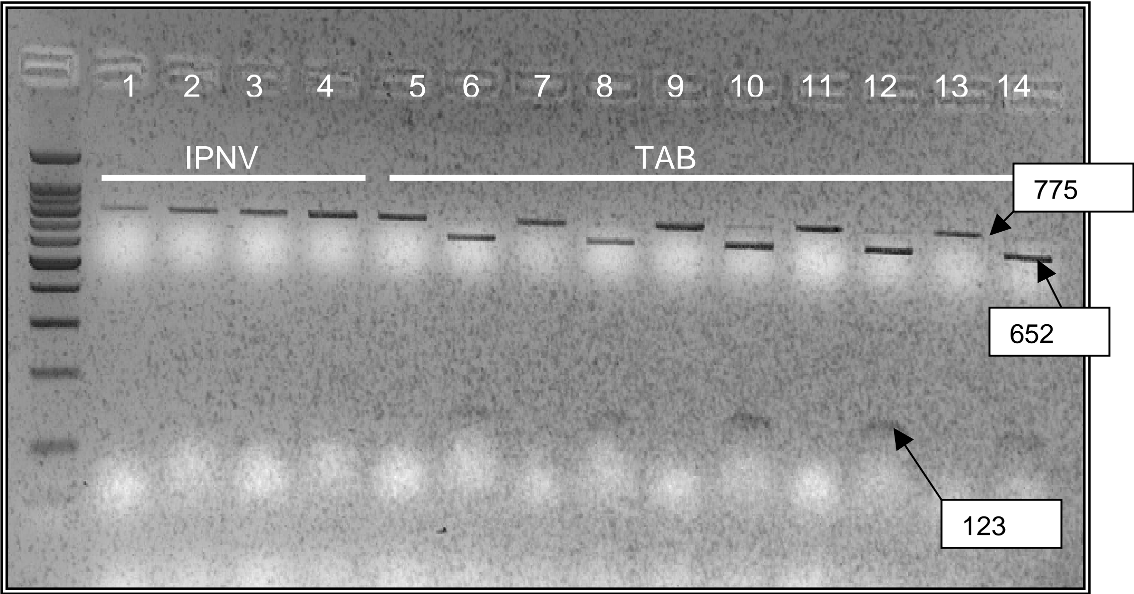


Table 1. Percentage similarities of Australasian isolates at the nucleotide and amino acid level.

Nucleotide		Amino acid			
		TAB 98	TAB 02	NZ 6	NZ 10
	TAB 98		99.0	98.0	99.0
	TAB 02	99.0		98.0	98.0
	NZ 6	94.0	94.0		98.0
	NZ 10	97.0	97.0	96.0	

Table 2. Structure of the pathogenicity trial conducted on Atlantic salmon and brook trout with Tasmanian aquabirnavirus.

Spp	No. of fish inoculated		
	TAB	IPN (Erwin)	Neg ctl
Brook trout	71	49 (56)	74
Atlantic salmon	76	106 (106)	60

Table 3. Summary of mortality in TAB/IPN pathogenicity trial.

Fish	Virus	SAN	Route	Total in gp	No. with disease	% with disease
BT	None	3272	IP	74	0	0
AS	None	3269	IP	60	0	0
BT	TAB	3293	IP	71	9	13
AS	TAB	3297	IP	76	1	1
BT	Erwin	3295	IP	49	13	27
BT	Erwin	3294	Bath	56	0	0
AS	Erwin	3298	IP	106	1	1
AS	Erwin	3296	Bath	106	0	0