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Isolation of the North American strain of viral hemorrhagic septicemia virus (VHSV) associated with epizootic mortality in two new host species of Alaskan marine fish

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ABSTRACT: Thousands of dead Pacific herring *Clupea pallasi*, Pacific hake *Merluccius productus* and walleye pollock *Theragra chalcogramma* were reported in Lisianski Inlet near Pelican, Alaska, USA, on August 1, 1998. The Pacific hake and pollock continued to die through the end of September. Virological examinations of dead fish identified the North American strain of viral hemorrhagic septicemia virus (VHSV) from all 3 species of fish as well as associated high virus titers and possible histopathological lesions. No other primary fish pathogens were detected and there were no apparent environmental causes for fish mortality. This is the first report of VHSV in 2 new Alaskan fish host species and of a natural epizootic associated with VHSV in which progressive mass mortality was observed simultaneously in herring and 2 other species of free-ranging marine fish.

KEY WORDS: VHSV · Epizootic · Mortality

INTRODUCTION

On August 1, 1998, thousands of dead and dying fish were reported throughout Lisianski Inlet near Pelican, Alaska, USA (Alaska Department of Fish and Game staff pers. comm.; Dick Walker, Pelican, pers. comm.) (Fig. 1). Small Pacific herring *Clupea pallasi* were affected first, but by August 5 and thereafter dead fish consisted mostly of small Pacific hake *Merluccius productus* and occasionally (10%) walleye pollock *Theragra chalcogramma*. Moribund fish had no apparent external lesions and behaved lethargically, floating upside down and swimming in circles. The fish mortality apparently peaked around mid August while lesser numbers of fish continued to die through the end of September. No abnormal changes in water quality were identified that would have caused such fish mortality, but the North American strain of viral hemorrhagic septicemia virus (VHSV) was detected in all 3 species of fish examined. Tissue samples of Pacific hake and pollock showed high virus titers with possible interstitial kidney necrosis and mild pancreatic necrosis. In this report we describe the detection of the North American strain of VHSV associated with epizootic mortality in 2 new Alaskan host species of marine fish and the possible epizootiological relationship with VHSV-infected herring.

MATERIALS AND METHODS

Source of fish. On August 1, 1998, samples of dead fish from Lisianski Inlet were collected, frozen and later forwarded to the Alaska Department of Fish and Game (ADF&G) fish pathology laboratory in Juneau. Additional fish samples were collected near Pelican on August 6, 1998 (Dick Walker pers. comm.), and sent unfrozen to the Juneau ADF&G pathology staff.

Virology and cell culture. Kidneys, spleens and small portions of liver were aseptically removed from

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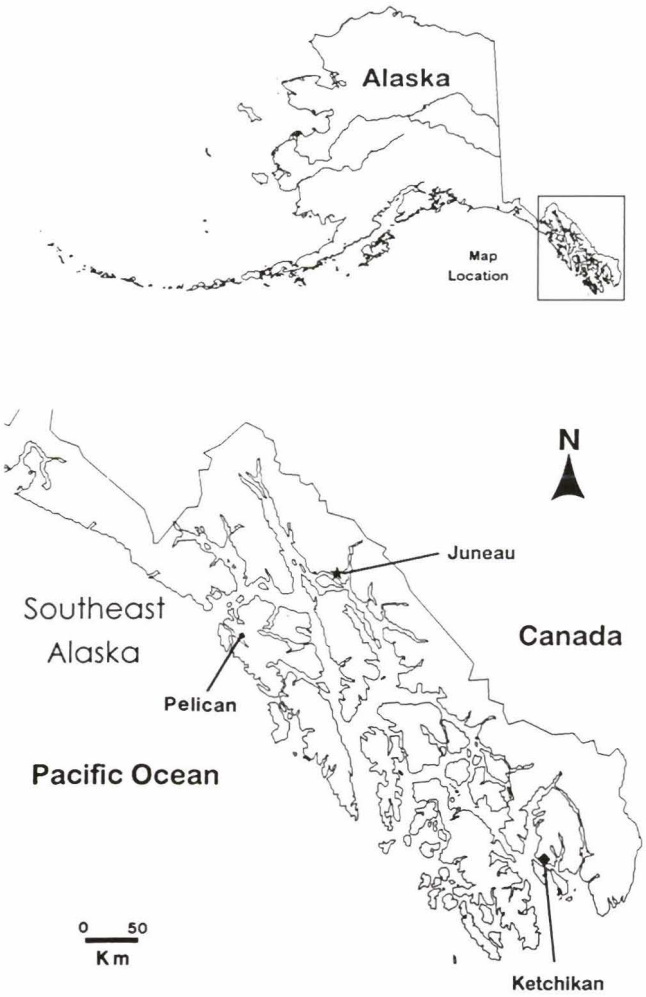


Fig. 1. Southeast Alaska, USA, and the village of Pelican on Lisianski Inlet, Chichagof Island

9 hake and 1 pollock received unfrozen on August 6 and from 6 Pacific herring, 12 hake and 1 pollock received frozen after collection on August 1. Tissues from the 6 herring were pooled together while the tissues from all other individual fish were processed as separate pools. Formulation of Eagle's minimum essential medium and growth of fish cell lines were according to standard procedures described by Meyers et al. (1994). Sample inoculation onto cell cultures (Meyers et al. 1994) began with homogenization of tissues in a Stomacher 80 (Seward Medical Ltd, London, UK) followed by centrifugation at 6000 × *g* for 20 min. Supernatants were inoculated in 0.1 ml volumes from undiluted samples (1:10) and 10^{-2} dilutions into duplicate wells of cells grown in 24-well plates. Inoculated plates were incubated in plastic containers with sealed lids at 15°C for 14 d. Cell cultures having no cytopathic effect (CPE) were blind-passaged onto fresh cell monolayers using 0.1 ml of cell suspension and supernatant from the first dilution well. The unfrozen fish samples were inoculated onto *Epithelioma papulosum cyprini* (EPC) cells (Fijan et al. 1983) and bluegill *Lepomis macrochirus* fry (BF-2) cells (Wolf et al. 1966) while the frozen fish samples were tested only with the EPC cell line. Remaining portions of all processed samples were frozen at –80°C. Estimates of virus titers on these original samples from 4 virus-positive unfrozen fish were determined after 1 freeze/thaw cycle according to the methods of Reed & Muench (1938). The minimum detection level for all samples was 50 infectious virus particles g^{-1} of pooled tissues.

Transmission electron microscopy (TEM). Both EPC and BF-2 cell lines were inoculated with tissues from the unfrozen fish. The EPC cells had been inoculated with pollock tissues while the BF-2 cells had received tissues from Pacific hake. Monolayers of EPC and BF-2 cells showing CPE were fixed overnight at 4°C in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.8). Cells were post-fixed in cacodylate-buffered 1% osmium tetroxide for 1 h at room temperature, dehydrated through a series of ethanol solutions and embedded in Spurr's resin. Ultrathin sections were mounted on 300 mesh copper grids and stained in 4% aqueous uranyl acetate and 2.6% lead citrate. Grids were examined with a Philips TEM 300 at 60 kV.

DNA probe and PCR. Extractions of mRNA from virus isolates of 3 Pacific hake and 1 pollock were tested using the nonradioactive DNA probes against VHSV and IHNV (infections hematopoietic necrosis virus) as described by Batts et al. (1993). The same pollock isolate, 1 of the 3 hake isolates and an isolate from the pooled Pacific herring were also examined by Bill Batts of the Western Fisheries Research Center in Seattle, Washington, using polymerase chain reaction (PCR). The primers used amplified fragments of the N gene (Einer-Jensen et al. 1995) producing a quantity of product specific for the North American strain of VHSV.

Bacteriology. Kidney tissues from the 9 unfrozen Pacific hake and 1 pollock were aseptically inoculated onto plates of tryptic soy agar (TSA) using standard bacteriological procedures. Plates were incubated at 20°C for 4 d and examined for microbial growth. Bacterial isolates were identified from biochemical reactions using the Minitek Differentiation System (Becton Dickinson and Company, Cockeysville, MD).

Necropsy and histology. Laboratory necropsies performed on 5 of the unfrozen Pacific hake included: gross external examination of all surfaces and orifices; microscopic examination of gill and skin wet mounts, Diff-Quik (Dade Diagnostics, Inc., Aquada, Puerto Rico) stained peripheral blood smears; gross internal examination of viscera with microscopic examination of spleen and gut wet mounts. Blood smears were

examined at 1000× magnification and considered negative for erythrocytic necrosis virus (ENV) if no cytoplasmic inclusions were observed in 30 fields. Complete necropsies were not performed on the fish samples received frozen due to severe post-mortem autolysis.

Only the fish received unfrozen were suitable for histological examination. Tissues were collected from 4 hake and 1 pollock that were not examined by necropsy and included gill, kidney, liver, pancreas, air bladder, stomach, pyloric cecae, intestine, rectum and heart. Tissues were preserved in Bouin's fixative for several days followed by transfer to 70% ethanol. Tissue dehydration, paraffin embedment and sectioning followed standard histological procedures. Cut sections on glass slides were stained with hematoxylin and eosin (H&E).

RESULTS

Virus isolation and identification

Diffuse CPE was evident in the highest plate dilutions of 10^{-3} for both EPC and BF-2 cells receiving individually pooled tissues from all 10 unfrozen fish (Table 1). The frozen fish produced CPE in EPC cells from 10 of 12 samples (Table 1) and required a blind-passage for definitive CPE to appear in 8 of these. Titration of the original samples from 3 hake and 1 pollock from the unfrozen group produced virus titers of 3.16×10^2 to 3.16×10^6 $TCID_{50}$ ml^{-1} (Table 1).

Ultrathin sections of EPC and BF-2 cells infected with virus isolates from hake and pollock confirmed the presence of rhabdovirus particles within vacuoles and at cell peripheries similar to cultured VHSV described from Pacific cod *Gadus macrocephalus* (Meyers et al. 1992) and Pacific herring (Meyers et al. 1994).

Extractions of mRNA from the 4 titrated virus isolates in Table 1 produced visible staining on nitrocellulose strips when reacted with the DNA probe specific for the N gene sequence of North American VHSV (Meyers et al. 1994). Results from PCR also identified the 3 virus isolates from hake, pollock and herring as North American VHSV (B. Batts pers. comm.).

Bacteriology

Bacterial growth was observed on 8 (including pollock) of 10 TSA plates inoculated with kidney tissues from the unfrozen fish. The organisms appeared to be mixed Gram-negative motile and nonmotile bacteria predominated by *Pseudomonas vesicularis*.

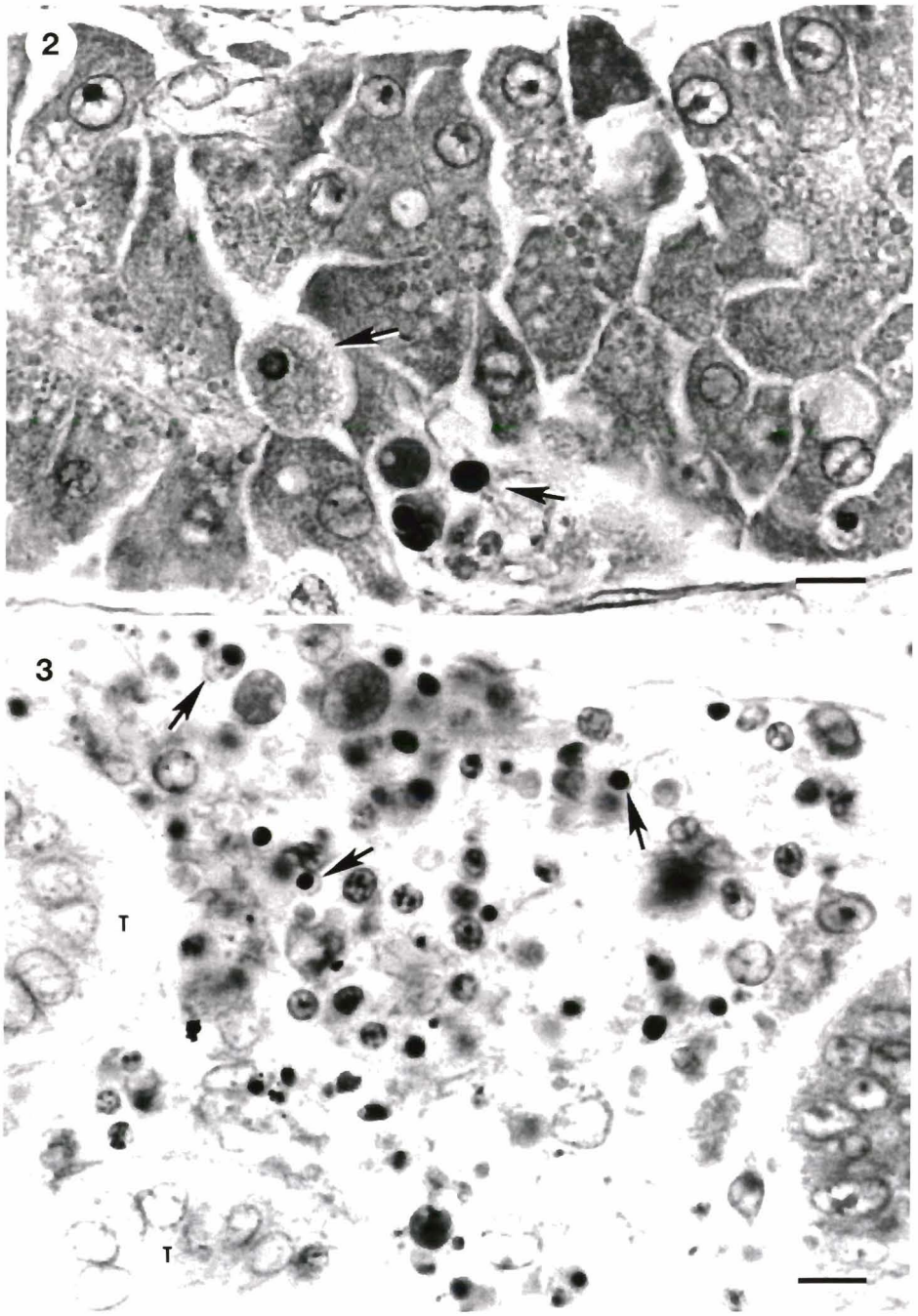
Table 1. Prevalences and titers of viral hemorrhagic septicemia virus (VHSV) in Pacific hake *Merluccius productus*, Pacific herring *Clupea pallasi*, and walleye pollock *Theragra chalcogramma* collected from an epizootic fish mortality near Pelican, Alaska, USA, on August 1 and August 6, 1998. nd: not done; VHSV values: number positive/number examined

Date/fish species	VHSV	Virus titers ($TCID_{50}$ ml^{-1})
August 1 (frozen)		
Pacific hake	8/10	nd
Pacific herring	1/1 (pooled)	nd
Pollock	1/1	nd
August 6 (unfrozen)		
Pacific hake	9/9	3.16×10^2
		3.16×10^5
		3.16×10^2
Pollock	1/1	3.16×10^6

Necropsy and histology

Mean total length and weight for the 9 hake in the unfrozen group were 222 mm and 66 g, respectively. The total length for the pollock was 285 mm with a weight of 147 g. None of these fish had grossly abnormal external or internal appearances. Complete necropsies of 5 hake showed occasional to numerous motile bacterial rods in skin scrapes of 4 fish, post-mortem autolysis of gill tissues with normal red coloration, aneurysms of gill lamellar capillaries in 2 fish, gastro-intestinal tracts devoid of food or fecal material with few helminth parasites, normal spleen wet mounts and normal appearance of peripheral blood cells with no evidence of ENV-type erythrocyte inclusion bodies.

Histological examination of the 4 hake showed microscopic changes including: hemosiderin-like deposits and congestion in the sinusoids and portal triads of the liver (3/4), in lamellar capillaries of the gills (2/4) and vessels of the pancreas (1/4) with minor congestion in the rete mirabile of the air bladder (3/4); minor single cell pyknosis and rounding of hepatocytes (1/4) and pancreatic acinar cells (1/4) (Fig. 2); minor to moderate pyknosis and karyorrhexis of kidney interstitial cells with slight nuclear swelling of tubular epithelium (4/4) (Fig. 3); minor to severe post-mortem autolysis of the gills (4/4), stomach (3/4), intestine (4/4) and rectum (4/4); diffuse fatty change of the liver (3/4); helminth parasites free within the lumens of the stomach (1/4) and intestine/rectum (4/4). Similar microscopic changes were observed in the tissues from the pollock except: kidney tissues were not present in sections; the rete mirabile of the air bladder was not congested; nematode worms were encysted in the liver capsule and pyknosis of hepatocytes was not apparent.



Figs. 2 & 3. *Merluccius productus*. Microscopic lesions in the pancreas and kidney of moribund Pacific hake infected with VHSV collected near Pelican, Alaska, August 1998; H&E, ×1250, bar lengths = 10 µm. Fig. 2. Rounding and pyknosis of pancreatic acinar cells (arrows). Fig. 3. Pyknosis and karyorrhexis of interstitial kidney cells (arrows) with minor nuclear swelling of tubular epithelium (T)

DISCUSSION

Results from the DNA probe and PCR confirmed that representative viral isolates from all 3 species of fish comprising the Pelican fishkill were members of the North American strain of VHSV. Until this report, VHSV in Alaska had only been isolated from Pacific cod (Meyers et al. 1992) and Pacific herring (Meyers et al. 1994). The Pacific herring species is an established marine reservoir for the virus in the Pacific Northwest (Meyers & Winton 1995, Amos et al. 1998), but isolation of VHSV from Pacific hake is a new host record for the virus. Although isolation of VHSV from pollock is also a new host record for Alaska, a recent report from Oregon described isolation of the virus from dying captive pollock as well as Pacific tomcod *Microgadus proximus* and Pacific cod originating from Puget Sound (P. Reno, Oregon State University, unpubl.).

Various possible water quality disturbances were investigated as potential causes of the Pelican fish mor-

tality, including nearby cannery effluent, dinoflagellate blooms and hydrogen sulfide gas from geothermal activity. However, field investigations and eyewitness accounts failed to substantiate any of these possibilities.

Dead fish had empty gastrointestinal tracts suggesting a chronic period of morbidity during which fish were not feeding. There were no gross clinical signs of septicemia to suggest a bacterial cause for the mortality in the 10 fish examined. Although *Pseudomonas vesicularis* was isolated from 8 of the unfrozen fish specimens, this organism is not known to be a primary fish pathogen. This bacterial species belongs to the unusual rRNA Group IV of pseudomonads and was originally isolated from freshwater leeches and aquatic environments (Krieg & Holt 1984). Consequently, this organism and other bacteria observed in fresh tissue preparations during necropsy were most likely mixed contaminants from the post-mortem autolysis that was evident in the more perishable tissues of the gills, skin and gut. Finally, the minor helminth parasite infestation observed should not have resulted in fish mortality and there were no obvious signs of anemia or associated erythrocytic inclusion bodies of ENV.

North American VHSV is extremely virulent for previously unexposed young-of-the-year herring (Kocan et al. 1997), but produces a more chronic stress-related infection in older herring (Meyers et al. 1994). Detection of VHSV in the Pelican fish was not considered incidental based on the high virus prevalences in both the unfrozen (100%) and frozen (83%) fish samples. Also, virus titers were originally 10 000 infectious particles ml^{-1} or more in all the unfrozen fish, with higher virus titers in 2 of the 4 titrated samples. Despite ultra-low storage temperatures of –80°C, 1 freeze-thaw cycle of samples can inactivate titers of North American VHSV by 1 to 3 orders of magnitude (ADF&G unpubl.). This inactivation would explain the 2 virus-negative samples from the frozen fish and the lower virus titers in those samples received unfrozen that were frozen once prior to titration.

Virus-infected fish also had histological evidence of necrosis in the kidney and pancreas which may not have been caused by post-mortem autolysis observed in other more perishable tissues. Whether these lesions were caused by VHSV infection will have to be investigated further by controlled virus exposure studies with hake and pollock. However, similar kidney and pancreatic lesions were reported for juvenile herring experimentally infected with VHSV (Kocan et al. 1997) and for the VHSV-infected marine fish species recently reported from Oregon (P. Reno pers. comm.). Although liver is a primary target organ of VHSV in herring, the minor hepatocyte pyknosis observed in the liver of only 1 hake was not convincing of virus infection. Should any or all of these lesions prove to be caused by VHSV, it is questionable whether the tissue damage observed was severe enough to result in mass fish mortality. The aneurysms found in the lamellae of 2 fish further suggest potentially significant gill lesions that may have been masked by post-mortem decomposition. The epitheliotropic nature of North American VHSV in Pacific cod (Meyers et al. 1992, 1994) and in herring (Meyers & Winton 1995, Kocan et al. 1997) offers some potential for epithelial hyperplasia or necrosis of gill tissues that may contribute to fish mortality. Nonetheless, additional testing of both healthy and moribund individuals of these 2 fish species from any future mass mortality or virus challenge experiments is warranted to establish conclusively whether mortality may be caused by VHSV infection.

Pacific herring were the most likely source of VHSV in the Pelican epizootic because they are established reservoirs for North American VHSV (Meyers et al. 1994, Meyers & Winton 1995, Hershberger et al. 1999). In addition, the juvenile herring tested were positive for VHSV and were among the first dead fish observed in Lisianski Inlet. Within a few days, these herring reportedly migrated out of the inlet. Pacific hake and pollock are pelagic continental shelf species that feed on any available forage fish including herring. It is plausible that VHSV was transmitted to the hake and pollock while feeding on dead and dying virus-infected herring. Although North American VHSV may have contributed to the population decline of Pacific herring in Prince William Sound, Alaska (Marty et al. 1998), this is the first report of a natural epizootic associated with VHSV in which progressive mass mortality was observed simultaneously in herring and 2 other species of free-ranging marine fish.

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