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Prevalence of and Associated Risk Factors for Shedding *Cryptosporidium parvum* Oocysts and *Giardia* Cysts within Feral Pig Populations in California

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Populations of feral pigs (*Sus scrofa*) may serve as an environmental reservoir of *Cryptosporidium parvum* oocysts and *Giardia* sp. cysts for source water. We conducted a cross-sectional study to determine the prevalence of and associated demographic and environmental risk factors for the shedding of *C. parvum* oocysts and *Giardia* sp. cysts. Feral pigs were either live-trapped or dispatched from 10 populations located along the coastal mountains of western California, and fecal samples were obtained for immunofluorescence detection of *C. parvum* oocysts and *Giardia* sp. cysts. We found that 12 (5.4%) and 17 (7.6%) of 221 feral pigs were shedding *C. parvum* oocysts and *Giardia* sp. cysts, respectively. The pig's sex and body condition and the presence of cattle were not associated with the probability of the shedding of *C. parvum* oocysts. However, younger pigs (≤8 months) and pigs from high-density populations (>2.0 feral pigs/km^2) were significantly more likely to shed oocysts compared to older pigs (>8 months) and pigs from low-density populations (≤1.9 feral pigs/km^2). In contrast, none of these demographic and environmental variables were associated with the probability of the shedding of *Giardia* sp. cysts among feral pigs. These results suggest that given the propensity for feral pigs to focus their activity in riparian areas, feral pigs may serve as a source of protozoal contamination for surface water.

The role of wild or feral mammals as possible nonpoint sources of *Cryptosporidium parvum* and *Giardia duodenalis* for surface water is a subject of renewed interest given outbreaks of waterborne disease (2, 5, 10, 12, 21, 28), the presence of *Cryptosporidium* oocysts and *Giardia* cysts throughout U.S. surface waters (17, 18, 25), and the impending Enhanced Surface Water Treatment Rule (ESWTR) proposed by the Environmental Protection Agency. Although there exists strong evidence that bovine-derived *C. parvum* is infectious to humans (6, 19, 24), the zoonotic potential of *C. parvum* from wild or feral mammals is currently unknown. The zoonotic potential of *G. duodenalis* from domestic, wild, or feral mammals remains inconclusive (7), in part due to the ongoing confusion regarding the taxonomy of this genus (20). Nevertheless, this debate over the zoonotic potential of these parasites is largely irrelevant for water districts attempting to comply with the ESWTR since current diagnostic procedures which are federally approved for detecting and enumerating *Cryptosporidium* oocysts and *Giardia* cysts in surface water cannot distinguish between zoonotic and nonzoonotic species of *Cryptosporidium* or *Giardia* (7, 11).

Source water protection is being advocated as a method for reducing *Cryptosporidium* and *Giardia* concentrations in surface water. One of the first steps in protecting source water from protozoal contamination is to identify mammalian populations which actively shed these protozoans and which focus their activity in riparian areas. Given the tendency of feral pigs to focus their activities near, and wallow and forage around, the margins of seeps, springs, ponds, and lakes during the summer months (4, 8, 15, 26), we measured the prevalence of fecal shedding of *C. parvum* oocysts and *Giardia* sp. cysts for feral pigs from central and northern California. In addition, we examined whether various host (pig) and environmental variables were associated with the shedding status of each pig in order to identify factors that could signify pig populations at high risk of shedding these protozoans in their feces.

MATERIALS AND METHODS

Selection of study populations. This study was conducted in the Coast Range in central and northern California from June to September 1995. There were two types of study sites: population research sites (population sites) and memorandum-of-understanding feral pig removal sites (removal sites). Population sites were in counties in California where information from hunter-killed feral pig tags returned to the California Department of Fish and Game (CDFG) indicated that feral pig populations were relatively abundant. Removal sites were located in areas where cooperating private-land owners or state resource personnel were removing feral pigs to reduce rooting damage.

Trapping of feral pigs and fecal-sample acquisition. At population sites feral pigs were live-captured in modified panel traps baited with fermented corn. If the pig weighed more than 20 kg, it was chemically immobilized with Telazol and xylazine hydrochloride (31) and the fecal sample was collected from the rectum. If the pig weighed less than 20 kg, it was manually restrained for fecal collection. At removal sites feral pigs were captured in panel traps or box traps (26) and dispatched by rifle or handgun. Fecal samples were collected during necropsy. At population sites and removal sites traps were set in the evening and captured animals were processed the following morning in accordance with the University of California Animal Care and Use Committee Guidelines and CDFG trapping permits. Fecal samples were refrigerated for 1 to 5 days until they could be transported to the laboratory.

Analysis of *C. parvum* oocysts and *Giardia* sp. cysts. Upon arrival at the laboratory, 5 g of each sample was washed through a no. 20 sieve and the suspension was centrifuged at 1,000 × *g* for 10 min. The supernatant was aspirated, and the pellet was resuspended in 2 ml of 10% formalin. Ten microliters of the suspension was smeared onto a commercially prepared glass slide and air dried overnight, and the direct immunofluorescence assay was performed according to the manufacturer's instructions (Merifluor *Cryptosporidium*/*Giardia* direct immunofluorescent detection kit; Meridian Diagnostics, Inc., Cincinnati, Ohio). The entire smear was examined at a magnification of ×400, and the sample was scored positive for *C. parvum* if it had one or more ~5- by 5-μm

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oocysts which fluoresced and positive for *Giardia* if it had one or more ~12- by 15-μm cysts which fluoresced.

To confirm the species of *Cryptosporidium* in the feral pig as *C. parvum*, a representative isolate was purified by using a modification of the method described by Balatbat et al. (3). One hundred microliters of the sample was suspended in 400 μl of TES (50 mM Tris HCl [pH 7.5], 5 mM EDTA, 50 mM NaCl) buffer and centrifuged at 2,000 × *g* for 3 min, and the supernatant was transferred to a microcentrifuge tube and centrifuged at 7,000 × *g* for 5 min. The pellet was resuspended in 5.25% sodium hypochlorite for 10 min on ice and washed in sterile distilled water. Oocysts were incubated at 60°C for 48 h in 500 μl of TEN (10 mM Tris HCl [pH 7.5], 1 mM EDTA, 10 mM NaCl) buffer containing 0.8% Sarkosyl and 200 μg of proteinase K per ml. DNA was extracted with phenol-chloroform-isoamyl alcohol (24:24:1), precipitated in a double volume of 100% cold ethanol, and resuspended in Tris-EDTA buffer.

PCR amplification was performed at final concentrations (amounts) of 1× PCR buffer, 2.0 mM $MgCl_2$, 200 mM each deoxynucleoside triphosphate, 0.4 μM primer specific for *C. parvum* (16), 2.5 U of *Taq* polymerase (Perkin-Elmer, Foster City, Calif.), 0.010 mg of bovine serum albumin (Sigma Chemical, St. Louis, Mo.) per ml, 20 μl of sample DNA, and 12 μl of sterile distilled water. Amplification was done for 35 cycles at 94°C for 20 s, 50°C for 20 s, and 72°C for 60 s, followed by 5-min extension at 72°C. The PCR product and size markers (DNA markers 50–1,000 bp; FMC, Rockland, Maine) were electrophoresed on 2% agarose gel in Tris-borate-EDTA buffer and visualized with ethidium bromide (0.5 μg/ml). Four hundred base pairs of the approximately 452-bp amplicon was sequenced by using the Taq FS sequencing reagent (Perkin-Elmer), 20 ng of the amplicon, and 20 pmol of the Laxer primer (16). The sequence reaction was analyzed on an automated DNA sequencer (Applied Biosystems ABI 377).

Given the ongoing confusion regarding the taxonomy and systematics of *Giardia* (20), DNA from feral pig *Giardia* was not sequenced since the information would be of marginal utility.

Demographic and environmental factors. We examined demographic and environmental factors potentially associated with the shedding of *C. parvum* oocysts and *Giardia* sp. cysts in the feces of feral pigs. These included age, sex, body condition (population sites only), presence of cattle, and two different measures of population density (number of feral pigs per square kilometer and number of hunter-killed-pig tag returns per square kilometer of occupied area). Upon capture each feral pig was sexed, aged by dentition (22), and assigned to one of three age categories: 0 to 8 months, 9 to 18 months, or older than 18 months. Body condition was assessed for feral pigs sampled only at population sites by using residuals from the regression relationship between body mass (in kilograms) and estimated body volume (in cubic centimeters): ln body mass = $-4.55 + 0.86 \cdot \ln$ body volume (adjusted $R^2 = 0.98$, $P < 0.0001$, $n = 118$). Body volume was estimated from sternal chest circumference and dorsal body, which is the length along the dorsal midline of the pig from the base of the skull to the base of the tail. Animals with positive residuals were categorized as being in relatively good condition, whereas those with negative residuals were categorized as being in relatively poor condition (29).

At population sites the density of feral pigs was estimated by dividing the population size estimate for the site (determined by using standard mark-recapture techniques) by the area sampled by traps (determined by tracking the movements of feral pigs by radiotelemetry) (30). Population sites were categorized as high or low density based on calculated population densities of ≥2.0 and ≤1.9 feral pigs/km^2, respectively. We also used information on hunter-killed feral pig tags returned to CDFG to assign a density category to all sampling sites for further analysis. Feral pigs are a game mammal in California, and the 1993 CDFG management program requires hunters to return a portion of the tags that are affixed to all hunter-killed feral pigs to CDFG. Hunter-killed feral pig tag returns (pig tag returns) include information on the approximate geographic location of each animal killed. These data were used to derive an index of feral pig population density based on the number of feral pig tag returns in each county from 1993 to 1996 divided by the estimated area (in square kilometers) occupied by feral pigs in that county. The area occupied by feral pigs in each county was estimated by calculating the area of all 7 1/2′ U.S. Geologic Survey quadrangles occupied by feral pigs in a county (excluding lakes and the Pacific Ocean) with the computer program ARC/INFO (ESRI, Redlands, Calif.) in a geographic information system. This density index was used to classify the county of each population and removal site as high or low density based on ≥0.81 or ≤0.80 pig tag/km^2 of occupied county area, respectively.

Risk factor analysis. The distribution of protozoal shedding in feral pigs was calculated for each demographic and environmental variable, with the associated odds ratios for levels within each variable calculated by using exact parameter estimation and two-sided 95% confidence intervals (23). When the maximum conditional likelihood estimates for the odds ratio did not exist (e.g., 0 in one of the cells), the median unbiased point estimate of the odds ratio was used (13, 23). Statistical models for the association between protozoal shedding in feral pigs and demographic and environmental variables were constructed by using exact conditional logistic regression (23), with a *P* value for inclusion in the model set at ≤0.05. Exact conditional procedures were used for hypothesis testing and for parameter and two-sided confidence interval estimation due to sparse and unbalanced data.

RESULTS

Fecal samples were collected from 221 feral pigs at 10 sites in seven counties (Colusa, Mendocino, Monterey, San Luis Obispo, Santa Clara, Santa Cruz, and Sonoma) in central and northern California. As reported elsewhere (30), estimated population densities were 1.2, 1.8, 2.1, 3.3, 1.7, and 1.9 feral pigs/km^2 at the Bradford Ranch (Mendocino County), Salt Lake Ranch (Colusa County), Austin Creek State Recreation Area (Sonoma County), Henry Coe State Park (Santa Clara County), Rancho San Carlos (Monterey County), and the Chimney Rock Ranch (San Luis Obispo County), respectively. Similarly, county pig tag densities were 0.32, 0.10, 0.69, 0.87, 0.19, 0.44, and 0.24 pig tag returns/km^2 of occupied county area in Mendocino, Colusa, Sonoma, Santa Clara, Santa Cruz, Monterey, and San Luis Obispo counties, respectively.

Twelve of 221 feral pigs were shedding *C. parvum* oocysts in their feces at the time of trapping (Table 1). A diagnosis of *C. parvum* was confirmed by sequencing 400 bp of the approximately 452-bp amplicon and finding 95.5% homology with the *C. parvum* GenBank sequence S74588 (16). The sequence obtained from the feral pig isolate was further confirmed as *C. parvum* by the appropriate location of and 100% sequence homology with two internal probes and three internal primers (3, 16). The sequence for feral pig *C. parvum* can be found in GenBank under accession number U96770.

Shedding of *C. parvum* oocysts was associated with age and population density of feral pigs but not sex, body condition, or the presence of cattle at the research site (Table 1). The odds for the shedding of *C. parvum* oocysts was three times greater for pigs ≤8 months compared to older animals (Table 1). We collapsed the categories 9 to 18 months and >18 months into one category (≥9 months) since both categories had 3% of pigs shedding protozoans. When the association between pig age and the likelihood of shedding *C. parvum* oocysts was controlled for population density, the odds for the shedding of *C. parvum* oocysts was four times greater (1/0.24 = 4.2) for pigs ≤8 months compared to older animals (Table 2). Feral pigs from removal sites with ≥2.0 feral pigs/km^2 were about 10 times more likely to be shedding *C. parvum* oocysts compared to animals from sites with ≤1.9 feral pigs/km^2 (Table 1). Similarly, the odds for shedding *C. parvum* oocysts was 12 times greater for feral pigs from counties with ≥0.81 pig tag return/km^2 of occupied land area compared to pigs from counties with ≤0.80 pig tag return/km^2 (Table 1). When this association between the density of pig tag returns and the likelihood of shedding *C. parvum* oocysts was controlled for pig age, the odds for the shedding of *C. parvum* oocysts was 12.5 times greater for feral pigs from counties with ≥0.81 pig tag return/km^2 of occupied land area compared to feral pigs from counties with ≤0.80 pig tag return/km^2 (Table 2).

Seventeen of the 221 feral pigs sampled were shedding *Giardia* sp. cysts in their feces at the time of trapping (Table 1). However, there were no detectable associations between the odds of the shedding of *Giardia* sp. cysts and any of the demographic or environmental variables that we examined.

DISCUSSION

We found that feral pigs in central and northern coastal California shed both *C. parvum* and *Giardia* in their feces during the months of June to September (Tables 1). Feral pigs are abundant in western California, and these data suggest that under appropriate environmental conditions feral pigs may contaminate nearby surface water with *C. parvum* oocysts and *Giardia* sp. cysts. In particular, feral pigs focus their activities

TABLE 1. Factors evaluated for an association with shedding of *C. parvum* oocysts and *Giardia* cysts by feral pigs in California

Factor[a]	*C. parvum*		*Giardia*	
	No. of pigs[b] (%)	Odds ratio (CI)[c]	No. of pigs[b] (%)	Odds ratio (CI)[c]
Age				
≤8 mo	7/62 (11)	1.0	4/62 (6)	1.0
≥9 mo	5/159 (3)	0.3 (0.09–0.99)	13/159 (8)	1.3 (0.4–5.6)
Sex				
Male	4/97 (4)	1.0	7/97 (7)	1.0
Female	8/124 (6)	1.6 (0.4–7.5)	10/124 (8)	1.1 (0.4–3.6)
Body condition				
Poor	0/52	1.0	7/52 (13)	1.0
Good	1/48 (2)	1.1 (0.2–∞)	3/48 (6)	0.4 (0.07–2.0)
Cattle at research site				
Absent	6/87 (7)	1.0	10/87 (11)	1.0
Present	6/134 (4)	0.6 (0.2–2.5)	7/134 (5)	0.4 (0.1–1.3)
Density of pigs at research site				
≤1.9	0/63	1.0	5/63 (8)	1.0
≥2.0	6/58 (10)	9.6 (1.3–∞)	7/58 (12)	1.6 (0.4–6.8)
Density of pig tags in county				
≤0.80	0/87	1.0	5/87 (6)	1.0
≥0.81	12/134 (9)	11.8 (1.9–∞)	12/134 (9)	1.6 (0.5–6.1)

[a] See "Demographic and environmental factors."
[b] Number positive/number sampled.
[c] Referent categories for the odds of shedding are assigned a value of 1.0. CI, exact 95% confidence interval. See "Risk factor analysis."

near, and wallow and forage around, the margins of seeps, springs, ponds, and lakes during the summer months (4, 8, 15, 26). In this study 5 and 8% of the feral pigs sampled during the summer were shedding *C. parvum* oocysts and *Giardia* sp. cysts, respectively. These prevalence estimates for fecal shedding of these protozoans may be too low due to prepatent infections and sporadic shedding of cysts or oocysts (34). Deposition of feces along these riparian habitats compared to defecating in nonriparian zones increases the likelihood that fecally shed protozoans enter surface water supplies, particularly under conditions of overland flow subsequent to intense rainfall. However, rainfall events of sufficient magnitude both to saturate soils and to create overland flow are rare during the Mediterranean summer months typical for this region of California. If the presence of feral pig populations leads to increased numbers of these parasites in local surface water supplies, additional water treatment requirements could be levied against the affected water district under the proposed ESWTR.

Shedding of *C. parvum* by feral pigs was strongly associated with two independent measures of population density. One density measure was derived from research on live feral pigs at specific sites, whereas the other was a county-wide estimate obtained from hunter-killed feral pigs. Both population density estimates indicated that once a threshold of density was exceeded, the risk of shedding *C. parvum* by individual feral pigs increased dramatically (Tables 1 and 2); no pigs from low-density populations were shedding, compared to 9 to 10% of pigs from high-density populations. Hence, the risk of environmental contamination by *C. parvum* from dense feral pig populations was twofold: not only were there more feral pigs per area unit serving as an environmental reservoir of *C. parvum* within high-density populations, but the prevalence of shedding was considerably higher among dense feral pig populations compared to less dense populations.

Younger feral pigs were more likely to be shedding *C. parvum* oocysts than were older pigs; a similar relationship has been reported for dairy cattle (1, 9), sheep (33), and horses (32). Feral pigs reproduce year round, but there is a peak in parturition in the spring and one in the fall (4, 26). Hence, these are the seasons in which the high-risk age group for shedding *C. parvum* oocysts (e.g., young pigs) will be especially common on watersheds. We found 3.6% (3 of 84) of adult feral pigs (>18 months) shedding *C. parvum* oocysts in their feces. This pattern of adult shedding is uncommon in U.S. adult dairy cattle (1) and adult horses (14, 32). Thus, as we remove livestock and human activity from sensitive watersheds in California, we may also need to consider feral pigs and other wildlife populations as potential threats to surface water quality.

Although the overall proportions of feral pigs shedding *Giardia duodenalis* and those shedding *C. parvum* were relatively similar for these coastal pig populations (8 versus 5%, respectively), the associated risk factors for shedding were

TABLE 2. Adjusted odds ratios for factors associated with shedding of *C. parvum* oocysts by feral pigs in California

Factor	Odds ratio (CI)[a]	*P*[b]
Age		0.02
≤8 mo	1.0	
≥9 mo	0.24 (0.5–0.9)	
Density of pig tags in county[c]		0.003
≤0.80	1.0	
≥0.81	12.5 (2.0–∞)	

[a] Referent categories for the odds of shedding are assigned a value of 1.0. CI, exact 95% confidence interval. See "Risk factor analysis."
[b] Tests the null hypothesis that regression coefficients are 0 (odds ratio = 1.0) by using the exact conditional scores test (23).
[c] See "Demographic and environmental factors."

very different for the two protozoans. Compared to *C. parvum* shedding, for which pig age and population density were strong risk factors, there were no clear associations between the shedding of *Giardia* cysts by feral pigs and the demographic and environmental variables we measured. *Giardia* cysts was shed by 6 to 8% of feral pigs regardless of age, indicating that the risk of contaminating associated surface water with cysts will be a function of climatic variables and how the pigs distribute themselves in riparian areas rather than the seasonal distribution of certain age groups on the watershed.

As we move toward watershed management as a strategy to protect source water quality from *C. parvum* or *G. duodenalis* contamination, we need to identify the primary environmental sources of these protozoans. Furthermore, we also need to understand how mammalian populations infected with these protozoans distribute themselves on a watershed and how climatic and geographic variables influence the risk of contamination. For example, although elk can shed *C. parvum* (27), they calve only in late spring (the dry season) and their activities focus less around water compared to feral pigs. Thus, on an animal-by-animal basis, elk may pose less of a threat to surface water quality than feral pigs. The challenge for source water protection is to integrate this diversity of information into a valid risk assessment and not to rely solely on interspecies comparisons of the levels of prevalence of fecal shedding as we attempt to unravel nonpoint sources of protozoal contamination.

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