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Development and validation of a TaqMan® PCR assay for the Australian abalone herpes-like virus

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ABSTRACT: The recent emergence of a herpes-like virus in both farmed and wild populations of abalone in Victoria, Australia, has been associated with high mortality rates in animals of all ages. Based on viral genome sequence information, a virus-specific real-time TaqMan assay was developed for detection and identification of the abalone herpes-like virus (AbHV). The assay was shown to be specific as it did not detect other viruses from either the *Herpesvirales* or the *Iridovirales* orders which have genome sequence similarities. However, the TaqMan assay was able to detect DNA from the Taiwanese abalone herpes-like virus, suggesting a relationship between the Taiwanese and Australian viruses. In addition, the assay detected <300 copies of recombinant plasmid DNA per reaction. Performance characteristics for the AbHV TaqMan assay were established using 1673 samples from different abalone populations in Victoria and Tasmania. The highest diagnostic sensitivity and specificity were 96.7 (95% CI: 82.7 to 99.4) and 99.7 (95% CI: 99.3 to 99.9), respectively, at a threshold cycle (C_T) value of 35.8. The results from 2 separate laboratories indicated good repeatability and reproducibility. This molecular assay has already proven useful in confirming presumptive diagnosis (based on the presence of ganglioneuritis) of diseased abalone in Victorian waters as well as being a tool for surveillance of wild abalone stocks in other parts of Australia.

KEY WORDS: Herpesvirus · Abalone · Real-time TaqMan assay · Diagnosis

INTRODUCTION

Herpes-like viruses have been identified in a number of different molluscan species in the USA, Europe and Australia/New Zealand. The presence of these viruses has often been associated with disease outbreaks involving substantial mortalities (Farley et al. 1972, Hine et al. 1992, Comps & Cochennec 1993, Renault et al. 1994a,b, Hine 1997, Hine & Thorne 1997). More recently, *Haliotis diversicolor* has been severely affected by disease outbreaks associated with a herpes-like virus in China and Taiwan (Wang et al. 2004, Chang et al. 2005). Histological and electron microscopic analyses have been the main tools for the detection and identification of these pathogens. The lack of suitable molluscan cell lines for the isolation and replication of these viruses *in vitro* has made further study of these viruses difficult. Therefore, the use of molecular biological techniques, such as polymerase chain reaction (PCR), gene sequencing and *in situ*

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hybridisation, has become an essential strategy for the detection and identification of herpesviruses in molluscan species (Renault et al. 2000, 2004, Arzul et al. 2001, Barbosa-Solomieu et al. 2005, Webb et al. 2007, Pepin et al. 2008, Renault 2008).

Commencing in December 2005 and continuing into 2006, an outbreak of disease occurred in hybrid abalone (*Haliotis laevigata* × *H. rubra*) farmed along western coastal Victoria, Australia. The disease was associated with the presence of neurotropic lesions (Hooper et al. 2007) and caused sudden and high levels of mortality. Electron microscopic analysis (Tan et al. 2008, A. D. Hyatt et al. unpubl.) indicated that the aetiological agent was a herpes-like virus. Since then the disease, abalone viral ganglioneuritis (AVG), has spread easterly and westerly along the Victorian coast, devastating wild abalone populations (Fig. 1). More recently in spring 2008, the same or similar virus was discovered in a fish processing plant in Tasmania.

The absence of rapid and sensitive molecular diagnostic techniques has impeded investigation of the disease and confirmation of the identity of the aetiological agent. To resolve this situation, initial studies were successful in concentrating the virus from diseased tissues (Tan et al. 2008), which facilitated sequencing of its genome (K. Savin et al. unpubl.) and development of PCR assays for virus detection and identification. The present paper describes the development, optimisation and validation of a real-time TaqMan assay for abalone herpes-like virus (AbHV) that enables not only early detection of infected abalone, but also surveillance of populations to allow better management of fisheries from all abalone-producing states in Australia.

MATERIALS AND METHODS

Origin and species of abalone. AbHV-infected wild blacklip abalone *Haliotis rubra* were obtained from Victorian coastal waters. Healthy farmed hybrid abalone (*H. laevigata* × *H. rubra*) (Great Southern Waters), as well as wild blacklip abalone from coastal waters of Tasmania and New South Wales, were used to obtain uninfected control tissues for the development and validation of the TaqMan assay. Presence and absence of infection was assessed by examination for overt clinical signs and for histopathological lesions in neural tissues. All abalone samples observed by histology during the course of the present study were also tested by TaqMan assay.

Samples from positive reference population with high prevalence of AVG. Thirty abalone samples were obtained from the State of Victoria, where outbreaks of AVG with high mortalities had been reported. For example, between January 2007 and June 2008, targeted sampling of moribund and dying abalone was performed from coastal waters of Victoria. Of these 30 samples, 22 were classified as positive and 8 as negative by histological examination. Another group of 18 samples were sourced from a Tasmanian fish processing plant experiencing a disease outbreak in abalone. Of these, 7 were positive by histological examination. Thus, the positive reference population was defined as abalone demonstrating both clinical signs and histopathology typical of AVG.

Samples from negative reference population with low prevalence of AVG. A total of 1625 abalone samples were obtained from open waters around the entire coast of Tasmania. These samples were considered to be from a reference population with an extremely low

Fig. 1. Abalone viral ganglioneuritis (AVG)-affected part of the Victorian coastline (dark shading). Initial outbreaks occurred in farms in the vicinity of Port Fairy. Subsequently, the disease was found in the wild population and has spread both easterly and westerly. The indicated range is current as of December 2009

prevalence, i.e. <1%, of disease. All samples originated, from abalone with no clinical signs of disease and were classified as negative by histological examination, i.e. absence of any ganglioneuritis. In addition, 11 samples were from a Tasmanian processing plant and 8 samples were from Victorian waters. All of these were negative for AbHV by histological examination and were included in the negative reference group.

Euthanasia and tissue dissection. Abalone were anaesthetised by placing them on ice covered with a paper towel for approximately 5 min to slow their metabolism. Using a scalpel, the body of the abalone was then cut longitudinally through the foot between the mantle and the distal surface of the foot. The pleuropedal ganglion and/or pedal nerve cords surrounded by some muscle tissue were dissected out and placed in 1.5 ml tubes for DNA extraction.

Histopathology. For histological examination, formalin-fixed tissues were processed through to paraffin blocks using standard procedures. Sections (5 µm thick) were cut from each block, processed and stained with haematoxylin and eosin prior to examination by light microscopy for histopathology, specifically the presence of ganglioneuritis (Hooper et al. 2007).

DNA extraction from abalone tissue. Nucleic acid from AbHV-infected and uninfected abalone tissues (approximately 20 mg of tissue including the target neural tissue surrounded by some muscle) was extracted using a QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. Nucleic acid, bound to minicolumns, was eluted and resuspended in a final volume of 100 µl of AE buffer (100 ± 20 ng μl^{-1}). DNA samples were not diluted to a specific concentration as we did not want to reduce the amount of viral DNA potentially present in samples. Samples were tested immediately after extraction or stored at −80°C until tested.

TaqMan assay design. AbHV genomic DNA sequences (K. Savin et al. unpubl.) allowed the development of virus-specific primers and probe using the Primer Express Software version 3.0 (PE Applied Biosystems). An ABI Prism® 7500 Fast Real-Time PCR System was used for the production, analysis and storage of data. Several primer and probe sets targeting different genomic sequences were obtained from the software 'highest scores' category and tested for specificity and sensitivity using DNA from infected and uninfected abalone and non-target pathogens (results not shown). The primer set ORF49 yielded the best results with regard to specificity and sensitivity and was chosen for further development of the diagnostic TaqMan assay. The ORF49 DNA sequence is derived from an AbHV partial genome sequence with similarity to that of *Ostreid herpesvirus 1* (GenBank accession no. AY509253) containing motifs V and VI, characteristic of SF2 helicases (Davison et al. 2005). The AbHV and 18S endogenous control primers and probe sequences are shown in Table 1.

The AbHV primers were used at a final concentration of 300 nM. The AbHV probe was used at a final concentration of 100 nM. Ribosomal DNA 18S primers and probe (Applied Biosystems) were used to validate the nucleic acid extraction procedure and the absence of PCR inhibitors. Both the 18S primers and the probe were used at a final concentration of 100 nM.

The reactions were carried out in 96-well plates using a 25 µl final reaction volume containing 12.5 µl TaqMan® Fast Universal PCR Master Mix (2X) (No AmpErase® UNG, Applied Biosystems), 2 µl (~100 ng μl^{-1}) extracted DNA sample and the reaction mix was made up to 25 µl using deionised water. The following thermal cycling conditions were used: 95°C for 59 s followed by 45 cycles of 95°C for 3 s and 62°C for 30 s.

All samples, including the no-template control, were tested in duplicate or triplicate to ensure repeatability. A sample was considered above the test background level when the change in fluorescence (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference dye), exceeded the threshold value (ΔR_n 0.1) that was arbitrarily set at the upper end of the exponential phase of the amplification curve. The cycle threshold (C_T) was defined as the cycle number at which a statistically significant increase in fluorescence output above background was detected. Based on the present study, samples were considered negative for AbHV when the C_T value was >35.8. Conservatively, samples with a C_T

Table 1. Primers and probes used to amplify/identify abalone herpes-like virus (AbHV) presence. Ribosomal DNA 18S primers and probe (Applied Biosystems) were used to validate the nucleic acid extraction procedure and the absence of PCR inhibitors. FAM: 6-carboxyfluorescein. TAMRA: 6-carboxytetramethylrhodamine

Primer/probe	Use	Sequence (5′–3′)
AbHV		
ORF49F	Forward primer	AACCCACACCCAATTTTTGA
ORF49R	Reverse primer	CCCAAGGCAAGTTTGTTGTT
ORF49Pr	Probe	6FAM-CCGCTTTCAATCTGATCCGTGG-TAMRA
18S rDNA		
18S Forward	Forward primer	CGGCTACCACATCCAAGGAA
18S Reverse	Reverse primer	GCTGGAATTACCGCGGCT
18S Probe	Probe	VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA

value <35.0 were considered clearly positive. Thus, based on this conservative estimate, there is an indeterminate range (C_T 35.0 to 35.8) and samples that yielded C_T values within this range were retested. A C_T value <40 was considered positive for the 18S Vic reporter dye. All abalone samples used for the present study produced C_T values <40 for the Vic reporter signal.

Analytical specificity. In order to demonstrate assay analytical specificity, DNA preparations from various non-target aquatic animal viruses as well as from an abalone infected with the parasite *Perkinsus olseni* were tested in duplicate.

Preparation of plasmid DNA standard and analytical sensitivity. In order to determine the sensitivity of the assay, the AbHV target amplicon (126 bp) was cloned into the pCR4 Blunt Topo vector (Invitrogen) according to the manufacturer's instructions. The recombinant plasmid from one clone was sequenced to confirm the presence of the amplicon. The plasmid, designated Topo-ORF49, was replicated in *Escherichia coli* DH5α and extracted using a QIAprep kit (QIAGEN) according to the manufacturer's instructions. The concentration of the plasmid Topo-ORF49 was determined in triplicate by UV spectrophotometry (using a wavelength of 260 nm and extinction coefficient of 50 ng cm ml^{-1}). The mass of a single plasmid DNA molecule was calculated using the formula 1 bp = 660 g mol^{-1}, the known size of the recombinant plasmid (4082 bp) and following the method outlined in the ABI manual of absolute real-time RT-PCR quantification (Applied Biosystems 2003). The TaqMan assay efficiency (E) of 98% was calculated from standard curves as the percentage of template molecules that was doubled during each cycle [$(10^{(-1/\text{slope})} - 1) \times 100$]. The supercoiled plasmid Topo-ORF49 was serially diluted 10-fold in DNAse-free sterile distilled water as well as in a solution of DNA extracted from known uninfected abalone tissue. The plasmid solutions were heated at 95°C for 5 min and put on ice for 5 min to separate the plasmid strands, which facilitates the TaqMan amplification process. A threshold value of ΔR_n of 0.1 was used for the testing of both serial dilutions.

Performance characteristics of the AbHV TaqMan assay. Analyses were performed using Microsoft Office Excel 2003 (11.8237.8221) SP3, and the Microsoft MedCal Version 9.6.4.0 using receiver operator characteristic (ROC) analysis, modified ROC curves, 2 × 2 table and frequency distributions. The true status of the sample was determined using histological examination as a reference standard together with epidemiological information and expert opinion to define high and low prevalence populations. Performance characteristics of the AbHV TaqMan assay were established using a total of 1673 abalone samples.

TaqMan assay precision. Establishment of intra- and inter-assay variability was performed using DNA samples obtained from 6 and 7 different abalone, respectively (OIE 2008): 6 confirmed AbHV-infected abalone (positive controls) from Victoria, and 7 negative controls from healthy uninfected abalone originating from New South Wales, where AbHV or AVG have never been reported.

RESULTS AND DISCUSSION

AbHV target amplicon

The expected amplicon size is 126 bp and its sequence (including the primers) is: AAC CCA CAC CCA ATT TTT GAG TGT AGG CGA ATA CAT TTG CTT TCT TAC CGC TTT CAA TCT GAT CCG TGG TTT CTT TAG TCG TTT TGA GAA TCT GTT TGC ATA AAG GAA CAA CAA ACT TGC CTT GGG. The target region sequence of the Taiwanese isolate is identical to the Australian isolate shown here.

Analytical specificity

In order to determine the specificity of the TaqMan assay, DNA extracted from a range of other aquatic animal herpesviruses as well as aquatic animal iridoviruses, which also possess a double-stranded DNA genome, were used as target in the assay. The TaqMan assay was shown to be specific for AbHV (Table 2). In addition, since the parasitic pathogen *Perkinsus olseni* is present in some abalone populations in Australia, the assay was also performed using *P. olseni* DNA as the target. The test yielded negative results (Table 2), which is essential when using the assay to gather accurate information from surveillance testing in regions where both aetiological agents can be prevalent. Viral DNA from the Taiwanese abalone herpes-like virus (Chang et al. 2005) was also tested and yielded positive results by the TaqMan assay. These data indicated at least some genomic similarity between the Taiwanese and Australian viruses (Table 2).

Analytical sensitivity

Since conventional cell culture methods for viral replication and quantification of viral titres is not possible for this virus, a molecular method, using the recombinant plasmid Topo-ORF49, was established to estimate the viral load in infected tissues. The TaqMan assay performed on 10-fold serial dilutions of a stock of the Topo-ORF49 plasmid detected the viral target

sequence down to 10^{-8} dilution, which is equivalent to 30 copies of recombinant plasmid, when the plasmid was diluted in either water or a solution of DNA extracted from uninfected abalone tissues (Table 3). Therefore, it appeared that the presence of excess non-specific DNA had little effect on the detection of the viral target sequence from recombinant plasmid. The dynamic range of the assay was estimated from standard curve assays and a linear correlation was obtained between template copy number and C_T value for over 4 $\log_{10}$ dilutions (Fig. 2).

Table 2. Abalone herpes-like virus (AbHV) TaqMan assay specificity. DNA samples with cycle threshold (C_T) values <35.8 are positive; those with C_T values ≥35.8 are negative. DNA concentration of virus samples ranged from 50 to 250 ng μl^{-1}. The Vic 18S signal was positive for all samples

Test sample	C_T value (mean ± SD)
Herpesviruses	
Oncorhynchus masou virus	>40
Ostreid herpesvirus 1	>40
Koi herpesvirus	>40
Pilchard herpesvirus	>40
Taiwanese abalone herpes-like virus	26.04 ± 0.4
Iridoviruses	
Epizootic haematopoietic necrosis virus	>40
Frog virus 3	>40
Red sea bream iridovirus	38.96 ± 0.04
Bohle iridovirus	39.98 ± 0.03
Lymphocystivirus	39.37 ± 0.19
Protozoan parasite	
Perkinsus olseni-infected abalone tissues	>40
Positive control	
Abalone experimentally infected with AbHV	19.68 ± 0.11
Negative controls	
Uninfected abalone tissue	>40
Water	>40

Samples from positive reference populations with a high prevalence of AbHV infection

In Victorian coastal waters, outbreaks of AbHV with high mortalities have been reported. For example, between January 2007 and June 2008, targeted sampling was performed on abalone from 4 different sites, Port Fairy, Portland on the western coast, and Flinders

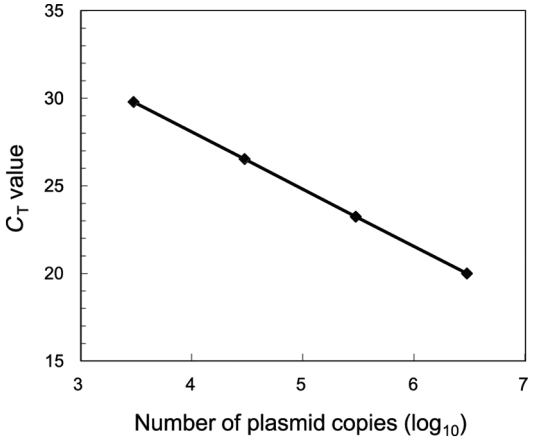

Fig. 2. Linearity of the abalone herpes-like virus (AbHV) TaqMan® assay using dilutions of the recombinant plasmid target. Plasmid dilutions (◆) were 3×10^6, 3×10^5, 3×10^4, and 3×10^3. Line represents the linear regression (the assay standard curve) of $\log_{10}$ copy number of the target gene (*x*-axis) versus cycle threshold (C_T) values (*y*-axis); slope = −3.26; *y*-intercept = 41.15; R^2 = 0.999; efficiency = 98%

Table 3. Abalone herpes-like virus (AbHV) TaqMan assay limit of detection using Topo-ORF49 plasmid DNA diluted in deionised water or uninfected abalone DNA. DNA samples with cycle threshold (C_T) values <35.8 are positive; those with C_T values ≥35.8 are considered negative. A threshold value of 0.1 was used for both tests. Tests 1 and 2 refer to both dilutions being performed in duplicate

Dilution factor	Plasmid copy number per reaction	C_T value (mean ± SD) for deionised water dilution		C_T value (mean ± SD) for uninfected abalone DNA dilution	
		Test 1	Test 2	Test 1	Test 2
10^{-2}	30000000	12.14 ± 0.09	11.99 ± 0.13	13.02 ± 0.03	13.28 ± 0.13
10^{-3}	3000000	13.88 ± 0.36	14.39 ± 0.00	16.35 ± 0.14	16.39 ± 0.02
10^{-4}	300000	17.55 ± 0.36	17.86 ± 0.62	18.94 ± 0.14	19.18 ± 0.18
10^{-5}	30000	22.27 ± 0.09	20.82 ± 0.54	22.14 ± 0.24	22.35 ± 0.04
10^{-6}	3000	24.77 ± 0.12	25.21 ± 0.23	25.12 ± 0.01	25.28 ± 0.04
10^{-7}	300	28.63 ± 0.26	29.00 ± 0.08	28.61 ± 0.09	28.62 ± 0.27
10^{-8}	30	30.44 ± 0.01	32.67 ± 0.60	34.90 ± 0.39	34.58 ± 0.82
10^{-9}	3	33.66 ± 0.54	36.39 ± 0.38	37.43 ± 1.06	38.79 ± 1.6
10^{-10}	0.3	36.88 ± 0.82	38.89 ± 1.23	39.09 ± 1.2	38.43 ± 0.34
10^{-11}	0.03	>40	>40	>40	39.03 ± 1.37
10^{-12}	0.003	>40	>40	>40	>40

Table 4. Assessment of abalone herpes-like virus (AbHV) infection of Victorian (Vic) and Tasmanian (Tas) abalone tissues by histological examination and by TaqMan assay at a threshold value of 35.8 for the cycle threshold (C_T) as a 2 × 2 table. Tas Plant: samples from a fish processing plant in Tasmania

Origin	TaqMan® PCR	Histopathology (+)	(–)	Total
Vic	(+)	21	2	23
	(–)	1	6	7
Tas Plant	(+)	7	2	9
	(–)	0	9	9
Tas	(+)	0	1	1
	(–)	0	1624	1624
Total		29	1644	1673

and Avalon on the central coast, and within Port Phillip Bay. Based on the presence of histopathology, there was a total of 29 samples from the positive reference populations; 22 were from Victoria and 7 from a processing plant in Tasmania that had experienced an AVG outbreak. All but one (which had a C_T value of 39.3) of these 29 samples were positive in the TaqMan assay at a 35.8 C_T cut-off (Table 4).

Samples from negative reference population with low prevalence of AVG

A total of 1625 abalone samples were obtained from open waters around the entire coast of Tasmania. These samples were considered to be from a reference population with an extremely low prevalence (<1%) of disease, based on nil reports of disease in wild abalone populations. All 1625 samples from this reference population had no clinical signs of disease and were classified as negative by histological examination, i.e. absence of any ganglioneuritis. In addition 11 samples were from a Tasmanian processing plant and 8 samples were from Victorian waters. All of these were negative (normal) based on histological examination (Table 4). When tested by the TaqMan assay, the majority of these samples (1507 samples, 91.7%) returned C_T values ≥40, while 134 samples (8.15%) yielded results with a C_T value ≥35.8 and <40, and 3 samples (0.18%) yielded C_T values below the threshold of 35.8.

ROC curve

ROC analysis was used to evaluate the discriminatory power of the TaqMan assay (Fig. 3). Of the 29 positive reference samples (based on the presence of ganglioneuritis), with 22 obtained from Victoria and 7 from a processing plant in Tasmania, the AbHV TaqMan assay classified 28 as positive, that is, having a C_T value <35.8. A histologically positive sample that was negative in the TaqMan assay came from Victoria and had a C_T value of 39.3. A summary of the data from the reference populations is presented in the 2 × 2 table (Table 4).

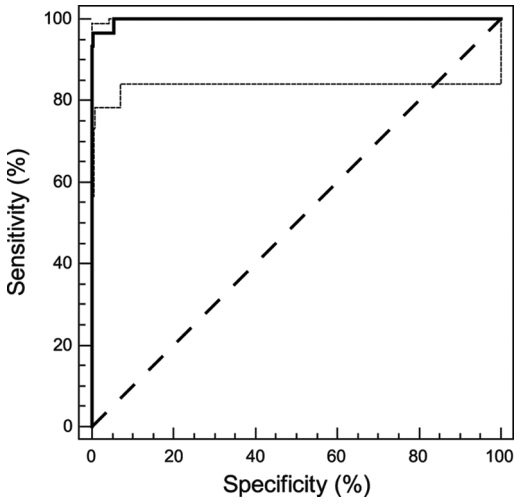

Fig. 3. Receiver operator characteristic (ROC) curve (solid line). The 95% confidence interval is indicated (fine dotted line). For reference, an ROC curve for a test that cannot discriminate between positive and negative samples is also shown (thick dashed line). The highest combined diagnostic sensitivity, 96.7 (95% CI: 82.7 to 99.4), and specificity, 99.7 (95% CI: 99.3 to 99.9), were obtained at a cycle threshold value (C_T) of 35.8 (see Fig. 5)

Of the 1644 negative reference samples (based on the absence of any lesions characteristic of ganglioneuritis), 8 samples were obtained from Victoria and 1636 came from Tasmania (11 from a processing plant and 1625 from open waters). The AbHV TaqMan assay classified 1639 of these samples as negative, i.e. C_T value >35.8. Five histologically negative samples yielded C_T values below this cut-off threshold in the TaqMan assay and therefore tested positive for the presence of viral DNA. These samples were from Victoria (2 samples with C_T values of 26.6 and 35.6) and Tasmania (2 samples from the processing plant with C_T values of 31.6 and 35.4, and 1 sample from Tasmanian open waters with a C_T value of 34.4).

The AbHV TaqMan assay had the highest diagnostic sensitivity and specificity at a C_T value threshold of 35.8, i.e. 96.7 (95% CI: 82.7 to 99.4) and 99.7 (95% CI: 99.3 to 99.9), respectively (Fig. 3). Specificity increased to 99.94% when only samples from the negative reference population from Tasmanian open waters were used for analysis, i.e. 1624/1625 samples were negative.

The area under the ROC curve (AUC) is a global summary statistic of the diagnostic accuracy. Plots for

diagnostic tests with perfect discrimination between negative and positive reference samples (i.e. no overlap of values of the 2 groups) pass through the coordinates 0 and 1, and represent 100% sensitivity and specificity. In accordance with an arbitrary guideline, one could distinguish between non-informative (AUC = 0.5), less accurate (0.5 < AUC ≤0.7), moderately accurate (0.7 < AUC ≤0.9), highly accurate (0.9 < AUC < 1) and perfect tests (AUC = 1) (Greiner et al. 2000). The AUC of the AbHV TaqMan assay was 0.998. Hence, this assay can be considered a highly accurate test.

Interactive dot diagram

The interactive dot diagram (Fig. 4) was used to illustrate the best separation (minimal false negative and false positive results) between the positive and negative samples as determined by histological examination. At a 35.8 C_T cut-off value, shown as a horizontal line in Fig. 4, the AbHV TaqMan assay produced 1 'false negative' in 29 positive samples and 5 'false positive' in 1644 negative samples as assessed in reference to histological examination.

Plot versus criterion

For a diagnostic test, the higher the cut-off, or in this case the C_T value, the higher the diagnostic sensitivity with a respective drop in the diagnostic specificity, resulting in more 'false positive' results. Higher diagnostic sensitivity is normally required for screening tests used in surveillance programs. On the other hand, a lower cut-off increases the diagnostic specificity with a respective drop in diagnostic sensitivity, resulting in a higher percentage of 'false negative' results. A higher specificity is normally required for a confirmatory test. Fig. 5 shows results for sensitivity and specificity for the AbHV TaqMan assay at all possible cut-off values.

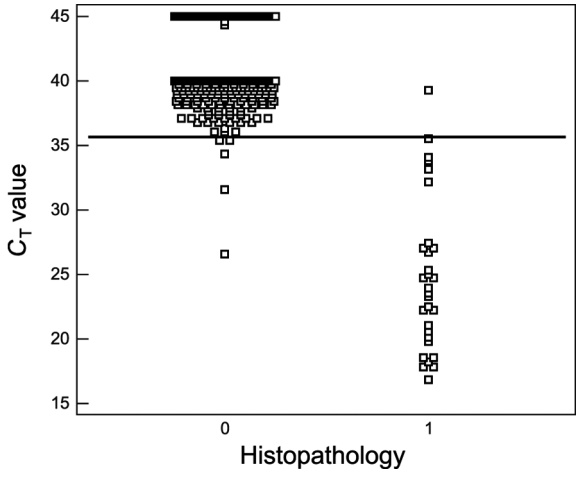

Fig. 4. Interactive dot diagram. This illustrates the best separation (minimal false negative and false positive results) between the positive and negative groups at a cut-off value of 35.8 for the cycle threshold (C_T) (horizontal solid line). For histopathology: 0 = negative, 1 = positive

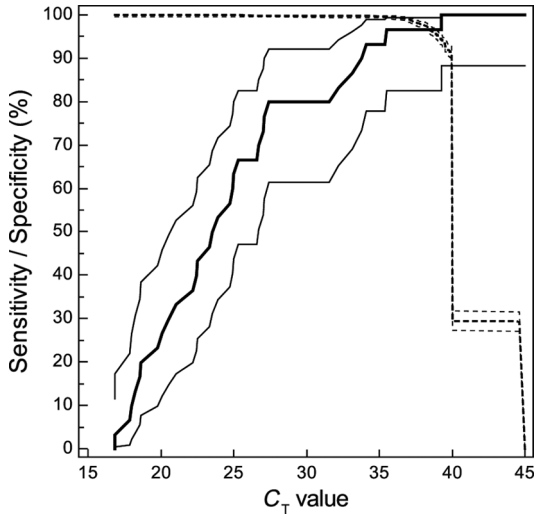

Fig. 5. Plot versus criterion graph. This illustrates the changes in diagnostic sensitivity (thick solid line) and specificity (thick dashed line), including their 95% confidence intervals (sensitivity: solid fine lines; specificity: dashed fine lines), at different cut-off levels for the cycle threshold (C_T)

Repeatability

Inter- and intra-assay variation of the AbHV TaqMan assay is summarised in Table 5. The mean C_T value for the positive control samples (C+) was 22 with a standard deviation of 2, a coefficient of variation of 10 and minimum and maximum values of 18 and 27, respectively. The mean C_T value for the negative control samples (C–) was 40 with a standard deviation of 3, a coefficient of variation of 7 and minimum and maximum values of 35 and 45, respectively (Table 5a, Fig. 6). Based on these results, preliminary upper and lower control limits using 1, 2 and 3 standard deviations were established for C+ and C– as shown in Table 5b.

Reproducibility

For nucleic acid detection tests it is important to obtain estimates for ruggedness and robustness at the early stages of assay development. The TaqMan assay

Table 5. (a) Summary statistics for positive (C+) and negative controls (C–) in the abalone herpes-like virus (AbHV) TaqMan assay (cycle threshold C_T values). (b) Upper and lower control limits for positive and negative controls in the AbHV TaqMan assay (see Table 3) (C_T values). LCL: lower control limit, UCL: upper control limit

(a) Statistics	C+	C–	
Mean	22	40	
Standard error	0	0	
Median	23	40	
Mode	23	40	
Standard deviation	2	3	
Sample variance	5	7	
Kurtosis	–1	0	
Skewness	0	1	
Range	9	10	
Minimum	18	35	
Maximum	27	45	
Coefficient of variation	10	7	
Count	106	98	
Confidence level (95.0%)	0	1	
(b) Control limits	1 SD	2 SD	3 SD
Positive control			
UCL	24	27	29
LCL	20	18	16
Negative control			
UCL	42	45	48
LCL	37	35	32

was transferred to the Biosciences Research Division, Department of Primary Industries Victoria (VicDPI) and parallel testing of 47 Victorian samples (DNA was extracted, halved and sent to both laboratories) was performed at both the Australian Animal Health Laboratory (AAHL) and VicDPI. Results indicate that AAHL consistently produced slightly lower C_T values, with mean C_T values for all samples being 29 for AAHL and 31 for VicDPI (Fig. 7). These data indicate a slightly increased sensitivity of the test when run under conditions at AAHL.

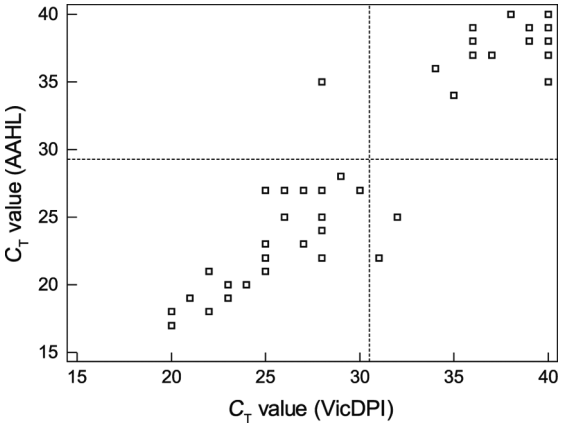

Fig. 7. Reproducibility of the abalone herpes-like virus (AbHV) TaqMan assay. Scatter diagram for results expressed as cycle threshold (C_T) values for 47 samples from Victoria using the AbHV TaqMan assay at the Australian Animal Health Laboratory (AAHL) (*y*-axis) and at the Department of Primary Industries Victoria (VicDPI) (*x*-axis). Note that the mean C_T value at AAHL (horizontal dashed line) is slightly lower than that at VicDPI (vertical dashed line)

In the absence of any other diagnostic test for comparative purposes, histological examination was the standard reference test used as the 'gold standard' test for comparison and in conjunction with epidemiological

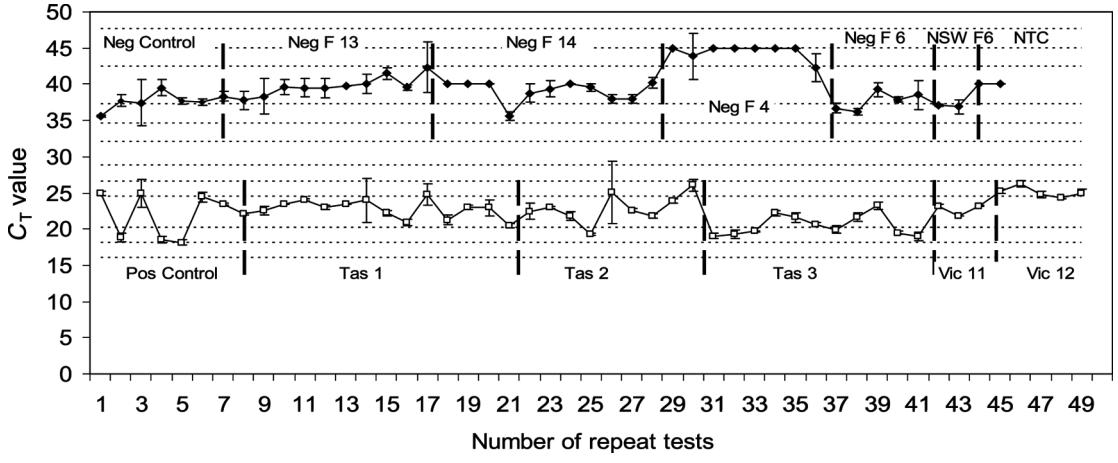

Fig. 6. Repeatability of the abalone herpes-like virus (AbHV) TaqMan assay. Inter- and intra-assay variation for different batches of positive and negative control samples (September to November 2008). Data points on each of the 2 plotted lines represent the mean values of the controls in duplicate or triplicate. Error bars represent 2 SDs. Also shown are 1, 2 and 3 SDs (⋯) after 49 runs with 6 positive samples (□) and 45 runs with 7 negative samples (◆). Inter- and intra-assay variation within the same batch did not include variation due to the nucleic acid extraction process. Neg: negative, NSW: New South Wales, NTC: no template control, Pos: positive, Tas: Tasmania, Vic: Victoria

information about reference populations, and expert advice was used as the presumptive test to determine the true status of the samples. However, it is likely that the presence of AbHV genomic DNA can be detected by the AbHV TaqMan assay before pathological changes become evident, i.e. the TaqMan assay can potentially detect subclinical infection. This circumstance may result in positive TaqMan results with corresponding negative histological examination. In the present analysis such data would be classified as false positive and would result in an underestimation of the diagnostic specificity of the TaqMan assay. Out of 1644 samples defined as negative by histological examination, 1639 were also negative in the TaqMan assay with C_T values ≥35.8 (the cut-off), but 5 samples gave C_T values <35.8 and were considered positive in the TaqMan assay. One explanation for these results is that the abalone were in the early, pre-clinical, stage of viral infection. Moreover, histopathology may remain long after the infection has cleared, even when viral or genomic material is no longer present in amounts detectable by the TaqMan assay. Samples belonging to this category would produce a positive histopathological assessment and a (false) negative TaqMan result and lead to an underestimation of the diagnostic sensitivity of the TaqMan assay. In addition, similar histopathological findings may be caused by other pathogens or environmental/toxic factors. Out of the 29 reference samples determined positive by histological findings, i.e. for the presence of ganglioneuritis, the AbHV TaqMan assay classified 28 as PCR-positive, that is, having a C_T value <35.8. In the present study, one sample was positive for histopathology and negative by real-time PCR with a C_T value of 39.3. During histological examination, it has been observed that lesions may occur discontinuously along the length of the neural tissue (result not shown), which may reflect localised/focal areas of infection. Thus, a histopathology-positive/PCR-negative (or vice versa) result may be a consequence of tissue sampling error due merely to chance. To minimise the effects of an imperfect reference standard, other information, such as epidemiological evidence of AbHV infection, was taken into consideration during the present study (e.g. prevalence and mortality were included in the selection of the sites where abalone were sampled). For example, the exclusive use of negative samples from Tasmanian open water abalone increased the specificity of the AbHV TaqMan to 99.94% (1624/1625 samples tested negative at a 35.8 C_T cut-off).

Thus, the preliminary performance characteristics of a new AbHV diagnostic PCR test were estimated by convenience sampling of a limited number of positive samples from a high prevalence population and a high number of samples from a low prevalence population. In addition, the reference status of the sample was determined by histological examination of all samples from the high prevalence population and some samples from the low prevalence population (Greiner & Gardner 2000). At a threshold of 35.8 C_T, the AbHV TaqMan assay had a diagnostic sensitivity and diagnostic specificity of 96.7 (95% CI: 82.7 to 99.4) and 99.7 (95% CI: 99.3 to 99.9), respectively (Fig. 3). Due to the limited number of positive samples, the 95% confidence intervals for the diagnostic sensitivity are relatively broad, and more samples from AbHV-infected abalone would be needed for a more robust estimate of this parameter. However, at the time of the initial disease outbreak only samples for histological examination were taken since no other diagnostic tests had been developed. The availability and convenience of the TaqMan assays as a diagnostic test has now reduced the need to undertake histological examination of routine samples. It is rare that samples for histology and PCR are taken from the same abalone.

Out of 1625 samples from a low AbHV prevalence population from Tasmanian open waters, 1498 samples (92.18%) returned C_T values ≥40, while 126 samples (7.75%) had C_T values ≥35.8 and <40, and 1 sample (0.06%) had a C_T value of 34.4, i.e. below the 35.8 C_T threshold.

If the purpose of the AbHV TaqMan assay is to be used as a screening test, such as for surveillance studies to demonstrate freedom from infection in live animals or animal products (OIE 2008), the C_T cut-off threshold should be adjusted towards a high sensitivity level, which can be achieved by selecting a relatively high C_T value.

The ultimate evidence for the usefulness of a new assay is its successful application in other laboratories and inclusion in national, regional or international control programmes. This requires acceptable robustness and ruggedness. Results for intra- and inter-assay variation at AAHL were satisfactory and preliminary upper and lower control limits were established. Coefficient of variation values of 10 and 7 for different batches of positive and negative controls respectively indicated good assay repeatability. When transferred and implemented at another laboratory (VicDPI), the assay also showed good reproducibility of results, with a range of positive and negative samples. The AbHV TaqMan® PCR is a suitable candidate assay for the detection of AbHV infection in abalone because of its reliable and accurate performance.

The development and validation of the AbHV TaqMan® PCR assay has provided a beneficial molecular tool for viral detection and identification as well as epidemiological studies. The TaqMan assay provides sensitivity and rapidity and allows for high through-put testing and for many purposes has replaced histological examination as the standard diagnostic test. Due to its high specificity, the assay facilitates the correct

assessment of the prevalence of AbHV in abalone populations. In addition, future infectivity trials will allow establishment of the earliest time point at which AbHV can be detected during the infection process.

The development of this PCR test now allows researchers and pathologists to address issues of an epidemiological nature, such as viral persistence in subclinical carriers (Arzul et al. 2002), viral presence in other potential hosts (e.g. other mollusc species), possible routes of viral transmission, viral tissue distribution and approximate viral load in abalone tissues. In addition, preliminary data showing that the Taiwanese herpes-like virus is detected by the TaqMan assay suggest at least some genomic similarity between the Australian and Taiwanese herpes-like viruses. Implementation of the TaqMan assay in Taiwanese laboratories would also support disease management strategies implemented by the Taiwanese fisheries authorities. Further analyses, including extended genome sequencing and phylogenetic analysis, will shed some light on the relationship of the 2 viruses.

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