A Critique on Infectious Salmon Anemia Virus Detection Capabilities of the Canadian Fish Health Protection Regulations

S.A. Goldes, MSc.
2011

INTRODUCTION

Atlantic salmon eyed eggs have been imported almost yearly into British Columbia during the period 1985 until 2010 from a number of countries including the USA, UK , Iceland and also from Atlantic Canada (BC Atlantic Imports). Source aquaculture facilities, except for more recent imports from Iceland (where the definition of lot was not achieved, however the rest of the procedures were the same) were certified free of specified piscine pathogens of concern according to testing protocols mandated in the Canadian Fish Health Protection Regulations (CFHPR). Immediately prior to shipment, eyed eggs were disinfected according to the CFHPR iodophor disinfection protocol. Certification and iodine egg disinfection together are the pillar's of Canada's defense against the introduction of exotic piscine diseases such as Infectious Salmon Anemia (ISA). In order to protect British Columbia's wild aquatic ecosystems and aquaculture industries these measures must provide a high level of security. Close scientific examination of these regulatory measures however raises concerns that in-practice, these measures fail to provide the high level of protection required. This discussion focuses on issues with (1) ISA detection using cell culture, (2) sample size, and (3) iodine surface disinfection, however there remain many other weaknesses.

(1) INFECTIOUS SALMON ANEMIA VIRUS DETECTION USING CELL CULTURE

Infectious salmon anemia was first reported in Norway in 1984 (Plarre *et al.*, 2005), coincidentally one year prior to the first importation of Atlantic salmon eyed eggs into BC from Scotland in 1985. Atlantic salmon eyed eggs were imported into BC almost yearly from the UK from 1985 to 1993, despite the fact that Norwegian ISAV epizootics were ongoing and also with the knowledge that detecting ISAV by cell culture was not possible (Mjaaland *et al.*, 1997). Thus during the early years of Atlantic salmon imports (1985 to 1995) the Canadian government understood that the CFHPR protocols were incapable of detecting ISAV. At that time it was naively thought that ISA was limited to Norway alone and that Scotland was sufficiently distant. Later this assumption was proven erroneous, because shortly after ISAV cell culture became possible in 1995 (Dannevig *et al.*, 1995a; Dannevig *et al.*,

1995b), ISAV was detected in: New Brunswick in 1997 (Mullins *et al.*, 1998), Scotland in 1998 (Rodger *et al.*, 1998) and Maine, USA in 2001 (Bouchard *et al.*, 2001), as well as several other countries. It is also worth mentioning, that despite the devastation ISA has caused aquaculture elsewhere, ISA is still not listed in the current CFHPR as a pathogen of concern.

The CFHPR specifies that the initial testing for viral pathogens of concern is to be done using cell culture. In this test diluted and filtered extracts of virus-infected tissue (or ovarian fluid) are placed on the surface of a single cell layer deep, carpet of fish cells. A laboratory worker visually inspects the fish cells (cell lines) for presence of abnormalities (referred to as cytopathic effect (CPE)) indicative of viral pathology over a period of 14-21 days. This visual analysis is very subjective, requires substantial experience and is likely to vary between individuals since there is no licensing or standardization of training requirements. Ultimately all virus detection depends on the knowledge of the virologist. Inexperience can lead to a high number of false negatives.

Although ISAV outbreaks started in 1984 in Norway, it wasn't until eleven years later that the virus could be detected in cell culture. In 1995 Dannevig's laboratory developed a cell line from Atlantic salmon head kidney tissue (SHK1) in which ISAV could be cultured and also caused CPE (Dannevig *et al.*, 1995a; Dannevig *et al.*, 1995b). As previously mentioned, the use of SHK1 cells led to the detection of ISAV in many countries. Shortly thereafter it was found that ISAV could be detected in chinook salmon embryo (CHSE214) as well as several newly developed cell lines: Atlantic salmon (AS), Atlantic salmon head kidney leukocytes (TO) (Bouchard *et al.*, 1999; Kibenge *et al.*, 2000; Nicholson *et al.*, 1973; Wergeland *et al.*, 2001). The CFHPR mandates that two of the following cell lines be used: rainbow trout gonad (RTG2), chinook salmon embryo (CHSE214), epithelioma papulosum cyprini (EPC) or fathead minnow (FHM). Of these cell lines, ISAV has only been cultured successfully in CHSE214 cells. Note that the CFHPR does not require the use of CHSE214 cells since other combinations of other specified cell lines could technically suffice. In the case of Atlantic salmon imports I believe CHSE214 cells were used, however not all records are available. The use of SHK1 cells was also not mandated by the CFHPR during the certification of Atlantic salmon source facilities, despite the fact that in 1997 the Office International des Epizooties (OIE) Aquatic animal manual specified that SHK1 cells were the cell line of choice for detection of ISAV [*in* (Bouchard *et al.*, 1999)]. SHK1 cells however do not always reliably detect ISAV. Twenty seven percent (27%) of isolates tested (Europe and Eastern Canada) did not grow in SHK1 cell lines (Mjaaland *et al.*, 2002). In addition CPE can be slow to develop and visually difficult to detect in SHK1 cell lines (Falk *et al.*, 1998). Thus even with the most sensitive cell line, false negatives are still a problem. Consequently, to detect ISAV by cell culture several cell lines (SHK1, TO, ASK, CHSE214) need to be used concurrently.

Atlantic salmon were tested using CHSE214 cells, however this cell line has its own issues with ISAV. First, not all genotypes of ISAV will grow in CHSE214 cell lines. Of thirteen ISAV isolates that caused CPE in SHK1 cells, only 53% also caused CPE in CHSE214 cell lines (Kibenge *et al.*, 2000). Another study found that of eight ISAV genotypes tested (included Norway, Scotland and Eastern Canada) only one (Canadian genotype) caused CPE in CHSE214 cells

(Munir *et al.*, 2004). In another study, SHK1 cell lines detected 30 positives, while CHSE214 cell lines detected only 13 (43%) (Merrill, 2003). In a two lab comparison using CHSE214 cell lines, one laboratory failed to detect any $ISAV^{+}$ positives while the other lab found all samples to be positive (Rolland *et al.*, 2005). In addition on CHSE214 cell lines, some ISAV isolates do cause CPE when the cell lines are first inoculated with tissue filtrate. However when the cells and supernatant from the apparently negative cell culture wells were removed and re-inoculated onto fresh cell lines, CPE occurred and as many as three more re-inoculations were necessary to classify samples as negative (Kibenge *et al.*, 2000). Re-inoculations, known as blind passes, are not required by the CFHPR, consequently samples with no initial CPE would be classified as negative. CPE also develops more slowly in CHSE214 as compared to AS and SHK1 cell lines. At 14 days post inoculation CPE was present in 93.8% of ISAV positive samples in SHK1 cells, but only 23% of ISAV positives samples using CHSE214 cell lines (Rolland *et al.*, 2005). This means that if CHSE214 cell lines are only kept for 14 days (as permitted under the CFHPR), then there would be a high number of false positives.

(2) SAMPLING

The CFHPR specifies the number of fish to be tested at each step during facility certification. Sample numbers are calculated by referring to the output of an algorithm developed by Ossiander in 1973 (Ossiander *et al.*, 1973). All sampling is done assuming that 5 percent of the fish in the population are at least carriers of the pathogen of concern. A carrier is any fish having more one or more ISA virus particles on board and who is otherwise healthy. Using five percent prevalence assumption, Ossiander's algorithm states that a sample of 60 fish should be taken from fish populations over 100,000 fish (Table 1). This is essentially the same sampling table presented in the CFHPR. Ossiander's sampling algorithm assumes that the diagnostic test can detect a carrier, which in fact is erroneous. In addition the CFHPR's assumption that five percent of the population are pathogen carriers needs to be revisited.

Implicit in the Ossiander algorithm is that the diagnostic test used to detect the pathogen is capable of detecting a carrier. First it is well known than no veterinary diagnostic test can achieve this extremely low level of sensitivity. Even the RTPCR test for ISAV may fail to detect carriers (Devold *et al.*, 2000). For ISAV detection by cell culture the lower limit of sensitivity is unknown both in the research laboratory and in a diagnostic laboratory. We do however know that in-practice the ability of different laboratories to detect ISAV using cell lines varies greatly. When two laboratories were blind-tested for their ability to detect ISAV by cell culture, the sensitivity in one of the laboratories ranged from 0.92 to 0.96, while the other laboratory had a perfect sensitivity of 1.00 (Nerette *et al.*, 2005). In another study, one laboratory failed to detect any ISAV in kidney tissue by cell culture (using SHK1 cell lines), whereas the other laboratory detected 25 ISAV positive fish in identical samples (Merrill, 2003). In a third

study, again a two laboratory comparison, it was found that the mean sensitivity of cell culture for ISAV was only 67 percent (McClure *et al.*, 2005). This data indicates that in some hands the sensitivity of ISAV detection using cell culture was very low. Consequently Ossiander's assumption of perfect test sensitivity is not realistic. By extension, if the test sensitivity is not perfect, then the sample size must be increased. I am not an epidemiologist, however sample size calculations which factor in test sensitivity have been developed (Humphry *et al.*, 2004). With current knowledge, the use of Ossiander's tables is outdated, thereby indicating that sample sizes specified in the CFHPR were/are too low.

The CFHPR mandates that samples sizes are calculated assuming five percent of the fish are carriers. The true prevalence of ISAV in wild and cultured fish populations is unknown, however in wild fish and healthy cultured fish the prevalence is likely to be lower than five percent. There is little published data on the prevalence of ISAV in asymptomatic fish at the farm level, however this data no doubt exists and should be used to generate a more realistic appraisal of prevalence. In Maine the prevalence of ISAV was 1.3 percent in cultured Atlantic salmon sampled from a region thought to be lightly infected (Gustafson *et al.*, 2008). In wild fish, distant from aquaculture facilities, the prevalence is likely to be lower than occurs on farms. In wild Scottish sea (brown) trout (*Salmo trutta*), the prevalence of ISAV was only 2.5 percent, when tested by cell culture. This data suggests that the five percent assumption is unrealistically low, leading again to insufficient sample size. Using Ossiander's table the appropriate sample size at the one percent level would be approximately 296 instead of the 60 (without correction for sensitivity) in a fish population over 100,000.

Finally it should be pointed out that farms have advance warning of their test dates, and it is well known amongst Fish Health Officials that it is rare to find moribund fish when they arrive to sample. This means the probability of detecting a pathogen is even further diminished since the most sensitive samples (moribund fish) are excluded from the sample. Eliminating prior notification would help increase the sensitivity of ISAV testing.

(3) Iodine Disinfection of Salmonid Eggs

The CFHPR and the Atlantic salmon Import Policy required that all Atlantic salmon eyed eggs imported into BC were/are surface disinfected with an iodine-containing solution (100 ppm active iodine for 10 minutes) just prior to shipment. It is generally felt that iodine disinfection is highly efficacious at killing egg-surface associated virus, however it is surprising how little research has been done to verify this assumption.

Iodine disinfection is applied only to the outside of the egg, and the iodine does not penetrate into the egg in sufficient quantity to kill pathogens within. This is a concern because some piscine pathogens including the bacteria *Renibacterum salmoninarum* (causative agent of bacterial kidney disease (BKD)) and *Flavobacterium*

psychrophilum (causative agent of coldwater disease) are known to be vertically transmitted inside the salmonid egg (Brown *et al.*, 1997; Evelyn *et al.*, 1984). Strong circumstantial evidence for the vertical transmission of ISAV comes from Chile. Salmonids are exotic to Chile, however live salmon were introduced into the wild in Chile in 1921 (Montero *et al.*, 2006). The importation of salmonid eggs for aquaculture began around 1985 and the Chilean aquaculture industry greatly expanded from 1996 on (Montero *et al.*, 2006). It is likely that earlier egg importations were not surface disinfected, however more recent importations likely were. Eggs were imported from North America or Europe. ISAV was isolated in Chile for the first time in farmed moribund coho (*Oncorhnychus kisutch*) in 2001 (Kibenge *et al.*, 2001), and in farmed Atlantic salmon in 2008 (Godoy *et al.*, 2008). Since ISAV's first detection, ISAV has decimated farmed salmon populations and has led to a dramatic collapse of a large number of Chilean aquaculture facilities (Asche *et al.*, 2009). The fact that these isolates originated from the northern hemisphere, suggests but does not prove vertical transmission took place.

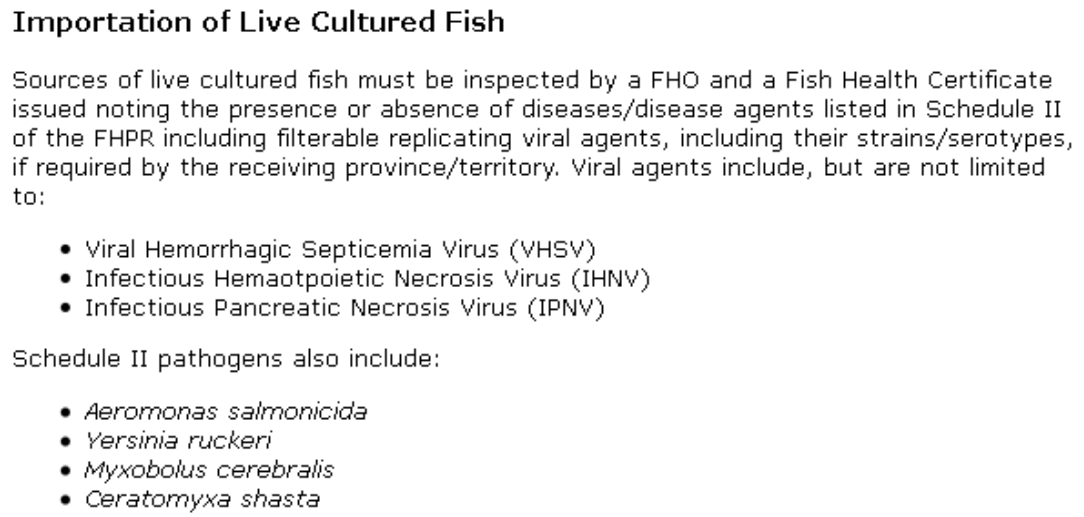
Originally iodine egg disinfection was adopted by aquaculture in the 1970's to kill pathogenic bacteria on the egg's surface (Tuttle-Lau *et al.*, 2010), particularly to prevent salmonid diseases such as furunculosis (*Aeromonas salmonicida*) and enteric redmouth disease (*Yersinia ruckeri*). At the time iodine disinfection was developed, little was known about viral diseases since viral detection methods were in their infancy. Over time iodine egg disinfection was assumed, but not proven, to be equally effective against viruses. Little attention was however given to research data that demonstrated otherwise, both for bacteria and viruses. Though iodine disinfection has greatly reduced the number of outbreaks of egg-surface associated bacterial diseases, it fails to kill all live bacteria present. Steelhead (*Oncorhynchus mykiss*) eggs were artificially coated with a piscine pathogenic bacterium known as *Flavobacterium psychrophilium*, and thereafter disinfected in 100 ppm iodine for 30 minutes (20 minutes longer than the CFHPR require). While the kill rate was high (98%), two percent of the bacteria survived (Brown *et al.*, 1997). This information shows that for bacteria, the iodine disinfection is incomplete. In the case of viruses the evidence on iodophors efficacy is similarly scant, but does indicate that iodine disinfection against piscine viruses is also incomplete. In one study ovarian fluid from CFHPR-certified $IHNV^-$ rainbow trout green (freshly spawned eggs) and eyed eggs was artificially loaded with 10^6 PFU/mL IHNV (equivalent to a moderate to high IHNV titer (load) as seen in feral sockeye (Mulcahy *et al.*, 1983)), the green eggs (n=300, four replicates per treatment for each of green and eyed eggs [normally thousands of eggs are disinfected at a time]) were then fertilized, and both green and eyed eggs were disinfected using the CFHPR protocol of iodine disinfection using 100 ppm iodine for 10 minutes (Goldes *et al.*, 1995). After disinfection, eggs were rinsed in pathogen-free water and finally placed in a suspension of gently stirred epithelioma papulosum cyprini (EPC) cells for 10 minutes. It was theorized that live cell associated virus would adhere to and infect the EPC cells. The suspended EPC cells were then placed into tissue culture plates and visually assessed for IHNV-mediated CPE. Iodine disinfection markedly (99.98%) reduced the number of live virions on the surface both green and eyed eggs, however it did not eliminate all live virus on the the surface of the eggs. In fact live IHNV was cultured in 75% of the treatment replicates in <u>both</u> green and eyed eggs. This data suggests that even when eggs were disinfected under the best of laboratory conditions, it fails to

eradicate IHNV on the egg surface. While iodine disinfection has not been shown to be complete under laboratory conditions, the protocol is likely to be much less effective under farm conditions. Freshly spawned eggs (green eggs) become contaminated with excess sperm, blood, bile and feces from the parents when fish are manually spawned in aquaculture. This material may carry ISA. In addition freshly spawned eggs have a 10 µm deep adhesive organic layer over the surface (Flugel, 1964). These organic materials may harbor virus and conceal its presence such that iodine cannot access and kill it. Organic matter also inhibits the disinfecting ability of iodine. The addition of 1% calf serum (organic material used in tissue culture media) to an aqueous solution of the commercial iodophor Betadine (iodine releasing disinfectant), the available iodine concentration dropped 62 percent, from 50 to approximately 19 ppm iodine (Elliott *et al.*, 1978). Consequently the efficacy of iodine disinfection of green eggs is likely to be less complete than with eyed eggs (which are much cleaner). Another practical problem with iodine egg disinfection is that thousands of eggs are disinfected at a time. When iodine levels were tested at the bottom of the egg mass being disinfected under hatchery conditions, it was found that iodine levels dropped 70% (from 105 to 30 ppm) (Chapman *et al.*, 1992). Consequently as practiced, the efficacy iodine disinfection likely varies greatly within each group of eggs disinfected. In summary this data indicates that iodine egg disinfection as specified under the CFHPR likely does not prevent the 'vertical' transmission of ISAV on the exterior of the egg, and given the limitations of iodine disinfection in practice the efficacy of iodine disinfection is questionable. It is also important to remember that iodine disinfection does not kill ISAV present inside the egg, and it is unknown whether ISAV is in this location. Given that some piscine pathogens are transmitted inside the egg, it should be conservatively assumed until otherwise proven that this is the case for ISAV.

CONCLUSIONS

Every precaution must be taken to prevent the introduction of ISA in BC's ecosystems. While research studies have shown little evidence for morbidity or mortality in pacific salmonids and other non-salmonids(McClure *et al.*, 2004; Nylund *et al.*, 1997; Rolland *et al.*, 2003; Snow *et al.*, 2002), this may not be the case in BC where native fish are immunologically naïve to ISAV and given current stressful environmental conditions (pollution, habitat destruction etc.). Stress plays a key role in causing disease. BC's fish populations are in danger and adding one more stressor (ISAV) may make a difference in the survival of endangered aquatic species and sub-populations. The data presented herein (variable sensitivity of CHSE214 cells, inadequate sample sizes, ineffectiveness of iodine disinfection etc.) suggests that the current CFHPR do not provide a high level of regulatory security against the introduction of ISAV into British Columbia.

Figure 1.



Importation of Live Cultured Fish

Sources of live cultured fish must be inspected by a FHO and a Fish Health Certificate issued noting the presence or absence of diseases/disease agents listed in Schedule II of the FHPR including filterable replicating viral agents, including their strains/serotypes, if required by the receiving province/territory. Viral agents include, but are not limited to:

- Viral Hemorrhagic Septicemia Virus (VHSV)
- Infectious Hemaotpoietic Necrosis Virus (IHNV)
- Infectious Pancreatic Necrosis Virus (IPNV)

Schedule II pathogens also include:

- *Aeromonas salmonicida*
- *Yersinia ruckeri*
- *Myxobolus cerebralis*
- *Ceratomyxa shasta*

Figure 1. Pathogens of concern listed in the CFHPR (http://www.dfo-mpo.gc.ca/science/enviro/aah-saa/regulation-reglements-eng.htm#xii)

Table 1.

Number of fish to be sampled when assumed prevalence of detectable infection is:		
Population Size	**5%**	**10%**
50	29	20
100	43	23
250	49	25
500	54	26
1,000	55	27
2,500	56	27
5,000	57	27
10,000	57	27
100,000	57	27
Over 100,000	60	30

Table 1. CFHPR specifications for sample size determination. Sample size required to detect one or more infected specimens in populations (lots) with an assumed minimum prevalence of detectable infection of 5 and 10%. Calculations are based on a 96 percent level of confidence (http://www.dfo-mpo.gc.ca/science/enviro/aah-saa/regulation-reglements-eng.htm#xii)

Literature Cited

Asche, F., Hansen, H., Rangnar, T., and Tveteras, S. (2009). The salmon disease crisis in Chile. *Marine Resource Economics* **24,** 405-411.

Bouchard, D., Keleher, W., Opitz, H. M., Blake, S., Edwards, K. C., and Nicholson, B. L. (1999). Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. *Dis Aquat Organ* **35**(2)**,** 131-7.

Bouchard, D. A., Brockway, K., Giray, C., Keleher, W., and Merrill, P. L. (2001). First report of infectious salmon anemia (ISA) in the United States. *Bulletin of the European Association of Fish Pathologists* **21**(2)**,** 86-88.

Brown, L. L., Cox, W. T., and Levine, R. P. (1997). Evidence that the causal agent of bacterial coldwater disease Flavobacterium psychrophilum is transmitted within salmonid eggs. *Diseases of Aquatic Organisms* **29,** 213-218.

Chapman, P. F., and Rogers, R. W. (1992). Decline in iodine concentration of iodophor during water hardening of salmonid eggs and methods to reduce this effect. *Progressive Fish-Culturist* **54,** 81-87.

Dannevig, B. H., Falk, K., and Namork, E. (1995a). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *J Gen Virol* **76 (Pt 6),** 1353-9.

Dannevig, B. H., Falk, K., and Press, C. M. (1995b). Propagation of infectious salmon anaemia (ISA) virus in cell culture. *Vet Res* **26**(5-6)**,** 438-42.

Devold, M., Krossoy, B., Aspehaug, V., and Nylund, A. (2000). Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. *Dis Aquat Organ* **40**(1)**,** 9-18.

Elliott, D. G., and Amend, D. F. (1978). Efficacy of certain disinfectants against infectious pancreatic necrosis virus. *Journal of Fish Biology* **12,** 277-286.

Evelyn, T. P. T., Ketcheson, J. E., and Prosperi-Porta, L. (1984). Further evidence for the presence of renibacterium salmoninarum in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection rate in water-hardened eggs. *Journal of Fish Diseases* **7,** 173-182.

Falk, K., Namork, E., and Dannevig, B. H. (1998). Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. *Dis Aquat Organ* **34**(2)**,** 77-85.

Flugel, H. (1964). Electron microscopic investigations on the fine structure of the follicular cells and the zona radiata of trout oocytes during and after ovulation. *Die Naturwissenschaften* **51**(23)**,** 564-565.

Godoy, M. G., Aedo, A., Kibenge, M. J., Groman, D. B., Yason, C. V., Grothusen, H., Lisperguer, A., Calbucura, M., Avendano, F., Imilan, M., Jarpa, M., and Kibenge, F. S. (2008). First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. *BMC Vet Res* **4,** 28.

Goldes, S. A., and Mead, S. L. (1995). Efficacy of iodophor disinfection against egg surface-associated infectious hematopoietic necrosis virus. *The Progressive Fish-Culturist* **57,** 26-29.

Gustafson, D. L. (1997). Ecological association of Tubifex tubifex in Montana waters. *In* "1997 Whirling Disease Symposium: Expanding the database: 1996 research progress reports", pp. 47-51. Whirling Disease Foundation, Bozeman, Montana.

Gustafson, L., Ellis, S., Bouchard, D., Robinson, T., Marenghi, F., Warg, J., and Giray, C. (2008). Estimating diagnostic test accuracy for infectious salmon anaemia virus in Maine, USA. *J Fish Dis* **31**(2)**,** 117-25.

Humphry, R. W., Cameron, A., and Gunn, G. J. (2004). A practical approach to calculate sample size for herd prevalence surveys. *Preventive Veterinary Medicine* **65,** 173-188.

Kibenge, F. S., Garate, O. N., Johnson, G., Arriagada, R., Kibenge, M. J., and Wadowska, D. (2001). Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. *Dis Aquat Organ* **45**(1)**,** 9-18.

Kibenge, F. S., Lyaku, J. R., Rainnie, D., and Hammell, K. L. (2000). Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. *J Gen Virol* **81**(Pt 1)**,** 143-50.

McClure, C. A., Hammell, K. L., Dohoo, I. R., and Gagne, N. (2004). Lack of evidence of infectious salmon anemia virus in pollock Pollachius virens cohabitating with infected farmed Atlantic salmon Salmo salar. *Dis Aquat Organ* **61**(1-2)**,** 149-52.

McClure, C. A., Hammell, K. L., Stryhn, H., Dohoo, I. R., and Hawkins, L. J. (2005). Application of surveillance data in evaluation of diagnostic tests for infectious salmon anemia. *Dis Aquat Organ* **63**(2-3)**,** 119-27.

Merrill, P. L. (2003). *International response to Infectious Salmon Anemia: prevention, control and eradication, New Orleans, LA, USA*.

Mjaaland, S., Hungnes, O., Teig, A., Dannevig, B. H., Thorud, K., and Rimstad, E. (2002). Polymorphism in the infectious salmon anemia virus hemagglutinin gene: importance and possible implications for evolution and ecology of infectious salmon anemia disease. *Virology* **304**(2)**,** 379-91.

Mjaaland, S., Rimstad, E., Falk, K., and Dannevig, B. H. (1997). Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (Salmo salar L.): an orthomyxo-like virus in a teleost. *J Virol* **71**(10)**,** 7681-6.

Montero, C., Konde, V., and Farinelli, F. (2006). A case study of salmon industry in Chile. *In* "Transfer technology for successful integration into the Global Economy" (J. Zhan, Ed.), Vol. UNCTAD/ITE/IIT/2005/12. United Nations conference on trade and development, Geneva.

Mulcahy, D., Pascho, R. J., and Jenes, C. K. (1983). Titre distribution patterns of infectious hematopoietic necrosis virus in ovarian fluids of hatchery and feral salmon populations. *Journal of Fish Diseases* **6,** 183-188.

Mullins, J. E., Growman, D., and Wadowska, D. (1998). Infectious salmon anaemia in salt water Atlantic salmon (Salmo salar) in New Brunswick, Canada. *Bulletin of the European Association of Fish Pathologists* **18**(4)**,** 110.

Munir, K., and Kibenge, F. S. (2004). Detection of infectious salmon anaemia virus by real-time RT-PCR. *J Virol Methods* **117**(1)**,** 37-47.

Nerette, P., Dohoo, I., Hammell, L., Gagne, N., Barbash, P., Maclean, S., and Yason, C. (2005). Estimation of the repeatability and reproducibility of three diagnostic tests for infectious salmon anaemia virus. *J Fish Dis* **28**(2)**,** 101-10.

Nicholson, B. L., and Byrne, C. (1973). An established cell line from the Atlantic salmon (Salmo salar). *Journal of the Fisheries Research Board of Canada* **30,** 913-916.

Nylund, A., Kvenseth, A. M., Kroosoy, B., and Hodneland, K. (1997). Replication of the infectious salmon anaemia virus (ISAV) in rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Fish Diseases* **20,** 275-279.

Ossiander, F. J., and Wedemeyer, G. (1973). Computer program for sample sizes required to determine disease incidence in fish populations. *Journal of the Fisheries Research Board of Canada* **30**(9)**,** 1383-1384.

Plarre, H., Devold, M., Snow, M., and Nylund, A. (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Dis Aquat Organ* **66**(1)**,** 71-9.

Rodger, H. D., Turnbull, T., Muir, F., Millar, S., and Richards, R. H. (1998). Infectious salmon anemia (ISA) in the United Kingdom. *Bulletin of the European Association of Fish Pathologists* **18**(4)**,** 115-116.

Rolland, J. B., Bouchard, D., Coll, J., and Winton, J. R. (2005). Combined use of the ASK and SHK-1 cell lines to enhance the detection of infectious salmon anemia virus. *J Vet Diagn Invest* **17**(2)**,** 151-7.

Rolland, J. B., and Winton, J. R. (2003). Relative resistance of Pacific salmon to infectious salmon anaemia virus. *J Fish Dis* **26**(9)**,** 511-20.

Snow, M., Raynard, R., Bruno, D. W., van Nieuwstadt, A. P., Olesen, N. J., Lovold, T., and Wallace, C. (2002). Investigation into the susceptibility of saithe Pollachius virens to infectious salmon anaemia virus (ISAV) and their potential role as a vector for viral transmission. *Dis Aquat Organ* **50**(1)**,** 13-8.

Tuttle-Lau, M. T., Phillips, K. A., and Gaikowski, M. P. (2010). Evaluation of the efficacy of iodophor disinfection of walleye and northern pike eggs to eliminate viral hemorrhagic septicemia virus. *In* "Fact Sheet", Vol. US Department of the Interior/US Geological Survey Fact Sheet: 2009-3107.

Wergeland, H. I., and Jakobsen, R. A. (2001). A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). *Dis Aquat Organ* **44**(3)**,** 183-90.