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ISOLATION OF PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS FROM A FLORIDA PANTHER

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ABSTRACT: Pseudorabies virus was isolated in cell culture from the brain tissue of a 3.5-year-old male Florida panther (*Felis concolor coryi*). The virus was not isolated from other tissues collected at necropsy. Based upon a nested polymerase chain reaction (PCR), the virus was determined to have the classical wild-type virulent genotype, glycoprotein I^+ (gI^+) and thymidine kinase$^+$ (TK^+).

Key words: Florida panther, *Felis concolor coryi*, pseudorabies virus, feral swine, *Sus scrofa*, viral ecology, endangered species, interspecies transmission.

INTRODUCTION

The endangered Florida panther (*Felis concolor coryi*) has been under close scientific scrutiny ever since the development of its recovery plan in 1981 (Maehr, 1992). Recently Roelke et al. (1993) reported the occurrence of antibodies to a wide range of infectious microorganisms including feline calicivirus, feline immunodeficiency-like virus and feline panleukopenia virus. Pseudorabies virus has been suspected of affecting the cougar population for a number of years (Roelke et al., 1993). However, neither seropositive cougars have been found, nor has pseudorabies been isolated from dead Florida panthers so far (Roelke et al., 1993). Roelke et al. (1993) suggested that if Florida panthers die as rapidly from pseudorabies virus infection as domestic cats (Dow and McFerran, 1963), then detecting the clinical phase, observing antibody production, or retrieving a carcass fresh enough to make an accurate histologic and virologic diagnosis would be very difficult.

We report the isolation of pseudorabies virus from a Florida panther found dead within 2 days of his last verified living location.

MATERIALS AND METHODS

A 3.5-yr-old male Florida panther (FP#29) was found dead on 27 May 1992 in Gum Swamp, in a mixed hardwood and conifer forest on private land in southern Hendry County, Florida, USA (26°21′N, 81°14′W). The panther had a radio collar and was alive on 25 May 1992 as determined by a radio fix. It was found approximately 1.7 km from that location. There was no sign of a struggle near the carcass and no obvious cause of death. The panther had been vaccinated for rabies (IMRAB®, Rhone Merieux, Athens, Georgia, USA) and feline panleukopenia, feline calicivirus and feline rhinotracheitis viruses (FEL-O-VAX® PCT, Fort Dodge Laboratory, Fort Dodge, Iowa, USA) with the last vaccination given in January 1991. Hair in the stomach was identified by the methods of Maehr et al. (1990). Histologic analysis of tissues was not feasible because of tissue autolysis.

Unfixed samples of the brain (cerebrum and cerebellum), heart, liver, spleen, and serum were collected and stored at −70 C for arbovirus isolation. Initial virus isolation was conducted at the Centers for Disease Control (CDC) Arbovirus Laboratory at Fort Collins, Colorado, USA.

Each tissue was thawed and a 5 to 7 mm^2 piece was ground with a mortar and pestle in enough diluent containing Medium 199 and penicillin (100 IU) and streptomycin (100 μg/ml) (Grand Island Biological Company, Grand Island, New York, USA) to obtain an approximate 10% suspension. The suspension was centrifuged at 2,000 × *g* for 15 min, the supernatant was collected, and 0.1 ml of each sample was

inoculated onto monolayer cultures of serially propagated Vero cells (American Type Culture Collection, Rockville, Maryland, USA) grown in six-well plastic plates and allowed to adsorb for 1 hr at 37 C. The serum specimen was inoculated (0.1 ml) directly onto Vero cell cultures. The cultures then were overlaid with nutrient medium containing 1% Noble agar (Difco, Detroit, Michigan, USA) and a 1:25,000 dilution of neutral red (Grand Island Biological Company) and incubated at 37 C in 5% CO_2 for 10 days or until plaques were observed. If plaques were seen, the cell cultures with plaques were harvested, 2 ml of diluent with 20% fetal calf serum (HyClone Laboratories, Logan, Utah, USA) was added, and the suspension was frozen at −70 C. An aliquot (0.1 ml) of the thawed suspension was inoculated onto additional Vero cell cultures (McLean et al., 1985). The initial Vero passage subsequently was inoculated onto additional Vero cells for the determination of cytopathogenic effect (CPE) and for the preparation of slides for immunofluorescence assay (IFA). The IFA test was used to screen virus isolates against the National Institutes of Health grouping immune reagents for the following groups of viruses: Group A, Polyvalent groups Anopheles A and B and Turlock, Group B, Bunyamwera, Group California, Polyvalent rabies and Herpes M23579 (Calisher et al., 1990). A second Vero passage was made and prepared for observation by electron microscopy (EM) (Zeller et al., 1989).

For EM, monolayer cultures of Vero cells were inoculated with a 1:100 dilution of virus. When CPE involved approximately 50% of the cells, the cells were sequentially fixed in 4% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.3) and 1% osmium tetroxide. Fixed cell pellets were dehydrated in a graded ethanol series and propylene oxide and embedded in Polybed 812 (Polysciences, Inc., Warrington, Pennsylvania, USA). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 410 transmission electron microscope (Philips Co., Eindhoven, The Netherlands).

Identification of the viral isolate was conducted at the Washington Animal Disease Diagnostic Laboratory (WADDL) at Pullman, Washington (USA). Frozen vials of the virus isolate were received from CDC packed on frozen CO_2. Upon receipt, the vials were thawed rapidly in a 37 C water bath and the virus suspension inoculated onto Crandell feline kidney cells (American Type Culture Collection). The virus produced CPE within 48 hr post-inoculation and subsequently was identified by direct IFA and virus neutralization using pseudorabies specific reagents provided by The National Veterinary Services Laboratory (NVSL, Ames, Iowa).

Confirmatory genotypic analysis was conducted at the NVSL and included nested polymerase chain reaction (PCR) and verification of the wild-type pseudorabies genotype by the method of Katz and Pedersen (1992). Briefly, virus samples were amplified in porcine kidney cells (PK-15, NVSL) and the viral DNA extracted (Katz and Pedersen, 1992). For each viral DNA specimen, first stage thymidine kinase (TK) and glycoprotein I (gI) gene segment DNA amplifications were conducted in separate tubes. Second stage TK and gI amplifications then also were conducted separately, using 1.5 μl of the first stage product as the second stage specimen. All reactions (50 μl) contained 1.25 units of Taq polymerase, 200 μm each of dATP, dGTP, dCTP and dTTP, 2.2 mM $MgCl_2$ and 25 moles of each primer diluted into a commercially available TRIS buffer, pH 8.3 (Perkin Elmer-Cetus Corp., Emeryville, California, USA). Glycerol was added to 10% (v/v) to increase primer annealing specificity as well as to reduce thermal degradation of the polymerase at the high denaturation temperature employed. First stage thermal cycling conditions were: 3 min at 99 C followed by rapid cooling to 4 C and rapid heating to 90 C for 3 min. This denatured the target DNA and maintained it above the primer annealing temperature, allowing for maximal specificity by minimizing initial nonspecific primer annealing. The polymerase was added during the 90 C step, after which followed five cycles at variable temperatures and time in seconds. The cycles consisted of 98 C (40 sec), 65 C (1 sec), 74 C (75 sec), and an additional 30 cycles of 98 C (20 sec), 65 C (1 sec) and 74 C (75 sec). Second state PCR conditions were identical except that the 74 C extension time was reduced to 40 sec.

Separate aliquots (10 μl) of each amplification reaction were subject to electrophoresis for 3 hr at 65 volts through 2.75% (w/v) agarose gels (Seaplaque, FMC Bioproducts Corp., Rockland, Maine, USA) both before and after incubation (37 C, 60 min) with 1 μl of Nco I, Sal I or Sac I restriction endonucleases (Boehringer Mannheim Corp., Indianapolis, Indiana, USA). Gels were immersed briefly in ethidium bromide solution and then photographed under ultraviolet illumination.

RESULTS

Upon presentation for necropsy, the panther weighed 50 kg, was in good physical condition, but the carcass was decomposed. There was red gelatinous blood

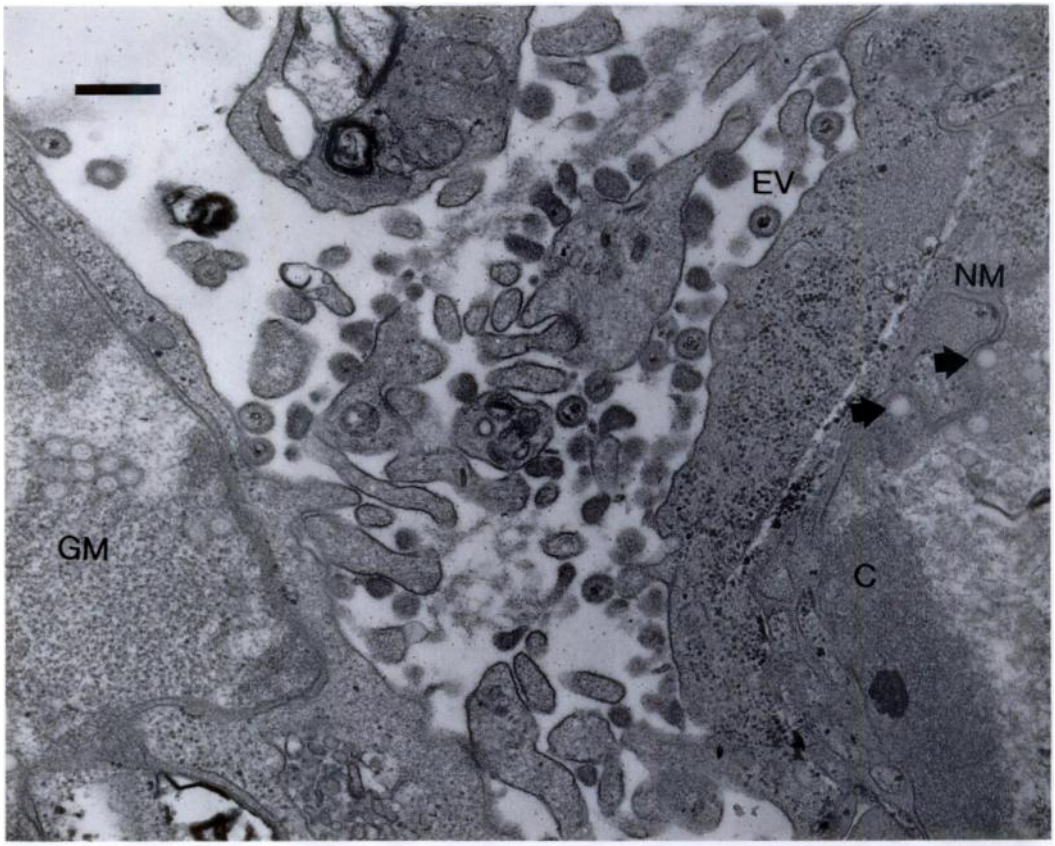

FIGURE 1. Electron microscopy of Vero cells 18 hr postinfection with pseudorabies virus isolated from Florida panther. Enveloped virion (EV), nuclear membrane (NM), chromatin (C), and granular material (GM). Acquisition of the viral envelope is occurring at the nuclear membrane (arrows). (×49,000) Bar = 400 nm. Uranyl acetate and lead citrate stain.

loosely adhered around the skin of the nares. The left testicle was within the scrotal sac while the right testicle had not descended normally. Dark red fluid was in peritoneal and thoracic cavities. The esophagus and stomach contained a moderate amount of dark red, slightly viscous fluid; a small amount of hair identified as originating from a wild swine also was in the stomach. The duodenum contained similar contents as the stomach and had multiple pinpoint to 3 mm round erosions through the mucosa; the remainder of the intestines contained mucus and a small amount of swine hair. Meningeal blood vessels were engorged with blood. The conclusions of the gross necropsy were a retained right testicle, mild to moderate multifocal erosions of the duodenum, and severe, diffuse autolysis of the body as a whole.

All unfixed tissue and serum samples of panther FP#29 were negative for virus isolation except the brain tissue, which yielded a virus in Vero cell culture. On day 5 following inoculation, small (about 1 mm) plaques began to appear and grew only slightly by day 7 when the cell sheet was harvested. The virus was successfully re-isolated in Vero cell culture from the original brain tissue with the same pattern of small plaques appearing by day 5. All of the IFA tests were negative for the arbovirus groups tested; therefore, EM was performed in an attempt to identify the virus and place it in a virus family. When

a second Vero passage was made for EM observation, unique CPE was noted at about 15 to 18 hr postinfection. The Vero cells were about 10 times their normal size with syncytia, rounding, and ballooning apparent. Virus maturation was very rapid, with all viral replication stages present within the first 18 hr postinfection (Fig. 1). Non-enveloped capsids appeared singly or in small groups scattered through the cell nucleus. Large numbers of particles were formed rapidly and synchronously and formed intranuclear viral crystals (Fig. 1); virus-like crystals could be seen as areas of fine granular material about 10 nm in diameter, which may make up the capsomeric protein. The single membrane particles seen in thin section were non-enveloped capsids and the double membrane forms were the enveloped virions. The acquisition of the envelope occurred at the nuclear membrane. Based on the EM photographs, the structure, and the estimated size of 155 to 175 nm of the virus, we propose that the isolate was a member of the Herpesviridae.

The proposed herpesvirus subsequently was identified at the WADDL as pseudorabies virus by IFA and virus neutralization. This finding was supported by virus neutralization studies conducted at NVSL. Based on the glycoprotein gI and thymidine kinase gene segment amplification analysis, the panther isolate was determined to be of the wild-type genotype gI^+ and TK^+ (Fig. 2).

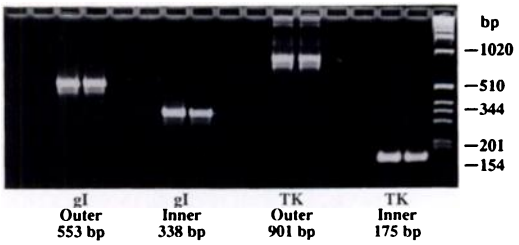

FIGURE 2. Photograph of glycoprotein I (gI) and thymidine kinase (TK) gene segment analysis of pseudorabies viral DNA recovered from the Florida panther isolate (WSU 92-10542). Both external (outer) and internal (inner) nested amplified DNA segments were characteristic and specific for the presence of a wild-type pseudorabies virus genome. Each polymerase chain reaction was conducted in duplicate. Molecular size standards; far right lane, bp: base pairs.

DISCUSSION

Although there have been prior serologic surveys of Florida panthers, pseudorabies virus antibodies have not been detected (Roelke et al., 1993). Thus, either no exposure had taken place, or panthers exposed to pseudorabies did not survive long enough to produce detectable antibodies; however, few deaths of Florida panthers have gone without explanation (Maehr et al., 1991). Feral swine in Florida are exposed to, and in all likelihood, are infected with pseudorabies virus (Pirtle et al., 1989). Recently Van der Leek et al. (1993) reported that feral swine populations throughout Florida were seropositive to pseudorabies virus, with a composite seroprevalence of 35% (range of 6% to 58%). Since feral swine are an important component in the diet of the Florida panther (Maehr et al., 1990), it is inevitable that some panthers will be exposed to pseudorabies virus from the ingestion of feral swine.

Because FP#29 was alive 2 days prior to the discovery of death, we believe that death was sudden. The pathologic finding of swine hair remnants in the digestive tract is evidence that the panther recently had ingested swine tissues. Feral swine probably were the source of the pseudorabies virus that was eventually isolated.

Our findings support the contention that pseudorabies virus can infect the Florida panther (Roelke et al., 1993). The isolation of pseudorabies virus from a dead Florida panther augments our understanding of the natural ecology of this virus since it had been speculated for a number of years that the virus may have a negative impact upon panther survival in the wild. However, because this is the first verified instance of panther mortality resulting from pseudorabies, its true impact on the population is unknown. In general, infectious

diseases have not been regarded as a major factor in Florida panther mortality (Maehr et al., 1991). Nonetheless, vaccination of Florida panthers with an inactivated pseudorabies vaccine should be considered during the routine captures of study animals in order to provide some level of immunity against this disease.

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