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Diagnostic test accuracy when screening for *Haliotid herpesvirus 1* (AbHV) in apparently healthy populations of Australian abalone *Haliotis* spp.

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ABSTRACT: The accuracy of 3 real-time PCR assays (ORF49, ORF66 and ORF77) and histopathology was evaluated for the purpose of demonstrating or certifying abalone free from *Haliotid herpesvirus 1* (AbHV), the causative agent of abalone viral ganglioneuritis. Analytically, all 3 qPCRs showed equivalent limit of detection (20 copies per reaction); however, ORF49 could not detect 2 of the AbHV genotypes. A selection of 1452 archive specimens sourced from apparently healthy abalone populations was screened using all 4 tests. In the absence of a perfect reference standard, a Bayesian latent class analysis was built to estimate diagnostic sensitivity (DSe), diagnostic specificity (DSp) and likelihood ratios of a positive (LR^+) and negative test result (LR^-) for each individual test and for all possible combinations of test pairs interpreted either in series or in parallel. The pair ORF49/ORF66 interpreted in parallel performed the best both analytically and diagnostically to demonstrate freedom from AbHV in an established population of abalone and to certify individual abalone free from AbHV for trade or movement purposes (DSe = 96.0%, 95% posterior credibility interval [PCI]: 82.6 to 99.9; DSp = 97.7%, 95% PCI: 96.4 to 99.4; LR^+ = 41.4, 95% PCI: 27.4 to 148.7; LR^- = 0.041, 95% PCI: 0.001 to 0.176). Histopathology showed very poor DSe (DSe = 6.3%, 95% PCI: 2.4 to 13.1) as expected since most infected abalone in the study were likely sub-clinical with limited pathological change. Nevertheless, we recommend histopathology when clinically investigating outbreaks to find potential, new, emerging AbHV genotype(s) that may not be detectable by either ORF49 or ORF66.

KEY WORDS: *Haliotid herpesvirus 1* · Real-time PCR · Histopathology · Sensitivity · Specificity · Bayesian latent class analysis

1. INTRODUCTION

Abalone herpesvirus (AbHV) is the causative agent of abalone viral ganglioneuritis (AVG) in Australian greenlip abalone *Haliotis laevigata*, blacklip abalone *H. rubra*, and hybrids of these 2 species (*H. laevigata* × *H. rubra*) (Tan et al. 2008, Savin et al. 2010). More recently the International Committee on Taxonomy of Viruses has re-classified AbHV as *Haliotid herpesvirus 1*, the type species of the genus *Aurivirus* in the Family *Malacoherpesvirus* (Adams et al. 2013). To maintain convention with the current World

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Organisation for Animal Health (OIE) Aquatic Animal Health Code and Manual of Diagnostic Tests for Aquatic Animals, AbHV will be used in this manuscript (OIE 2018a). Abalone infected with AbHV present a wide clinical spectrum ranging from apparently healthy (carriers) to severe neurological manifestations with lethargy, mouth swelling and protrusion, inward curling of the foot, and ultimately death (Hooper et al. 2007, Ellard et al. 2009). Outbreaks of AVG associated with AbHV-like particles have also been reported in Jiukong abalone *Haliotis diversicolor supertexta* in China and Taiwan (Wang et al. 2004, Chang et al. 2005). Regardless of the abalone species, mortality rates associated with AVG can reach up to 90% (Wang et al. 2004, Chang et al. 2005). Given the significant production losses caused by the virus and the need to protect those zones and countries free from the infection, AbHV has been listed as a notifiable aquatic animal disease by the OIE (OIE 2018a). This entails that AbHV-infected countries or zones notify OIE, and the method(s) of detection should be properly evaluated and validated to show fitness-for-purpose (OIE 2018b).

Several detection methods for AbHV have been developed: histopathology (Hooper et al. 2007), electron microscopy (Tan et al. 2008), *in situ* hybridization (Mohammad et al. 2011), and real-time PCR (qPCR) (Corbeil et al. 2010). Corbeil et al. (2010) designed the qPCR based on a partial genome sequence of the first Australian AbHV genotype sourced from the first reported AVG outbreak in Victoria in December 2005 (genotype Vic1). The test used the best performing set of primers and probe, referred to as open reading frame (ORF) 49, targeting the putative motifs V and VI, characteristic of SF2 helicases. At the time, the ORF49 qPCR detected all known genotypes including a recently emerged 2008 Tasmanian isolate (genotype Tas1). However, AVG outbreaks in Tasmania from 2009 to 2011 revealed new AbHV genotypes that could not be detected consistently by ORF49 (Ellard et al. 2009). Genome sequence analysis identified these isolates as genotypes for which ORF49 has poor affinity (Cowley et al. 2011). However, 2 other sets of primers/probes, ORF66 and ORF77, previously considered by Corbeil et al. (2010), showed good affinity for the Tasmanian genotypes Tas2 and Tas3.

The primary objective of this study was to assess the diagnostic accuracy of the 2 alternative sets of primers/probe, ORF66 and ORF77, in comparison with ORF49 when screening apparently healthy populations of Australian abalone (i.e. no overt AVG outbreak) to demonstrate or document freedom from AbHV infection as defined by OIE (2018a). Histopathology was also evaluated as it is routinely used in diagnosis and surveillance of abalone diseases. A secondary objective of this study was to evaluate all possible combinations of test pairs to further reduce potential misclassifications. This report follows the Standards for Reporting of Animal Diagnostic Accuracy Studies (STRADAS)-aquatic published by Gardner et al. (2016).

2. MATERIALS AND METHODS

2.1. Analytical characteristics of real-time PCR assays

2.1.1. Analytical evaluation

The analytical sensitivity (ASe), analytical specificity (ASp), and repeatability of the 3 real-time assays (ORF49, ORF 66 and ORF77) were estimated as follows. For ASe, or limit of detection, positive-control plasmids were designed to include the nucleotide sequence between the forward and reverse primer for each assay using a reference genotype of AbHV-Vic1. Plasmids were produced commercially (Integrated DNA Technologies), and based on transcript length and concentration, determined by Qubit, stock plasmids were made to 10^9 or 10^8 copies μl^{-1} in molecular-grade water. Ten-fold dilutions of each plasmid were prepared in molecular-grade water and negative abalone DNA (50 ng μl^{-1}). Five replicates for each 10-fold dilution in each diluent were tested, with at least 3 replicates required to test positive for a dilution to be considered to test positive. For ASp, DNA from the different AbHV genotypes (Vic1, Tas1, Tas2, Tas3, Tas4 and Tas5) as well as a selection of herpesviruses, iridoviruses and parasites was tested. For test repeatability, positive-control plasmids were repeatedly tested over multiple runs by multiple operators. The coefficient of variation (CV = SD C_T / mean C_T) was calculated to assess the relative amplitude of the within- and between-operator variability.

2.1.2. Real-time PCR assays

Real-time PCR reactions in a final volume of 25 µl contained 2 µl template nucleic acid, 12.5 µl TaqMan Fast Universal PCR Master Mix (Life Technologies) and a final concentration of 300 nM for each AbHV-specific primer, 100 nM for the AbHV-specific probe

(see Table S1 in the Supplement at www.int-res.com/articles/suppl/d136p199_supp.pdf) and 1000 nM for each 18S primer and 250 nM for the 18S probe. Specimens were deemed positive if a typical amplification curve crossed the fluorescence threshold of 0.1 after a run of 50 cycles. The qPCR assays were performed in a 7500 Fast Real-Time PCR System (Life Technologies) and analysed with the 7500 software (v2.3).

2.2. Diagnostic characteristics of AbHV tests

2.2.1. Study population

Evaluation material was sourced from the Tasmanian Department of Primary Industry, Parks, Water and Environment Animal Health Laboratory (DPIPWE-AHL) archived tissue specimens collected and previously tested as part of DPIPWE disease investigation, surveillance and health certification activities. The archives included Tasmanian greenlip *Haliotis laevigata*, blacklip *Haliotis rubra*, and hybrid *H. laevigata* × *H. rubra* abalone sampled from wild and farm populations between 2008 and 2012. To fit the intended purpose, only submissions originating from abalone populations showing no evidence of AVG-associated mortality at the time of sampling were included in the evaluation. Additional requirements were that individual abalone should have material suitable for both histopathology and qPCR testing and an identifiable source. From an available pool of 285 submissions (5369 abalone), 211 submissions fitted the inclusion criteria (4363 abalone), and a random subset of individual abalone was selected from 94 submissions for this study (1452 abalone). The 94 submissions were chosen such that all 10 Tasmanian emergency biosecurity regions were represented in the study, and individual abalone were selected within each submission using a formal random process. Where >1 species was present within a particular region, selection was intentionally biased so all species were covered. Selected abalone were grouped according to their source (i.e. farmed, wild or live-holding facility) into 4 source populations with varying expected prevalence (Table 1).

2.2.2. Specimen collection

At the time of submission, a duplicate set of 1 cm cubes of soft tissue containing neural ganglion tissue was dissected from the buccal area and fixed in 95% (v/v) analytical-grade ethanol. One duplicate was processed for routine molecular testing, whilst the second was stored at −80°C as part of the tissue archive used in this evaluation. Additional soft tissue was debrided of excess muscle tissue and fixed in 10% seawater-buffered formalin. Formalin-fixed tissue was embedded in paraffin blocks using standard histological techniques (Howard et al. 2004) and stored until processing for histopathology.

2.2.3. Histopathological examination

Histopathological examinations were performed at the DPIPWE-AHL, accredited by the Australian National Association of Testing Authorities (NATA) under the ISO/IEC 17025 standards. Thin (5 µm) sections were processed using standard histological procedures, stained with haematoxylin and eosin and mounted. Histological slides were examined by qualified veterinary pathologists using light microscopy for the presence of ganglioneuritis lesions consistent with descriptions outlined within Hooper et al. (2007). These examinations were conducted as part of the routine diagnostic work, and no formal measures were taken to mask histopathologists to the source of the specimens; however, no other test result was available at the time for result comparison.

Table 1. Summary of source population characteristics of the abalone used for the evaluation of the diagnostic test accuracy when detecting *Haliotid herpesvirus 1* (AbHV)

Source population	Description	No. specimen selected	% Blacklip (number)	% Greenlip (number)	% Hybrid/ Unknown (number)	Sampling period
1	Wild abalone direct from marine environment	703	92.7 (652)	7.3 (51)	0.0 (0)	2008 and 2011
2	Wild abalone via processor	440	81.1 (357)	17.5(77)	1.4 (6)	2008 to 2011
3	Cultured abalone direct from farm	138	14.5 (20)	50 (69)	35.5 (49)	2008 to 2012
4	Wild abalone direct from mother boat	171	60.8 (104)	8.2 (14)	31.0 (53)	2008–2009 and 2011
Total		1452	78.7 (1,143)	14.5 (211)	7.4 (108)	2008 to 2012

2.3.4. Real-time PCR assays

The archived ethanol-fixed tissues were sent for qPCR testing to the OIE Reference Laboratory for infection with abalone herpesvirus at the CSIRO Australian Animal Health Laboratory (AAHL) - Fish Diseases Laboratory, also NATA accredited according to ISO/IEC 17025 requirements. Specimens were trimmed to obtain a sub-sample with visible ganglion and extracted using the MagMAX-96 Viral RNA Isolation Kit (Life Technologies) on the MagMAX Express-96 Magnetic Particle Processor (Life Technologies). Nucleic acids were eluted in 50 µl of elution buffer. Nucleic acid extracts were tested with primers and probe specific for 18S ribosomal RNA (Applied Biosystems) to validate the extraction procedure. Extracts were tested for AbHV as described above (Section 2.1). AbHV-positive controls, in the form of a plasmid containing the sequence specific to each qPCR assay, and a no-template control were included in each assay plate. Positive and negative controls must have generated the expected results for the test run to be considered valid, with acceptance limits for positive controls set at the mean ± 2 SD generated from 10 previous test runs.

Specimens were tested in duplicate and deemed positive if at least 1 duplicate generated a typical amplification curve. No cycle threshold (C_T) cut-off values were used. No formal measures were taken to mask laboratory operators to the source of the specimens; however, no other test results were accessible for comparison.

2.3.5. Statistical analysis

The diagnostic sensitivity (DSe) and specificity (DSp) were estimated using latent class analysis (LCA; Hui & Walter 1980) because none of the accessible reference methods were assumed diagnostically perfect (i.e. gold standard). The likelihood ratio of a positive test result [LR^+ = DSe / (1 − DSp)], and likelihood ratio of a negative result [LR^- = (1 − DSe) / DSp] were also estimated to enhance the comparison and selection of tests to best fit purpose (Caraguel et al. 2015). LRs combine the DSe and DSp and better reflect the strength of the evidence provided by given test results. Potential further accuracy gains were explored by calculating within the model the combined accuracy for each possible pair of tests using the interpretation of results either in parallel or in series (Dohoo et al. 2012).

LCA was implemented in a Bayesian framework to allow for modelling flexibility with the open-source software OpenBUGS v.3.2.3 rev1012 (Thomas et al. 2006). This facilitated the exploration of conditional dependence between pairs of tests by adding covariance terms in infected and non-infected abalone (γ_{Se} and γ_{Sp}, respectively) (Georgiadis et al. 2003). DSe, DSp, and prevalence model parameters were given a uniform prior distribution between 0 and 1 [Beta(1,1)], while conditional covariance parameters were given uniform prior distribution ranging between their lower and upper limits (Lγ and Uγ) as calculated in Gardner et al. (2000). An initial burn-in of 10 000 iterations was run to allow for Markov Chain Monte Carlo (MCMC) convergence, and the subsequent 50 000 iterations were sampled for posterior estimations and inferences. Data goodness-of-fit was assessed by comparing the deviance information criterion (DIC) of models including conditional dependence with the DIC of the reference model without conditional dependence. A reduction in DIC > 3 was considered a significant data fitness improvement (Spiegelhalter et al. 2002). The final model was selected as the model with the lowest DIC and the least number of parameters. MCMC convergence of the final model was assessed visually using the Gelman-Rubin diagnostic plots with 3 different sets of initial values following Toft et al. (2007a) recommendations. A thinning of 10 (every 10th iteration sampled for posterior estimation) was used to reduce estimates' autocorrelation along the MCMC. Comparisons of posterior estimates were conducted by estimating the Bayesian posterior probabilities (BPP), defined as the proportion of sampled iterations where the tested hypothesis was true, e.g. $DSe_1 > DSe_2$, and interpreted at the 5% threshold (Toft et al. 2007b). The assumption of constant DSe and DSp across study populations was assessed by comparing estimates when removing one population at the time from the final model. The OpenBUGS code for the final model is accessible in the Figshare knowledge repository (doi:10.25909/5c416aaf948cf).

3. RESULTS

3.1. Analytical characteristics of real-time PCR assays

The ASe of each assay was at least 20 plasmid copies per reaction when diluted in host DNA, and the amplification efficiency ranged between 90 and 110% (Table 2). All 3 assays revealed acceptable

Table 2. Analytical sensitivity and efficiency of the *Haliotid herpesvirus 1* (AbHV) real-time PCR assays using the primers/probe sets ORF49, ORF66 or ORF77

Plasmid copies	ORF49 qPCR C_T value (mean ± SD)		ORF66 qPCR C_T value (mean ± SD)		ORF77 qPCR C_T value (mean ± SD)	
	Water	Abalone DNA	Water	Abalone DNA	Water	Abalone DNA
2×10^8	11.85 ± 0.09	10.39 ± 1.28	11.04 ± 0.09	10.18 ± 0.14	11.42 ± 0.21	11.07 ± 0.18
2×10^7	15.64 ± 0.19	14.73 ± 0.25	14.9 ± 0.08	13.57 ± 0.15	15.64 ± 0.09	13.57 ± 2.76
2×10^6	19.01 ± 0.28	18.20 ± 0.19	18.21 ± 0.07	17.14 ± 0.05	18.84 ± 0.40	18.26 ± 0.61
2×10^5	22.48 ± 0.77	21.71 ± 0.06	21.5 ± 0.06	20.36 ± 0.85	22.58 ± 0.08	22.19 ± 0.90
2×10^4	25.44 ± 1.06	24.96 ± 0.08	25.3 ± 0.25	24.16 ± 0.58	26.04 ± 0.31	26.54 ± 1.77
2×10^3	29.52 ± 0.91	28.39 ± 0.51	28.73 ± 1.27	28.49 ± 0.30	30.21 ± 1.11	29.60 ± 3.31
2×10^2	34.39 ± 2.14	32.54 ± 0.76	32.99 ± 1.32	31.45 ± 1.51	34.83 ± 0.99	32.81 ± 4.19
2×10^1	Not detected	34.35 ± 2.31	37.08 ± 1.35	36.54 ± 0.34	38.61 ± 0.53	38.18 ± 1.07
2×10^0	Not detected	Not detected	Not detected	38.01 ± 0.18	Not detected	Not detected
2×10^{-1}	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Efficiency	94.2%	97.3%	93.7%	94.5%	90.9%	90.8%

ASp as none of the non-target pathogens were detected (Table 3). The ORF66 and ORF77 could detect all 7 known AbHV genotypes; however, ORF49 was not able to detect the Tas2 and Tas3 AbHV genotypes. The assays were highly repeatable with CV < 3.2% within or between operators (see Table S2 in the Supplement).

3.2. Diagnostic characteristics of AbHV tests

3.2.1. Test results

A summary of the cross-tabulated results of the 1452 selected abalone across the 4 tests is reported in Table S3 in the Supplement. All 4 tests agreed on a negative test result in 1331 (91.7%) abalone and on a positive test result in only 1 (0.07%), which suggested overall a low AbHV prevalence in the 4 study populations. Substantially fewer positive (n = 5, 0.3%) abalone were found by histopathological analysis compared to ORF77 (n = 48, 3.3%), ORF66 (n = 79, 5.4%) and ORF49 (n = 80, 5.5%). The observed agreement was very high (~ 95%) among all 4 tests due mainly to the high proportion of negative specimens.

3.2.2. Final model estimates

The model with conditional dependence between ORF66 and ORF77 was selected as final because its DIC was within 3 units from the lowest DIC model and had the smallest number of parameters (Table S4 in the Supplement). This model had significant covariance between ORF66 and ORF77 in both infected and non-infected abalone, representing 41.3% and 60.0% of the maximum possible covariance, respectively. There was no evidence of lack of MCMC convergence based on a visual assessment of the Gelman Rubin diagnostic plots. When removing one population at the time from the final model, no major changes in DSe and DSp were observed (Table S5 in the Supplement). There was no evidence of major violation of the model assumption of constant DSe/DSp across study populations.

Prevalences across source populations varied from 1.0% to 8.9%. Final accuracy estimates of the tests interpreted individually, in parallel and in series are reported in Table 4. When interpreted individually, ORF49 had significantly the best DSe (90.04%) and, therefore, the lowest LR^- (0.100). Histopathology had the best DSp (99.95%); however, due to its very poor DSe (6.30%), its LR^+ (122.7) was lower than the ORF49's LR^+ (425.4), although not significantly. This pattern suggests that a positive ORF49 result was the strongest single test evidence of AbHV being present.

The combination of ORF49 and ORF66 interpreted in parallel improved the DSe (96.0%) and LR^- (0.041) compared to ORF49 alone (Table 4). A negative result with both ORF49 and ORF66 was the best evidence of AbHV absence. However, when interpreting in-parallel, DSp and LR^+ decreased substantially regardless of the test combination.

When interpreted in series, most pairs of tests reached excellent DSp (> 99.99%) but their DSe collapsed (Table 4). Pairs of tests including ORF49 had the largest LR^+, but because ORF49 could not detect the AbHV genotypes Tas2 and Tas3 (Table 3), these

Table 3. Analytical specificity of the *Haliotid herpesvirus 1* (AbHV) real-time PCR assays using the primers/probe sets ORF49, ORF66 or ORF77. '–': not detected

Sample	ORF49 qPCR C_T value	ORF66 qPCR C_T value	ORF77 qPCR C_T value
Herpesviruses			
AbHV Vic1	28.52	28.69	29.81
AbHV Tas1	23.82	27.09	24.68
AbHV Tas2	–	24.33	25.53
AbHV Tas3	–	23.51	24.90
AbHV Tas4	24.76	25.21	26.55
AbHV Tas5	24.70	24.88	26.21
AbHV Taiwan	21.59	22.15	25.02
Salmonid herpesvirus 2	–	–	–
Ictalurid herpesvirus 1	–	–	–
Cyprinid herpesvirus 1	–	–	–
Cyprinid herpesvirus 3	–	–	–
Ostreid herpesvirus 1	–	–	–
Pilchard herpesvirus	–	–	–
Iridoviruses			
Epizootic haematopoietic necrosis virus	–	–	–
Frog virus 3	–	–	–
Bohle iridovirus	–	–	–
Mahafee road virus	–	–	–
European catfish virus	–	–	–
Red sea bream iridovirus	–	–	–
Lymphocystis disease virus	–	–	–
Parasites			
Perkinsus olseni	–	–	–
Bonamia exitiosa	–	–	–
Positive control plasmids			
AbHV ORF49 qPCR plasmid NQC-1	25.71	–	–
AbHV ORF49 qPCR plasmid NQC-2	32.58	–	–
AbHV ORF66 qPCR plasmid NQC-1	–	24.86	–
AbHV ORF66 qPCR plasmid NQC-2	–	32.18	–
AbHV ORF77 qPCR plasmid NQC-1	–	–	25.32
AbHV ORF77 qPCR plasmid NQC-2	–	–	32.97
Negative controls			
Uninfected abalone tissue	–	–	–
Molecular grade water	–	–	–

particular genotypes would never be detected when interpreting ORF49 in series with any other test.

4. DISCUSSION

In the context of demonstrating or certifying freedom from AbHV, the candidate abalone populations or stocks are apparently healthy and not experiencing an overt AVG outbreak. However, if AbHV is present, it is possible that, at the time of sampling, few individual abalone may experience clinical or pathological changes indicative of AVG even without prior evidence of active disease at the population level. The studied abalone were selected from apparently healthy sources to represent the spectrum of AbHV infection when demonstrating or certifying freedom. This resulted in low prevalences of AbHV across the study populations (<10%). The low number of infected abalone in these sources could have impaired the precision of the DSe estimation, but this was accounted for by using a large sample size (n = 1452). The few infected abalone in the study were likely sub-clinical with low viral load and non-homogeneous distribution of viral particles. Indeed, except for ORF49, the tests showed poor DSe (<60%), including histopathology (6.3%) which can only detect the advanced stages of the infection. The much higher DSe of ORF49 (90.04%) could not be explained by its ASe, which was equivalent to the other qPCRs (Table 2), or its ASp, which was lower than the other 2 qPCRs (Table 3). Corbeil et al. (2010) reported a slightly higher DSe for ORF49 (DSe = 96.7%, 95% CI: 82.7 to 99.4) when using abalone with both clinical signs and typical AVG histopathology as their reference infected population. It is reasonable to expect that the other tests evaluated in this study would also perform better when testing clinically infected abalone from disease events. The estimates of this report should be used primarily in the context of demonstration or certification of freedom.

The demonstration, and later the maintenance, of freedom requires routine monitoring of the target population to gather evidence that the infection is absent and remains absent (OIE 2018c). The accumulation of negative evidence over time increases and maintains the confidence that the infection is absent; however, a single positive finding, even false, eliminates it. Therefore, when demonstrating freedom, it is generally accepted that the detection protocol should be selected to minimise the probability of

Table 4. Posterior model estimates (median with 95% posterior credibility interval [PCI]) of diagnostic sensitivity and specificity, likelihood ratios of a positive and of a negative test result when detecting *Haliotid herpesvirus 1* (AbHV) with histopathology or qPCR using the primers/probe sets ORF49, ORF66 or ORF77, and their pairwise combination interpreted in parallel or in series. Within a column and a given type of interpretation (isolation, in parallel or in series), test parameters that share the same uppercase letter were not significantly different from each other at the 5% level

Diagnostic tests	Diagnostic sensitivity (%)			Diagnostic specificity (%)			Likelihood ratio of positive result			Likelihood ratio of negative result		
	Median	PCI 2.5th percentile	PCI 97.5th percentile	Median	PCI 2.5th percentile	PCI 97.5th percentile	Median	PCI 2.5th percentile	PCI 97.5th percentile	Median	PCI 2.5th percentile	PCI 97.5th percentile
Interpreted in isolation												
Histopathology	6.3	2.4	13.1	100.0[a]	99.7	100.0	122.7[a,b]	17.1	3573.0	0.938	0.870	0.977
qPCR-ORF49	90.0	59.2	99.7	99.8[a]	99.0	100.0	425.4[a]	78.9	11 800.0	0.100	0.003	0.410
qPCR-ORF66	59.1	47.6	71.5	97.9[b]	96.9	99.7	28.7[b]	17.7	217.5	0.418	0.291	0.535
qPCR-ORF77	33.7	23.5	45.5	98.6[b]	97.8	99.6	24.2[b]	13.4	87.1	0.672	0.552	0.776
Interpreted in parallel												
Histopathology & qPCR-ORF49	90.1[a]	61.1	99.7	97.7[b]	96.4	99.4	39.0[a]	26.1	113.8	0.095[a]	0.003	0.392
Histopathology & qPCR-ORF66	61.8	50.8	73.6	97.9	96.8	99.6	29.1[a,c]	18.3	175.9	0.390	0.269	0.503
Histopathology & qPCR-ORF77	38.2	27.7	49.9	98.5	97.7	99.5	26.1[a,c]	15.1	80.6	0.628	0.509	0.733
qPCR-ORF49 & qPCR-ORF66	96.0	82.6	99.9	97.7[a]	96.4	99.4	41.4[c]	27.4	148.7	0.041	0.001	0.176
qPCR-ORF49 & qPCR-ORF77	93.5	72.3	99.8	98.3[a,b]	97.3	99.3	55.7[b,c]	34.5	118.6	0.066	0.002	0.280
qPCR-ORF66 & qPCR-ORF77	67.4[a]	55.8	79.5	97.4[a,b]	96.2	99.4	25.6[a,b]	16.7	107.5	0.335[a]	0.209	0.454
Interpreted in series												
Histopathology & qPCR-ORF49	5.5	1.8	12.3	100.0	100.0	100.0	62 940.0[b]	3557.0	5 270 000.0	0.945	0.877	0.982
Histopathology & qPCR-ORF66	3.7	1.4	8.0	100.0[a]	100.0	100.0	4041.0[a]	513.0	155 000.0	0.963	0.920	0.986
Histopathology & qPCR-ORF77	2.1	0.7	4.8	100.0[a]	100.0	100.0	3167.0[a]	399.0	107 500.0	0.979	0.952	0.993
qPCR-ORF49 & qPCR-ORF66	51.9	33.3	66.7	100.0[a]	100.0	100.0	13 980.0[a,b]	2637.0	452 600.0	0.481	0.333	0.667
qPCR-ORF49 & qPCR-ORF77	29.4[a]	17.1	42.8	100.0[a]	100.0	100.0	10 930.0[a,b]	2028.0	317 800.0	0.706[a]	0.572	0.829
qPCR-ORF66 & qPCR-ORF77	25.4[a]	16.5	36.4	99.2	98.5	99.9	31.2	15.0	330.0	0.752[a]	0.641	0.842

finding a false-positive result. This is done by choosing the test or a combination of tests that has the highest LR^+ to optimise the strength of evidence provided by a positive finding (Caraguel et al. 2015). To maximise the strength of a positive finding, one may recommend running ORF49 in combination with another test to interpret their results in series. The interpretation in series classifies an abalone as positive if both tests are positive, or alternatively, as negative if at least 1 test is negative (Dohoo et al. 2012). However, ORF49 could not detect Tas2 or Tas3 AbHV genotypes (Table 3), and it would be impossible in theory for these 2 genotypes to yield a positive with ORF49 as well as with another test. No other test used individually or in combination provides a clear alternative to optimise the LR^+. Therefore, to standardise laboratory protocols, we recommend using the same detection protocol for demonstration and for certification of freedom (see below).

The certification of freedom from infection involves screening individual animals (or products) before they are translocated to a free population (OIE 2018c), and only animals testing negative are granted entry to minimise the risk of infection introduction (i.e. ruling-out). When certifying freedom, the detection protocol should be selected to minimise the probability of a false-negative result. The test or the combination of tests with the lowest LR^- should be selected to optimise the evidence provided by a negative finding (Caraguel et al. 2015). In this instance, we recommend running the pair ORF49 and ORF66 (in separate reactions) and interpreting their results in parallel (LR^- = 0.041, Table 4). When interpreting in parallel, an abalone is classified as positive if at least 1 test is positive, or alternatively, as negative if both tests are negative (Dohoo et al. 2012).

It is unknown if ORF49 or ORF66 will detect any new emerging AbHV genotype. We therefore recommend continuing the use of histopathology to investigate clinically suspect AbHV cases to detect any new genotypes in the future that are potentially not detectable by these qPCRs.

5. CONCLUSIONS AND RECOMMENDATIONS

We recommend the use of both the ORF49 and ORF66 primers/probe sets (in separate assays) and the interpretation of their results in parallel when demonstrating freedom from AbHV in an abalone population or when certifying individual abalone free from AbHV for trade or movement purposes. Histopathology may be useful in routine disease diagnosis to confirm clinically suspect cases and find any new emerging AbHV genotype(s) potentially not detectable by either ORF49 or ORF66.

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