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 × 10^6 and 1 × 10^6 oocysts per 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 (odds ratio=1.8) and contact with cattle (odds 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 sheeps 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 oocysts 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]. Infections 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 cases the 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 by oocysts. Oocysts, the infective stages, can be transported for long distances in water due to their low specific gravity [11]. Oocysts are environmentally resistant 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 lambs that 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 limited although 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, little is 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).

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 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 compared 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 44,100 oocysts/yearling/day, and 349,800 to 1,049,400 oocysts/ewe/day by infected (Table 3).
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 associated with sheep age and lambs shed significant higher concentrations of oocysts than adult ewes, the risk factor analysis was performed 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 (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* ^0.030×10 = 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 significant ranch-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 of 60 and 90 day old, respectively (Figure 2). Actual 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 of 37.4% (61/163) and 38.3% (41/107) were observed in lambs of 60 and 90 day old, respectively. 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.

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

*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.

<table>
<thead>
<tr>
<th>Ranch</th>
<th>L</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>week</td>
<td>-10</td>
<td>-5</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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% (2/311) 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.

<table>
<thead>
<tr>
<th>Sheep age</th>
<th>Body weight range (kg)</th>
<th>Prevalence (positive/total samples) (%)</th>
<th><strong>Mean (±SD) of oocysts/g feces</strong></th>
<th>Estimated oocysts shedding per day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Total&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lamb</td>
<td>3 - 35</td>
<td>121/395 (30.6)</td>
<td>25,434.5 (108,493)</td>
<td>7,791.4 (61,015)</td>
</tr>
<tr>
<td>Yearling</td>
<td>30 - 45</td>
<td>5/31 (16.1)</td>
<td>258.6 (419)</td>
<td>39.2 (175)</td>
</tr>
<tr>
<td>Ewe</td>
<td>40 - 120</td>
<td>12/372 (3.2)</td>
<td>10,785.5 (17,775)</td>
<td>349.8 (3,616)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Arithmetic mean of numbers of oocysts shed per gram of positive fecal sample adjusted by the percent recovery of the DFA.

<sup>b</sup>Arithmetic mean of numbers of oocysts shed per gram of all fecal sample adjusted by the percent recovery of the DFA.

<sup>c</sup>Estimated daily oocysts shedding calculated using the mean oocysts/g found in the total population<sup>b</sup> 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.

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>P value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact with cattle&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.77</td>
<td>0.030</td>
</tr>
<tr>
<td>Access to surface water&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.83</td>
<td>0.0001</td>
</tr>
<tr>
<td>Number of ewes in the flock</td>
<td>0.99</td>
<td>0.024</td>
<td>0.99</td>
</tr>
</tbody>
</table>

<sup>a</sup>Contact with cattle is defined as the use of a pasture recently grazed by cattle or currently sharing a pasture with cattle.

<sup>b</sup>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.

<sup>c</sup>Referent category.

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

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>P value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep age</td>
<td>Ewes&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Yearlings</td>
<td>5.1</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Lambs</td>
<td>15.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Pasture size (acres)</td>
<td>0.99</td>
<td>0.042</td>
<td>0.98</td>
</tr>
<tr>
<td>Contact with cattle&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.82</td>
<td>0.008</td>
</tr>
<tr>
<td>Access to surface water&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.58</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Contact with cattle is defined as the use of a pasture recently grazed by cattle or currently sharing a pasture with cattle.

<sup>b</sup>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.

<sup>c</sup>Referent category.

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 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.
by sheep. The mean concentrations of oocysts shed by lambs, yearlings, and adult ewes were approximately 2.5×10⁶, 260, and 1.1×10⁴ 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 2.8×10⁶ oocysts/g feces at 4-5 days post inoculation and up to 1.5×10⁷ 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 - 9.2×10⁴ oocysts by beef cattle [41], 5000 - 4.2×10⁶ oocysts by dairy calves [42], 5.8×10⁴ oocysts by horses [43], up to 3.7×10⁵ oocysts by pigs [44], and up to 2.8×10⁷ 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 6.8×10⁶ and 1.0×10⁷ 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 with the increased number of ewes in a flock. This is 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 study based 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 (Figure 1). 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 farm visit, 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 on grazing 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 a fluorescent microscope (Olympus BX60) and oocysts were counted. Concentrations of oocysts in feces were calculated as follows:

\[
\text{Oocysts} / g = \frac{\text{Oocyst count in (10 fmale suspension}}{\text{fecal suspension weight}} \times \text{fetal smear weight} \times \text{percent recovery}
\]

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 of sheep 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 methods 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* ≤ 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* ≤ 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 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, EpInfo 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:

\[ EF = \frac{PR - 1}{P (PR - 1)} \]

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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