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Review Article

Intensity Characterization of Fecal Shedding of cryptosporidium and Risk Factors In Sheep Farms In California, USA

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Abstract

An epidemiological study was conducted to investigate the prevalence of *Cryptosporidium* spp. and intensity of fecal shedding of oocysts in sheep and to identify risk factors in sheep farms in California, USA. A total of 798 fecal samples from 372 adult ewes, 31 yearlings, and 395 lambs were collected from 16 ranches in central and northern California. Quantitative detection of *Cryptosporidium* oocysts in feces were performed using a direct immunofluorescent assay. The overall prevalence of *Cryptosporidium* spp. was 30.6% (121/395) in lambs, 16.1% (5/31) in yearlings, and 3.2% (12/372) in adult ewes. High prevalence was observed in 60-day old [37.4% (61/163)] and 90-day old [38.3% (41/107)] lambs. Infected lambs and adult ewes shed up to 6.8 $\times 10^6$and 1 $\times 10^6$oocystsper animal per day, respectively. Farm management practices, flock, and sheep information were collected from each ranch during each sampling event and used for statistical analysis of risk factors associated with the prevalence of fecal shedding of *Cryptosporidium* oocysts. Access to surface sources of drinking water (odd ratio=1.8) and contact with cattle (odd ratio=3.8) significantly increased the fecal shedding of oocysts in sheep of all ages. The odds of fecal shedding of oocysts in lambs decreased as the number of ewes increased in the flock and the odds of fecal shedding of oocysts in adult ewes decreased as the pasture size increased. Fecal shedding of *Cryptosporidium* oocysts was not significantly associated with diarrhea in sheep.

Importance: *Cryptosporidium* is an important parasite infects a wide range of vertebrates including livestock. *Cryptosporidium* infection in sheep impacts animal health and fecal shedding of oocysts impacts environmental health. Studying the prevalence and risk factors of fecal shedding *Cryptosporidium* in sheep is an important part of controlling the infection and protecting the health of sheep and the environment. This work not only determined variable prevalence of *Cryptosporidium* in lambs, yearlings and ewes in sheep farms and shedding intensity and environmental loading rate, but also identified accessing to surface water and contacting with cattle increased the odds of sheep infection.Incorporating this information to beneficial management practices can reduce the fecal shedding of *Cryptosporidium* in sheep and subsequently reduce environmental loading of this parasite.

INTRODUCTION

Cryptosporidium spp. is a coccidian parasite with a worldwide distribution and public health relevance [1]. Several genetically distinct species and genotypes can be transmitted from infected animals to humans [2,3]. Infections in immune-competent peoplecan be asymptomatic or a self-limiting diarrhea. However, people with immune deficiencies may develop a chronic and life-threatening infection [4]. nfections in animals can also be asymptomatic or with gastrointestinal symptoms such as malabsorption and diarrhea [5], which can lead to decreased growth rates and in severe casesthe death of neonatal animals [6,7]. There are no highly effective therapeutic treatments for cryptosporidiosis in humans [8,9] and no cost-effective treatment for animals [10].

Cryptosporidium spp. can be transmitted by direct contact with infected humans and animals or ingestion of food or water contaminated byoocysts. Oocysts, the infective stages,can be transported for long distances in water due to their low specific gravity [11]. Oocysts are environmentallyresistant and can remain infectious for long periods of time in favorable environmental conditions. For example, oocysts can persist in cool water or moist cool environments protected from solar UV light for six months or longer [2,12]. Infected young animals such as dairy calves and lambs [13] and wildlife such as California ground squirrels (*Spermophilus beecheyi*) can shed very high numbers of oocysts into the environment [14], elevating the risk of transmission to other animals and humans if the species of *Cryptosporidium* is zoonotic [15].

Presently little is known about the prevalence and intensity of infection and environmental loading levels of *Cryptosporidium* in commercial sheep production systems in the western United States [16]. California has more than 4,200 sheep operations that

ranked third and 575,000 sheep and lambsthat ranked second in the USA [17]. Zoonotic transmission of *Cryptosporidium* by direct or indirect contact with lamb feces as source of oocysts infectious to humans has been documented [18]. Surface water draining from agricultural and livestock operations into municipal waterways is a potential source of human exposure to these pathogens [19,20]. However, evidence of transmission of *Cryptosporidium* from sheep to humans via contaminated water is limitedalthough it is possible under certain environmental conditions [21,22]. For example, sheep flocks grazing California foothills have the potential of contaminating watersheds with *Cryptosporidium* oocysts, especially during winter when annual precipitation can elute feces into surface waterways via storm runoff [23]. Coincidentally, the rainy season in California overlaps with the lambing season from October to March.Lastly, littleis known about which management practices and environmental conditions may impact the prevalence of *Cryptosporidium* infection levels in flocks of sheep. The objectives of this study were to determine the prevalence, estimate the environmental loading rate of *Cryptosporidium* spp. [24] and identify management practices associated with the odds of infection in commercial sheep production ranches across California.

RESULTS

Prevalence of *Cryptosporidium* in sheep

In total 798 fecal samples were collected from 372 adult ewes, 31 yearlings and 395 lambs respectively. For samples collected during the first sampling visit at each ranch, 11 of 16 ranches had one or more animals test positive to *Cryptosporidium* oocysts in their feces. For samples collected from the second sampling visit, all ranches had one or more animals test positive for *Cryptosporidium*. The overall prevalence of *Cryptosporidium* was 17.3% (138/798) which increased from 7.6% (29/380) from the first sampling to 26% (109/418) at the second sampling, most likely due to the increase of younger lambs during the second sampling. The prevalence of *Cryptosporidium* in lambs, yearlings, and adult ewes was 30.6% (121/395), 16.1% (5/31), and 3.2% (12/372), respectively (Table 3). The occurrence of oocyst shedding was not significantly associated with diarrhea in sheep ($P = 0.72$). The etiological fraction for *Cryptosporidium*-associated diarrhea for the study population was 0.008, which indicates that only 0.8% of individuals with diarrhea were associated with fecal shedding of oocysts. Typical for many livestock species, lambs and yearlings were about 5 and 10 times more likely to shed *Cryptosporidium* oocysts compared to adult ewes. The prevalence of *Cryptosporidium* peaked around the second month [37.4% (61/163)] and third month [38.3% (41/107)] of age (Figure 2), with the prevalence being lower in older animals. The prevalence of *Cryptosporidium* in adult sheep before and after lambing seasons was not significantly different ($P = 0.42$). Highest prevalence [32.0% (16/50)] was observed in ranch no. 5 located in the Sonoma county while the lowest prevalence [0 (0/30)] occurred in ranch no. 16 located in the Contra Costa county, both were extensive grazing operations in the San Francisco Bay area. The mean prevalence of *Cryptosporidium* infection was 16.6% (94/563) in rotational grazing operations (no. 1-4, 6, 8. 10, and 12-15), 22.1% (29/131) in extensive grazing operations (no. 5, 7, and 16), 10.2% (5/49) in dry lot (no.9), and 18.2% (10/55) in dry lot or mixed dry lot-rotational grazing operations (no.11) (Table 1).

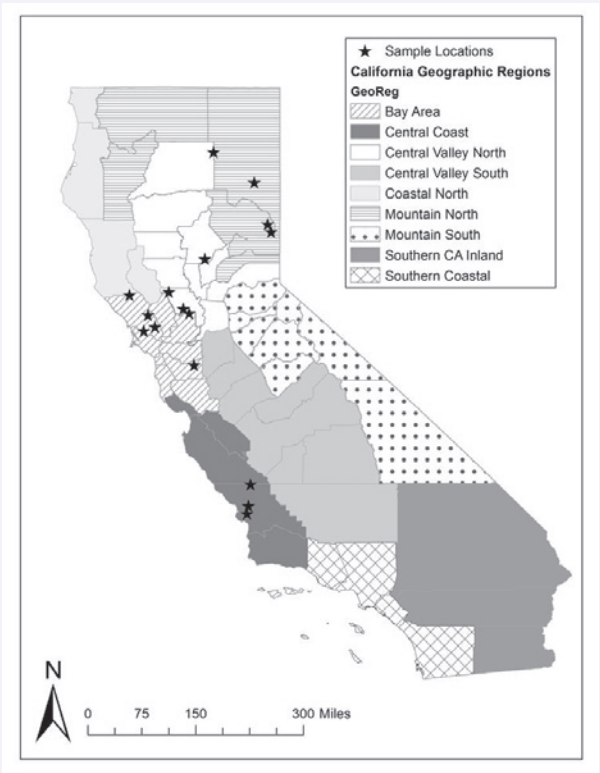

Figure 1 Sample locations in this study.

Intensity of fecal shedding and environmental loading of oocysts

The percent recovery of the DFA method for detection of oocysts from sheep feces was determined to be 43.4% and the detection limit was 2.3 oocysts/g feces in this study. Using this percent recovery, the adjusted average oocyst concentrations were 25,434.5; 258.6; and 10,785.5 oocysts/g feces in positive samples from lambs, yearlings and adult ewes respectively, and data were 7,791.4; 39.2; and 349.8 oocysts/g feces in all samples (both positive and negative samples), respectively. The intensity of fecal shedding of oocysts was significantly associated with sheep age ($P<0.0001$). Lambs were 24 times more likely to shed high concentrations of oocysts compare to adult ewes (Figure 3). In positive samples, the intensity of fecal shedding of oocysts by adult sheep increased from an average of 0.5 oocysts/g feces prior to lambing to 127.8 oocysts/g feces after lambing ($P<0.0001$), or about a 250-fold increase. The environmental loading rate was defined as the total number of infective forms of a pathogen produced by an animal per day [16,24] (Table 3). Using a range of average body weights, daily fecal production as 2.5% of body weight [25] and mean fecal oocyst concentrations, the daily fecal load of oocysts shed into the environment by infected animals were estimated to range from 584,355 to 6,817,475 oocysts/lamb/day, 29,400 to 44,100oocysts/yearling/day, and 349,800 to 1,049,400 oocysts/ewe/day by infected (Table 3).

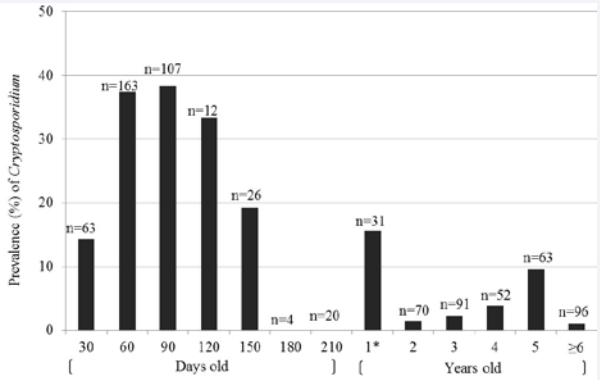

Figure 2 Prevalence (%) of *Cryptosporidium* spp. in sheep stratified by age (* This age block includes sheep of 8 to 12 month old; n = total number of samples representing each age group).

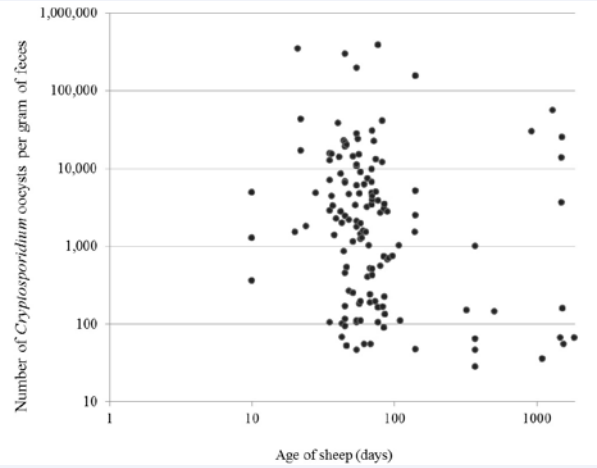

Figure 3 Intensity of fecal shedding of *Cryptosporidium* oocysts as a function of age of sheep.

Risk factors associated with *Cryptosporidium prevalence* and intensity of shedding oocysts

Multivariable analysis of the association between farm management practices and shedding oocysts by lambs and ewes. Because the intensity of oocysts shedding was significantly association with sheep age and lambs shed significant higher concentrations of oocysts than adult ewes, the risk factor analysis was performed for first for lambs and then for all sheep adjusted for age. When including significant variables into a multivariable model, we found that contact with cattle (odds ratio=1.77) and access to surface water (odds ratio=3.83) were risk factors significantly associated with fecal shedding of oocysts in lambs. The odds of shedding oocysts was 1.8 times greater in lambs that had been in contact with cattle compared to lambs without contact with cattle (P=0.030). Lambs that had access to surface water such as a pond, wetland or creek, or had received drinking water from any of these water sources had 3.8 higher odds of shedding oocysts compared to lambs that had no access to surface water ($P \leq 0.0001$). Lastly, the number of adult ewes in a flock was negatively associated with the odds of shedding oocystsby lambs (odds ratio=0.99, P = 0.024) (Table 4). Specifically, for each additional 10 ewes in the flock, the odds of shedding oocysts by lambs was reduced by 2% ($e^{-0.0013\times10}$ =0.98). For adult ewes, contact with cattle was the only risk factor significantly associated with shedding oocysts among all other variables evaluated (data not shown). Ewes had 9.2 higher odds of shedding oocysts if they had contact with cattle compared with ewes that had no contact with cattle (P=0.005). All other variables were not significant in the multivariable analysis if contact with cattle was included.

Multivariate analysis of Cryptosporidium infections in the sheep flock with farm management practices. To determine farm management factors associated with fecal shedding of *Cryptosporidium* oocysts in all sheep, we used a multivariable analysis which was adjusted for age of sheep because age was identified as the biological factor associated with *Cryptosporidium* fecal shedding. When sheep had contact with cattle, the odds of shedding *Cryptosporidium* oocysts was 1.8 times greater (odds ratio=1.82, P=0.008) compared to sheep that had no contact with cattle. If a sheep had access to surface water such as a pond, wetland or creek, or received drinking water from any of these sources, the odds of shedding oocysts was 3.6 times greater (odds ratio=3.58, P<0.0001) compared to sheep with no access to surface water. Pasture size was negatively associated with the odds of sheep shedding oocysts; for every 10 acre increase in pasture size, there was a 10% ($e^{-0.0098\times10}$ =0.90) reduction in the odds of fecal shedding oocysts in the flock (P = 0.042) (Table 5). Finally, when adding the variable of ranch ID as a random effect to the mixed-effects logistic regression model, ranch ID did not have a significant effect on the overall analysis, meaning that there were no significantranch-level random effects associated with the odds of shedding oocysts (P>0.05).

DISCUSSION

Prevalence of *Cryptosporidium* in sheep in California

Contact with sheep farms was based on convenience selection and farm enrollment was based on willingness to participate in the study by farm owners. Approximately 85% of contacted ranchers agreed to participate in the study. *Cryptosporidium* oocysts were detected in feces of sheep from all the study ranches during the lambing seasons in four different geographical regions in California. The overall prevalence of *Cryptosporidium* in this study period was 30.6% (121/395) in lambs, 16.1% (5/31) in yearlings, and 3.2% (12/372) in adult ewes. Highest prevalence of 37.4% (61/163) and 38.3% (41/107) were observed in lambs of 60 and 90 day old, respectively (Figure 2). Actual prevalence could be higher because the percent recovery of the DFA method was determined to be 43.4% in this study, hence positive samples with concentrations lower than limit of detection (2.3 oocysts/g feces) were unlikely to be detected. Similar prevalence of *Cryptosporidium* in lambs (32.2%)was reported in the east coast of USA although the age group for the peak prevalence was less than 14 days old [26]. The prevalence of *Cryptosporidium* in sheep determined in our work was also comparable to studies conducted in Western Australia where 26% of sheep were positive of *Cryptosporidium* [27], and Norway where 15-24% prevalence of

TABLE 1: Information on enrolled sheep ranches and prevalence of *Cryptosporidium* in these ranches.

Ranch	County	Region	Operation type[a]	Flock size	Prevalence of *Cryptosporidium*
1	Sonoma	Bay area	Rotational grazing	90	10.2% (5/49)
2	Yolo	Central valley north	Rotational grazing	124	21.6% (11/51)
3	Yolo	Central valley north	Rotational grazing	282	16.0% (8/50)
4	Yolo	Central valley north	Rotational grazing	111	13.7% (7/51)
5	Sonoma	Bay area	Extensive grazing	1120	32.0% (16/50)
6	Santa Rosa	Bay area	Rotational grazing	58	16.7% (8/48)
7	Mendocino	Bay area	Extensive grazing	386	25.5% (13/51)
8	Plumas	Mountain north	Rotational grazing	190	19.2% (10/52)
9	Plumas	Mountain north	Dry lot	41	10.2% (5/49)
10	Lassen	Mountain north	Rotational grazing	223	13.0% (7/54)
11	Lassen	Mountain north	Dry lot/Rotational grazing	367	18.2% (10/55)
12	San Luis Obispo	Central coast	Rotational grazing	123	14.5% (9/62)
13	San Luis Obispo	Central coast	Rotational grazing	91	14.5% (8/55)
14	San Luis Obispo	Central coast	Rotational grazing	283	26.7% (16/60)
15	Butte	Central valley north	Rotational grazing	784	16.1% (5/31)
16	Contra Costa	Bay area	Extensive grazing	9085	0% (0/30)

[a] Rotational grazing: systematic rotation of the flock between two or more paddocks or pastures; Extensive grazing: grazing natural forages over an extensive area not partitioned into paddocks; Dry lot: the flock is confined on a wooden, concrete or relatively bare earthen floor and feed is provided.

Table 2: Timeline chart indicating the first (1) and second (2) sampling visits and the onset of lambing (L) at each sheep ranch.

Ranch																																				
1													L						1															2		
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3													L							1											2					
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week				-10				-5					1				5				10					15				20					30	

Cryptosporidium was found in 40-70 days old lambs [28]. A higher peak prevalence (76.2%) of *Cryptosporidium* in young lambs (8-14 days old) was reported in Zaragoza, Spain [29]. Diarrhea was observed in 3% (12/395) lambs, 6% (2/31) yearlings and 1.6% (6/372) adult ewes throughout our study period, which was not significantly associated with shedding of oocysts. Although diarrhea can be a common symptom of *Cryptosporidium* infection, asymptomatic infections of *Cryptosporidium* are common. For example, asymptomatic *Cryptosporidium* infections have been observed in calves [30,31]; in red deer hinds and calves [32]; in pigs [33,34]; in dogs [35,36] and in sheep [37,38]. In addition, symptoms of *Cryptosporidium* infection are related to age of animal and stage of infection [39], with younger animals more vulnerable to clinical infection due to the weakness of immune

Table 3: Overall prevalence and estimated intensity of fecal shedding of *Cryptosporidium* oocysts in sheep in California.

Sheep age	Body weight range (kg)	Prevalence (positive/total samples) (%)	Mean (±SD) of oocysts/g feces		Estimated oocysts shedding per day[c]
			Positive[a]	Total[b]	
Lamb	3 - 35	121/395 (30.6)	25,434.5 (108,493)	7,791.4 (61,015)	584,355 - 6,817,475
Yearling	30 - 45	5/31 (16.1)	258.6 (419)	39.2 (175)	29,400 - 44,100
Ewe	40 - 120	12/372 (3.2)	10,785.5 (17,775)	349.8 (3,616)	349,800 - 1,049,400

[a] Arithmetic mean of numbers of oocysts shed per gram of positive fecal sample sad justed by the percent recovery of the DFA.
[b] Arithmetic mean of numbers of oocysts shed per gram of all fecal sample sad justed by the percent recovery of the DFA.
[c] Estimated daily oocysts shedding calculated using the mean oocysts/g found in the total population([b]) sampled and based on an estimated daily fecal output per animal of 2.5% of body weight.

Table 4: Multivariate logistic regression model for farm factors associated with the odds of lambs shedding *Cryptosporidium* oocysts.

Factor		OR	*P* value	95 % confidence interval	
Contact with cattle[a]	No[c]	1.0	--	--	--
	Yes	1.77	0.030	1.05	2.97
Access to surface water[b]	No[c]	1.0	--	--	--
	Yes	3.83	0.0001	2.10	7.00
Number of ewes in the flock		0.99	0.024	0.99	0.99

[a] Contact with cattle is defined as the use of a pasture recently grazed by cattle or currently sharing a pasture with cattle.
[b] Access to surface water is defined as the presence of a lagoon, pond, wetland or creek in the pasture where the flock is currently present, or receiving drinking water from any of these water bodies.
[c]Referent category.

Table 5: Multivariate logistic regression model for farm factors associated with the odds of sheep (lambs, yearlings, and ewes) shedding *Cryptosporidium* oocysts.

Factor		OR	*P* value	95% confidence interval	
Sheep age	Ewes[c]	1.0	--	--	--
	Yearlings	5.1	0.005	1.65	15.8
	Lambs	15.5	0.0001	8.26	29.2
Pasture size (acres)		0.99	0.042	0.98	0.99
Contact with cattle[a]	No[c]	1.0	--	--	--
	Yes	1.82	0.008	1.17	2.84
Access to surface water[b]	No[c]	1.0	--	--	--
	Yes	3.58	0.0001	1.97	6.48

[a] Contact with cattle is defined as the use of a pasture recently grazed by cattle or currently sharing a pasture with cattle.
[b] Access to surface water is defined as the presence of a lagoon, pond, wetland or creek in the pasture or enclosure where the flock is currently present, or receiving drinking water from any of these water bodies.
[c]Referent category.

system in younger animals. About 50% of the 798 animals sampled in our study were either yearlings (n=31) or adults (n=372). Among the 395 lambs, only 16% (63/395) of lambs were 30 days old and the remaining 84% (332/395) were >60 days old (Figure 2). Moreover, our cross-sectional sampling did not monitor the dynamic stages of infection that might be associated with symptoms of *Cryptosporidium* infection including diarrhea. These may explain why diarrhea was not significantly associated with *Cryptosporidium* infection in our enrolled flocks during the study period.

Quantitative shedding and loading of *Cryptosporidium* oocysts by sheep

Our study not only determined the prevalence of *Cryptosporidium* in sheep but also quantified the concentrations of fecal oocysts and estimated the daily environmental loading

by sheep. The mean concentrations of oocysts shed by lambs, yearlings, and adult ewes were approximately 2.5×10^4, 260, and 1.1×10^4 oocysts/g feces, respectively. Little is known about intensities of shedding oocysts by naturally infected sheep on farms due to limited availability of quantitative detections of oocysts in sheep flocks. An early experimental study reported lambs shed 2.8×10^6 oocysts/g feces at 4-5 days post inoculation and up to 1.5×10^7 oocysts/g feces at 8 days post inoculation [40]. Although we detected lower fecal oocyst concentrations in naturally infected lambs than that in experimentally infected lambs, the differences could be due to the dose of oocysts lambs exposed to and differences in the stage of infection between studies. As might be expected, the mean concentrations of oocysts in our study exhibited large standard deviations indicating that intensity of oocyst shedding was highly variable among individual sheep and across different age groups (Table 3). Depending on species of livestock and infections status, daily loading rates of *Cryptosporidium* oocysts vary among livestock species, ranging from 3.9 - 9.2×0^3 oocysts by beef cattle [41], 5000 - 4.2×10^9 oocysts by dairy calves [42], 5.8×10^7 oocysts by horses [43], up to 3.7×10^7 oocysts by pigs [44], and up to 2.8×10^7 oocysts by sheep [44]. Focusing on lambs and adult ewes, the two major age groups in our study, the daily shedding loads were estimated to be up to 6.8×10^6 and 1.0×10^6 oocysts per infected lamb and adult ewe, respectively. Interestingly, we found fecal concentrations of oocysts in adult ewes increased ~250-fold after lambing commenced compared to ewes prior to lambing in this study, which could be associated with exposure to higher doses of oocysts in the environment shed by infected lambs which is similar to observations by other researchers [45]. Results suggested sheep including lambs and adult ewes shed significant loads of *Cryptosporidium* oocysts into the environment, especially during the lambing season. These age groups must be taken in special attention for farm management programs, due to they represent the polluting group with oocysts for remaining groups.

Risk factors associated with infection and shedding of *Cryptosporidium*

In order to determine risk factors potentially contributing to the likelihood of infection of *Cryptosporidium* in sheep in studied farms, we evaluated on-farm practices including: stocking densities in permanent dry lots, grazing rotation rates, size of grazing pastures, supplemental feeding, moving newborn lambs with ewes to a new and clean pasture, the use of anti coccidial or anthelmintic drugs, lambing in a barn or a pasture, access to surface water, and contact with cattle, etc. Multiple risk factor analysis indicated that access to surface water as a source of drinking water and contact with cattle were predominant factors associated with *Cryptosporidium* shedding in sheep of all ages (Tables 4 and 5). Results suggested that sheep had access to surface sources of drinking water were at higher risk of ingesting oocysts in water from the same flock, other livestock in upstream of watersheds, or other sources of oocyst contamination such as wildlife. On the other hand, because *Cryptosporidium* species in cattle (e.g.,*C. parvum* and *C. bovis*) are also infectious to sheep, this may explain why contact with cattle increased the risk of *Cryptosporidium* infection in sheep. Interestingly, the odds of fecal shedding of *Cryptosporidium* oocysts by lambs decreased with the increased number of ewes in a flock. This is might be due to a variety of possible reasons, such as collinearity between the number of ewes and pasture size or reduced contact rates between infected lambs and susceptible animals for flocks with larger numbers of ewes relative to lambs. Although stocking density in permanent dry lots was not significantly associated with shedding of *Cryptosporidium* in this study which is in controversy to a study on dairy calves [46], we found that the odds of shedding of *Cryptosporidium* oocysts by adult ewes was lower for flocks on larger pastures. This result indicated that sheep grazing on relatively larger pastures or on extensive acres of rangeland was associated with reduced odds of infection with *Cryptosporidium* in a flock.

The significance of managements in lambing seasons

It has been reported that greater intensity and frequency of *Cryptosporidium* oocyst shedding beyond the neonatal period is associated with reductions of growth in sheep [47]. Therefore management practice promoting lambs growth is of significance in order to improve production efficiency and reduce cryptosporidiosis in lambs. Lambs are present on most California ranches only on a seasonal basis, primarily between November and May. Hence, the lambing season can overlap with the rainy seasons from October through March in California. Because the higher concentrations of *Cryptosporidium* oocysts shed by lambs can directly result in higher environmental loading of oocysts, farm management strategies should be primarily directed towards reducing environmental contamination by feces from lambs during the rainy and runoff season. Management practices to reduce the transport of fecal oocyst loads from livestock into watersheds have been described previously [23,48]. These include retaining manure in stock piles or lagoons for extended periods of time for confined populations, placing supplemental feed away from surface waterways, removing livestock from sensitive grazing locations such as riparian corridors at least 2 to 4 weeks before the onset of the rainy season, and creating vegetative buffer zones down slope of grazed locations to reduce the risk of overland flow and runoff from grazed pastures. Other farm management strategies to prevent fecal contamination of watersheds may include rotational grazing to better distribute the oocyst load on watersheds and/or fencing off streams to prevent animal access during the lambing seasons [22].

This cross-sectional study focused on the important period immediately preceding and subsequent months following the lambing seasons which overlaps with the rainfall season in California. Our results indicate that the high concentrations of fecal shedding of oocysts during lambing season by both lambs and ewes can be a source of environmental loading and elevate the risk of watershed contamination of *Cryptosporidium* if adequate beneficial management practices are not sufficiently practiced by the sheep manager. Minimizing sheep access to surface drinking water sources and reducing contact with cattle were associated with reduced levels of *Cryptosporidium* infection in sheep, which if causal would lead to reduced environmental loading and a lower risk of watershed contamination in California. It is critical to determine the species and genotypes of *Cryptosporidium* that are being shed in the feces of sheep in order to assess the public health impacts of *Cryptosporidium* infection in sheep [27].

Genotyping of *Cryptosporidium* from positive sheep samples stratified by farms and sheep ages will be reported in follow up publication.

MATERIALS AND METHODS

Study farms and sample collections

Through collaborations with livestock and natural resource advisors of the University of California Cooperative Extension, 16 sheep ranches located in Northern and Central California (Figure 1) were enrolled in this studybased on ranch owners' voluntarily participation (Table 1). The 16 sheep ranches were located in four different geographical regions in California. Four ranches were located in the Mountain North region, four in the Central Valley North region, five in the San Francisco Bay Area, and three in the Central Coast region (Figure1). The climates vary across these four regions ranging from colder Mountain North with an extended winter season to the Central Coast with its more Mediterranean climate and warm summers. The average annual cumulative precipitation ranges between 15 to 30 inches in the Mountain North, Central Valley North and Central Coast and 30 to 80 inches in the Bay Area. We visited each ranch twice (with the exception of two ranches that enrolled late in the study), either before, during or after the lambing seasons between November 2009 and May 2010 (Table 2). During each farmvisit, 20 to 30 fecal samples were collected per rectum from individual animals based on random selection of adult ewes, yearlings and lambs when available. A total of 798 fecal samples were collected from individual animals including 372 adult ewes, 31 yearlings, and 395 lambs. Fecal samples were placed on ice immediately after collection and remained on ice during transportation to the laboratory at University of California in Davis. The sampling was approved by the Institutional Animal Care and Use Committee of the University of California Davis.

Collection of information of risk factors

During each farm visit, a questionnaire was administered to collect farm management, environmental, flock, and individual animal factors potentially associated with the occurrence and intensities of *Cryptosporidium* in sheep. The questionnaire collected information ongrazing management (the pasture area, forage composition, and rotations); drinking water (source and the method of water delivery); contact with cattle(use of a pasture recently grazed by cattle or currently sharing a pasture with cattle); general animal health management (helminth and coccidian control, vaccination, etc.); reproduction and lambing management (breeding schedules, lambing location, separation of ewe/lamb pairs, etc.);flock demographics (breed, population size, density, number of ewes and lambs); and individual animal factors (age, sex, breed, body condition score, and diarrhea scores). For flocks in permanent dry lots, questionnaire also included the corral dimensions, manure management, and type of concentrates and forages.

Detection of oocysts from fecal samples

Samples were stored at 4°C upon arrival at the laboratory and processed for detection of *Cryptosporidium* oocysts within one week of collection. Quantitative detection of *Cryptosporidium* oocysts was performed using a direct immunofluorescent assay (DFA) as described previously [24]. Briefly, approximately 5 grams of fecal material were homogenized in 40 ml of phosphate buffer saline (PBS) solution and filtered through 4-layer gauze to remove large fragments of fibers followed by centrifugation at 1000 g for 10 min. The supernatant was discarded by aspiration and the sediment of fecal pellet was resuspended with PBS at 1:1 ratio of volumes. The fecal suspension was homogenized, weighed, and then 10 µl were smeared onto wells of pre-treated slides (Waterborne Inc. New Orleans, LA). Smears on slides were weighed, dried, and stained with *Cryptosporidium* specific FITC-antibodies (Waterborne, Inc., New Orleans, LA). Slides were examined using afluorescent microscope (Olympus BX60) and oocysts were counted. Concentrations of oocysts in feces were calculated as follows:

$$\text{Oocysts}/\text{g} = \frac{\dfrac{\text{Oocyst count in10µl fecal suspension}}{\text{fecal smear weight}} \times \text{fecal suspension weight}}{\text{fecal sample weight} \times \text{percent recovery}}$$

The percent recovery was the percentage of oocysts that can be recovered in fecal samples by the DFA method. It was determined by spiking either 5×10^2, 5×10^3,5×10^4,5×10^5 and 5×10^6 oocysts of wild-type bovine *C. parvum* into 5 grams ofsheep fecal material which tested negative for *Cryptosporidium* oocysts, with five replicates per oocyst concentration. Samples spiked with oocysts were processed the same way as above and numbers of recovered oocysts were used to estimate percent recovery of the DFA methodas described previously (49).

Statistical analysis

All risk factors were first screened for a univariate association with the presence or absence of *Cryptosporidium* oocysts in sheep feces, using a cutoff value of $P \leq 0.20$ based on the Wald or likelihood ratio test to retain the variable for evaluation in a multivariate logistic regression model. A forward stepping algorithm was used to construct the logistic regression model, with a cutoff value of $P \leq 0.05$ based on the likelihood ratio or Wald test for inclusion in the model. Because we sampled different age groups of sheep from 16 ranches, we initially used a mixed effects logistic regression model with fecal shedding of oocysts (0/1) as the outcome variable, risk factors as fixed effects, and flock ID as a group or random effect for the possibility that the odds of oocyst shedding between animals was correlated within flock. If the group effect was found to be not significant ($P > 0.05$) in the full model, the term was dropped and the model reverted to ordinary (fixed effects only) logistic regression. The Stata 11 (Statistic Data Analysis, Texas) was used for logistic regression analysis. Chi square and 2×2 table analysis was performed using StatCalc, EpiInfo 7.0.9.7 (Centers for Disease Control and Prevention, GA, USA). The etiological fraction (EF), defined as the proportion of cases of watery feces associated with fecal shedding of oocysts, was determined from the cross-sectional data using the following equation:

$$EF = \frac{p(PR-1)}{p(PR-1)+1}$$

Where p was the proportion of sheep that were shedding oocysts at the time of sampling, and PR was the prevalence ratio, calculated as the prevalence of watery feces in sheep shedding oocysts divided by the prevalence of watery feces in sheep not shedding oocysts.

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