Pseudorabies Virus Shedding and Antibody Production in Invasive Wild Pigs in California

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ABSTRACT: Pseudorabies virus (PrV) is a herpesvirus endemic in invasive wild pigs in the United States. The virus has the potential to spill over into domestic herds and wildlife causing extensive morbidity and mortality. We surveyed 35 wild pigs from Kern County, California for evidence of exposure to PrV using serological analysis and for viral shedding using quantitative PCR. All 29 individuals that had sufficient sera to screen for antibody production via serological assay were positive. Two of 35 individuals were found to be shedding virus via genital mucosa. An additional 5 individuals were suspected to be shedding virus either in genital mucosa or circulating virus in their bloodstream, but these results were not definitive. The prevalence of viral shedding for PrV in Kern County, California (6%) suggests that native wildlife are susceptible to spillover of this virus which is deadly to carnivore species.

Wild pigs (*Sus scrofa*) have been in California since the 1920s when Eurasian boar were introduced as game, and domestic pigs were released and became feral (Waithman et al. 1999, Mayer and Brisbin 2008). Wild pigs are considered a game mammal by California Department of Fish and Wildlife, yet throughout much of the United States (US) wild pigs are viewed as an exotic invasive species and classified as feral livestock, nuisance wildlife, or as an invasive exotic mammal because of their destructive

potential to native flora, fauna, and personal property. In addition, wild pigs harbor many pathogens that are damaging to native wildlife, livestock, and public health. Wild pigs are known to carry multiple protozoan, bacterial and viral agents including *Toxoplasma gondii*, *Brucella* spp., *Leptospira* spp., porcine parvovirus, porcine reproductive and respiratory syndrome virus, porcine circovirus, and pseudorabies virus (Cleveland et al. 2017).

Pseudorabies virus (PrV) is an alphaherpesvirus endemic to wild pigs throughout the US (Pedersen et al. 2013). Typical serological prevalence ranges from 2 to 64% in invasive wild pigs in the US and its territories (Cleveland et al. 2017; Musante et al. 2014). The virus causes mild symptoms in adult domestic and wild pigs, but unweaned piglets have significant morbidity and mortality associated with infection (Hahn et al. 1997; Müller et al. 2001). In 2004, the US swine industry concluded a successful vaccination effort for domestic pigs that rid the industry of the disease. Wild pigs pose the threat of reintroduction of PrV back into commercial herds.

In addition to threatening the livestock industry, PrV can spillover into wildlife species where it is deadly to carnivore species (Müller et al. 2001). Mortalities due to PRV infection have been documented in raccoons (*Procyon lotor*; Thawley and Wright 1982; Platt et al. 1983), bears (Schultze et al. 1986; Zanin et al. 1997) canids (Caruso et al. 2014; Verpoest et al. 2014), and the endangered Iberian lynx (*Lynx pardinus*; Masot et al. 2017). PRV is also a significant cause of mortality in the endangered Florida panther (*Puma concolor coryi*; Glass et al. 1994; M. Cunningham pers. comm.). Transmission occurs from eating infected tissue as a result of predation, when scavenging species feed on swine carcasses, or when hunters feed raw meat to hunting dogs. In California, black bears (*Ursus americanus*) and cougars (*Puma concolor*) have been documented to depredate wild pigs and are thus at risk of contracting PrV.

Like other herpesviruses, PrV produces a lifelong infection in swine that can reactivate during periods of stress. As the virus reactivates from a latent state, it begins to circulate and shed from mucous glands in the mouth, nose, and genitalia; it can also circulate in the blood (Hernández et al. in review). Animals can thus sporadically shed and transmit the virus throughout their lifetime. While most studies of wildlife diseases estimate the number of animals that have been exposed to a pathogen and are producing antibodies to the virus (serological analysis), few studies actually estimate the number of infectious animals that are actively shedding the pathogen. Knowledge regarding the prevalence of PrV viral shedding is important in understanding the risk of transmission to native wildlife. In this study, we surveyed wild pigs from Kern County, California for evidence of exposure to and shedding of PrV.

STUDY AREA

The study was conducted on a 1,100 km^2 privately owned cattle ranch located approximately 100 km north of Los Angeles, California in the Tehachapi Mountains in Kern County, California. This mountain range ran southwest to northeast, was bordered by the Grand Central Valley and Mojave Desert, and formed a linkage between the Coast and Sierra Nevada Ranges. Due to its unique geographic location, the ranch hosted a diverse assemblage of vegetation communities including oak savannahs and woodlands, conifer forests, and riparian corridors.

METHODS

From June 2016 through January 2017, biological samples were collected from wild

pigs that were collared for an animal movement study. Pigs were trapped using a corral style trap with panels and a swing head gate. A heavily modified squeeze chute originally designed for sheep and goats was attached to the head gate with tie down straps. The door to the trap and one end of the squeeze chute were opened and pigs were herded into the squeeze chute one at a time. For adult animals only, approximately 40 ml of blood was drawn from the jugular vein, and the mouth, nose, and genital region were swabbed. Sex and ear tag number were recorded. All animals were handled by USDA APHIS WS Operations personnel. Animal handling followed established protocols and was approved by the National Wildlife Research Center Institutional Animal Care and Use Committee (IACUC protocol: QA-2521).

Serology

To assess PrV antibody production, we collected sera from whole blood. Whole blood was immediately placed into Covidien® serum separator tubes (Covidien AG, Dublin, Ireland). Samples were refrigerated at 4 C as soon as possible after collection, and centrifugation occurred within 12 hours of collection. Sera were aliquoted into 2-mL Corning® cryovials (Corning Incorporated, Lowell, Massachusetts, USA) and labeled with a unique barcode for each wild pig. Samples were frozen for up to a month prior to shipment on ice packs to the Kentucky Federal Brucellosis Laboratory. Sera were screened using the PrV-gB enzyme-linked immunosorbent assay per the manufacturer's recommendations (ELISA; IDEXX Laboratories, Westbrook, Maine, USA).

Viral shedding

We used the detection of viral genetic material to infer viral shedding of PrV in wild pigs. Although the detection of viral genetic material does not necessarily reflect pathogen viability in tissues, a previous study of PrV found that when viral DNA was detected, live virus was also recovered from the same tissues and was indicative of infectious material (Müller et al. 2001). Whole blood (0.5 mL) was stored immediately in 1 mL mammalian lysis buffer (Qiagen, Valencia, CA, USA) in the field. Nasal, oral, and genital swabs were collected and stored in 1.5 mL mammalian lysis buffer. Samples were immediately refrigerated at 4 C or kept on ice packs, transported to the University of Florida and stored at -80 C until DNA could be extracted. Due to logistical constraints, we were not able to collect every sample type from every animal.

For downstream analyses of viral DNA shed into blood and mucous, we extracted DNA from these biological samples using previously published methods (Hernández et al. in review). We used previously published primers and a probe targeting the 5' coding region of the PrV glycoprotein B (*gB*) gene (also known as *UL27*) in order to detect PrV DNA in all sample types. All reaction conditions were used as in Sayler et al. (*in press*). To control for false negatives due to low sample yield, we used a commercially available nucleic acid internal control (VetMax Xeno Internal Positive Control DNA, Applied Biosystems, Foster City, CA). Assays were also run with negative controls (molecular grade water) and extraction controls (i.e., no template controls) to detect false positives due to contamination. The cutoff value for this qPCR assay was 39 Cq (threshold cycle), which corresponded to the average Cq for the detection of 10 copies of PrV DNA which represented the lower limit of detection of the assay (Sayler et al. *in press*). PCR amplification that cycled after this threshold value was considered a negative result. PCR-positive samples were confirmed in triplicate

Table 1. Results of serology and qPCR for 7 positive and suspected positive invasive wild pigs from Kern County, California.

Pig ID	Date collected	Sex	Serology (+/-)	qPCR Positive/ Suspected Positive	qPCR Ave. Cq value[a]
1	7/13/2016	F	+	Suspected Positive	34 (1/3)
2	12/8/2016	F	+	Suspected Positive	38 (1/3)
3	12/13/2016	M	+	Suspected Positive	39 (1/3)
4	7/12/2016	F	+	Suspected Positive	34 (1/3)
5	11/8/2016	M	+	Suspected Positive	35 (1/3)
6	11/15/2016	F	+	Positive	37.6 (3/3)
7	12/21/2016	M	+	Positive	38.3 (3/3)

[a] Numbers in parentheses refer to the number times successful amplification occurred in triplicate samples.

when at least two-thirds of the replicates were PrV DNA positive.

RESULTS
Serology was conducted when enough sera was collected for testing on 29 of 35 individuals, and all samples tested positive for the presence of antibodies to PrV. We performed PrV-gB qPCR assays on 145 samples collected from 35 animals; three individuals were sampled twice. qPCR was conducted on 37 blood, 38 nasal, 38 oral, and 32 genital samples. We detected PrV DNA above the threshold of detection (Cq=39) in three of three replicate tests for two genital samples from two unique individuals. One sample came from a female wild pig collected in November 2016. The other positive sample came from an adult male who was not shedding virus in July 2016, but tested positive for shedding in December of the same year. Five additional samples (3 genital and 2 blood samples) tested positive initially, but those results were not replicated upon additional testing and were therefore considered suspected positives without confirmation (Table 1). Each positive or suspected positive came from a unique animal. No animal had a positive or suspected positive result in > 1 sample type.

DISCUSSION
All animals that were tested for antibody production were seropositive (n=29) suggesting that all animals had been exposed to PrV and were possible carriers of the virus. This is the highest prevalence of PrV exposure reported to date in the US. It is not surprising that we found two individuals (6% prevalence) which were shedding virus, and 5 additional animals (20% prevalence) which were suspected to be positive for viral shedding. All of these animals had levels of circulating virus that were near the limit of detection of the assay (Sayler et al. in press), which is the likely reason we had multiple suspected positives that could not be confirmed. The Cq values of our samples further suggest that virus was circulating in animals at a low level which is consistent with a herpesvirus that has reactivated and is recirculating in an animal.

The percentage of wild pigs shedding virus (6-20%) was similar to previously published studies of viral shedding from animals in Europe (5.5% in Spain; González-Barrio et al. 2015 and 18.7% in Italy; Verin et al. 2014) and in the US (0-60% in Florida; Hernández et al. in review). In these areas, PrV has been documented to kill endangered and threatened species such as the Iberian lynx (Masot et al. 2017) and Florida Panther (Glass et al. 1991). The route of spillover transmission to wildlife has been linked to consumption of infected prey or carcasses. Contributing factors that may promote PrV transmission via scavenging includes the disposal of unwanted parts of wild pigs carcasses in 'gut pits' or at harvest sites (Gioeli and Huffman 2012). PrV has been

shown to remain intact in the environment for one to two weeks (Sobsey and Meschke, 2003; USDA Animal and Plant Health Inspection Service 2008; Paluszak et al. 2012) and may facilitate additional opportunities for PrV to spill over into wildlife via environmental exposure.

MANAGEMENT IMPLICATIONS
All wild pigs in our study had been exposed to PrV and had the potential to be carriers of the virus. While the two positive animals were circulating virus in the fall, virus reactivation can occur at any time of year by any carrier animal. Suspected positive animals were found in July, November, and December; thus, native wildlife on this cattle ranch are potentially exposed to PrV throughout the year. Native wildlife on this property that are susceptible to disease from PrV include 13 species of carnivores. Carnivores that have the potential to be exposed via scavenging include raccoon, badger (*Taxidea taxus*), bobcat (*Lynx rufus*), cougar, coyotes (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), red fox (*Vulpes vulpes*), the endangered San Joaquin kit fox (*Vulpes macrotis*), and black bear. Black bear and cougar have been documented to prey on wild pigs on the property. Given the threat of PrV to native wildlife including threatened and endangered species, control of the wild pig population may be warranted and care should be taken by hunters to dispose of offal and carcasses in a manner that does not allow carnivores or companion animals to scavenge the remains.

Surveillance for viral shedding provides a more comprehensive indication of the risk of transmission of PrV from pig to pig and from pigs to wildlife. Like previous studies (Hernández et al. in review), we found evidence of viral shedding from multiple tissue types. For a more accurate estimation of viral shedding, we recommend that biosamples from multiple origins (oral, nasal, genital, and blood) be collected from each animal to provide an accurate representation of viral shedding in the population.

LITERATURE CITED
Caruso, C., A. Dondo, F. Cerutti, L. Masoero, A. Rosamilia, S. Zoppi, V. D'Errico, C. Grattarola, P.L. Acutis, and S. Peletto. 2014. Aujeszky's Disease in red fox (*Vulpes vulpes*): phylogenetic analysis unravels an unexpected epidemiologic link. Journal of Wildlife Diseases 50:707–710.

Cleveland, C.A., A. Denicola, J.P. Dubey, D.E. Hill, R.D. Berghaus, and M.J. Yabsley. 2017. Survey for selected pathogens in wild pigs (*Sus scrofa*) from Guam, Marianna Islands, USA. Veterinary Microbiology 205:22-25.

Gioeli KT and J. Huffman. 2012. Land managers' feral hog management practices inventory in Florida. Proceedings of the Florida State Horticultural Society 125:2012.

Glass C.M., R.G. McLean, J.B. Katz, D.S. Maehr, C.B. Cropp, L.J. Kirk, A.J. McKeiman, and J.F. Evermann. 1994. Isolation of pseudorabies (Aujeszky's disease) virus from a Florida panther. Journal of Wildlife Diseases 30:180–184.

González-Barrio, D., M.P. Martín-Hernando, and F. Ruiz-Fons. 2015. Shedding patterns of endemic Eurasian wild boar (*Sus scrofa*) pathogens. Research in Veterinary Science 102:206-211.

Hahn, E., G. Page, P. Hahn, K. Gillis, C. Romero. 1997. Mechanisms of transmission of Aujeszky's disease virus originating from feral swine in the USA. Veterinary Microbiology 55:123–130.

Hernández, F., K.A. Sayler, C. Bounds, M.P. Milleson, A.N. Carr, and S.M. Wisely. 2018. Pseudorabies virus (PrV) shedding in feral swine (*Sus scrofa*) populations of Florida. Journal of Wildlife Diseases. 54:45–53.

Masot, A.J., M. Gil, D. Risco, O.M. Jiménez, J.I. Núñez, and E. Redondo. 2017. Pseudorabies virus infection (Aujeszky's disease) in an Iberian lynx (*Lynx pardinus*) in Spain: a case report. BMC Veterinary Research 13:6. doi:10.1186/s12917-016-0938-7

Mayer, J.J. and I.L. Brisbin. 2008. Wild pigs in the United States: their history, comparative morphology, and current status. University of Georgia Press.

Müller, T.F., J. Teuffert, R. Zellmer, F.J. Conraths. 2001. Experimental infection of European wild boars and domestic pigs with pseudorabies viruses with differing virulence. American Journal of Veterinary Research 62:252–258.

Musante, A.R., K. Pedersen, and P. Hall. 2014. First reports of pseudorabies and winter ticks (*Dermacentor albipictus*) associated with an emerging feral swine (*Sus scrofa*) population in New Hampshire. Journal of Wildlife Diseases 50:121-124.

Paluszak, Z., A. Lipowski, and A. Ligocka. 2012. Survival rate of Suid Herpesvirus (SuHV-1, Aujeszky's disease virus, ADV) in composted sewage sludge. Polish Journal of Veterinary Science 15:51–54.

Pedersen, K., S.N. Bevins, J.A. Baroch, J.C. Cumbee Jr, S.C. Chandler, B.S. Woodruff, T.T. Bigelow, and T.J. DeLiberto. 2013. Pseudorabies in feral swine in the United States, 2009–2012. Journal of Wildlife Diseases, 49:709-713.

Platt, K.B., D.L. Graham, and R.A. Faaborg. 1983. Pseudorabies: experimental studies in raccoons with different virus strains. Journal of Wildlife Diseases 19:297–301.

Sayler, K.A., T. Bigelow, S. Swenson, C. Bounds, F. Hernández, and S.M. Wisely. Development of a rapid, simple and specific qPCR assay for detection of pseudorabies in domestic swine herds. Journal of Veterinary Diagnostic Investigation. In press. doi: 10.1177/1040638717706593

Schultze, A.E., R.K. Maes, and D.C. Taylor. 1986. Pseudorabies and volvulus in a black bear. Journal of the American Veterinary Medical Association 189:1165–1166.

Sobsey, M.D. and J.S. Meschke. 2003. Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin. Report for the World Health Organization, Geneva, Switzerland.

Thawley, D.G. and J.C. Wright. 1982. Pseudorabies virus infection in raccoons: A review. Journal of Wildlife Diseases 18:113–116.

United States Department of Agriculture, Animal and Plant Health Inspection Service. 2008. Pseudorabies (Aujeszky's disease) and its

eradication: a review of the U.S. experience. Technical Bulletin No. 1923.

Verin, R., P. Varuzza, M. Mazzei, and A. Poli. 2014. Serologic, molecular, and pathologic survey of pseudorabies virus infection in hunted wild boars (*Sus scrofa*) in Italy. Journal of Wildlife Diseases 50:559–565.

Verpoest, S., A.B. Cay, O. Bertrand, M. Saulmont, and N. De Regge. 2014. Isolation and characterization of pseudorabies virus from a wolf (*Canis lupus*) from Belgium. European Journal of Wildlife Research 60:149–153.

Waithman, J.D., R.A. Sweitzer, D. Van Vuren, J.D. Drew, A.J. Brinkhaus, I.A. Gardner, and W.M. Boyce. 1999. Range expansion, population sizes, and management of wild pigs in California. The Journal of Wildlife Management 63:298-308.

Zanin, E., I. Capua, C. Casaccia, A. Zuin, and A. Moresco. 1997. Isolation and characterization of Aujeszky's disease virus in captive brown bears from Italy. Journal of Wildlife Diseases 33:632–634.