Vol. 42: 11–15, 2000 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published August 10

A dual infection of infectious salmon anaemia (ISA) virus and a togavirus-like virus in ISA of Atlantic salmon *Salmo salar* in New Brunswick, Canada

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ABSTRACT: Two viruses, infectious salmon anaemia (ISA) virus and a novel togavirus-like virus, were isolated from ISA disease outbreaks that were first reported as a new syndrome, haemorrhagic kidney syndrome (HKS) affecting farmed Atlantic salmon *Salmo salar* L. on the East coast of Canada. Laboratory confirmation of ISA diagnosis was initially complicated by isolation of only the togavirus-like agent using the CHSE-214 cell line. Here we demonstrate that a clinical sample from a disease outbreak of ISA contained a mixture of ISA virus and togavirus-like virus. Reverse transcriptase-polymerase chain reaction (RT-PCR) confirmed the presence of both viruses during serial passage of cultures in SHK-1 and CHSE-214 cells. Virus harvested at passage level 3 in both cell lines caused high mortalities and severe gross pathology consistent with ISA virus infection in experimentally inoculated Atlantic salmon parr (~35 g) in freshwater, beginning 12 d post inoculation. ISA virus was detected by virus isolation from kidney and liver tissues of all dead or moribund fish tested. A comparison of virus isolation, 1-step procedure RT-PCR and RNA dot-blot hybridization for detection of ISA virus (ISAV) in fish tissues showed virus isolation to have 100% sensitivity, followed by RT-PCR (66 and 28% sensitivity in kidney and liver, respectively), with RNA dot-blot hybridization as the least sensitive method (20 and 10% sensitivity in kidney and liver, respectively). No togavirus-like virus was detected in these samples by virus isolation. Moreover, another togavirus-like virus isolate grown in CHSE-214 cells in the absence of any other detectable pathogen was non-pathogenic in experimentally inoculated fish. This study confirms that the original ISA outbreaks in New Brunswick, Canada, were caused solely by ISAV.

KEY WORDS: ISA virus · Togavirus-like virus · Haemorrhagic kidney syndrome · Infectious salmon anaemia · Atlantic salmon

INTRODUCTION

Since July 1996, the salmon aquaculture industry of New Brunswick, Canada, has been struggling with infectious salmon anemia (ISA) (Byrne et al. 1998, Mullins et al. 1998). The pathological findings in Atlantic salmon *Salmo salar* L. with ISA include severe anemia, leucopenia, ascites, and haemorrhagic liver necrosis (Evensen et al. 1991). Because of a distinctly different manifestation of the disease in the early stages in the Bay of Fundy, in New Brunswick, the disease was initially termed haemorrhagic kidney syndrome (HKS) (Byrne et al. 1998). While much work was reported on the isolation and pathogenicity of ISA virus from outbreaks of ISA in New Brunswick (Mullins et al. 1998, Bouchard et al. 1999, Jones et al. 1999, Lovely et al. 1999), the presence of a novel togavirus-like virus that was commonly isolated from affected fish during the original ISA outbreaks and its possible role in the disease remained obscure. The present study was primarily designed to clarify the role of ISA virus and the togavirus-like virus in ISA outbreaks in farmed Atlantic salmon on the East coast of Canada. We describe here the analysis of a clinical sample (designated HKS-36)

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from 1 of the original outbreaks of ISA that had a dual viral infection demonstrated by cytopathic effects (CPE) of a togavirus-like virus on the CHSE-214 cell line and CPE of ISA virus on the SHK-1 cell line. In addition, because the RT-PCR method presented the only confirmatory diagnostic method with which to differentiate between togavirus-like virus CPE and ISA virus CPE in CHSE-214 cells, it was essential to establish the sensitivity of RT-PCR for ISA virus in fish tissues. Thus in this report, a 1 tube, 1-step procedure RT-PCR, and RNA dot-blot hybridization are compared to virus isolation for detection of ISA virus in fish tissues.

MATERIALS AND METHODS

Virus isolates and virus culture. ISA virus isolate 'Site A 98', togavirus-like virus isolate U14785(#6), and virus sample HKS-36 containing a mixture of ISA virus and togavirus-like virus used in this study were obtained from clinical outbreaks of ISA in farmed Atlantic salmon in the Bay of Fundy, New Brunswick, and the presence of viable virus was checked by inoculation of both salmon head kidney (SHK-1) cell line (Dannevig et al. 1995) and Chinook salmon embryo (CHSE-214) cell line (Fryer et al. 1965). The cell culture harvests were also tested by RT-PCR for presence of ISA virus and togavirus-like virus nucleic acids. Virus propagation and infectivity titrations were performed as previously described (Kibenge et al. 2000). For virus isolation from tissues, the cell monolayers in 24-well tissue culture plates were inoculated with 100 µl well^{-1} of tissue homogenates diluted 1:50 in serum-free medium and filtered through 0.45 µm syringe filters prior to use. Samples from fish inoculated either with ISA virus 'Site A 98' or HKS-36 viruses grown in CHSE-214 cells were inoculated on CHSE-214 cells. Samples from fish inoculated with HKS-36 viruses grown in SHK-1 cells were inoculated on SHK-1 cells. Cultures that were difficult to read or that were negative by CPE were passaged on fresh monolayers and/or were confirmed by RT-PCR.

RT-PCR. Viral RNA was extracted from 250 µl volumes of either tissue homogenates or cell culture lysates using TRIZOL LS reagent (Canadian Lite Technologies) following the manufacturer's protocol. The RT-PCR primers and conditions used to detect ISA virus by RT-PCR were as previously described (Kibenge et al. 2000). Molecular studies are currently underway to more specifically classify the togavirus-like virus isolates. However, the limited genetic sequence data obtained from a togavirus-like virus cDNA clone M107 were used to design specific RT-PCR primers which would yield a 330 bp product. The RT-PCR cycling conditions consisted of 1 cycle of cDNA synthesis and predenaturation at 55°C for 30 min and 94°C for 2 min, followed by 40 cycles each consisting of denaturation at 94°C for 15 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min using the Titan™ One Tube RT-PCR System kit (Roche Molecular Biochemicals) in a PTC-200 DNA Engine Peltier thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA).

RNA dot-blot hybridization. RNA dot-blot hybridization followed standard procedures (Kibenge 1992). Total RNA was processed from homogenized fish tissues using the TRIZOL method as described above. The radiolabeled DNA probe was prepared using the ISA virus RT-PCR product purified from low-melting agarose gels (Sambrook et al. 1989). The DNA probe was radiolabeled using the Random Priming System (Canadian Life Technologies) and 50 µCi of [α-^{32}P]deoxycytidine triphosphate (sp act approx. 3000 Ci mmol^{-1}) according to manufacturer's instructions.

Experimental infection of fish. The Atlantic salmon used in this study were Saint John River Atlantic salmon parr obtained from AquaBounty Farms, Prince Edward Island, Canada, with no record of ISA virus or togavirus-like virus isolation. The experimental procedures used in this study were performed in accordance with the guidelines of the Canadian Council on Animal Care (Olfert et al. 1993). A total of 405 fish were randomly assigned to treatment groups and tanks. The tanks were fibreglass-reinforced plastic tanks of 110 l capacity. Daily water temperature was maintained at 10.0 ± 1.0°C throughout the study. Fish belonging to each group were individually removed from the study tank and anaesthetised by immersion in an aerated solution of tricaine methane sulphonate (MS-222) (100 mg l^{-1}). Each fish was challenged by intraperitoneal inoculation (0.25 ml) and by oral gavage (0.25 ml). Control fish received freshly prepared cell culture maintainance medium. Infected fish, each received $10^{3.5}$ $TCID_{50}$/0.5 ml of virus inoculum. There were 113 fish in the uninfected control tank. All virus-inoculated tanks contained 75 fish each except for the tank with togavirus-like virus isolate U14785 (#6), which contained 67 fish. Fish were observed daily for mortality, abnormal behaviour, and external lesions. Moribund fish were euthanised prior to post-mortem examination. All mortalities were necropsied and the kidney and liver tissues were collected for virus detection.

RESULTS

Fig. 1 shows the RT-PCR result of the virus preparations used to inoculate the experimental fish. The ISA virus isolate 'Site A 98' (Fig. 1, lanes 7 and 8) which produced CPE typical for ISA virus in both SHK-1 and

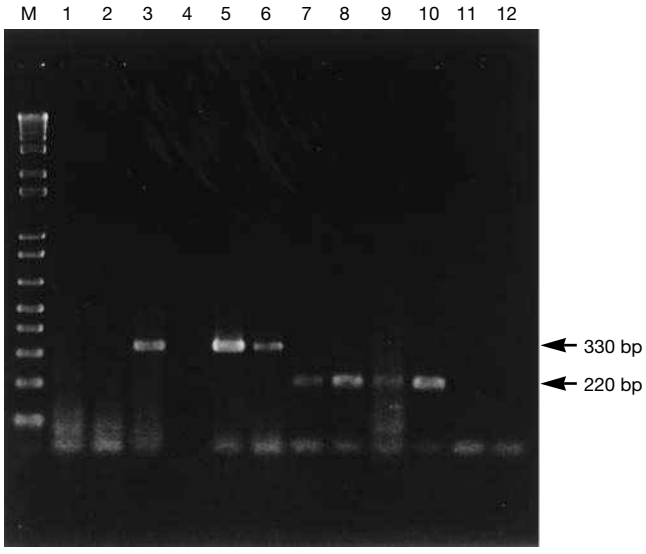

Fig. 1. Agarose gel electrophoresis of RT-PCR products from CHSE-214 and SHK-1 cells infected with ISAV and/or togavirus-like virus. Lane M contains 1 kb plus DNA ladder (Canadian Life Technologies); lanes 1 to 6 are RT-PCR products with togavirus-like virus-specific primer pair M107F1/R1 and target RNA from 'Site A 98' virus-infected CHSE-214 cells, 'Site A 98' virus-infected SHK-1 cells, HKS-36 virus-infected CHSE-214 cells, HKS-36 virus-infected SHK-1 cells, U14785(#6) virus-infected CHSE-214 cells, and U14785(#6) virus infected SHK-1 cells, respectively; lanes 7 to 12 are RT-PCR products with ISAV-specific primer pair FA-3/RA-3 and target RNA as listed for lanes 1 to 6

CHSE-214 cell lines (Kibenge et al. 2000) was free of contamination with togavirus-like virus (Fig. 1, lanes 1 and 2). The togavirus-like virus isolate U14785(#6) shown to be free of contamination with ISA virus (Fig. 1, lanes 11 and 12) produced CPE in CHSE-214 cells but was non-cytopathic in SHK-1 cells as evidenced by the positive RT-PCR results (Fig. 1, lanes 5 and 6, respectively). The togavirus-like virus CPE in CHSE-214 cells developed slowly, appearing anywhere from 8 to 28 d post inoculation (DPI) and consisted of numerous highly refractile syncytia and rounded individual cells. Virus sample HKS-36, like ISAV isolate 'Site A 98', produced CPE in both the SHK-1 and CHSE-214 cell lines. However, the CPE in the SHK-1 cell line was similar to that produced by ISA virus isolate 'Site A 98' whereas the CPE in the CHSE-214 cell line was similar to that produced by togavirus-like virus isolate U14785(#6). The HKS-36 CPE in the respective cell lines did not change with up to 4 serial passages (P4). Examination of these passages by RT-PCR showed the presence of both ISAV and togavirus-like virus nucleic acids in both SHK-1 and CHSE-214 passaged preparations. In Fig. 1, it can be clearly seen that HKS-36 passaged in CHSE-214 cells contained a mixture of togavirus-like virus (Fig. 1, lane 3) and ISA virus (Fig. 1, lane 9). Since the virus titers used to determine the inoculum dose were based on end-point CPE, the $10^{3.5}$ $TCID_{50}$/0.5 ml of the HKS-36 preparation in SHK-1 cells refers to ISA virus titer whereas that in CHSE-214 cells refers to togavirus-like virus titer.

No sign of sickness and no mortality were observed in the control fish. In the group inoculated with togavirus-like virus isolate U14785(#6), only 1 mortality was observed 18 DPI, which was a result of trauma rather than virus infection. Within the remaining infected tanks, those fish which became moribund or were removed as mortalities appeared to deteriorate suddenly, becoming lethargic and unresponsive to stimuli and dying within 24 h of the initial observation. Mortality started at 12 DPI (15 DPI for the group inoculated with HKS-36 grown in CHSE-214 cells), peaked between 14 to 18 DPI, and stopped at 28 DPI. The cumulative daily mortality for all experimental groups is shown in Fig. 2. Overall, inoculation with $10^{3.5}$ $TCID_{50}$/0.5 ml virus resulted in 58.7% mortality with 'Site A 98', 64% mortality with HKS-36 grown in SHK-1 cells, and 61.3% mortality with HKS-36 grown in CHSE-214 cells. Gross lessions characteristic of ISA (Thorud & Djupvik 1988, Evensen et al. 1991, Speilberg et al. 1995, Totland et al. 1996) were observed in all moribund fish and mortalities neropsied. These ISA lesions mimicked gross pathology lesions that have been observed in Atlantic salmon retrieved from ISA-infected cage sites in the Bay of Fundy (Mullins et al. 1998, O'Halloran et al. 1999).

Table 1 summarizes the results of virus detection by virus isolation, RT-PCR, and RNA dot-blot hybridization in kidney and liver tissues from fish that died at 12 to 15, 17, and 21 DPI representing the beginning, mid-

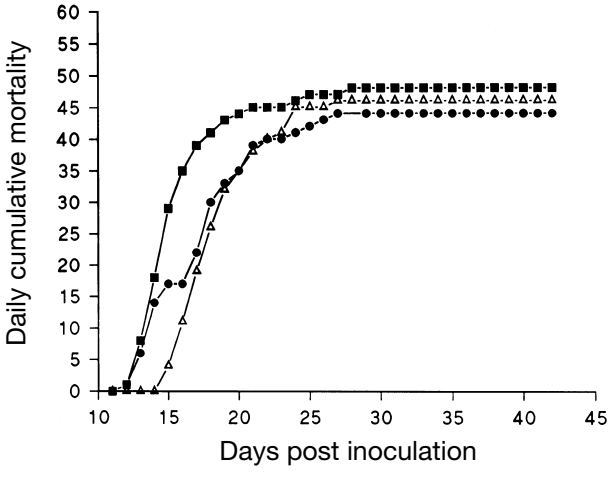

Fig. 2. Percent cumulative mortality of Atlantic salmon in freshwater after intraperitoneal and oral inoculation (total dose $10^{3.5}$ $TCID_{50}$) with different ISAV isolates. (■) HKS-36 virus grown in SHK-1 cells; (▲) HKS-36 virus grown in CHSE-214 cells; (●) 'Site A 98' virus grown in CHSE-214 cells

Table 1. Comparison of 3 different methods used to detect presence of virus (no. fish positive/total tested) in kidney (Kid.) and liver (Liv.) tissues of Atlantic salmon that died following experimental inoculation with different virus preparations. DPI: days post inoculation, ND: not done

Virus preparations	DPI	RT-PCR			RNA dot-blot hybridization			Virus isolation					
								CHSE-214 cells[a]			SHK-1 cells[a]		
		Kid.	Liv.	Total	Kid.	Liv.	Total	Kid.	Liv.	Total	Kid.	Liv.	Total
'Site A 98'	12–13	3/6	0/6	3/6	0/6	0/6	0/6	6/6	6/6	6/6	ND	ND	ND
	15	5/8	2/8	5/8	3/8	0/8	3/8	8/8	8/8	8/8	ND	ND	ND
	17	3/5	1/5	4/5	0/5	0/5	0/5	5/5	5/5	5/5	ND	ND	ND
	21	2/3	0/3	2/3	0/3	0/3	0/3	3/3	3/3	3/3	ND	ND	ND
HKS-36	12–13	8/8	3/8	8/8	5/8	2/8	6/8	ND	ND	ND	8/8	8/8	8/8
(SHK-1)	14	8/10	1/10	9/10	4/10	0/10	4/10	ND	ND	ND	10/10	10/10	10/10
	17	1/6	3/6	3/6	0/6	3/6	3/6	ND	ND	ND	4/6	6/6	6/6
	20	1/1	0/1	1/1	0/1	0/1	0/1	ND	ND	ND	1/1	1/1	1/1
HKS-36	17	4/7	4/7	6/7	0/7	0/7	0/7	0/7	0/7	0/7	7/7	7/7	7/7
(CHSE-214)	21	3/4	2/4	4/4	0/4	1/4	1/4	0/4	0/4	0/4	4/4	4/4	4/4

[a]Inoculated cells showing positive CPE were further confirmed by RT-PCR

dle, and end of the mortality period, respectively. ISA virus was re-isolated from kidney and liver tissues of all dead or moribund fish tested. In contrast, no togavirus-like virus was recovered in CHSE-214 cell monolayers inoculated with samples from fish inoculated with HKS-36 virus grown in CHSE-214 cells. A comparison of virus isolation, 1-step procedure RT-PCR and RNA dot-blot hybridization for detection of ISA virus showed virus isolation to be the most sensitive method, displaying 100% sensitivity. Direct ISAV RT-PCR on tissues performed particularly poorly on liver tissues. Overall RT-PCR had a sensitivity of 78% (45/58 fish). However, when considering kidney and liver tissues separately, the sensitivity dropped to 66% (38/58 kidneys) and 28% (16/58 livers) for kidney and liver tissues, respectively. RNA dot-blot hybridization was the least sensitive of the 3 methods with an overall sensitivity of only 29%, and as with RT-PCR, fewer positives were detected among liver tissues (10%) than with the kidney (20%) tissues.

DISCUSSION

The togavirus-like virus has been suspected to play a role in the aetiology of ISA in the Bay of Fundy because the virus was isolated from fish with the characteristic HKS lesions from several sites in the early stages of the outbreak. In addition, preliminary transmission studies using infected CHSE-214 cell cultures showing togavirus-like virus CPE resulted in high mortalities in adult Atlantic salmon, with gross lesions consistent with classical ISA. Moreover, others have reported difficulties of reproducing all of the gross and microscopic lesions of ISA (Jones et al. 1999, Lovely et al. 1999). The experimental reproduction of ISA mortality and gross pathology with the CPE-positive CHSE-214 preparation containing togavirus-like virus and in which the ISA virus presence could be demonstrated by RT-PCR, and the lack of any clinical disease in fish inoculated with CPE-positive CHSE-214 preparations containing only togavirus-like virus in the present study, conclusively demonstrate the lack of virulence of the togavirus-like virus and confirm that the original ISA outbreaks in New Brunswick were solely caused by ISA virus.

The best method for detecting ISA virus in both kidney and liver of experimentally infected fish was virus isolation followed by RT-PCR, which was more reliable in the kidney. The lower sensitivity of RT-PCR compared to virus culture in the present study is most likely due to the smaller fraction of the sample used in RT-PCR compared to virus isolation. These results are in agreement with those of Rimstad et al. (1999), who used a 2 tube, 2-step RT-PCR in a time course study to demonstrate that wide tissue dissemination of the virus occurred after 13 DPI. The poor sensitivity of RT-PCR in liver tissues in the present study was not due to absence of virus since all samples were positive by virus isolation. The low sensitivity of RT-PCR in the liver tissues could have been due to higher levels of degradation of RNA extracted from this tissue. It was observed that even those samples which were strongly positive if RT-PCR was carried out immediately after dissolving RNA in distilled deionized water would either give a weak RT-PCR product or no product at all if the RT-PCR was repeated on the same sample a day later compared to only a change in the intensity of the

RT-PCR product in kidney samples. The difference could be that there is more RNase activity in liver tissues compared to the kidney (Jayanthi & Van Tuyle 1992). RNA dot-blot hybridization was not very sensitive as it picked up very few positive samples both in the kidney and liver, and this could be due to the low levels of viral RNA present in the small samples used. But because the results of RT-PCR and RNA dot-blot hybridization are influenced by the type of sample, primers, and probes used, further studies are needed for the comparison of the detection methods.

Acknowledgements. The technical assistance of Patricia McKenna is greatly appreciated. Financial support for this work was provided by a strategic grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada and AVC Inc.

LITERATURE CITED

Bouchard D, Keleher W, Opitz HM, Blake S, Edwards KC, Nicholson BL (1999) Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. Dis Aquat Org 35:131–137

Byrne PJ, MacPhee DD, Ostland VE, Johnson G, Ferguson HW (1998) Haemorrhagic kidney syndrome of Atlantic salmon, *Salmo salar* L. J Fish Dis 21:81–91

Dannevig BH, Falk K, Namork E (1995) Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J Gen Virol 76:1353–1359

Evensen Ø, Thorud KE, Olsen YA (1991) A morphological study of the gross and light microscopic lesions of infectious anaemia in Atlantic salmon (*Salmo salar*). Res Vet Sci 51:215–222

Fryer JL, Yusha A, Pilcher KS (1965) The *in vitro* cultivation of tissue and cells of Pacific salmon and steelhead trout. Annu NY Acad Sci 126:566–586

Jayanthi GP, Tuyle GC (1992) Characterization of ribonuclease P isolated from rat liver cytosol. Arch Biochem Biophy 296:264–270

Jones SRM, MacKinnon AM, Groman DB (1999) Virulence and pathogenicity of infectious salmon anaemia virus isolated from farmed salmon in Atlantic Canada. J Aquat Anim Health 11:400–405

Kibenge FSB (1992) Differential detection of infectious bursal disease virus serotypes using cDNA probes to VP2 coding region. Am J Vet Res 53:1337–1342

Kibenge FSB, Lyaku J, Rainnie D, Hammell KL (2000) Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. J Gen Virol 81:143–150

Lovely JE, Dannevig BH, Falk K, Hutchin L, MacKinnon AM, Melville KJ, Rimstad E, Griffiths SG (1999) First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome. Dis Aquat Org 35: 145–148

Mullins JE, Groman D, Wadwoska D (1998) Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. Bull Eur Assoc Fish Pathol 11: 110–114

O'Halloran JL, L'Aventure JP, Groman DB, Reid AM (1999) Infectious salmon anaemia in Atlantic salmon. Can Vet J 40:351–352

Olfert ED, Cross BM, McWilliam A (1993) A guide to the care and use of experimental animals, 2nd edn. Bradda Printing Services Inc, Ottawa

Rimstad E, Falk K, Mikalsen AB, Teig A (1999) Time course tissue distribution of infectious salmon anaemia virus in experimentally infected Atlantic salmon *Salmo salar*. Dis Aquat Org 36:107–112

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Speilberg L, Evensen Ø, Dannevig BH (1995) A sequential study of the light and electron microscopic liver lesions of infectious salmon anaemia in Atlantic salmon (*Salmo salar* L.). Vet Pathol 32:466–478

Thorud K, Djupvik HO (1988) Infectious anaemia in Atlantic Salmon (*Salmo salar* L.). Bull Eur Assoc Fish Pathol 18: 109–111

Totland GK, Hjeltnes BK, Flood PR (1996) Transmission of infectious salmon anaemia (ISA) through natural secretions and excretions from infected smolts of Atlantic salmon *Salmo salar* during their presymptomatic phase. Dis Aquat Org 26:25–31

Editorial responsibility: Jo-Ann Leong, Corvallis, Oregon, USA

Submitted: February 17, 2000; Accepted: May 22, 2000
Proofs received from author(s): July 20, 2000