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NOTE

First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome

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ABSTRACT: Haemorrhagic kidney syndrome (HKS), a serious disease affecting Atlantic salmon on the east coast of Canada, was determined to be caused by infectious salmon anaemia virus (ISAV) through the isolation of the pathogen on the SHK-1 (salmon head kidney) cell line and confirmation by ISAV-specific immunofluorescent antibody test (IFAT) and reverse transcriptase polymerase chain reaction (RT-PCR). In addition, the defining histopathology of HKS could be reproduced following the injection of material that rendered challenged fish ISAV-positive by cell culture in the absence of any other detectable pathogen. Preliminary nucleotide sequence comparison does not suggest any direct epidemiological connection between the Canadian and Norwegian isolates.

KEY WORDS: Haemorrhagic kidney syndrome (HKS) · Infectious salmon anaemia (ISA) · Atlantic salmon

During summer 1996, elevations in mortality and morbidity were recorded among Atlantic salmon reared in the Bay of Fundy, New Brunswick, Canada. This new disease condition, referred to as haemorrhagic kidney syndrome (HKS) (Byrne et al. 1998), was initially characterised on the basis of histopathological lesions of which the necrosis of kidney tubules and associated interstitial haemorrhage were considered pathognomonic. Initial diagnostic testing, however, did not reveal a specific aetiologic agent(s). This study outlines the process by which infectious salmon anaemia virus (ISAV) was determined to be the cause of HKS, and is the first report that this pathogen has been identified outside of Norway.

Materials and methods. ***Microbiological analysis:*** Kidney tissue was removed from market size (3 kg average) Atlantic salmon from net pens containing fish experiencing elevated mortality which had been identified as HKS-positive (Site A) (D. D. MacPhee pers. comm.). Homogenates were prepared in Hank's balanced tissue culture medium (10% w/v) by grinding equal portions of front, middle and hind kidney (ca 1 g total, 0.33 g each section) in a glass mortar with a motorised Teflon pestle. For bacteriological evaluation, individual homogenates were swabbed onto brain heart infusion (BHI) agar supplemented with 2% NaCl in addition to SKDM-2 (selective kidney disease medium) agar (Austin et al. 1983). For virological analysis, homogenates were further diluted 5-fold in Hank's medium, centrifuged for 15 min at 2500 rpm (1141 × *g*) and 4°C, and filtered through 0.45 µm membranes. One hundred µl of the filtrate was applied to individual wells containing a monolayer of salmon head kidney cells (SHK-1) grown to 80% confluence in 24-well plates using the culture conditions of Dannevig et al. (1995). Following inoculation, the filtrates were adsorbed for 1 h at 15°C. The cell line CHSE 214 (coho salmon embryo, ATCC no. CRL-1681; American Type Culture Collection, Rockville, MD) was also inoculated with the same volume of tissue homogenate and incubated in the same manner as the SHK-1 cells with the exception that the culture medium was MEM (mimimum essential medium) with Earle's salts, 10% FBS (fetal bovin serum), 0.01 M HEPES and 2 mM L-glutamine (all reagents from Life Technologies). All inoculated cultures were incubated for up to 30 d at 15°C, or until any cytopathic effects (CPEs) were observed. Cells from wells in which CPEs were evident were scraped off and kept at 4°C pending inclusion in virulence assays or confirmatory analysis.

Polymerase chain reaction (PCR): Due to the high prevalence of *Renibacterium salmoninarum* in the

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New Brunswick aquaculture industry (Griffiths et al. 1996), kidney tissues were screened using a PCR probe as described by Miriam et al. (1997). In addition, to address the possibility of the involvement of an unculturable bacterium in HKS, a PCR method for the universal detection of bacteria was used incorporating the primers p515f and p806r complementary to segments of the 16S rDNA gene as described by Relman (1993). Briefly, 50 µl of 10% homogenate were allowed to dry onto 0.7 cm no. 30 glass fibre discs (Schleicher and Schuell, Keene, NH), washed in a 1.5 ml microtube with 1 ml of tissue culture grade water (Life Technologies) and extracted with 200 µl of Instagene matrix (BioRad Laboratories, Hercules, CA). The forward primer, p515f was further modified at the 5' end with a GC-rich 40 base pair (bp) sequence that enabled the separation of similarly sized 16S rDNA fragments by denaturing gradient gel electrophoresis (DGGE) as described by Muyzer et al. (1993).

Immunofluorescent antibody test (IFAT): IFAT analysis was performed on SHK-1 cells showing CPE using a monoclonal antibody (Mab 3H6F8) as described by Dannevig et al. (1995).

Reverse transcriptase PCR (RT-PCR): RT-PCR analysis was performed on SHK-1 cell cultures showing CPEs using the primer pair amplifying a 155 bp fragment as described by Mjaaland et al. (1997). In addition, for the purposes of SHK-1 CPE confirmatory testing and nucleotide sequence comparisons, the Canadian laboratory (Research and Productivity Council, RPC, New Brunswick) used an alternative primer set based on the nucleotide sequence of the ISAV specific clone 14 from genomic segment no. 8 of the ISAV genome (Mjaaland et al. 1997) identified with the OLIGOs program (National Biosciences, Inc. [NBI], Plymouth, MN). The primers p268f sense (cgagcgacgatgactctctac) and p879r antisense (acccaaatatcccattcaaca) were used with the EZ rTth one-step RT-PCR system (Perkin Elmer Applied Biosystems, Foster City, CA) incorporating 2.5 mM manganese ion, 0.5 µm primer and 0.3 mM dNTP to yield a 592 bp fragment. Amplification conditions were 60°C for 60 min, 94°C for 2 min, followed by 40 cycles of 1 min at 94, 55 and 72°C, and completed by a final extension step of 72°C for 7 min. Amplified product was analysed by electrophoresis on a non-denaturing 8% acrylamide minigel and visualised by silver staining as previously described (Miriam et al. 1997).

Sequencing comparison: The 592 bp fragment amplified with primers p268f sense and p879r antisense was sequenced using automated DNA sequencing devices: an ABI Prism 377 in Norway and a 310 Genetic Analyser in Canada (both devices Perkin Elmer Applied Biosystems). Alignment of the nucleotide sequences of the Fundy (Canadian) and the Glesvaer (Norwegian) ISAV strains was performed using the BestFit Program (Wisconsin Package, Genetics Computer Group, Madison, WI).

Transmission electron microscopy (TEM): TEM was performed on thin sections of fixed pellets of infected SHK-1 cells as described by Doane & Anderson (1987).

Virulence studies: As an initial virulence study (Trial 1), 20 Atlantic salmon parr-smolts (average weight 25 g) were held in freshwater and intraperitoneally (i.p.) injected with 100 µl of supernatant from SHK-1 cell cultures infected by the then unknown cytopathic agent following 2 subpassages of material collected from Site A. Fish were maintained in 1 m diameter tanks at 11°C at an inflow flow rate of 5 l min^{-1} freshwater. Kidney tissue samples were prepared as homogenates and applied to the SHK-1 and CHSE 214 cell lines as described above.

In Trial 2, Atlantic salmon (200 to 400 g) held in seawater (10°C) at an inflow rate of 10 l min^{-1} were injected i.p. with 1 ml of filtrates of 10 or 5% homogenates (n = 11 and 10, respectively) or with 2.5% unfiltered homogenate (n = 8) prepared from kidney and gill tissues from 1+ sea year-cultured Atlantic salmon (n = 5) that showed the lethargy characteristic of HKS. Subsequent histopathological examination confirmed the existence of HKS lesions in the original kidney (D. Groman, Atlantic Veterinary College, pers. comm.). The homogenates were screened for bacterial and other viral pathogens and were found to be negative. Controls received an i.p. injection of 1 ml of 0.89% saline (n = 5). Individual treatment groups were held in separate tanks.

Results. ***Microbiological studies:*** The first opportunity to examine fish from the site originally identified as suffering from HKS was in February 1997. Subsequent samples from cages experiencing elevated mortality were also collected from the same site in May and June. With the exception of *Renibacterium salmoninarum*, no other bacterial pathogens could be identified as common to fish from cages affected by HKS, although during May and June it was possible to identify *Vibrio salmonicida* in occasional samples by culture. Use of the universal bacterial PCR method and DGGE did not reveal any DNA sequences that were suggestive of an intracellular bacterium (with the exception of *R. salmoninarum*) although it was possible to amplify and resolve 16S rDNA sequences that were identical to *V. salmonicida* in addition to other unidentified *Vibrio* species in occasional samples.

Upon application of kidney tissue filtrates to the SHK-1 cell line, it was noted that the inocula could elicit a cytopathic effect (CPE) between Days 7 and 21 of incubation at 15°C. It was determined by PCR, however, that *Renibacterium salmoninarum* could be found in the SHK-1 cells even following 0.22 µm filtra-

tion, suggesting that the bacterium can multiply intracellularly in this cell line. *R. salmoninarum* activity could be excluded upon further filtration, suggesting that the smaller bacteria were eventually removed. In the absence of a positive PCR result for *R. salmoninarum* it was still possible to produce the characteristic CPEs on the SHK-1 cell line, indicating the involvement of another filterable agent.

TEM of the culture supernatants of the infected SHK-1 cells failed to reveal any evidence for viral particles. Upon sectioning of the infected cell pellets, however, it was possible to observe frequently-pleomorphic particles that were spherical (ca 100 nm), ovoid, or kidney-shaped or appeared as long fibrillar structures. Superficially, the particles were identical to previously published electron micrographs of ISAV (Hovland et al. 1994, Dannevig et al. 1995), prompting the dispatch of material to NVI (National Veterinary Institute) in Oslo for confirmatory analysis.

Infected SHK-1 cells resulting from the inoculation of kidney tissue filtrates from Site A, in addition to material from 2 other sites (Sites B and C) subsequently reported to be afflicted with HKS, were positively identified as ISAV using IFAT and RT-PCR at NVI . The cell lysates (diluted 1:10) exhibited CPE 3 to 4 d following inoculation of SHK-1 cells and were observed to exhibit behaviour similar to that of the indigenous isolate, including the staining pattern by IFAT with the anti-ISAV Mab 3H6F8. The nucleotide sequence of the 592 bp amplified product from segment no. 8 of the Canadian isolate (GenBank accession no. AF109304) revealed 87% homology to the equivalent sequence of the Norwegian Glesvaer strain (accession no. Y10404).

Virulence studies: In the freshwater challenge study (Trial 1) 4 of the fish died between 17 and 21 d post-injection. The primary gross pathological lesion for this group of fish was petechial haemorrhage of pyloric caecae and mesenteric fat. The injected agent was reisolated from kidney tissue filtrates as judged by the CPE that was observed following inoculation of SHK-1 cells. One of the lysates was confirmed as ISAV by IFAT and RT-PCR as previously described. Representative tissue samples were sent to the University of Guelph (Canada) for histological analysis but the results were inconclusive for HKS (H. Ferguson, Ontario Veterinary College, pers. comm.).

In the marine challenge study (Trial 2) incorporating the 200 to 400 g salmon in saltwater, accumulated mortality reached 82% at Day 45 for fish receiving the 10% filtered homogenate, 100% for the 5% filtered homogenate, and 50% for the 2.5% unfiltered homogenate. No mortality occurred in the control group receiving saline. Gross pathology of the mortality was limited to ventral petechiae and blood-tinged ascites. Histopathology in concordance with HKS was detected in all mortalies (H. Ferguson pers. comm.). The CPE-causing agent was reisolated in SHK-1 cells from all dead fish and ISAV was confirmed by RT-PCR from each isolation.

Discussion. In summer 1996, a disease was reported to be affecting Atlantic salmon based on elevated mortality and the existence of hitherto unreported histopathology (Byrne et al. 1998). At that time, the disease could not be attributed to any specific agent despite extensive efforts to detect potential bacterial or viral pathogens. At the time of the reports in 1996, ISAV was a significant pathogen of the Norwegian salmon farming industry. To rule out the possibility of ISAV involvement in HKS, it was necessary to determine whether it could be isolated using the SHK-1 cell line (Dannevig et al. 1995), which was not included in the study of Byrne et al. (1998). Our first opportunity to analyse tissue material from an HKS-positive site using the SHK-1 cell line was in February 1997. Subsequently, the agent was shown to be ISAV by a monoclonal-antibody-based IFAT and RT-PCR.

The first opportunity to fulfil Koch's postulates came in April 1997 in the freshwater trial. However, at that time it was not possible to reproduce the clinical pathology of HKS. Histopathology characteristic of HKS consisting of tubular necrosis and interstitial haemorrhage was subsequently reproduced in the challenge study using 200 to 400 g salmon in the marine challenge (Trial 2) with tissue homogenate that was later determined to be ISAV positive in the absence of any other pathogen. This successful transmission of the clinical pathology of HKS through the injection of tissue homogenates from affected fish also confirmed the findings of S. Jones et al., AquaHealth Ltd, Prince Edward Island, Canada (pers. comm.).

Although the pathological changes found to be typical for HKS are not published in the Norwegian literature (e.g. Evenson et al. 1991), subsequent communication has revealed that HKS-like changes have been found in a number of tissue samples collected from ISAV outbreaks in Norway (T. Poppe & T. Håstein pers. comm.). It is possible that the pathological changes found in HKS are typical of an earlier manifestation of the infection, that they are more prevalent under the environmental conditions peculiar to aquaculture on the east coast of Canada, and that a variant of ISAV has been identified or that the Saint John stock of Atlantic salmon differ from Norwegian stocks in their susceptibility to infection.

The origin of ISAV infection on the east coast of Canada is a contentious issue. The preliminary nucleotide sequence comparison reported here, however, suggests that there are no direct epidemiological connections to the Glesvaer ISAV isolate. To confirm this observation, additional nucleotide sequence data

from different geographic isolates would be necessary to interpret the epidemiological significance of the sequence homology found in this study. Alternatively, the recent identification of ISA in Canada may merely reflect the time required for the infectious load to increase to the point where disease and mortality became noticeable.

In conclusion, the current study has demonstrated that HKS can be reproduced in seawater-reared Atlantic salmon inoculated with tissue homogenates containing the New Brunswick isolate of ISAV, and suggests that further studies are now warranted on the potential for variation in geographic isolates with further implications for disease management and regulatory issues.

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