Experimental transmission of dermal sarcoma to the sauger *Stizostedion canadense*

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ABSTRACT: Walleye dermal sarcoma virus (WDSV) has been identified as the causative agent of a benign neoplasia of walleye *Stizostedion vitreum*, walleye dermal sarcoma (WDS). We conducted an experimental transmission regimen to determine if WDSV is capable of inducing dermal sarcoma in the closely related sauger *S. canadense*. Nearly all of young-of-the-year saugers (96%) inoculated with filtrates of spring-collected tumors developed dermal sarcomas, while all of the inoculated walleyes developed tumors. Most of the sauger tumors were limited to the skin, but invasive tumors, similar to those previously observed in experimental walleyes, were observed in some fish.

KEY WORDS: Dermal sarcoma Sauger Neoplasia Retrovirus

INTRODUCTION

Two species of the percid genus *Stizostedion*, walleye *S. vitreum* and sauger *S. canadense*, are found in North America (cited in Billington et al. 1991). Allozyme differences and mitochondrial DNA restriction length polymorphisms suggest that walleyes and saugers diverged 3 to 8 million years ago (Billington et al. 1991). Walleyes and saugers are often found in the same bodies of water and are interfertile as are their hybrid off-spring, saugeyes (Stroud 1948, Clayton et al. 1973, Billington et al. 1988, Ward & Berry 1995). Saugeyes may represent approximately 10% of the sauger-walleye population in some lakes (Nelson & Walburg 1977, Van Zee et al. 1996).

Walleye dermal sarcoma (WDS) is a commonly observed benign skin lesion of walleye (Walker 1969, Yamamoto et al. 1976, 1985, Bowser et al. 1988, Martineau et al. 1990). WDS is seasonal, lesions being present from late fall until the spring, when their regression is nearly complete (Bowser et al. 1988, Bowser &

Wooster 1991). Walleye dermal sarcoma virus (WDSV) has been molecularly cloned from tumor tissue and subsequently shown to be a complex retrovirus (Martineau et al. 1992, Holzschu et al. 1995). WDS has been transmitted experimentally to young-of-the-year (YOY) walleye by intramuscular injection of cell-free filtrates of tumor homogenates (Martineau et al. 1990). While some skin diseases, e.g. lymphocystis, have been documented for walleyes and saugers, there are no published reports of saugers in North America presenting WDS (Amin 1979). In this study, we used our laboratory transmission protocol to establish that infectious WDSV can induce dermal sarcoma in the sauger. The pathology of the lesions is described along with documentation of WDSV presence in the experimentally induced tumors.

METHODS

Fish. YOY walleye and saugers were obtained from the LaSalle Hatchery of the Illinois Department of Natural Resources, La Salle, IL, USA. Fish were fed swim-up fathead minnow fry *Pimephales promelas* and later frozen guppies *Poecilia reticulata*, ad libitum during the study.

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Preparation of cell-free inoculum. Cell-free filtrates were prepared from composite samples of WDS collected in the spring of 1996, and from normal walleye skin. The preparation of cell-free filtrates has been described (Bowser et al. 1997). Briefly, tissues collected from adult walleyes were immediately placed on dry ice and stored at -80°C . Tumor tissues were thawed, homogenized with a tissue grinder and suspended at a 1:3 (weight:volume) dilution in sterile 0.01 M phosphate-buffered saline (PBS; pH 7.2). The suspension was sonicated for 1 min in the cold at 20 kHz and 28 W and centrifuged at $1200 \times g$. The supernatant was passed through a $0.45 \mu\text{m}$ porosity membrane filter to produce the WDS cell-free filtrate for injection. Skin from yearling walleyes was used to prepare a normal skin (control) cell-free filtrate by the same procedure employed to prepare tumor filtrates.

Experimental transmission. The fish were kept in 37 l glass aquaria with flowing 15°C dechlorinated municipal water and aeration. All fish were acclimated for 1 wk after being stocked in the aquaria. Due to the limited number of fish available, the following study design was used: 28 saugers, in 3 aquaria, were injected with tumor filtrate; 17 saugers, in 2 aquaria, were injected with a normal walleye skin filtrate; 9 walleyes, in 2 aquaria were injected with tumor filtrate while 6 walleyes were injected with the normal skin filtrate. The 14 wk old fish (walleyes, 53 mm mean total length; saugers, 46 mm mean total length) were injected with 0.1 ml of cell-free filtrate into the right epaxial musculature. Fish were maintained in the aquaria for 24 wk and observed daily for deaths or adverse signs. At 24 wk post-inoculation, all fish were euthanized with tricaine methane sulfonate (MS-222) and examined grossly for tumors. In an independent experiment reported elsewhere (Bowser et al. 1997), walleyes originating from Oneida Lake, New York, USA, received the same tumor filtrate and control inocula. These fish from New York serve as an additional control to the present study. Fish were fixed in 10% neutral buffered formalin and 4 cross-sections per fish were embedded in paraffin. Thin sections were stained with hematoxylin and eosin stains for histological examination.

Significant differences in percent incidence of tumors between groups of fish were determined with a chi-squared test (STATISTIX for Windows 1.0, Analytical Software, Tallahassee, FL, USA) at the end of the 24 wk study.

PCR amplification of WDSV *gag* sequences from experimental tumors. During the examination of fish at 24 wk, tissue samples from control fish and samples that included tumors along with uninvolved tissue were taken and frozen at -80°C . The samples were thawed and added to 1 ml of digestion buffer, 0.5%

sodium dodecyl sulphate (SDS), 100 mM EDTA, 50 mM Tris pH 8, and $500 \mu\text{g ml}^{-1}$ proteinase K. Following incubation at 55°C overnight, $10 \mu\text{g}$ of yeast tRNA (Sigma, St. Louis, MO, USA) was added and the samples were extracted with buffer saturated phenol:chloroform, precipitated with ethanol, and taken up in $100 \mu\text{l}$ of TE (10 mM Tris pH 8.0, 1 mM EDTA). One μl of these samples was added to standard polymerase chain reactions (PCR) containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl_2 , 200 μM deoxyribonucleotide triphosphate (dNTP), and primers at 0.5 μM . The mixtures were incubated at 96°C for 5 min; 2.5 units of *Thermus aquaticus* (TAQ) polymerase were added (Gibco BRL, Grand Island, NY, USA), and incubation continued at 94°C for 30 s, 53°C for 30 s, 72°C for 30 s for 25 cycles, followed by 72°C for 5 min. The forward primer 5'ATCCCATGGATTCTAGTCTC begins at position 989; the reverse primer 5'GACCGAGTCAGAACTCCGTC begins at position 1495 in the *gag* gene of the WDSV genome (Holzschu et al. 1995). Amplification products were separated by agarose gel electrophoresis and visualized following staining with ethidium bromide (Sambrook et al. 1989).

RESULTS

Experimental transmission

We have used an established WDS transmission protocol to determine if saugers will support dermal sarcoma (Bowser et al. 1990). Survival of the saugers ranged from 82 to 89%; the survival of the Illinois walleyes was lower, 55% to 66% (Table 1). Both walleyes and saugers presented dermal sarcoma within weeks of inoculation with dermal sarcoma cell-free filtrates prepared from Oneida Lake, NY, walleyes. Transmission was very efficient, with 96% and 100% of the saugers and walleyes presenting tumors, respectively (Table 1). In a parallel experiment using the

Table 1 *Stizostedion canadense* and *S. vitreum*. Incidence of walleye dermal sarcoma (WDS) in saugers and walleyes from Illinois injected intramuscularly with cell-free tumor filtrates (WDS) or cell-free filtrates of normal skin (Control). Percentage of fish developing dermal sarcoma in all groups challenged with cell-free tumor filtrate was significantly different from control groups of fish ($p > 0.05$; chi-squared test)

Fish	Inoculum	Survival/no. inoculated	Tumor positive
Sauger	WDS	25/28	24/25 (4/24 invasive)
	Control	14/17	0/14
Walleye	WDS	5/9	5/5
	Control	4/6	0/6

same inoculum (Bowser et al. 1997), 100% of 12 wk old walleyes from New York presented WDS. There were no significant differences ($p > 0.05$) in the incidence of experimentally induced dermal sarcoma between the groups of Illinois saugers, Illinois walleyes and New York walleyes. Most of the saugers presented superficial lesions typical of WDS (Martineau et al. 1990). Four of the saugers presented invasive tumors similar to those seen following injection of 12 wk old walleye fingerlings from New York (Earnest-Koons et al. 1996, Bowser et al. 1997). There was no significant difference ($p > 0.05$) in the incidence of invasive tumors between the saugers (17%) and New York walleyes (5%) (Bowser et al. 1997). The Illinois walleye did not develop invasive tumors, but the small number of fish available for this study does not allow for a meaningful comparison.

Grossly and histologically, the tumors appeared identical to those described previously for WDS (Martineau et al. 1990, Earnest-Koons et al. 1996). The

tumors, most numerous near the site of injection, appeared grossly as small (1 to 3 mm in diameter), slightly raised masses on the surface of the skin. Fig. 1 shows histological examples of the superficial tumors observed on saugers and walleyes. Figs. 2, 3 & 4 show sections of invasive tumors of saugers. In Fig. 2 neoplastic cells can be seen that have invaded musculature near the skull. Figs. 3 & 4 are of a neoplasm that has invaded and displaced a large portion of the musculature surrounding the vertebral column. Additionally, neoplastic cells have invaded the spinal column causing erosion of the vertebrae.

PCR detection of viral DNA in tumors

Since WDSV is thought to be the etiological agent of dermal sarcoma, tissue samples were taken at the time of necropsy for DNA preparation and PCR of viral sequences (Fig. 5). A number of nonspecific products

Fig. 1 *Stizostedion canadense* and *S. vitreum*. Histological sections of YOY sauger or walleye following intramuscular injections of a cell-free filtrate of walleye dermal sarcoma from an adult walleye. (A) Dermal sarcoma on a young sauger typical of most dermal sarcomas observed during the study, being superficial in nature. LL: lateral line. (B) Dermal sarcoma on a young walleye following intramuscular injection of a cell-free filtrate of walleye dermal sarcoma. Tumor is similar to (A) in its superficial nature

Fig. 2. *Stizostedion canadense*. Histological section of YOY sauger following intramuscular injection of a cell-free filtrate of walleye dermal sarcoma from an adult walleye. Note the invasive nature of the dermal sarcoma. Neoplastic cells have invaded through the skin into the underlying musculature (arrows) in the region of the skull (S) and brain (B)

were amplified by the *gag* primer pair from both tumorous and control tissues from saugers and walleyes. However, the 495 basepair (bp) viral specific product is only observed in the tumorous tissue samples, lanes 1, 2, and 3 for saugers and lane 7 for wal-

leye. This band is not present from tissues of control saugers, lanes 4, 5, and 6, or control walleyes, lane 8. Because many of the tumors were very small and difficult to isolate, most of the tumor samples also contained a large portion of uninvolved tissue. We attribute the weakness of the viral specific bands and the number of nonspecific bands to there being many cells present in the samples that did not harbor viral target DNA. The same phenomenon was observed when using primer pairs derived from the *env* gene (data not shown).

DISCUSSION

Walleye and sauger are closely related species that are known to hybridize in nature (Stroud 1948, Clayton et al. 1973, Billington et al. 1988, Ward & Berry 1995). Approximately 10% of the fish sampled from 2 Missouri River reservoirs (USA) were identified by morphological traits as F1 walleye-sauger hybrids (Nelson & Walberg 1977). These results were supported by isozyme analysis of 4 loci that also identified 10% of the percids from Lewis and Clark Lake in South Dakota, USA, as hybrids (Van Zee et al. 1996). Interestingly, while walleyes and saugers are interfertile and their ranges largely overlap, there are no published reports of saugers presenting a common disease of walleyes, dermal sarcoma. Since there are no reports of saugers that cohabitate water with walleyes presenting dermal sarcoma, it was of interest to determine if the sauger is naturally resistant to dermal sarcoma.

Fig. 3. *Stizostedion canadense*. Histological section of YOY sauger following intramuscular injection of a cell-free filtrate of walleye dermal sarcoma from an adult walleye. The neoplasm has invaded and replaced much of the musculature surrounding the vertebral column (V). Neoplastic cells have also invaded the spinal column causing an erosion of the bone of the vertebrae (arrows)

Fig. 4. *Stizostedion canadense*. Histological sections of YOY sauger following intramuscular injection of a cell-free filtrate of walleye dermal sarcoma from an adult walleye (high magnification). Note the area of erosion of the spinal column by neoplastic cells (arrows)

We used an established tumor transmission protocol to investigate the susceptibility of saugers to WDS (Martineau et al. 1990). Our results clearly show that dermal sarcoma can be experimentally transmitted to saugers and that WDSV is present in the lesions. It should be noted, however, that experimental transmission of dermal sarcoma to saugers may not be representative of infection in the wild. We injected the saugers with an inoculum that contains a massive dose of viral particles that may have overwhelmed any resistance the saugers may have to WDSV. Therefore, while we have determined that saugers are susceptible to virally induced dermal sarcoma, further experiments are warranted. Investigations using dilutions of inocula and different routes of transmission need to be made to determine the relative susceptibility of saugers and walleyes to WDSV induced dermal sarcoma.

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Fig. 5. PCR amplification of a WDSV *gag* sequence from sauger and walleye tumors. Lanes 1–3: amplification products from dermal sarcomas collected from 3 individual saugers; lanes 4–6: amplification products from 3 uninoculated sauger controls; lane 7: amplification products from a walleye dermal sarcoma; lane 8: amplification products from an uninoculated walleye control; lane 9: no DNA template control; lane 10: 100 basepair ladder (BRL). The arrow points to the *gag* specific WDSV amplification product. The sizes of the DNA products are shown on the right

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