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EVIDENCE OF PSEUDORABIES VIRUS SHEDDING IN FERAL SWINE (*SUS SCROFA*) POPULATIONS OF FLORIDA, USA

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ABSTRACT: Feral swine (*Sus scrofa*) are a pathogen reservoir for pseudorabies virus (PrV). The virus can be fatal to wildlife and contributes to economic losses in the swine industry worldwide. National surveillance efforts in the US use serology to detect PrV-specific antibodies in feral swine populations, but PrV exposure is not a direct indicator of pathogen transmission among conspecifics or to non-suid wildlife species. We measured antibody production and the presence of PrV DNA in four tissue types from feral swine populations of Florida, US. We sampled blood, nasal, oral, and genital swabs from 551 individuals at 39 sites during 2014–16. Of the animals tested for antibody production, 224 of 436 (51%) feral swine were antibody positive while 38 of 549 feral swine (7%) tested for viral shedding were quantitative polymerase chain reaction (qPCR)-positive for PrV. The detection of PrV DNA across all the collected sample types (blood, nasal, oral, and genital [vaginal] swabs) suggested viral shedding via direct (oronasal or venereal), and potentially indirect (through carcass consumption), routes of transmission among infected and susceptible animals. Fourteen of 212 seronegative feral swine were qPCR-positive, indicating 7% false negatives in the serologic assay. Our findings suggest that serology may underestimate the actual infection risk posed by feral swine to other species and that feral swine populations in Florida are capable of shedding the virus through multiple routes.

Key words: Aujeszky's disease, ELISA, feral swine, qPCR, spill over, *Suid alphaherpesvirus 1*, transmission.

INTRODUCTION

In the US, feral swine (*Sus scrofa*) are one of the most-common exotic invasive ungulates, and they have dramatically expanded their range and abundance since their first introduction in the early 1500s by European conquistadors (Wood and Barret 1979). The rapid spread of the species poses serious disease threats to native ecosystems and livestock industries (Meng et al. 2009). Ongoing national serologic surveillance efforts provide useful information about the geography of disease prevalence (Pedersen et al. 2013), yet additional information is needed to understand the actual risk posed by persistently infected feral swine as amplifiers of multihost pathogens that may affect naïve native wildlife as well as commercial species.

Feral swine harbor and transmit more than 65 agents of diseases (Seward and VerCauteren 2004). One of those agents is the pseudorabies virus (PrV) (also known as *Suid alphaherpesvirus 1* or Aujeszky's disease virus), a pathogen of domestic and feral swine (Mettenleiter 2000) that is highly adapted to a single reservoir host, but it is capable of spilling over to multiple susceptible species where it has a very high fatality rate (King et al. 2012). The PrV usually causes mild symptoms in adult swine but causes significant morbidity and mortality in unweaned piglets (Hahn et al. 1997; Müller et al. 2001). Once feral swine are infected, the virus establishes a lifelong latent infection accompanied by relatively decreased levels of neutralizing antibodies (Ruiz-Fons et al. 2007; Pedersen et al. 2013). Asymptomatic, persistently in-

fected feral swine can experience sporadic recrudescence (i.e., reactivation and circulation of PrV virions), often brought on by stress (Tozzini et al. 1982; Capua et al. 1997). After reactivation, the virus may be shed in mucosa that provides transmission routes via oronasal (Tozzini et al. 1982; Pirtle et al. 1989) and venereal (Romero et al. 1997, 2001) contact. Indirect routes of infection may operate through contact with fomites or ingestion of contaminated carcasses by other feral swine or carnivores (Hahn et al. 1997; Müller et al. 2011; Pannwitz et al. 2012).

The PrV also causes rapidly fatal infections in carnivores and livestock (Müller et al. 2011). Mortalities due to PrV infection have been reported in raccoons (*Procyon lotor*; Thawley and Wright 1982; Platt et al. 1983), bears (*Ursus* spp.; Schultze et al. 1986; Zanin et al. 1997), and canids (Canidae; Caruso et al. 2014; Verpoest et al. 2014). Pseudorabies virus is also an emerging health threat to the endangered Florida panther (*Puma concolor coryi*), which preys on feral swine and likely consumes hunter-killed carcasses (Glass et al. 1994; M. Cunningham pers. comm.). Globally, PrV can cause significant economic losses to commercial producers (Müller et al. 2011). Although PrV was eliminated from commercial herds in the US in 2004, the expansion of persistently infected, free-living feral swine and the continued maintenance of transitional herds (i.e., small production facilities or outdoor swine farms that mix feral and commercial herds) increases the risk of reintroduction of PrV into commercial herds (Corn et al. 2004; US Department of Agriculture [USDA] 2008; Florida Department of Agricultural and Consumer Services [FDACS] 2016).

In spite of the demonstrated conservation and economic impacts PrV can cause, there is still a paucity of empirical data on the epidemiology of recrudescence and occurrence of viral shedding in free-ranging feral swine populations in the US. This information is essential to understanding the epidemiologic role of feral swine as a transmitter of the virus among conspecifics and to other nonsuid species. The majority of research on PrV has focused on seroprevalence (Nettles 1991; Gaskamp et al. 2016) or the prevalence of PrV DNA in tissues, such as nervous ganglia and tonsils, of latently infected feral swine (Lari et al. 2006; Chiari et al. 2015; Moreno et al. 2015; see Supplementary Table S1). Only a few studies, however, have reported on the prevalence of PrV shedding in nasal, oral, or genital secretions of free-ranging wild pigs, and all of those studies involved native Eurasian wild boars (*S. scrofa*) from Italy and Spain (Verin et al. 2014; Gonzalez-Barrio et al. 2015; see Supplementary Table S1). The prevalence of viral shedding by feral swine in the US has not been previously reported.

In the US, feral swine have been observed in at least 38 states (Bevins et al. 2014), including newly invaded regions in the upper Midwest and long-established populations in focal regions of the Southeast (Müller et al. 2011; Barrios-Garcia and Ballari 2012; Bevins et al. 2014). Regionally, the feral swine population of Florida is estimated to have between 500,000 and one million individuals (Giuliano 2010; FDACS 2016), second only to Texas. Previous studies demonstrated that Florida feral swine populations have been persistently exposed to PrV (van der Leek et al. 1993), but researchers have not explored the dynamics of pathogen shedding or the factors that predict the shedding of PrV in free-ranging feral swine populations. In this study, we explored the prevalence of animals shedding PrV in Florida and the potential role of host individual traits (i.e., age, sex) as modulators of the shedding of PrV in the feral swine populations. Based on PrV-specific antibodies and PrV DNA data, we highlight the relevance of detecting PrV shedding through multiple routes (blood, oronasal, and genital) as a predictor of pathogen spread among feral swine and spillover to other species.

MATERIALS AND METHODS

Sampling of feral swine

From January 2014–March 2016, we sampled 551 feral swine at 39 sites across the state of

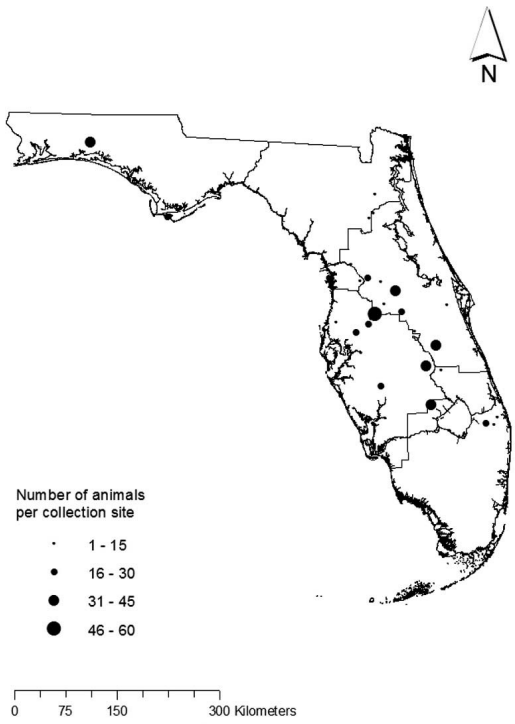

FIGURE 1. Numbers of feral swine (*Sus scrofa*) collected per site through the state of Florida, USA (2014–16) for serologic and molecular testing for pseudorabies virus.

Florida (Fig. 1). Animals were opportunistically sampled as part of a national feral swine disease monitoring effort led by the USDA, Animal Plant and Health Inspection Service, Wildlife Services, National Wildlife Disease Program. Feral swine were either trapped and euthanized during animal-control efforts or shot year-round by hunters in federal and state wildlife management areas, military bases, and private properties. Hunted animals were typically sampled at hunter check-stations. Data recorded for each animal included sex, age, and GPS location in decimal degrees using WGS84 projection. Each animal was categorized as adult (≥1 yr), subadult (2 mo–1 yr), or juvenile (<2 mo) using body size, reproductive traits, and tooth eruption (Matschke 1967). To assess PrV antibody production and viral shedding, we collected blood, nasal, and oral swabs from both male and female feral swine. Genital swabs were collected from females only, based on the role of females as sexual transmitters of PrV (Verin et al. 2014). Due to logistic constraints, we collected each sample type from only a subset of the individuals.

PrV serology

Once feral swine were euthanized, 35 mL of whole blood were collected via cardiac puncture or orbital draw and immediately placed into Covidien® serum separator tubes (Covidien AG, Dublin, Ireland). Samples were immediately refrigerated at 4 C after collection, and centrifugation occurred within 12 h of collection. Sera were aliquoted into 2 mL Corning® cryovials (Corning Incorporated, Lowell, Massachusetts, USA) and labeled with a unique barcode for each feral swine. Samples were refrigerated for up to 4 d prior to shipment on ice packs to the University of Georgia Veterinary Diagnostic Laboratory in Tifton. Sera were screened using the PrV-gB enzyme-linked immunosorbent assay (ELISA) per the manufacturer's recommendations (IDEXX Laboratories, Westbrook, Maine, USA). This assay has a diagnostic sensitivity of 99%.

Viral DNA preparation and detection

Whole blood (0.5 mL) and nasal, oral, and genital swabs were placed in 1 mL mammalian lysis buffer (Qiagen, Valencia, California, USA) in the field and immediately refrigerated at 4 C after collection or kept with ice packs. Samples were transported to the University of Florida, Gainesville, and stored at –80 C until DNA could be extracted. For quantitative polymerase chain reaction (qPCR) analysis, DNA was extracted from blood and swabs using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions, which were slightly modified to maximize the concentration of recovered DNA. Modifications to the protocol included vigorous mixing of the samples prior to extraction, increasing the amount of starting material (i.e., 200 µL for blood and 300 µL for swabs), and a longer incubation period during final DNA elution (i.e., up to 15 min with shaking). The concentration of recovered nucleic acids was quantified using the Epoch Microplate Spectrophotometer running the Gen5 software, version 2.09 (BioTek Instruments, Winooski, Vermont, USA). Recovered DNA was stored at –20 C until further processing.

We used 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 various sample types (Sayler et al. 2017). To control for false negatives, we used a commercially available nucleic acid internal control (VetMAX™ Xeno™ Internal Positive Control DNA, Applied Biosystems, Foster City, California, USA). Assays were also run with negative controls (molecular grade water) and extraction controls (i.e., no template controls) to eliminate detection of false positives due to contamination.

The PrV-gB qPCR assays were performed on the ABI 7500 fast thermocycler by using Brilliant III Ultra-Fast qPCR Master Mix (Agilent, Santa Clara, California, USA) with 2 μL of template DNA, 0.4 μL of PrV-gB forward primer at 20 μM, 0.4 μL of PrV-gB reverse primer at 20 μM, and 0.4 μL of PrV-probe at 10 μM. Cycling conditions were set as follows: 95 C for 3 min followed by 40 cycles of 95 C for 15 s and 60 C for 30 s. The cutoff cycle value for this qPCR assay was 35 quantification cycles (Cq), which corresponded to the average number of Cq for the detection of 10 copies of PrV DNA and represented the lower limit of detection of the assay (Sayler et al. 2017). Any Cq values >35 were considered a negative result. Positive qPCR samples were confirmed by triplicating the assay (i.e., if at least two-thirds of the replicates had Cq values ≤35).

Statistical analyses

For all statistical analyses, we only included data with conclusively positive or negative diagnostic results (n=510) and if the animal age (39 juveniles, 47 subadults, 424 adults) and sex (237 males, 273 females) were known. We used single logistic regression models to assess the relationships between the probability of shedding PrV (positive or negative)—both overall and route-specific shedding—and host age and sex. Because we did not collect genital swabs from males, we only included female data to test the relationship between the probability of genital PrV shedding and age. We used a Kruskal-Wallis nonparametric test to evaluate differences among the Cq values of PrV DNA-positive blood, nasal, oral, and genital swabs because Cq value distribution was found to violate the assumption of normality (Shapiro-Wilkes test, $P<0.05$). We assessed statistical significance at an alpha level of 0.05. Statistical analyses were performed using the software R v. 3.2.2. (R Core Team 2015).

RESULTS

We performed PrV-gB qPCR assays on 439 blood, 498 nasal, 408 oral, and 196 genital samples (Table 1). We detected PrV DNA in 7% (38/549; confidence interval (CI)=5–9%) of feral swine in either blood, nasal, oral, or genital swabs (across all the tested feral swine). The probability of shedding PrV was not influenced by age (juvenile vs. subadult: β_1 0.64, z-value 0.88, P=0.38; juvenile vs. adult: β_1 −1.03, z-value −1.86, P=0.06) or sex (β_1 0.59, z-value 1.55, P=0.12 for data including 33 PrV DNA-positive feral swine with known age and sex; Table 1).

The prevalence of route-specific (blood, nasal, oral, genital) PrV shedding was not significantly related to age or sex of feral swine (β_1 coefficients and z-scores of all logistic regression models had $P>0.05$). Animals of all age classes exhibited relatively similar PrV shedding prevalence in blood, nasal, oral, and genital swabs (except oral swabs that were all PrV DNA-negative for juveniles; Table 1). Although we detected PrV DNA at similar prevalence among all sample types (Table 1), no animal was found to shed the pathogen through more than one route simultaneously. Average Cq values were similar among PrV DNA-positive blood, nasal, and oral samples and were close to the cutoff value (35 Cq) determined for our qPCR assay, suggesting that animals were shedding at low levels close to the limits of detection of our assay. The Cq values were not statistically different among PrV DNA-positive blood, nasal, oral, and genital samples (Kruskal-Wallis test; χ^2=5.04, df=3, P=0.17). Mean Cq values of all PrV DNA-positive tissue types were closer to the cutoff value (35 Cq) determined for our qPCR assay except for the PrV DNA-positive genital samples mean (±SE) of 30.2 (3.4) Cq (Table 1). This lower mean was driven by one PrV DNA-positive genital sample with a Cq value of 20.6.

We found that 51% (224/436, CI=47–56%) of the feral swine that were tested for both PrV exposure and shedding exhibited PrV-specific antibodies in their serum. About 6% (13/224, CI=3–10%) of the seropositive animals were also positive for PrV DNA in either blood, nasal, or oral swabs (see Supplementary Table S2) while 94% (211/224, CI=90–97%) of seropositive animals were PrV DNA-negative. We found 7% (14/212, CI 4–11%) of the seronegative animals were PrV DNA-positive in either blood, oronasal, or genital swabs (see Supplementary Table S2) while 93% (198/212, CI=89–96%) of the seronegative animals were PrV DNA-negative.

TABLE 1. Percentages of blood, nasal, oral, and genital swabs in feral swine populations of Florida, USA (2014–16) that tested positive for pseudorabies virus (PrV). Only values for individuals with known age and sex are included. Mean (±SE) values of quantification cycle (Cq) corresponding to PrV DNA-positive blood, nasal, oral, and genital swabs are indicated.[a]

	Percent positive (no. positive/no. tested)				
Tissue	Juveniles	Subadults	Adults	Total	Cq
Blood					35.3 (0.4)
Males	5 (1/20)	4 (1/24)	1 (2/156)	2 (4/200)	
Females	0 (0/16)	0 (0/20)	3 (7/203)	3 (7/239)	
Total	3 (1/36)	2 (1/44)	2 (9/359)	3 (11/439)	
95% CI	1–14	1–12	1–5	1–4	
Nasal					35.8 (0.3)
Males	0 (0/18)	9 (2/23)	1 (2/190)	2 (4/231)	
Females	0 (0/14)	4 (1/22)	1 (2/231)	1 (3/267)	
Total	0 (0/32)	7 (3/45)	1 (4/421)	1 (7/498)	
95% CI	—	1–18	0–2	1–3	
Oral					35.1 (0.5)
Males	0 (0/21)	6 (1/16)	1 (2/138)	2 (3/175)	
Females	0 (0/16)	0 (0/19)	4 (7/198)	3 (7/233)	
Total	0 (0/37)	3 (1/35)	3 (9/336)	2 (10/408)	
95% CI	—	1–15	1–5	1–5	
Genital					30.2±3.4
Females	0/14	6 (1/18)	2 (4/164)	3 (5/196)	
Total	0/15	6 (1/18)	2 (4/164)	3 (5/196)	
95% CI	—	0–27	1–6	1–6	

[a] CI = confidence interval; — = not applicable.

DISCUSSION

Our study is a unique report on PrV shedding prevalence in free-ranging feral swine populations in the US and particularly in Florida. Considering the estimated Florida feral swine population to be between 500,000 and one million individuals (Giuliano 2010; FDACS 2016), our findings suggest that 35,000 to 70,000 feral swine may be shedding PrV through blood, nasal, oral, or genital routes. These findings have serious implications for wildlife conservation and for the livestock industry.

We detected PrV DNA in all tissue types we collected (blood, nasal, oral, and genital), suggesting different potential routes of intraspecific and interspecific pathogen transmission. Although the detection of PrV genetic material does not necessarily reflect pathogen viability, we assumed that PrV DNA detection constituted a reliable indicator of virus shedding through multiple routes in feral swine. Previous studies of PrV have detected both virions and viral DNA in up to 70% of tissues of PrV-infected individuals (Müller et al. 2001). Indeed, for multiple herpesviruses, the detection of viral DNA in peripheral tissues has been determined to be an accurate reflection of actual viral shedding (Scinicariello et al. 1993; Burgesser et al. 1999).

We report the detection of virus in the blood of free-ranging feral swine. The presence of PrV in the blood of feral swine may represent a source of pathogen transmission to scavenging animals and carnivores (USDA 2008; Müller et al. 2011; Pannwitz et al. 2012). Cannibalism on PrV-infected feral swine was reported as a potential route of pathogen infection (Hahn et al. 1997). In addition, deaths of Florida panthers (Glass et al. 1994; M. Cunningham pers. comm.) and other species (Zanin et al. 1997; Verpoest et al. 2014) have been linked to consumption of

infected prey or offal. Contributing factors that may promote PrV infection through scavenging would be the disposal of offal of feral swine carcasses in 'gut pits', commonly conducted by managers at public and private hunting areas of Florida (Gioeli and Huffman 2012), and the above-ground disposal of carcasses left by animal control personnel. Pseudorabies virus has been shown to remain intact in the environment for 1–2 wk (Sobsey and Meschke 2003; USDA 2008; Paluszak et al. 2012) and may facilitate additional opportunities for PrV to infect wildlife that feed on feral swine carcasses.

The prevalence of PrV shedding in nasal (1%) and oral (3%) mucosa was similar to the 0% and 1% values, respectively, reported by González-Barrio et al. (2015). Previous research suggests that the oronasal route was an important route of direct transmission (Tozzini et al. 1982; Pirtle et al. 1989), and our study confirmed that viral shedding occurred via these routes. Social gregariousness may increase contact among females and offspring within the maternal group, favoring respiratory-oral contact routes of infection (Vicente et al. 2005). The risk of direct oronasal contact between feral and domestic swine is one of the main potential routes of reinfection of commercial and transitional herds for which management does not practice proper biosecurity (USDA 2003; Corn et al. 2004; Verin et al. 2014).

We found that 3% of females shed PrV through the vaginal tract, which was similar to PrV shedding rates (2%) reported for female wild boar in Spain (González-Barrio et al. 2015) and lower than shedding rates (16%) reported for female wild boar in Italy (Verin et al. 2014). Venereal contact is one of the major routes of transmission among free-ranging feral swine, and our findings support previous experimental evidence of sexual transmission between captive feral and domestic swine (Romero et al. 1997, 2001). The polygynous and promiscuous mating behaviors enhance opportunities of PrV transmission in free-ranging feral swine populations (Romero et al. 2001; Delgado-Acevedo et al. 2010) and may account for the rapid increase in prevalence of this pathogen in Florida (Corn et al. 2004). This pattern of increase has direct implications for the livestock industry because of the presence of transitional herds in areas of high prevalence. Livestock holders have introduced and bred feral swine into feeder pig herds (i.e., transitional herds), enhancing the opportunities for the introduction of PrV back into commercial swine herds (USDA 2008).

Simultaneous data obtained from serology and qPCR tests highlight the limitations of serologic data to accurately detect infected animals or to assess transmission dynamics. We found that 7% (14/212) of animals shed the virus but were serologically negative, suggesting that detection of PrV-specific antibodies alone does not accurately detect infected individuals (Lutz et al. 2003; Ruiz-Fons et al. 2007). False negatives derived from serologic testing could have serious implications for pork producers who may rely on serologic testing to understand the status of individuals for sale or transportation. The 7% of false negatives in our serology data surpassed the sensitivity threshold of the ELISA (99%), which suggests that the number of false negatives were not just simply a function of assay sensitivity.

Multiple factors may have caused the pattern of false negatives in serology. After a primary infection, there is a delay in the production and circulation of antibodies (Gilbert et al. 2013), which might explain the detection of PrV DNA in blood, oronasal, and genital secretions in animals that were not producing detectable levels of antibodies (Ruiz-Fons et al. 2007). Antibodies to PrV can be detected 15 d (or later) in wild boars after contact with experimentally infected wild boars (Müller et al. 2001) and after a delay of up to 8 wk in female feral swine after contact with naturally infected male feral swine (Romero et al. 1997).

The detection of serologic false negatives may also be explained by immunosuppression followed by reactivation of the virus. Reactivation of herpesviruses in latently infected animals has been associated with stress-related factors that cause depression of the host's immune response and trigger PrV

shedding through oronasal and venereal routes (Romero et al. 1997; Müller et al. 2001). An additional factor affecting serologic false negatives may be related to virus pathogenicity. Strains of PrV from wild boar and feral swine are presumed to be more attenuated than are strains of domestic swine; strains from feral animals did not cause clinical signs or produce a humoral response in exposed individuals (Hahn et al. 1997; Müller et al. 2001). Although serologic assays provide cost-effective evidence of past exposure to pathogens and important epidemiologic information, they provide limited knowledge about the PrV potential infection status of individuals (Gilbert et al. 2013).

We did not find a significant influence of age or sex in the overall or route-specific virus shedding in feral swine. We did find that the prevalence of PrV-positive Florida feral swine was similar to that of wild boars in Spain (González-Barrio et al. 2015). These authors suggested a role of females as spreaders of disease owing to the frequent oronasal contact among individuals within female groups. Juveniles have also been found to have a high shedding prevalence in Italian wild boars, probably associated with the occurrence of vertical transmission from infected sows to piglets (Verin et al. 2014). In Florida, feral swine of all age and sex classes were found to shed PrV virus, suggesting that transmission was not confined or determined by any one demographic group.

Our findings suggest that viral shedding occurs in approximately 7% of the population and that the route of infection is variable. Serology alone may underestimate the actual infection risk of individuals and, thus, the likelihood of pathogen transmission to other species. Because the pathogen may be shed through multiple specific-routes (blood, oronasal, genital), the analysis of different tissue types will facilitate a more-accurate assessment of PrV infection status in feral swine and allow for a more-thorough assessment of risk to susceptible hosts including commercial livestock, companion or working animals, and wildlife.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2017-04-071.

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