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[Communication]

Evaluation of a Reverse Transcriptase Polymerase Chain Reaction Test and Virus Isolation on Field Samples Collected for the Diagnosis of Infectious Hematopoietic Necrosis Virus in Cultured Atlantic Salmon in British Columbia

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Abstract.—Infectious hematopoietic necrosis virus (IHNV) has been found to cause disease in cultured salmon of the Pacific Northwest region of North America. Diagnosis of IHNV by virus isolation (VI) can take over 2 weeks. Recently, a rapid reverse transcriptase (RT) polymerase chain reaction (PCR) test on fish tissues has been used for diagnosis. Test performances of the VI and RT PCR assays were compared using samples collected in the field. The effect of different storage conditions (tissue frozen with or without RNAlater [Ambion, Inc., Austin, Texas] versus fresh tissue) on the diagnostic tests was also evaluated. Based on the limited number of samples tested, the operating characteristics of RT PCR were very similar to those of VI; therefore, this method is likely suitable for testing field samples for IHNV. The ability of the tests to identify a positive fish ranged from 74% to 89%. Freezing samples at −80°C before testing did not negatively affect the performance of RT PCR or VI. However, due to reduced test performance, RNAlater frozen storage is not recommended without further investigation.

Infectious hematopoietic necrosis virus (IHNV) has been found to cause disease in wild and cultured salmon and trout and is native to wild salmonids of the Pacific Northwest region of North America (Troyer et al. 2000). In the summer of 2001, an IHNV outbreak was diagnosed in farmed Atlantic salmon *Salmo salar* in British Columbia (BC). Over a period of 22 months, infection spread to 36 salmon farms at which over 12 million fish died or were culled (Saksida 2006).

Clinical signs of IHNV in Atlantic salmon include severe petechial and ecchymotic hemorrhaging in the viscera, bloody ascites, and pale livers and gills (Traxler et al. 1998). These signs are not pathognomonic for IHNV, as other more common diseases such as furunculosis and vibriosis have similar presentations. As a result, laboratory testing is required to confirm suspicion of IHNV. The lack of a quick and

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TABLE 1.—Estimation of diagnostic Sensitivity (Se) and specificity (Sp) of a diagnostic test using a 2 × 2 table. Sensitivity = $a/a + c$. Specificity = $d/b + d$.

	True disease status		
Test results	Diseased	Not diseased	Total
Positive	a	b	$a + b$
Negative	c	d	$c + d$
Total	$a + c$	$b + d$	$a + b + c + d = n$

effective diagnostic tool was a significant contributing factor in the spread of the disease; an average of 15.7 d lapsed between suspicion of the disease and laboratory diagnosis (Saksida 2006). At the time of the outbreak, IHNV was diagnosed using virus isolation (VI) on tissue culture, followed by either an indirect fluorescent antibody test or a reverse transcriptase (RT) polymerase chain reaction (PCR) to confirm IHNV from the cell cultures (Hostnik et al. 2002).

Recently, an RT PCR based on a test developed at the U.S. Geological Survey's Western Fisheries Research Center (Batts and Winton 2000) has been performed at the BC Provincial Animal Health Centre (AHC) in Abbotsford. The IHNV RNA is extracted directly from infected fish tissue, reducing the time required for diagnosis. Initial laboratory testing of this RT PCR and preliminary field trials have been very encouraging, and the time between sample collection and diagnosis is less than 48 h. Despite such results, both VI and RT PCR diagnostic tests must be validated and thoroughly assessed on samples collected under field conditions.

Studies of IHNV have compared VI with RT PCR (Miller et al. 1998; Barlic-Maganja et al. 2002; Bergmann et al. 2002; Hostnik et al. 2002). Bergmann et al. (2002) applied the tests to experimentally infected rainbow trout *Oncorhynchus mykiss*. The other studies (Miller et al. 1998; Barlic-Maganja et al. 2002; Hostnik et al. 2002) applied the tests to field samples collected from rainbow trout farms but did not present the most important test performance parameters (Noordhuizen et al. 2001): diagnostic sensitivity (Se) and specificity (Sp). The diagnostic Se is the proportion of diseased animals that test positive. The diagnostic Sp is the proportion of nondiseased animals that test negative. Computations of these two parameters in the simplest situation are presented in Table 1. A test's diagnostic Se and Sp are different from (1) analytical sensitivity, which measures the lowest level of detectable pathogen the assay can identify as the pathogen, and (2) specificity, which measures the assay's ability to correctly identify a specific pathogen, thus minimizing the test's cross-reactivity with other pathogens (McClure et al. 2005). Some of the above reports have evaluated analytical sensitivity, but none have presented the tests' diagnostic Se and Sp. In addition, there are no reports of Se and Sp in the testing of farmed Atlantic salmon.

Until these two tests are validated, the screening test protocol for early detection of IHNV remains inefficient because the VI in cell culture must incubate for 7–10 d prior to diagnosis. The virus has the potential to spread within a site and to other sites during the period between fish sampling and diagnosis. A screening protocol using RT PCR would minimize the time to diagnosis and early harvest of infected fish.

The objectives of this study were twofold. The first was to estimate diagnostic Se and Sp of the VI and RT PCR tests to determine their reliability for detecting IHNV in farmed Atlantic salmon sampled in the field. The second objective was to evaluate the effects of different storage conditions (freezing with and without RNAlater [Ambion, Inc., Austin, Texas] versus fresh tissue testing) on diagnostic test performance.

Methods

Field Collection

Sample collection was conducted in September 2003. Samples were taken from three BC Atlantic salmon farms that represented different prevalence populations: (1) a farm undergoing an IHNV epizootic (fish with high IHNV prevalence; average weight = 1.7 kg), (2) a farm that had recently experienced an epizootic (fish with low IHNV prevalence; average weight = 5.0 kg), and (3) a farm that never had experienced an outbreak (fish with no IHNV; average weight = 1.5 kg). From farm 1, 50 fresh mortalities or moribund fish were sampled from 6 affected cages via cage removal by a mortality diver and an uplift system. From farm 2, 50 fish from 9 affected cages were taken by the mortality diver. From farm 3, 50 healthy fish from 1 pen were seined at the IHNV-negative site. Locations of the farms are shown in Figure 1.

All living fish were euthanized with a sharp blow to the head. All fish were examined for any gross clinical signs of disease, which were recorded for each fish. Using aseptic technique, the anterior kidney and spleen were excised and subdivided into six subsamples, each approximately 1 cm^3 when enough tissue was available. One spleen sample and one kidney sample were placed together into four Whirl-Pak bags (Nasco, Fort Atkinson, Wisconsin). The two remaining samples were placed in screw-top tubes containing RNAlater. The samples were coded using a randomly generated series of numbers so that their origin was unknown to the laboratory testers.

All samples were transported on ice to the Pacific

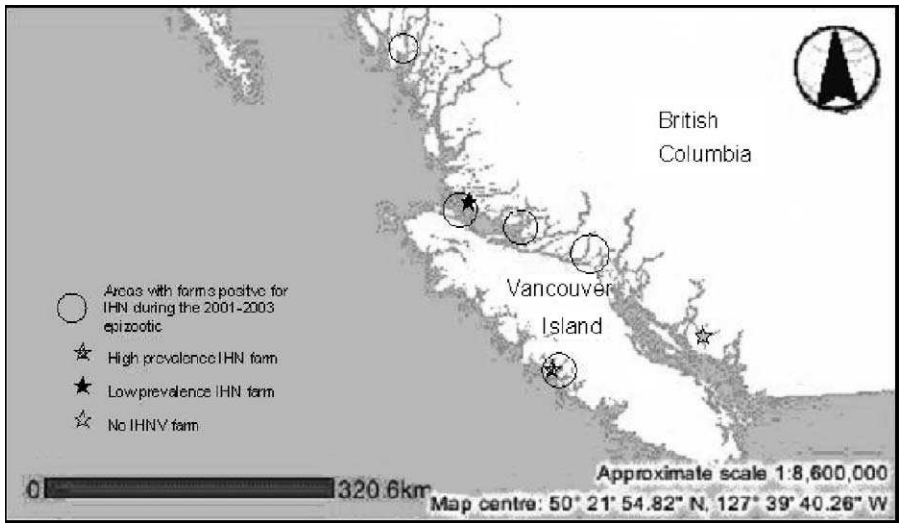

FIGURE 1.—Map of Atlantic salmon farm sites in BC that tested positive for IHNV infection during a 2001–2003 epizootic, and locations of three farms (high, low, and zero IHNV prevalence) sampled for a comparison of reverse transcriptase PCR and virus isolation tests.

Biological Station (PBS) in Nanaimo, British Columbia, on the day of sampling. One fresh tissue sample was kept on ice until tested by VI, and one was placed in an −80°C freezer for VI testing on frozen tissues. Samples (two neat and one frozen in RNAlater) from each fish tested by VI at PBS were shipped overnight on ice by courier to the AHC. One fresh sample was tested by RT PCR at the AHC immediately upon arrival. One fresh sample and one preserved in RNAlater were stored in an −80°C freezer at the AHC until RT PCR testing. Testing was coordinated so that all frozen samples were tested by VI and RT PCR at the same time to avoid a difference in storage time between laboratories. All frozen samples (with and without RNAlater) were tested within 3 months of collection. Sampling is summarized in Table 2.

Laboratory Testing

Cell culture and virus isolation.—The epithelioma papulosum cyprini (EPC) cell line was used for the detection of IHNV (Fijan et al. 1983). Uninfected monolayers were cultured at 15°C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California). Stock cells were split into multiwell plates for assay 24 h prior to use. Tissue was assayed for IHNV by 1:10 dilution of the tissue samples with Earle's balanced salt solution with antibiotics followed by homogenization with a Polytron generator (Brinkman Instruments Co., Rexdale, Ontario). Homogenates were centrifuged for 10 min at 2,000 × gravity, and 10-fold dilutions of the supernatants were inoculated on preformed monolayers of EPC cells (Traxler et al. 1999). After 1 h of absorption at 15°C, the monolayers were overlain with MEM containing methyl cellulose. The cultures were examined every second day for cytopathic effects typical of IHNV and were fixed and stained after a 7–10-d incubation at 15°C. The virus was quantified by counting plaques, and titers were calculated as plaque-

TABLE 2.—Summary of the number of fish sampled from each farm and the storage methods used 50 RNAlater samples taken for virus isolation (VI), but were not tested due to concern with infectivity of virus.

	Farm 1		Farm 2		Farm 3	
Storage method	RT PCR	VI	RT PCR	VI	RT PCR	VI
Fresh tissues	50	50	50	50	50	50
Frozen tissues	50	50	49[a]	50	50	50
Tissues frozen in RNAlater	50		50		50	

[a] Originally 50 samples, but one sample was accidentally destroyed.

forming units per milliliter per gram of tissue (Burke and Mulcahy 1980).

Reverse transcriptase PCR.—The RT PCR protocol was similar to that of Winton (2006), but thermocycling was optimized for the assay. Samples were extracted to obtain total RNA using the RNeasy extraction method (Qiagen, Mississauga, Ontario). Reverse transcription and external amplification were performed using Ready-to-Go RT PCR Beads (Amersham–GE Healthcare, Baie d'Urfe, Quebec). A previously sequenced IHNV was used as a positive control, and the negative control was a no-template assay. Positive and negative controls were used with all sample PCR testing. Briefly, the reverse transcription was carried out at 48°C for 45 min, followed by initial denaturation at 95°C for 2 min. Thermocycling (a total of 50 cycles) followed with denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Fifty cycles were used to maximize the analytical sensitivity of the assay. Final extension was for 7 min at 72°C. The amplification product was 786 base pairs (bp).

After completion of the external amplification protocol, a secondary "nesting" assay was completed using a set of primers that further amplified a sequence within the external bp target (external primers: 5′-TCAAGGGGGGAG TCCTCGA-3′, 5′-CACCG-TACTTTGCTGCTAC-3′; nesting primers: 5′-TTCGCAGATCCCAACAACAA-3′, 5′-GCGCA-CAGTGCCTTGGCT-3′). The nesting assay amplified a 323-bp product, and the amplification protocol consisted of 1 cycle at 95°C for 5 min; 50 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min.

Statistical Analysis

Interpretation of the test results.—Results of the VI were considered positive if reported as a low, medium, or high titer and negative if reported as no growth. The results of the RT PCR were considered positive if reported as either positive or weak positive.

Operating characteristics of a diagnostic test.—As Table 1 shows, the calculations for diagnostic Se and Sp are straightforward, assuming that the IHNV infection status of each fish is known. However, with IHNV, as with most other viruses, there is no known perfect test (gold standard) that correctly identifies infected and noninfected fish 100% of the time. Therefore, a gold standard must be defined to complete the estimations for Se and Sp. For this study, two different gold standards were defined.

Gold standards.—The first gold standard (GS clin–history) for IHNV detection was defined by the farm's health status based on observed clinical signs of disease, previous diagnosis and detection of IHNV, and the medical history of the farm as provided by the attending veterinarian. Fish that were IHNV positive were either freshly dead or dying fish from farm 1, which was experiencing high mortality due to clinical IHNV. Fish that were IHNV negative came from farm 3, which had no suspicion of IHNV during the period of the epizootic and was in a completely different water body than any of the outbreak areas shown in Figure 1. We present Se and Sp for both RT PCR and VI using this gold standard.

The second gold standard (GS VI×2) utilized all fish from farms 1–3 and was based on the fresh or frozen samples analyzed by VI. Fish that were IHNV positive were those that tested positive on VI in either fresh or frozen tissue. Fish that were IHNV negative were those that tested negative on VI in both fresh and frozen tissues. Because the GS VI×2 was based on virology results, the Se and Sp were estimated with this gold standard for the RT PCR test only.

Evaluating freezing and RNAlater storage effects.—McNemar's exact test was used to test for a difference in the proportion of positive test results between fresh and frozen samples (Dohoo et al. 2003). The effect of using frozen tissues instead of fresh tissues was evaluated for both the VI and RT PCR. In addition, the effect of freezing in RNAlater versus freezing without RNAlater was evaluated for RT PCR. Significant results ($P < 0.05$) indicated a storage effect on a test's ability to detect the virus.

Results

Test Sensitivity and Specificity

The Se and Sp of VI and RT PCR using the two different gold standards are presented in Table 3. Although infectivity of some viruses has been maintained in RNAlater at room temperature (Uhlenhaut and Kracht 2005), VI of IHNV was not performed on tissues stored in RNAlater at −80°C, as the infectivity of the virus frozen in preservative is unknown.

Freezing and RNAlater Storage Effects

The 2 × 2 tables for frozen versus fresh tissues and frozen tissue with and without RNAlater are presented in Table 4. Freezing had no significant effect on the results of the RT PCR (McNemar's exact test $P = 1.00$) or VI (McNemar's exact test $P = 0.38$). However, there were more positive results for VI when the tissues were frozen than when they were fresh (Table 4). For the RT PCR, the proportion of positive results was significantly lower (McNemar's exact test $P = 0.01$) for tissue

TABLE 3.—Sensitivity and specificity of virus isolation (VI) and RT PCR for different storage methods and two different gold standards. N/A = not applicable because gold standard is based on the results of the virus isolation test with fresh and frozen tissues.

		GS clin–history		GS VI×2 (all farms)	
Test	Storage method	Farm 1 Se	Farm 3 Sp	Se	Sp
VI	Fresh	0.80 (0.66–0.90)	1 (0.93–1.00)	N/A	N/A
	Frozen	0.86 (0.73–0.94)	1 (0.93–1.00)	N/A	N/A
RT PCR	Fresh	0.88 (0.76–0.95)	1 (0.93–1.00)	0.89 (0.76–0.96)	0.92 (0.86–0.97)
	Frozen	0.88 (0.75–0.95)	1 (0.93–1.00)	0.89 (0.75–0.96)	0.92 (0.86–0.97)
	Frozen in RNAlater	0.74 (0.60–0.85)	1 (0.93–1.00)	0.82 (0.68–0.92)	0.98 (0.93–1.00)

stored frozen in RNAlater than for tissue stored frozen without preservative.

Discussion

In our study, both VI and RT PCR appeared to perform mildly well. The range of Se for both tests was 0.74–0.89, indicating that the tests missed over 10% of positive fish. For both tests, Sp was perfect when compared with the first gold standard, GS clin–history. The Sp of the RT PCR test was imperfect for all three farms based on GS VI×2. Using the two gold standards has led to bias in the results (Brenner and Gefeller 1997). Defining infection status on samples from perfectly healthy farms and highly diseased farms can introduce bias in two different ways. First, some of the fish in farm 1 (high prevalence) may have died from causes other than IHNV (e.g., failure to thrive, predator wounds). These fish would probably have tested negative, although the gold standard would have inappropriately counted them as IHNV positive rather than negative. This misclassification would falsely reduce the Se and Sp of the tests. Second, if a fish was selected from a site that had just started to show signs of IHNV and if the fish had a very low viral titer, it might not test positive on the available tests, resulting in a loss of sensitivity. Because fish were chosen from only farm 1 for positive fish and only from farm 3 for negative fish, test performance results using this gold standard might be better than those produced by an assessment using the entire spectrum of disease states naturally present in production populations (i.e., no infection, early population infection, subclinical population infection, rapid propagation of infection, maximal morbidity–mortality of the outbreak, and recovery; McClure et al. 2005). The GS VI×2 incorporated a low–moderate prevalence group that would help minimize the bias based on population selection. Unfortunately, bias may exist due to the assumption that VI on both fresh and frozen samples would yield the true disease status of each fish.

Test characteristics of RT PCR on fresh or frozen samples were very similar to those of the VI test currently used to diagnose IHNV. The rapidity of using RT PCR directly on fish tissues is vital for Atlantic salmon farms given the short time from exposure to onset of clinical signs and mortality. The shorter

TABLE 4.—Effects of freezing and storing frozen in RNAlater on the diagnostic test results from all three farms (VI = virus isolation).

			Result from comparison tissue (in bold)		
Test	Tissue type	Result	Positive	Negative	Total
			Fresh tissue		
VI	Frozen	Positive	40	4	44
		Negative	1	105	106
		Total	41	109	150
			Fresh tissue		
RT PCR	Frozen	Positive	45	2	47
		Negative	2	100	102
		Total	47	102	149
			Tissue frozen in RNAlater		
RT PCR	Frozen	Positive	37	10	47
		Negative	1	101	102
		Total	38	111	149

diagnosis time will permit earlier management changes and reduction in the spread of disease. It should be noted that these results are based on a small number of fish from only three farms. More samples from many different farms would help reduce the standard error of the Se and Sp estimates and may confirm that RT PCR is an appropriate test for diagnosing IHNV.

Although based on a small number of samples, there was a significant difference between the Se of the RT PCR stored frozen with and without RNAlater using GS clin–history. The Se of the RT PCR frozen in RNAlater using GS VI×2 was less than that of the other tests. Many (8 of 45) samples that had negative RT PCR results when stored frozen in RNAlater were positive on the VI test. RNAlater is an RNA preservative used to protect against RNA degradation by RNases released by the cell. Because it should preserve the viral RNA, the Se of the RT PCR test should be maximized (Blacksell et al. 2004; Uhlenhaut and Kracht 2005). Unfortunately, in our study, this was not the case. Why this particular storage method reduced the Se of the RT PCR is unclear.

McNemar's exact test was used to determine whether there was a significant effect of freezing the samples (with and without RNAlater) before testing relative to testing fresh samples. The only significant difference was between frozen samples stored with and without RNAlater and tested by RT PCR, as discussed above. However, it is interesting that VI identified more positive samples in frozen tissues than in fresh tissues. Hostnik et al. (2002) found a somewhat opposite effect in that repeated freezing and thawing reduced the viral titer in the VI cell culture supernatants until IHNV was undetectable in VI after 13 freeze–thaw cycles. It is possible that IHNV was not homogenously distributed throughout the spleen and kidney in our fish and that not all pieces sectioned contained equal amounts of virus, accounting for differences in test results on the same fish. Because there was no significant difference between frozen and fresh samples tested by VI, freezing at −80°C (without RNAlater) appears to be a suitable method of storage if samples cannot be tested immediately.

Based on the limited number of samples tested, the operating characteristics of the RT PCR were very similar to those of VI; therefore, this method is likely suitable for field testing of fish for IHNV. Freezing samples before testing does not negatively affect the ability of RT PCR or VI to identify positive samples. However, due to reduced Se of the RT PCR test, RNAlater frozen storage is not recommended without further investigation. In the future, repeatability of the tests at the same laboratory should also be evaluated. A larger number of samples collected from numerous farms would also decrease the uncertainty in the estimates and allow for new methods of analysis, including test performance estimates modeled without the use of a gold standard.

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References

Barlic-Maganja, D., M. Strancar, P. Hostnik, V. Jencic, and J. Grom. 2002. Comparison of the efficiency and sensitivity of virus isolation and molecular methods for routine diagnosis of infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus. Journal of Fish Diseases 25:73–80.

Batts, W. N., and J. R. Winton. 2000. Diagnostic manual for aquatic animal diseases, part 2. Office International des Epizooties, Paris.

Bergmann, S. M., E. Ariel, H. F. Skall, D. Fichtner, H. J. Schlotfeldt, and N. J. Olesen. 2002. [Comparison of methods for detection of an infection with different isolates of infectious haematopoietic necrosis virus (IHNV)]. Berliner und Muenchener Tieraerztliche Wochenschrift 115:385–389.

Blacksell, S. D., S. Khounsy, and H. A. Westbury. 2004. The effect of sample degradation and RNA stabilization on classical swine fever virus RT-PCR and ELISA methods. Journal of Virological Methods 118:33–37.

Brenner, H., and O. Gefeller. 1997. Variation of sensitivity, specificity, likelihood ratios and predictive values with disease prevalence. Statistics in Medicine 16:981–991.

Burke, J., and D. Mulcahy. 1980. Plaquing procedure for infectious hematopoietic necrosis virus. Microbiology 39:872–876.

Dohoo, I. R., W. Martin, and H. Stryhn. 2003. Veterinary epidemiologic research, 1st edition. AVC, Charlottetown, Prince Edward Island, Canada.

Fijan, N., D. Sulimanovic, M. Bearotti, D. Musinie, L. D. Zwillenberg, S. Chilmonczyk, J. F. Vantherot, and P. deKinklin. 1983. Some properties of epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Annals of Virology 134:207–220.

Hostnik, P., D. Barlic-Maganja, M. Strancar, V. Jencic, I. Toplak, and J. Grom. 2002. Influence of storage temperature on infectious hematopoietic necrosis virus detection by cell culture isolation and RT-PCR methods. Diseases of Aquatic Organisms 52:179–184.

McClure, C. A., K. L. Hammell, I. R. Dohoo, H. Stryhn, and L. J. Hawkins. 2005. Application of surveillance data in

evaluation of diagnostic tests for infectious salmon anemia. Diseases of Aquatic Organisms 63:119–127.

Miller, T. A., J. Rapp, U. Wastlhuber, R.W. Hoffmann, and P. J. Enzmann. 1998. Rapid and sensitive reverse transcriptase–polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cells. Diseases of Aquatic Organisms 34:13–20.

Noordhuizen, J. P. T. M., K. Frankena, M. V. Thrusfield, and E. A. M. Graat. 2001. Application of quantitative methods in veterinary epidemiology, 2nd edition. Wageningen Press, The Netherlands.

Saksida, S. M. 2006. Infectious haematopoietic necrosis epidemic (2001 to 2003) in farmed Atlantic Salmon *Salmo salar* in British Columbia. Diseases of Aquatic Organisms: 72:213–223.

Traxler, G. S., E. Anderson, S. E. LaPatra, J. Richard, B. Shewmaker, and G. Kurath. 1999. Naked DNA vaccination of Atlantic salmon *Salmo salar* against IHNV. Diseases of Aquatic Organisms 38:183–190.

Traxler, G. S., M. L. Kent, and T. T. Poppe. 1998. Viral diseases. Pages 36–45 *in* M. L. Kent and T. T. Poppe, editors. Diseases of seawater netpen-reared salmonid fishes. Canadian Department of Fisheries and Oceans, Science Branch, Nanaimo, British Columbia.

Troyer, R. M., S. E. LaPatra, and G. Kurath. 2000. Genetic analyses reveal unusually high diversity of infectious haematopoietic necrosis virus in rainbow trout aquaculture. Journal of General Virology 81:2823–2832.

Uhlenhaut, C., and M. Kracht. 2005. Viral infectivity is maintained by an RNA protection buffer. Journal of Virological Methods 128:189–191.

Winton, J. R. 2006. Diagnostic manual for aquatic animal diseases 2006, part 2. Office International des Epizooties, Paris.