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Serologic Survey for Selected Infectious Diseases in Raccoons (*Procyon lotor*) in Indiana, USA

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ABSTRACT: The objective of this study was to characterize the antibody prevalence of important livestock and domestic animal pathogens in raccoons (*Procyon lotor*) trapped and sampled in 39 forest patches in north-central Indiana, USA, between 2004 and 2005. A total of 459 serum samples were tested for antibodies to *Leptospira* serovars, 512 for *Canine distemper virus* (CDV) antibodies, and 340 for antibodies to *Porcine reproductive and respiratory syndrome virus* (PRRSV). In total, 47, 16, and 0% of the samples were seropositive for at least one *Leptospira* serovar, CDV, and PRRSV, respectively. Most of the positive *Leptospira* results were to serovars grippotyphosa (36%), autumnalis (22%), and hardjo (22%). No statistically significant correlation was found between antibody prevalence estimates for different *Leptospira* serovars. A significant association was found between body weight and antibodies for *Leptospira* serovars and CDV. In addition, age (adult vs. juvenile) was significantly associated with the presence of CDV antibody, with adults exhibiting a higher prevalence than juveniles. This study confirmed that raccoons in Indiana, USA, are exposed to different *Leptospira interrogans* serovars and CDV and that age and weight are associated with the presence of antibodies for both pathogens.

Key words: *Canine distemper virus*, *Leptospira interrogans*, *Porcine reproductive and respiratory syndrome virus*, *Procyon lotor*, raccoon.

The raccoon (*Procyon lotor*), a mesocarnivore, has a wide distribution throughout most of North and Central America. Raccoons are abundant in landscapes characterized by a diversity of habitat types (Oehler and Litivatis, 1996) and, thus, have responded positively to human-mediated changes in land-use patterns, particularly urbanization and habitat fragmentation caused by agriculture (Prange et al., 2003). The raccoon has been reported to be one of the most important wildlife species associated with damage to agricultural crops (Hamberg et al., 2007), which undoubtedly is related to the increased abundance of this species (Beasley and Rhodes 2007, 2008). Apart from the ecologic and agriculture risks previously mentioned, the recent rise in raccoon abundance is also alarming from an epidemiologic perspective because of the increased potential for transmission of disease from raccoons to domestic species, especially livestock. Numerous diseases have been reported in raccoons, including leptospirosis and canine distemper, both of which can be transmitted to domestic animals. Several studies have found that raccoons were seropositive to *Leptospira interrogans* serovars grippotyphosa and icterohaemorrhagiae (Shotts et al., 1975; Mitchell et al., 1999; Richardson and Gauthier, 2003; Junge et al., 2007), and Mitchell et al., (1999) isolated *L. interrogans* serovar grippotyphosa from 6% of 82 urine samples obtained from seropositive animals. Reilly (1970) reported that raccoons inoculated intraperitoneally with *L. interrogans* grippotyphosa showed no clinical symptoms of the disease despite the presence of serum antibodies, leptospiremia, and leptospiruria. Epizootics of canine distemper also have been reported to occur sporadically among free-ranging raccoons (Hoff et al., 1974; Cranfield et al., 1984; Roscoe, 1993). Although *Canine distemper virus* (CDV) can cause high mortality in raccoons (Hoff et al., 1974; Roscoe, 1993), antibody prevalence

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studies have documented that it also can circulate widely in a population leaving many survivors (Mitchell et al., 1999). Thus, the available evidence suggests that not only are raccoons likely to be wildlife reservoirs for CDV but also that different strains of this virus, with different levels of virulence, affect raccoon populations (Lednicky et al., 2004). The importance of CDV to animal health surveillance programs should not be overlooked because CDV-infected raccoons may exhibit signs similar to those in raccoons with clinical rabies. The similarity of clinical signs for these two diseases can complicate decisions involving surveillance programs designed to detect the entrance of raccoon rabies into states, like Indiana, USA, which currently are free of the disease. Surprisingly, there is only a single study reporting surveillance results for these two diseases in the Midwest, and this study was conducted more than a decade ago (1989–1993) in Illinois, USA (Mitchell et al., 1999). Another important livestock disease agent is the *Porcine reproductive and respiratory syndrome virus* (PRRSV), which causes an estimated $560 million loss annually to the US swine industry (Neumann et al., 2006). Although it is well documented that PRRSV can be transmitted via direct and indirect routes, such as infected pigs, fomites, and airborne spread (Mortensen et al., 2002), the role of the wildlife-livestock interface in the transmission and the epidemiology of the disease has been evaluated only in Mallard ducks (*Anas platyrhynchos*; Trincado et al., 2004). From a biosecurity perspective, the abundance of raccoons in the vicinity of swine production justifies a study to assess whether raccoons have any role in the transmission of PRRSV. Hence, the objective of our study was to characterize the antibody prevalence of *L. interrogans*, CDV, and PRRSV in raccoons inhabiting an agriculturally fragmented landscapes located in north-central Indiana, USA.

The 1,165-km^2 study area was located in the Upper Wabash River Basin (UWB) in north-central Indiana, USA, encompassing portions of Grant, Huntington, Miami, and Wabash counties. Approximately 96% of the land area within the UWB was privately owned, 71% of which was in agricultural use. The research area is within the main concentration of dairy and swine farms in Indiana, USA. Only 13% of the basin was forested, and all contiguous forest tracts within the study area were confined to major drainages where frequent flooding or locally steep topography made the land unsuitable for crop production. Live trapping of raccoons was conducted from March through May during 2004 and 2005 in 39 forest patches distributed throughout the study area. Forest patches were selected based on their size and degree of isolation in an effort to encompass the full range of the distribution of these variables in the study area. Further information about trapping and sampling procedures are detailed elsewhere (Beasley and Rhodes, 2008).

All laboratory procedures were performed at the Indiana Animal Health Diagnostic Laboratory (West Lafayette, Indiana, USA). Detection of the antibody titer to *Leptospira* serovars was performed using microscopic agglutination tests (MAT; Shotts, 1976; Bolin, 1996). Briefly, the MAT was performed by mixing serum samples in serial dilutions with live *Leptospira interrogans* (serovars bratislava, canicula, icterohaemorrhagiae, pomona, autumnalis), *Leptospira kirchneri* (serovar grippotyphosa), and *Leptospira borgpetersenii* (serovar hardjo). Serum samples were serial diluted 10-fold and tested. The results of the MAT were read by dark-field microscopy, and the microagglutination titer was determined as the reciprocal of the highest dilution with 50% agglutination.

The indirect immunofluorescent antibody assay (IFA) for PRRSV was performed as described in details by Nelson et al (1994), with some modification using PRRSV M145–infected cells grown on Teflon-matted, 10-well dot slides. Serum

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samples were serially diluted twofold in Milk dilution blocking solution at pH 7.4 (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland, USA). One aliquot of 50 µl from each dilution was applied to a single well with cells including positive and negative samples on an antigen slide and incubated at 37 C for 30 min. Cells were washed in phosphate-buffered saline for 1–2 min and rinsed in deionized water after each of the incubation steps. Positive reaction was visualized by applying optimally diluted protein A conjugated with fluorescein isothiocyanate (Invitrogen, South San Francisco, California, USA) at the concentration of each corresponding well and incubated for 30 min at 37 C. Antibody titers for individual samples were determined as the reciprocal of the highest dilutions in which specific fluorescence staining was observed under a fluorescent microscope. Antibodies to CDV were determined in the same manner as that described above for PRRSV, with some modification. The CDV was propagated using the A72 (*Canis familiaris*) cell line from American Type Culture Collection (ATCC, Manassas, Virginia, USA) in minimum essential medium with 8% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Missouri, USA), supplemented with 8% FBS (Sigma).

All statistical procedures were performed using statistical software SAS (version 9.1, 2004; SAS Institute, Cary, North Carolina, USA). The correlation between seropositivity to different *Leptospira* serovars was assessed using Spearman correlation. To assess the association between test results (positive/negative) and sex, age, weight, forest patch, and their respective second-order interaction terms (forest patch×sex, and trapping location×age), we used a mixed logistic-regression model (PROC GLIMMIX). This procedure was selected to adjust for the variability between forest patches and possible lack of independence within forest patches observations. Forest patch was considered both as a block and as a random effect. Test results (positive/negative) were used as the outcome of interest, and all other variables were introduced into the model as explanatory variables. Raccoon age was categorized into two levels: juvenile or adult. Raccoon weight was first plotted, and approximately normal distribution was assessed, using PROC INSIGHT in SAS. Weight was analyzed as a continuous variable and as four indicator variables based on the variable quartiles (25th, median, and 75th) using dummy codes. This categorization was performed to relax the linearity assumption and for an easier interpretation of the odd ratios (OR). Variables that had $P \leq 0.15$ in the bivariate analysis were included in the multivariable analysis. The best model to describe the association between the outcome and the independent variables was determined using Akaike's information criterion (AIC) as a measure of goodness of fit.

A total of 459 raccoons (55% male, 50% adult) were tested for *Leptospira* spp. antibodies. Of the 459 raccoon sera tested for *Leptospira* spp, 47% were positive for at least one *Leptospira* serovar. The distribution of the serovars is shown in Table 1. Average body weight for seropositive and seronegative raccoons was 4.9 kg (SD=1.1, maximum [max]=8.2, minimum [min]=1.1) and 4.7 kg (SD=1.1, max=7.9, min=2.0), respectively. In bivariate analysis, body weight used as a continuous variable had a significant association with the presence of *Leptospira* antibodies. Similarly, when weight was categorized into four categories based on the 25th, median, and 75th percentiles, animals with body weights of 4.0–4.6, 4.6–5.6, and >5.6 kg were 1.2 (OR 95% confidence interval [CI], 0.65–2.22; $P=0.56$), 1.8 (OR CI, 1.1–3.1; $P=0.02$), and 1.8 (OR CI, 1.1–3.0; $P=0.03$) times more likely to be seropositive to at least one of the serovars, respectively, than raccoons with body weight <4.0 kg. *Leptospira borgpetersenii* serovar hardjo was

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TABLE 1. General distribution of *Leptospira* serovars among 459 raccoons captured and sampled in 25 forest patches between 2004 and 2005 in northern Indiana, USA.

Leptospira serovars	Positive	%	95% CI[a]
Autumnalis	102	22	18–82
Bratislava	38	8.3	6–94
Grippotyphosa	167	36	32–68
Hardjo	101	22	18–82
Icterohaemorrhagiae	8	1.7	1–99
Pomona	27	6	4–96
Overall[b]	217	47	42–58

[a] 95% CI = 95% confidence interval.

[b] Individuals with at least one serovar positive or all serovars negative.

the only *Leptospira* serovar where a significant association between antibodies and body weight >5.6 kg (OR=1.7; CI, 1.5–21.3; $P=0.01$) was detected. In the bivariate analysis, test results were not associated with sex or forest patch. Significant association was found between seropositivity and age (juvenile vs. adult). Adult raccoons were twice (OR CI, 1.04–2.17) more likely to be seropositive to at least one of the *Leptospira* serovars. In the multivariable analysis, where the dependent variable was a positive antibody result for any of the *Leptospira* serovars, none of the tested interaction terms were found to be statistically significant, and the final model included only body weight with four categories (AIC=644). No correlation was found between seropositivity for different serovars among individuals.

A total of 512 animals (48% females, 27% adults) were tested for CDV. Of these, a total of 81 (16%) were positive for antibodies to CDV. Average body weight for raccoons that were positive and negative to CDV antibody was 5.0 kg (SD=1.1, max=7.2, min=3.1) and 4.8 kg (SD=1.1, max=8.7, min=1.1), respectively. In the bivariate analysis, significant association was found between the presence of CDV antibodies and weight categories (4.6–5.6 kg and >5.6 kg) but not with body weight as a continuous variable. Animals with a weight category of 4.6–5.6 and >5.6 were 2.0 (CI=1.0–4.0) and 2.6 (CI=1.0–5.0) times more likely to be CDV-seropositive than animals with body weight <4 kg, respectively. Significant association was also found between age (adult vs. juveniles) and seropositivity. Adult animals were 2.7 (CI=1.7–4.4) times more likely to be seropositive than juveniles. Sex and forest patch did not have a significant association with CDV antibody status. None of the tested interaction terms was found to be statistically significant. The best model to describe the association between CDV seropositivity and other variables included age and body weight presented in four categories (AIC=441). A total of 456 animals were sampled for both diseases. An association was found between CDV and *Leptospira* serovars (OR=0.35, CI=0.21–0.6). The association with CDV was maintained when analyzing separately the seropositivity for *L. interrogans* serovars grippotyphosa (OR=0.23, CI=0.12–0.46) and autumnalis (OR=0.36, CI 0.17–0.77). A total of 340 (56% males, 62% adults) raccoons were tested for PRRSV. None of these samples exhibited positive results.

The large sample size reported here is the major strength of the current study. The cross-sectional study design, however, is a potential drawback of the study, which did not allow us to evaluate the effect of season on prevalence as well as body weight. Although the geographic distribution of our raccoon samples is relatively small (~1,100 km^2), they originate from a highly intensive agriculture area with a high concentration of cattle and swine farms (www.nass.usda.gov). Very similar *Leptospira interrogans* antibody prevalence (48%) was reported by Mitchell et al. (1999) from the neighbor state of Illinois, USA. It is likely that this similarity in *Leptospira interrogans* prevalence between this study and Mitchell et al. (1999) is because both studies were conducted in rural areas in the midwestern United States. In contrast to the current study,

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Junge et al. (2007) found higher antibody prevalence of *L. interrogans* serovar icterohaemorrhagiae than the current study (8.9% vs. 1.7%), whereas *L. interrogans* grippotyphosa seroprevalence was lower (6.3% vs. 36.4%). These differences might be because Junge et al. (2007) focused on urban raccoons, whereas Mitchell et al. (1999) and our study focused on rural populations. Mitchell et al. (1999) concluded that the bacteria continuously cycles in the raccoon populations and exposure is more likely to be sporadic, occurring in areas that temperature and moisture can sustain the spirochete.

Mitchell et al. (1999) reported CDV antibody prevalence of 23%, which is more in accord with our findings (16%). Junge et al. (2007), however, reported higher CDV antibody prevalence (54%) in raccoons from urban areas. This large difference in prevalence rates between these studies may be due to the higher probability of contact with dogs in urban areas and, hence, higher rates of CDV transmission to raccoons than that found in agriculture areas. Although Junge et al. (2007) did not find any coinfection association between CDV and *Leptospira* serovars, the current study results (OR<1.0) has shown a negative (protective) association between the two diseases. However, the nature of this association is not clear.

Our observed association of age and CDV antibodies is in sharp contrast to the findings of Hoff et al. (1974) and Roscoe (1993). A similar association with age was found by Mitchell et al. (1999) for *Leptospira* serovars and CDV. This association can be explained by the fact that older animals potentially have been exposed to the pathogen longer and, therefore, have developed higher antibodies titers. Also, as speculated by Mitchell et al. (1999), juvenile home ranges are smaller than adult animals and, hence, the expected exposure to the pathogen should be lower. Similar to our study, the authors did not found any association between sex and antibody prevalence for either pathogen. Nevertheless, using the same hypothesis about young vs. adults exposure, male exposure should be significantly higher because their home ranges are nearly twice as large as females.

Although the high antibody prevalence is alarming and suggests that raccoons might be a reservoir for *Leptospirosis interrogans* and CDV, a series of key questions should be addressed before determining the significance of an infected wild animal species as a reservoir host for any disease. These include the nature of the infection in individual animals, the dynamics of infection in the population, the geographic and local distribution of the wildlife host, and the interaction of the wildlife host with domestic animals (Corner, 2006). The answers to these questions are, however, beyond the scope of the current study. The epidemiologic importance of an animal as a reservoir is only confirmed when active pathogen shedding occurs through urine, feces, saliva, or air drops. Mitchell et al. (1999) isolated *Leptospira* grippotyphosa from five of 82 urine samples (6%) from antibody-positive raccoons, indicating the potential role of raccoons as reservoir for this pathogen. Hamir et al. (2001), however, detected the *Leptospira* spirochete in the kidneys of 24% and 17% of examined raccoons in Pennsylvania, USA, and Oregon, USA, respectively. The temporal and quantitative patterns of this active shedding should also be characterized to determine the epidemiologic importance of raccoons as reservoirs. Currently, our research group is working to assess the presence of these pathogens in raccoon urine.

In the current study, for the first time to our knowledge, a substantial number of raccoons were tested for another important livestock disease agent: PRRSV. It is important to emphasize that PRRSV is endemic in Indiana, USA, which is the fourth largest swine-industry state in the United States. Our power calculation indicates that, with the sample sizes used, our probability to detect a difference as

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low as 6% in seropositivity prevalence for PRRSV was 70%. Thus, the negative results suggest with reasonable confidence that raccoons probably do not play a major role as disease reservoirs for this pathogen.

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