Immunohistochemistry and pathology of multiple Great Lakes fish from mortality events associated with viral hemorrhagic septicemia virus type IVb

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ABSTRACT: A novel viral hemorrhagic septicemia virus (VHSV) (genotype IVb) has been isolated from mortality events in a range of wild freshwater fish from the Great Lakes since 2005. In 2005 and 2006, numerous new freshwater host species (~90 fish from 12 different species) were confirmed to have VHSV by cell culture and reverse transcriptase polymerase chain reaction. A prominent feature observed in infected fish were the petechial and ecchymotic haemorrhages on the body surface and in visceral organs, as well as serosanguinous ascites; however, many fish had few and subtle, gross lesions. Histologically, virtually all fish had a vasculitis and multifocal necrosis of numerous tissues. Excellent correlation was found between the presence of VHSV IVb antigen detected by immunohistochemistry and the pathological changes noted by light microscopy. Intact and degenerate leukocytes, including cells resembling lymphocytes and macrophages, also had cytoplasmic viral antigen. By contrast, renal tubules and gonadal tissues (ovary and testis), were strongly immunopositive for VHSV IVb, but no lesions were noted.

KEY WORDS: Viral hemorrhagic septicemia virus · VHSV IVb · Muskellunge · Freshwater drum · Walleye · Round goby · Immunohistochemistry · Pathology

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INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is an aquatic *Rhabdovirus* that affects many species of both marine and freshwater fish (Skall et al. 2005). Four genotypes of VHSV have been identified around the world based on N and G genes (Einer-Jensen et al. 2006, Nishizawa et al. 2006). Genotype I strains affect both freshwater and marine fish in Europe and have the greatest economic impact, since they produce clinical disease and mortality in the rainbow trout *Oncorhynchus mykiss*. Genotypes II and III are restricted to the Baltic Sea and North Atlantic Ocean

and are associated with mortality events in wild fish (Benmansour et al. 1997, Nishizawa et al. 2006). Genotype IV has been found in the Pacific Ocean, North America, Japan, and Korea, predominantly in marine fish (Hedrick et al. 2003).

Initial detection of VHSV IVa in North America occurred in spawning marine salmonids in the Pacific northwest in 1988 (Winton et al. 1991, Meyers & Winton 1995). The isolates from eastern North America are genotypically distinct from other genotype IV strains and have been designated VHSV IVb (Elsayed et al. 2006, Winton et al. 2008). VHSV IVb has been isolated from 4 species of fish, one of which was the brook trout

Salvelinus fontinalis in Atlantic Canada in 2001 (Gagne et al. 2007). The virus was initially found in the Great Lakes, and more specifically in the freshwater drum *Aplodinotus grunniens* in Lake Ontario, in the spring of 2005 (Lumsden et al. 2007); however, it was present in the muskellunge *Esox masquinongy* in Lake St. Clair, Michigan, USA, since at least 2003 (Elsayed et al. 2006). Most recently, VHSV IVb has been detected in the round goby *Neogobius melanostomus* in the waters of Lake Ontario and the St. Lawrence River in the summer of 2006 (Groocock et al. 2007). Since 2005, the total number of species that are affected in the United States has risen to 28 (Winton et al. 2008), including several commercially and recreationally important fish such as the walleye *Sander vitreus* and the yellow perch *Perca flavescens*.

Infection occurs most commonly between 4 and 14°C, and to date, spring mortality events are most common in the Great Lakes region. Acute clinical signs commonly described for VHSV include skin darkening, anemia, exophthalmia, and epidermal hemorrhages sometimes with ulceration (King et al. 2001, Einer-Jensen et al. 2006). Internal lesions consist of petechial and/or ecchymotic hemorrhages potentially located in most visceral organs and serosanguinous ascites (Skall et al. 2005). Histological changes are typically focal to multifocal degeneration and necrosis of a variety of tissues, although the pattern varies between species and genotype of the virus (Marty et al. 1998, Ishikki et al. 2001, Brudeseth et al. 2005, Lumsden et al. 2007), and there can also be a vasculitis (Wolf 1988, Lumsden et al. 2007). Given the range of species affected and the different genotypes identified, the variability of histological lesions is not surprising.

Descriptions of VHSV IVb light microscopic lesions in wild fish are not routinely performed, although there are exceptions (Groocock et al. 2007, Lumsden et al. 2007). Diagnosis of VHSV is more commonly made by molecular techniques as surveys of the Great Lakes and the surrounding watersheds continue. In addition, most of the fish submitted to diagnostic labs are either not suitable for examination due to decomposition or freezing or because the laboratory does not routinely perform light microscopy. It is debatable whether the molecular diagnosis of VHSV necessarily equates to the cause of mortality, and correlation of the presence of the virus in combination with gross and microscopic lesions is critical to make that link. Since VHSV has become very topical in the Great Lakes basin and the molecular tests used are very sensitive (Hope et al. 2010), there is a possibility that every mortality event from which VHSV nucleic acid or virus is obtained will be ascribed to the virus. Immunohistochemistry (IHC) is routinely used to localize a pathogen to a lesion pattern and has been used previously for other geno-

types of VHSV (Evensen & Olesen 1997, Brudeseth et al. 2005, Dale et al. 2009).

The purposes of this study therefore were (1) to document the species collected from the lower Great Lakes and the St. Lawrence River from which VHSV IVb has been isolated between 2005 and 2006, (2) to describe the distribution of the light microscopic lesions, and (3) to evaluate the distribution of viral antigen using IHC.

MATERIALS AND METHODS

Fish from Guelph. Forty-eight fish were obtained in 2005 and 2006: 10 freshwater drum, 7 muskellunge, ~15 round goby, and 1 American eel *Anguilla rostrata* from the Bay of Quinte and eastern Lake Ontario; and 11 freshwater drum, 1 white crappie *Pomoxis annularis*, 1 smallmouth bass *Micropterus dolomieu*, and 2 bluegill *Lepomis macrochirus* from Lake St. Clair, Ontario, Canada. The majority were collected alive and submitted the same day, although some of the muskellunge and the eel were found dead and had been frozen. In addition to the fish caught in 2006, freshwater drum from the Bay of Quinte mortality event (Lumsden et al. 2007), 2 muskellunge, and numerous gobies caught in 2005 were used for nested reverse transcriptase polymerase chain reaction (nRT-PCR) and IHC.

Fish from Cornell. Most fish were received from Lake Ontario and the St. Lawrence River. All fish were moribund, freshly dead (fish with red gills) or frozen. Moribund fish were euthanized with an overdose of MS-222 (tricaine methane sulphonate, Western Chemical). The samples consisted of 16 round gobies, 3 smallmouth bass, 1 muskellunge, and 1 burbot *Lota lota*. Additionally, 2 walleye *Sander vitreus* were received from a mortality event in Conesus Lake, the westernmost of the New York State Finger Lakes.

Pathological examination. Wet mounts were prepared from gill biopsies and skin scrapes from all fish. Tissues were fixed in 10% neutral buffered formalin (0.1 M phosphate-buffered saline with formalin) at pH 7.4, processed by routine methods, sectioned at 5 µm and stained with hematoxylin and eosin (H&E). Giemsa and Brown and Brenn-modified Gram stains were performed on selected tissues. The spleen and kidney were cultured aseptically, streaked on cytophaga and tryptic soy agar (Difco Laboratories), and aerobically incubated at ~20°C for 48 to 72 h. Selected tissues, including liver, kidney, heart, and spleen from fish from the Bay of Quinte and Lake St. Clair were pooled and submitted to the Animal Health Laboratory, University of Guelph, for mouse inoculation tests to detect botulinum toxin.

Virus detection. The spleen, kidney, liver, and heart from each fish sampled were pooled and were subjected to virus isolation and nRT-PCR. Virus isolation from fish processed by Cornell was performed as described (USFWS 2004). Fish examined at Guelph were processed according to Al-Hussinee et al. (2010). The nRT-PCR assay was performed as described (Al-Hussinee et al. 2010). The thermocycler conditions, primers, and visualized PCR products were used as recommended (USFWS 2004). Control specimens performed with each nRT-PCR run included a positive control using VHSV-infected epithelioma papulosum cyprini (EPC) cells and negative controls using tissue extracted from known uninfected fish as well as extraction (no tissue) and amplification (no template) controls. Bands of the expected size were excised from the gel, the cDNA was purified (Qiagen), and the PCR products were eluted in 10 µl of 10 mM Tris-HCl (pH 8.5). For the second round of PCR, the first-round products were re-amplified and re-purified under the same conditions as for the first round. DNA sequencing was performed by the Guelph Molecular Supercentre, University of Guelph, Canada (3730 DNA Analyzer, Applied Biosystems).

Preparation of rabbit polyclonal antibody to VHSV, and IHC. VHSV IVb isolated from freshwater drum (U13653, Lumsden et al. 2007) was purified as described (Al-Hussinee et al. 2010). Briefly, virus grown on EPC monolayers with complete cytopathic effect (CPE) were clarified by centrifugation and then applied to glycerol cushions (Granzow et al. 2001, Betts et al. 2003). The resulting pellets were suspended in 100 µl of TNE buffer (10 mM Tris-HCl pH 8.0, 10 mM sodium chloride, and 1 mM EDTA pH 8.0; Granzow et al. 2001) applied to a previously prepared 10 to 60% (5% increments) continuous sucrose gradient (Fernandez-Alonso et al. 1998, Granzow et al. 2001).

To prepare the rabbit antibody, 2 rabbits were injected with purified virus as described (Al-Hussinee et al. 2010). Pre-immune serum prepared in a similar manner was also available from both rabbits before immunization. Animal experimentation was performed according to the Animal Care Guidelines of the University of Guelph.

IHC was performed as described (Al-Hussinee et al. 2010). Briefly, formalin-fixed tissues were deparaffinized, sectioned at 5 µm, and mounted on Snowcoat X-tra glass slides (Surgipath) to air dry. After re-hydration, endogenous peroxidase activity was blocked using 0.3% (v/v) hydrogen peroxide, and all tissue sections were irradiated in 0.1 M citric acid (Evensen & Olesen 1997). Sections were overlaid with blocking solution (Labvision Corporation, Medicorp) followed by rabbit-anti-VHSV diluted at 1:500 in phosphate buffered saline (PBS, pH 7.4) and

then with biotinylated goat anti-rabbit IgG, a streptavidin-peroxidase conjugate and AEC chromagen (Zymed Laboratories, Invitrogen). Finally tissues were counterstained with Mayer's hematoxylin. All steps occurred at room temperature (~20°C). The same dilutions of pre-immune rabbit serum or PBS alone were applied to sections as negative controls. Tissues from healthy (as determined by virus isolation and light microscopy) walleye and muskellunge were also used for IHC.

RESULTS

Bacteriology and mouse inoculation

At Guelph, no significant bacterial growth was present from any of the fish on any of the media used. All mouse inoculation tests for botulinum toxin were negative. At Cornell, the 15 May submission of smallmouth bass had dermal erosions from which *Aeromonas sobria* were cultured, but no bacteria were isolated from the posterior kidneys of the same fish. All other submissions at Cornell had no significant bacterial growth on any of the media used.

Gross pathology

Guelph samples. Fish presented in very good (14 of 17 fish) to good (3 of 17 fish) post-mortem condition. The heart, swim bladder, spleen, liver, kidney, gills, skin, and axial musculature had gross lesions of varying severity. Wet mount examinations revealed mild to moderate lamellar fusion in a few fish, but no organisms were identified from either gill biopsies or skin scrapes. Four out of 9 freshwater drum had few epidermal hemorrhages at the base of pectoral fins and around the anus. Mild to moderate serosanguinous ascites and visceral petechial to patchy hemorrhages were found on the gastric, intestinal and hepatic serosal surface, and one freshwater drum exhibited multifocal hepatic pallor. The crappie had mild epidermal hemorrhages. By contrast, 5 drum, the smallmouth bass, muskellunge, and the bluegill, had no significant external lesions. The bluegill had mild ascites and splenomegaly. The round gobies were autolysed.

Cornell samples. The most notable gross pathological findings in the initial submission of round gobies have previously been described (Groocock et al. 2007). Subsequent round goby submissions to Cornell exhibited similar gross lesions including petechial hemorrhages in the muscle tissue, gonads, and liver. Some smallmouth bass had large (2 to 4 cm diameter) dermal erosive lesions on the flanks (see 'Bacteriology and

mouse inoculation'), and all had hemorrhagic areas on the swim bladder and liver. The walleye had multiple hemorrhagic areas on the flanks and fins of both fish. Other lesions found on few to several fish and considered to be incidental, included branchial *Trichodina* sp., small numbers of dermal monogenes, and encysted digenes and cestodes in the internal organs.

Light microscopy

Tissues examined include gills, heart, spleen, kidney, liver, eye, gonads, gastrointestinal tract, swim bladder, skin, musculature, and less commonly brain. The histological changes of freshwater drum (Lumsden et al. 2007) and round goby (Groocock et al. 2007) collected in 2005 were described previously.

Freshwater drum. The freshwater drum received in 2006 had lesions that were similar in tissue distribution as described previously (Lumsden et al. 2007). All fish examined had a cell-rich vasculitis of varying duration and severity (Figs. 1 & 2). The majority of drum also had a moderate, diffuse epicarditis accompanied by mild to occasionally a moderate, multifocal necrotizing myocarditis, a diffuse necrotizing enteritis, as well as mild multifocal necrotizing lesions in many tissues including the spleen and renal interstitium. In contrast to the infected freshwater drum described previously (Lumsden et al. 2007), the brains were not affected, and the lesions present were more acute and not nearly as severe.

Muskellunge. Moderate to severe vasculitis was prominent in the liver and spleen. Several fish had a moderate, diffuse epicarditis accompanied by a mild, multifocal necrotizing myocarditis (Fig. 3). There was a moderate, diffuse necrotizing enteritis with increased eosinophilic granular cells and mixed leukocytes including macrophages in the lamina propria, epithelium, and within the intestinal lumen. Mild to occasionally moderate, multifocal necrosis with edema, fibrin exudation, and variable leukocyte accumulations were also found throughout the renal interstitium and spleen in all fishes examined. The splenic and renal lesions, as for those seen in the drum, were often subtle. The swim bladder of several fish had an acute diffuse air sacculitis of moderate severity. Muskellunge collected at Cornell University had similar lesions but in general had a less severe vasculitis.

Bluegill, smallmouth bass, and crappie. A moderate to severe vasculitis was present in the liver and spleen of all 3 species, while the pancreas, kidney, and swim bladder retes were also affected in the bluegill. A diverse range of inflammatory cell types had infiltrated into all 3 layers of the blood vessel wall. Scattered acute necrotizing lesions associated with the vasculitis were

sporadically (1 or 2 fish only) seen in many tissues, e.g. eyes, pseudobranch, and gills. Lesions were often most prominent surrounding venules. All 3 of these species also had an epicarditis, myocarditis, splenitis, nephritis, and enteritis (Fig. 4) similar to that noted in the drum and muskellunge, although the severity varied between individual fish. Large foci of hepatic necrosis were notable lesions in the smallmouth bass (Fig. 5), bluegill, and crappie, which was in contrast with the drum and muskellunge, in which only single-cell to mild focal necrosis was occasionally noted. The pancreas was not commonly affected in any species; however, there was evidence of exocrine epithelial regeneration in the bluegill. The crappie and smallmouth bass had a prominent, acute to chronic, moderate, diffuse air sacculitis.

Walleye. The notable lesions seen in this species were a marked diffuse and severe, cell-poor fibrinoid vasculitis, most prominent in the venules of the liver. Hepatic sinusoids were expanded by clear spaces (edema) and there were numerous small foci of hepatic necrosis, often oriented adjacent to vessels, which were associated with few lymphocytes and macrophages. Renal interstitial necrosis with a loss of progenitor cells and concomitant exposure of the reticuloendothelial network and sinusoid expansion (edema) were present. At least one fish had patchy branchial necrosis of filaments and lamellae, and these lesions were infiltrated with densely packed filamentous bacteria consistent with *Flavobacterium columnare*.

Incidental lesions

Lesions that were judged to be incidental or the result of a separate process(es), included multiple granulomas typical of metazoan migration or encystment in the intestinal lamina propria of the bluegill and smallmouth bass, the renal interstitium of the freshwater drum, and the skeletal muscle and liver of the muskellunge. There were nematodes in the lamina propria and intestinal lumen of the bluegill and prespore myxosporeans in the renal tubules of the bluegill and some muskellunge. The crappie had encysted trematodes in the wall of the stomach, while the smallmouth bass had intestinal luminal trematodes. There was mild branchial lamellar fusion and hyperplasia of the gills of several fish.

Immunohistochemistry

In general, staining of fish tissues collected from New York State was relatively faint compared with that in fish collected from Canada.

Freshwater drum. A strongly immunopositive reaction was seen within the cytoplasm of myocardial cells in all drum examined. The often dramatic inflammatory response that was present in all layers of the heart in the drum from 2005 contained little viral antigen. In contrast, the macrophage and lymphocytes present in the heart of more acutely affected drum from 2006 were immunopositive. Abundant viral antigen was detected within the cy-

Fig. 1 to 5. Fig. 1. *Aplodinotus grunniens*. Hepatic venule of a freshwater drum. The venule wall is markedly expanded with large numbers of strongly immunopositive necrotic and intact inflammatory cells, and is elevated from surrounding tissue by fluid. The cytoplasm of a few intact intraluminal leukocytes is also stained. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 2. *Aplodinotus grunniens*. Immunohistochemistry of the meninges of a freshwater drum from the Bay of Quinte showing a marked diffuse meningitis with severe edema and a vasculitis. The blood vessel endothelium is strongly immunopositive as are extravasated and intravascular leukocytes. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 3. *Esox masquinongy*. Muskellunge with focally severe necrotizing myocarditis with a marked lymphohistiocytic infiltrate (H&E). Inset (a): Moderate multifocal punctate staining of myofibres. Inset (b): Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 4. *Lepomis macrochirus*. Immunohistochemistry of the intestine of a bluegill. The apical intestinal epithelium and rare intra-epithelial leukocytes are intensely immunopositive, as are capillary endothelium and intravascular leukocytes. There is light staining of collagen, eosinophilic granular cells, and mucosal goblet cells. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 5. *Micropterus dolomieu*. Large focus of hepatic necrosis (*) in the liver of a smallmouth bass (H&E). Inset (a): Immunohistochemistry demonstrating intense staining of necrotic hepatocytes (to the left) with lesser staining of the cytoplasm of scattered morphologically normal hepatocytes. Inset (b): Pre-immune rabbit serum replaced rabbit-anti-VHSV

toplasm of endothelial cells of blood vessels in numerous tissues but most prominently in the meninges (Fig. 2), tunica albuginea of ovary (Fig. 6), seminal vesicles (Fig. 7), epicardium, spleen, gastrointestinal tract, hepatopancreas (Fig. 1), and the choroidal rete of the eye.

There was moderate, scattered, single-cell necrosis of hepatocytes and exocrine pancreatic cells observed in the majority of drum, and affected cells contained cytoplasmic viral antigen as did infiltrated inflammatory cells, most numerous macrophages. Many melanomacrophage centers contained viral antigen. The reticuloendothelial cells of the splenic ellipsoids and infiltrated leukocytes were immunopositive for VHSV. Renal tubular epithelial cells had moderate amounts of viral antigen present. Immunopositive staining was detected in a few fish within the mucosa, submucosa, and muscularis layers of the gastrointestinal tract, and was associated with mild to moderate necrosis. Some sporadic eosinophilic granular cells appeared to contain intra-cytoplasmic viral antigen. In the ovary of freshwater drum, only ova in the latter stages of development exhibited positive staining, most prominently the outer layer of the oocyte (Fig. 6). Scattered leukocytes within the mesovarium had cytoplasmic staining.

Crappie, bluegill, and smallmouth bass. The most significant histological changes in the crappie were seen in the myocardium and liver, which contained moderate cytoplasmic viral antigen as did isolated inflammatory cells, located between trabeculae and in sinusoids. The splenic foci of necrosis were not associated with viral antigen, but cells of the white pulp and inflammatory cells together with scattered ellipsoids contained intracytoplasmic viral antigen. The endothelial cells of blood vessels did not have detectable viral antigen present. In the smallmouth bass, the prominent foci of hepatic necrosis were strongly immunopositive for VHSV (Fig. 5). The focus of pseudo-branch necrosis as well as the infiltrated inflammatory cells was intensely immunopositive (Fig. 8). Viral antigen was not detected within the exocrine pancreas in this fish; however, it was found within the cytoplasm of scattered inflammatory cells in the mucosa, lamina propria, and submucosal layers of the gastrointestinal tract. In bluegill, the strongest reaction for VHSV was in leukocytes. The latter were most prominent within the lumen of blood vessels in hepatic tissue, brain, and the coelomic cavity. Hepatocellular necrotic foci and the scant macrophage-rich infiltrates were strongly immunopositive. VHSV was prominent within the cytoplasm of epithelial cells of the gastrointestinal tract and in the lamina propria and submucosal endothelium as well as within scattered leukocytes (Fig. 4). The necrotic branchial lamellar epithelial and pillar cells were associated with moderate immunopositive staining.

Muskellunge. The myocardium of the muskellunge was the primary tissue targeted by VHSV based on

both the histopathological changes and presence of viral antigen. The myocardium and infiltrated macrophages had abundant intracytoplasmic viral antigen (Fig. 3). Only a small amount of viral antigen was noted in the splenic ellipsoids, in scattered reticuloendothelial cells, and in intact and necrotic leukocytes (Fig. 9) in muskellunge examined from Cornell. Small to moderate amounts of viral antigen were found in necrotic hepatocytes, endothelium, and infiltrated leukocytes. Moderate staining was noted in the interlamellar branchial epithelial cells, while the lamellar epithelium was stained lightly. The gastric gland epithelium and submucosal blood vessel endothelium were lightly immunopositive. In contrast, moderate amounts of viral antigen were present in similar locations in the tissues of fish from Canada. The apical portions of most renal tubular cells were moderately to strongly immunopositive, while there was mild staining of the interstitium and renal glomeruli (Fig. 10). Moderate, multifocal staining of maturing ova was present as well as sporadic staining of cells in the mesovarium and also the endothelial cells of blood vessels.

Walleye. There was moderate, multifocal, intracytoplasmic immunopositive staining in the renal tubular epithelial cells (Fig. 11) and light staining of hepatocytes (Fig. 12). The often dramatic vasculitis seen with light microscopy in both tissues was almost completely unstained, however. Limited viral antigen was noted in leukocytes surrounding some granulomas. Light to moderate staining of leukocytes within the branchial central venous sinus was noted as well as light staining of the cytoplasm of the branchial epithelium.

Round goby. There was a moderate degree of intracytoplasmic immunopositive staining within the renal tubular epithelial cells of the kidney. Sporadically, there was limited cytoplasmic staining of endothelial cells of some blood vessels in the spleen. Hepatocytes had small amounts of viral antigen present within their cytoplasm, as did the venular and sinusoidal endothelium (Fig. 13). In gill sections, the majority of staining was within the most distal of lamellar epithelial cells.

No viral antigen was detected in any tissues, including oocytes, using pre-immune antiserum, or with a rabbit polyclonal antibody raised against killed whole-cell *Flavobacterium psychrophilum* (Ostland et al. 1997). Viral antigen was not detected in tissues obtained from healthy walleye and muskellunge using the rabbit polyclonal antibody to VHSV employed above.

Virus isolation and nRT-PCR

All fish described from both Cornell and Guelph were virus-isolation positive except the American eel

and round gobies from Guelph, which were not suitable for virology due to autolysis, but these 2 species were positive by nRT-PCR. Every isolation of virus from cell culture was confirmed by nRT-PCR. The product of nRT-PCR amplification in all cases was of the expected size (950 bp in the first round of PCR, 558 bp in the second round of PCR) for VHSV when visualized by gel electrophoresis. Neighbour-joining distances of the sequence alignments of the second round of PCR confirmed identity with the

North American and Japanese isolates of genogroup IV (data not shown). There was 99% similarity between the VHSV isolates obtained in 2005 and 2006 (Lumsden et al. 2007). The isolates from muskellunge obtained in different years were 100% identical.

The supernatants of several Guelph isolates from CPE-positive second passage were subject to transmission electron microscopy. In all cases, numerous bullet shaped virions were seen when negatively stained with uranyl acetate (data not shown).

Figs. 6 to 9. Fig. 6. *Aplodinotus grunniens*. Immunohistochemistry of a freshwater drum ovary with maturing oocytes, which contain copious immunopositive material in contrast to the undeveloped oocytes. Larger oocytes, which may be undergoing atresia (*) are also stained. Inset (a): The oocyte capsule is diffusely and lightly stained, but there is strong punctate staining of yolk. There is also intense staining of the capillary endothelium (arrows). Inset (b): Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 7. *Aplodinotus grunniens*. Large venule and testis of a freshwater drum. There is intense staining of hypertrophied and ragged venular endothelium, and the vessel lumen is filled with swollen macrophages that have phagocytosed immunopositive material. Less commonly, intact leukocytes with cytoplasmic staining are present. Rare mesenchymal cells (resemble smooth muscle) are also immunopositive. The interstitium of the testis is diffusely and moderately stained compared to the spermatozoa and spermatids. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 8. *Micropterus dolomieu*. Immunohistochemistry of the pseudobranch of a smallmouth bass. There is an intense focus of necrosis with intact and degenerate epithelial and inflammatory cells, both of which are immunopositive. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV. Fig. 9. *Esox masquinongy*. Immunohistochemistry of the spleen of a muskellunge with light to moderate staining of ellipsoids and moderate staining of white pulp cells and larger leukocytes. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV

DISCUSSION

In this study, VHSV type IVb was confirmed in several wild freshwater species (American eel, white crappie, smallmouth bass, bluegill, burbot, and walleye) collected from the lower Great Lakes from mortality events during the springs of 2005 and 2006. The detection of VHSV in walleye from Conesus Lake in this study also confirms the presence of VHSV type IVb for the first time outside the contiguous Great Lakes. IHC using a rabbit antiserum to purified VHSV IVb largely correlated the pathological changes noted by light microscopy with the presence of viral antigen in infected tissues. The 2 excep-

tional tissues where viral antigen was abundant but lesions were slight or absent were in the gonads and renal tubules.

The majority of fish that were confirmed as infected with VHSV, and which were collected during mortality events, had extensive hemorrhaging. However, many of the muskellunge had only subtle internal hemorrhages, and several fish, including all fish collected from Lake St. Clair in 2006 that were used in this study (smallmouth bass, white crappie, freshwater drum, and a bluegill), had no external gross lesions and only mild internal hemorrhages. These fish, however, had light microscopic lesions and abundant viral antigen present in tissues. In contrast, there have been several

Figs. 10 to 13. **Fig. 10.** *Esox masquinongy*. Immunohistochemistry of the kidney of a muskellunge with intense staining of the apical epithelium of renal tubules, which are largely intact (top). Inset (a): Pre-immune rabbit serum replaced rabbit-anti-VHSV. Inset (b): The glomerular mesangium and rare interstitial cells are lightly stained and the endothelium of 2 capillaries is moderately stained (arrows). **Fig. 11.** *Sander vitreus*. Kidney of a walleye with a massive influx of inflammatory cells into the markedly expanded vessel wall. The endothelium is not apparent or absent (H&E) (left). Inset: Moderate staining of the tubular epithelium but limited or no staining of the markedly expanded and severely inflamed vessel wall (right). Pre-immune rabbit serum result not shown. **Fig. 12.** *Sander vitreus*. Liver of a walleye that has a subtle but diffuse fibrinoid vasculitis of hepatic venules with few intact and degenerate leukocytes in the wall of the vessel (H&E). Inset: Immunohistochemistry and sparse staining of hepatocytes with minimal staining of endothelium. **Fig. 13.** *Neogobius melanostomus*. Liver of the round goby with very light staining of venular endothelium, of sinusoids and less commonly of hepatocytes. Inset: Pre-immune rabbit serum replaced rabbit-anti-VHSV

instances of detection of VHSV IVb from asymptomatic fish over the last several years (Elizabeth Wright, Ontario Ministry of Natural Resources, pers. comm.). Similarly, VHSV IVa was isolated from healthy shiner perch and sticklebacks caught close to the coastal area of British Columbia, Canada (Kent et al. 1998). In addition, other agents may contribute to mortality, and at least one of the walleye examined in the present study had a severe necrotizing branchitis associated with filamentous bacteria consistent with *Flavobacterium columnare*. In concert with the largely undefined environmental factors that influence the severity of lesions and mortality, as well as species variability, it is evident that the presentation of VHSV IVb in Great Lakes fish can be protean.

Given the relative lack of pathological description available for fish affected by VHSV in North America, IHC can be a valuable tool to detect viral antigen in infected tissues and to correlate with histological changes. Only limited numbers of fish presented in a suitable state of preservation were available for this study. This is due to the difficulties associated with identification of a mortality event, for appropriate fish to be collected in a timely fashion by trained personnel (if available), and to be transported to the lab.

Vasculitis was one of the most dramatic lesions observed in VHSV positive cases from 2005 and 2006. While multiple tissues were affected, including most dramatically hematopoietic tissues and the meninges, IHC revealed viral antigen in the endothelium confirming this tropism of VHSV IVb. Similarly, vasculitis was a dominant lesion in natural infections of freshwater drum (Lumsden et al. 2007), and endothelial viral antigen has been noted in experimentally infected rainbow trout using more than one genotype of VHSV (Evensen et al. 1994, Al-Hussinee et al. 2010). The often extensive, gross hemorrhages observed on serosal surfaces correlated with the presence of viral antigen in blood vessels and tissues, but there were exceptions. Walleye had vascular lesions in the blood vessels of the kidney and liver; however, only weak staining for viral antigen was present. There are several possible reasons for this, including poor or excessive fixation of tissue or chronicity of the lesion in which the virus is no longer present while the inflammatory response remains. In contrast, the atrium and ventricle, both the compact and spongy layers, of most fish examined had significant lesions and were immunopositive for VHSV. It has been previously shown that VHSV III can produce cardiac lesions in experimentally infected hirame *Paralichthys olivaceus* (Isshiki et al. 2001) and turbot *Scophthalmus maximus* (Brudeseth et al. 2005). This was also true of fathead minnow and rainbow trout experimentally infected with VHSV IVb (Al-Hussinee et al. 2010).

Numerous epithelial cell types were targets of VHSV IVb. Multifocal hepatocellular necrosis was the most striking light microscopic lesion in smallmouth bass and crappie. In smallmouth bass, necrosis and inflammation of the pseudobranch were also found. These are novel lesions for a natural VHSV infection and have not been previously described to our knowledge, so it was critical to correlate the lesions with the presence of viral antigen.

Renal tubular epithelial cells also contained viral antigen, most prominently in muskellunge in the present study, and this was also noted in experimentally infected fathead minnow (FHM) and rainbow trout (Al-Hussinee et al. 2010). In a few of the cases from Cornell only, viral antigen was identified in the glomerular mesangium. The presence of intra-glomerular and intra-tubular viral antigen is presumably correlated to the shedding of virus in urine, which has been reported previously (Neukirch & Glass 1984). Entry of VHSV via the gill has been well demonstrated (Yamamoto et al. 1992, Brudeseth et al. 2002). Viral antigen in several fish species was present within lamellar epithelial cells along with mild lesions. The presence of intra-epithelial viral antigen for at least 38 d following experimentally infection of rainbow trout and FHM (Al-Hussinee et al. 2010), as well as in fish in the terminal or chronic stages of natural infection, suggest that the gill is also an exit route for the virus. Enteritis of variable severity was present among most of the fish examined, and viral antigen was commonly present in the lamina propria but not in the epithelium. In addition, no lesions other than those associated with *Aeromonas sobria* in smallmouth bass were noted in the skin of infected fish, and no or very minimal viral antigen was seen.

Several fish rhabdoviruses, such as pike fry rhabdovirus (Bootsma et al. 1975), spring viremia of carp (Bekesi & Csontos 1985), VHSV (Mulcahy & Pascho 1984), and infectious hematopoietic necrosis virus (Mulcahy et al. 1984) can be present in ovarian fluids during spawning; however, vertical transmission for any fish rhabdovirus has yet to be definitively established. In the current study, mature but not immature oocytes of the freshwater drum and muskellunge contained viral antigen. This finding is in agreement with the pattern of viral antigen in the oocytes of FHM experimentally infected with VHSV IVb (Al-Hussinee et al. 2010). The presence of viral antigen alone does not necessarily imply that VHSV IVb can be transmitted vertically. The naturally infected fish in the present study did not survive infection and would not have reproduced; however, some experimentally infected FHM survived, and the majority of naturally infected fish in the Great Lakes populations likely also survived. The gonads are highly vascular, particularly

during spawning, so the presence of viral antigen within the blood vessels of the gonad was not unexpected, and as already stated, VHSV has been found in ovarian fluid (Mulcahy & Pascho 1984). The majority of viral antigen was also found in atretic oocytes, which would not have been fertilized. Additionally, oocytes contain maternal immunoglobulin (Castillo et al. 1993) and innate defense molecules (Wang et al. 2008) that might eliminate infectious agents. Further studies will be required to determine if VHSV IVb has a reproductive impact in survivors, or if vertical transmission can occur in some situations or species.

Leukocytes, including cells morphologically resembling intact and degenerating or necrotic macrophages and lymphocytes, were numerous in the lesions noted here as well as in experimentally infected rainbow trout and FHM (Al-Hussinee et al. 2010), and these cells contained abundant viral antigen. Similar findings were also noted 15 d after experimental infection of turbot (genotype III) (Brudeseth et al. 2005). A high viral (genotype Ia) titer ($5.2 \log \text{TCID}_{50} \text{ ml}^{-1}$) was detected in rainbow trout kidney macrophages and blood leukocytes at 4 and 6 d post inoculation, respectively (Tafalla et al. 1998). This is in agreement with Estepa et al. (1992), who found that the virus titre (genotype Ia) in trout macrophage doubled after 7 d post *in vitro* infection. Involvement of kidney and spleen leukocytes in virus phagocytosis was also identified previously in rainbow trout (Chilmonczyk et al. 1995). While the relationship of macrophages (and other leukocytes) and viral replication is not completely clear, it is apparent that the leukocytes of naturally and experimentally infected fish (Al-Hussinee et al. 2010) contain abundant VHSV IVb antigen.

VHSV IVb will continue to be detected in the Great Lakes and surrounding waters and will continue to present a challenge for fisheries management and fish culture. The ultimate impact of the virus on fish populations will vary markedly with fish species, environmental conditions influencing clinical presentation, if any, and potentially with future variation of the virus. Sequencing of the PCR product from all 5 of the Canadian VHSV strains obtained in the present study demonstrated a variation of only 1 or 2 bases in the 558 bp product (not shown). More extensive sequencing of multiple virus genes is underway by other researchers and will be important to monitor genetic changes, which may be reflected in epidemiological impacts. Given the large number of Great Lakes fish species infected but variably affected by VHSV IVb, and the influence of other organisms in mortality events, it is important to describe the gross and microscopic lesions of naturally infected fish, and to determine those that are associated with VHSV.

Acknowledgements. Funding was provided by the Natural Sciences and Engineering Council of Canada (Fish Pathology Laboratory [FPL], University of Guelph) and by the Ontario Ministry of Natural Resources (FPL and Fish Health Laboratory). The FPL is part of the Canadian Cooperative Wildlife Health Centre (CCWHC). This publication in part is also a resulting product from Project R/FTD-10 funded under award NA07OAR4170010 from the National Sea Grant Program of the US Department of Commerce's National Oceanic and Atmospheric Administration, to the Research Foundation of State University of New York on behalf of New York Sea Grant. The statements, findings, conclusions, views, and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations (Cornell University). L. Shirose (CCWHC) was responsible for the collection and delivery of the fish from Lake St. Clair. J. Casselman (Queens University) collected and delivered the muskellunge and eels from eastern Lake Ontario.

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