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A Cross-Sectional Study of prevalence and Species of *Cryptosporidium* spp. in Preweaned Calves and Associated Management Risk Factors on Dairies in Central California, USA

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Abstract

Cryptosporidium spp. are major protozoal parasites infecting dairy cattle and cryptosporidiosis is a leading cause of morbidity in dairy calves. The objective of this study was to determine the prevalence and intensity of *Cryptosporidium* spp. infections associated with farm management risk factors and genotypes of *Cryptosporidium* spp. in preweaned dairy calves by conducting a cross-sectional study on eight dairies in the San Joaquin Valley of California. Fecal samples were collected from preweaned calves and evaluated for oocysts shedding load using immune fluorescent assay, and genotype using PCR and sequencing of a fragment of 18S rRNA gene. Information on management factors that contained categories of questions related to calf housing, feeding and management were also collected from the study farms. Statistical analysis of variables associated with *Cryptosporidium* spp. shedding was conducted using a multivariable logistic regression model.The overall prevalence of *Cryptosporidium* spp. in preweaned calves across all dairies was 56.0% [342/610]. Among 105 isolates of *Cryptosporidium* spp. successfully genotyped, 86.7% were determined to be C. *parvum*, 12.4% as C. *bovis*, and 0.9% as C. *ryanae* respectively. Calves shedding *Cryptosporidium* spp. Oocysts had 1.6 times the odds of being 16-31 days of age on the day of sampling compared to other age groups. A *Cryptosporidium* spp. positive calf had 8.3times higher odds of exposure to milk bottles cleaned 2 to 3times/week compared to negative calves that were exposed to bottles cleaned at every feeding. Positive calves also had 2.4 higher odds of exposure to bottles cleaned exclusively with disinfectant compared to bottles cleaned with disinfectant and water than negative calves. Cleaning bottles with only hot water was found to be protective against *Cryptosporidium* spp. shedding in calves compared to bottles cleaned with both water and disinfectant. Results of this study provided updated information on *Cryptosporidium* spp. genotypes infecting preweaned calves on California dairies and approaches to reducing oocysts shedding in calves.

INTRODUCTION

Cryptosporidiosis is a diarrheal disease caused by *Cryptosporidium* spp. Diarrhea caused by cryptosporidiosisis a leading cause of morbidity in dairy calves [1]. Although, cryptosporidiosis typically causes mild, self-limiting disease in calves, in some cases, it can lead to fulminating diarrhea, severe dehydration, and death if concurrent gastrointestinal infections exist [2]. Many calves can be asymptomatic carriers that shed infective oocysts into the environment for 3-14 days[3]. *Cryptosporidium*spp. oocysts persist in moist and shaded environments common on most dairy settings,which can lead to infection in newborn calves through environmental transmission. Reported effective disinfectants include ammonium hydroxide, UV light, hydrogen peroxide and 5% ammonia solutions [4]. Oocysts shed into waterways by cattle grazing in riparian areas is also a topic of public health and marine mammal conservation interest [5]. The role of *Cryptosporidium* spp. as a waterborne zoonotic disease has led to restrictions on cattle grazing along waterways, and implementation of vegetative buffers in these regions to reduce concentrations of oocysts reaching the water[6].

Previously we have characterized age, geographic, and temporal distribution of fecal shedding of *C. parvum* oocysts in cow-calf herds[7] and associations of herd composition, stocking

rate, and duration of calving season with fecal shedding of *C. parvum* oocysts in beef herds [6] both in California. We found that calf shedding of *C. parvum* oocystsis highest in the first 30 days of life [2,7], and peaks around 7-15 days of age in calves [7]. We also identified failure of passive transfer in calves and duration of calf contact with the dam as associated risk factors for *C. parvum* shedding [7]. *Cryptosporidium* spp. infection during the neonatal period has led many researchers to evaluate the role of the periparturient dam as a source of transmission of *Cryptosporidium* spp. to newborn calves [3,8] and to identify on-farm management practices that increase or decrease the odds of *Cryptosporidium* spp. shedding which is important for improving calf health and decreasing zoonotic risk through environmental contamination [4]. In one of our earlier studies we found no detectable shedding of *C. parvum* oocysts by periparturient dairy cattle in three dairies in California [9]. There is a need to assess more detailed information on management factors associated with shedding of *Cryptosporidium* spp. oocysts in preweaned calves on California dairies with the typical western style of confinement dairy production.

On the other hand, during the past decade the classifications of *Cryptosporidium* spp. have been consistently updated, including the descriptions of species/genotypes in cattle that are either zoonotic [human infective] or non-zoonotic [non-human infective] [10-15]. For example, although many of the previous isolations of *Cryptosporidium*spp. in cattle were classified as *C. parvum* which is a zoonotic species shed by livestock, wildlife and humans, other species of *Cryptosporidium* spp., such as *C. ryanae* that are generally not infectious to humans [non-zoonotic] were also describedin cattle [16]. Therefore, it is necessary to update the prevalence of different species of *Cryptosporidium* spp. in preweaned calves on California dairies.

The objectives of this cross-sectional study were: 1] to determine the prevalence and intensity of *Cryptosporidium* spp. infections; 2] to update major *Cryptosporidium* spp. species; and 3] to identify key farm management risk factors that are associated with *Cryptosporidium* spp. infection in pre weaned dairy calves. Our hypotheses were that the prevalence of *Cryptosporidium* spp. on dairies is associated with farm management practices and that multiple speciesof *Cryptosporidium* spp. infect preweaned calves.

MATERIALS AND METHODS

Study population

A convenience sample of eight dairies located in the San Joaquin Valley of California was chosen for the study following the recommendation of local Cooperative Extension Advisors and voluntary participation by the farms in the region. Each of the eight dairies was assigned a letter ID [A through H respectively] to protect their location and production information. Each dairy was visited twice on a different day between July and September 2012 to collect fresh fecal samples from calves housed at each respective dairy to analyze for *Cryptosporidium* spp. oocysts.A minimum of 15g of fresh feces was collected per rectum using a lubricated gloved hand by field staff at each farm. A new glove was used between each calf. Each sample was transferred into a polyethylene cup with lid and placed in a cooler prior to transport back to laboratory at UC Davis. Samples were then refrigerated at 4°C until analysis. The sampling protocol was approved by the UC Davis Institutional Animal Care and Use Committee [IACUC]. In order to collect information of farm management factors, a survey questionnaire was administered during dairy visits by asking questions to farm managers and farm workers directly involved in day-to-day calf care activities. The survey questionnaire contained closed and open-ended questions grouped into categories of calving and maternity area, and calf housing, feeding and management practices. Questions in the categories of calving and maternity pertained to type of bedding, frequency and method of cleaning. The second category, focusing on calf feeding and management, included questions regarding source of colostrum and feeding practices used at each dairy, along with husbandry questions. The survey also collected demographic information including primary breeds, rolling herd average, and herd size for each dairy.

Detection of *Cryptosporidium* spp. oocysts

Samples were processed within 48 hours for detection of *Cryptosporidium* spp. oocysts. A 5g of feces was homogenized in PBS and filtered through four-layer gauze secured on a strainer into a 50ml tube to remove large fibrous particles, and centrifuged at 1000 × g for 10 minutes. The supernatant was discarded and the sediment re-suspended in an equal volume of deionized water. Then 10µl of the fecal suspension was smeared onto a glass slide and air-dried, then a direct immunofluorescent assay [DFA] was performed on each slide to detect oocysts [17,18]. The entire slide was examined under 400× magnifications for identification and quantification of *Cryptosporidium* spp. oocysts using a fluorescent microscope [Olympus BX60].

Genotyping of *Cryptosporidium* spp.

A subset of microscopic positive fecal samples from different farms were subjected to genotyping of *Cryptosporidium* spp. A 0.2 g of feces were exposed to 5 cycles of freeze [−80°C] and thaw [+70°C] then used for DNA extraction by using the DNA Stool Mini Kit [Qiagen] according to the manufacturer's instructions. A nested PCR was performed using primers and reaction conditions amplifying a fragment of ~ 830 bp of the 18S rRNA gene according to methods previously described [19,20]. A DNA template of *C. parvum* isolated from calves from a local dairy farm and a negative control without DNA template were included. PCR products were verified by electrophoresis in 2% agarose gel stained with ethidium bromide. Products of the secondary PCR were purified using a Qiaquick spin columns [Qiagen] and sequenced at the UC Davis DNA Sequencing Facility using an ABI 3730 capillary electrophoresis genetic analyzer [Applied Biosystems Inc., Foster City, CA]. Primers of the secondary PCR were used for sequencing at both forward and reverse directions. Consensus sequences were generated from the forward and reverse sequences of each sample using Vector NTI Advanced 11 software [Invitrogen Corporation, Carlsbad, CA]. BLAST analyses were performed to compare the sequences to existing *Cryptosporidium*spp. sequences in the GenBank using the default settings of National Center for Biotechnology Information [NCBI] online blasting tool [https://blast.ncbi.nlm.nih.gov/Blast.cgi].

Statistical analysis

Survey management variables: A total of 54 management

questions were asked in the survey. These question variables were organized into three blocks [calf housing, calf feeding, calf management] for interpretation. Analyses were performed in R 3.3.2 [21]using the 'tidyverse'and 'rms' packages.

Univariate analysis: All open-ended questions were converted to categorical questions in the data cleaning stage. Categorical variables werelabeled as factors to create dummy variables in model building. The survey questions represented nominal categorical independent variables to be included in model building. Calf age was a continuous variable to start and assessed for normality using a basic scatterplot. Later calf age was converted from a continuous to a categorical variable by dividing it into 4 categories that represented clinically relevant intervals of time with regard to risk of oocysts shedding. The binary outcome of *Cryptosporidium* spp. oocysts status [Positive=1; Negative=0] was used as the dependent variable to calculate odds ratios using logistic regression. All nominal categorical variables were cross-tabulated with *Cryptosporidium* spp. outcome status to evaluate the frequency of responses for each answer choice to ensure an adequate count to conduct chi-square analysis. Levels of a categorical variable were collapsed to improve frequency counts if there were fewer than five responses for a given answer. Any survey question that lacked variability in the answer selection across dairies was excluded from further evaluation. A question was considered to lack variability if all dairies had the same response. Within each management block, a chi-square test for independence was conducted on all categorical variables cross-tabulated [one-at-a-time] with the outcome to determine if a relationship existed between each independent variable and the *Cryptosporidium* spp. oocysts status. Univariate logistic regression was used to calculate an odds ratio to measure the association between each categorical exposure variable and the outcome of *Cryptosporidium* spp. oocysts shedding status.

Model building: Following univariate analysis, the list of candidate variables to be included in the model selection process was further narrowed using the following criteria: 1] hypothesized biologic relevance to *Cryptosporidium* spp. oocyststsshedding, 2] previously shown to have a statistical association with *Cryptosporidium* spp. status in prior studies, and 3] P-value < 0.1 on univariate analysis. The remaining variables were then ranked from highest to lowest in significance based onthe p-value from univariate analysis and offered to an age-adjusted multivariable fixed-effects logistic regression model. A forward-stepping algorithm was used for model building in which the most significant variable was offered first, the model was re-fitted and the remaining candidate variables evaluated for significance, prior to the addition of the second most significant variable, and so on until all significant variables were included in the model. The significance of the likelihood ratio test was used to determine goodness-of-fit between each nested model in addition to Akaike information criterion [AIC]. Coefficient estimates and 95% confidence intervals were calculated using the Wald's X^2 statistic. To account for significant clustering of independent variables at the farm level, robust standard errors were calculated for the final model.

Multi-collinearity: For all categorical variables in the final model, collinearity was assessed using the chi-square test of independence between all covariates. Variables with a significant p-value on this test [<0.05] were considered collinear, and only one of the two variables were retained in the model. In addition, the variance inflation factor [VIF] was also calculated to assess for multi-collinearity between age and the categorical variables. Any variable with a VIF higher than 10 was removed from the final model.

RESULTS

Overall prevalence and load of shedding of *Cryptosporidium* spp. oocysts

The average total herd sizefor the eight dairieswas1,743 animals. In total 654 calves were sampled with an average number of 82 calves sampled at each farm [ranged between 80 and 83]. Ages of all calves sampled were between 0-60 days except for two calves that were 64 and 87 days old respectively. These two calves were both negative for *Cryptosporidium* spp. oocysts in feces.The highest proportion of calves positive for *Cryptosporidium* spp. oocysts were 16-31 days old [63.5 %] followed by 0-15 daysold [52.0 %], 32-46 days old [50.5 %] and >46 days old [40.0 %] [Figure 1]. The overall point prevalence of *Cryptosporidium*spp. shedding across all eight dairies was 56.0% [342/610], with DairyFhaving the highest point prevalence of 87.8% [65/74; 8 NAs], and Dairy A with the lowest point prevalence at 37.3% [31/83; 0 NAs]. Figure 2 depicts the number of *Cryptosporidium* spp. oocysts shed in feces based on calf age [days] across alldairies. There appears to be clustering of higher oocyststsshedding by positive calves in the age range of 4-15 days old, likely due to the onset of shedding after the pre-patency period.

Risk factors associated with shedding of *Cryptosporidium* spp. oocysts

Of the 54 question variables collected in the survey, 11 were significantly associated with *Cryptosporidium* spp. shedding on univariate analysis [Table 1]. The prevalence of shedding oocysts varied between risk factors and across levels of a factor. The final multivariable fixed-effects model and odds ratios for all significant variables in the modelare included in Table 2. The variables included in the final model were chosen based on having a combination of the lowest AIC and being statistically significant on likelihood ratio test during the forward-stepping procedure which corresponded to an improvement in goodness-of-fit. Calves aged 16-31 days were statistically significantly associated with increased odds of shedding *Cryptosporidium* spp. oocysts compared to younger [0-15 days old] or older [>46 days old] calves. The management practices pertaining to the frequency of cleaning milk bottles for each calf and the method for disinfecting the bottles were both significantly associated with the odds of shedding *Cryptosporidium* spp.oocysts.

Genotyping of *Cryptosporidium* spp.

In total 105 fecal samples microscopically positive for *Cryptosporidium* spp. from the eight dairies were successfully genotyped by PCR and sequencing a fragment of the SS rRNA gene. According to BLAST analysis completed by September 20, 2018, 86.7% [91/105] isolates were determined as *C. parvum*,

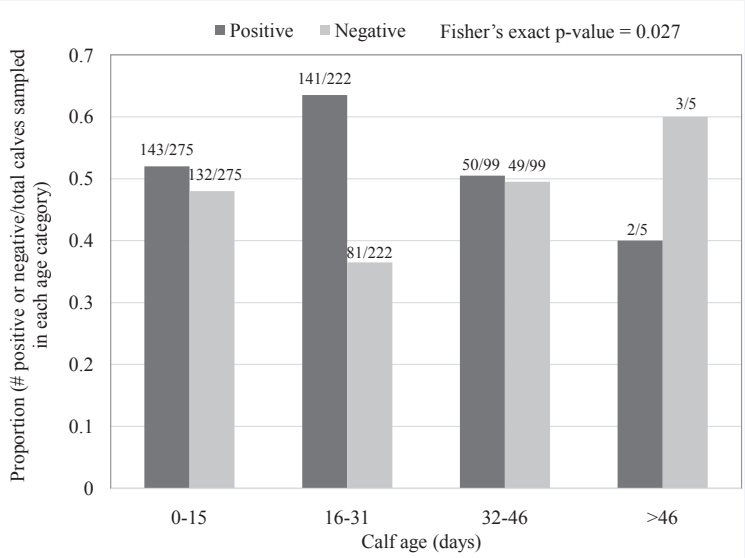

Figure 1 Proportion of calves positive and negative for *Cryptosporidium* spp. oocysts in each age category.

Table 1. Prevalence and intensity of *Cryptosporidium* spp. oocysts shedding in calf fecal samples for each significant management factor identified on univariate analysis.

Factor	No. of positive calves/no. of sampled calves (% positive)	OR (95% CI)	Mean no. oocysts/g in fecal samples [a]	
			Positive	Total
Age (Days)	-	-	-	-
0-15	143/275 (52.0)	1.0	80,329	41,771
16-31	141/222 (63.5)	1.6 (1.1, 2.3)	75,920	48,220
32-46	50/99 (50.5)	0.9 (0.6, 1.5)	21,197	10,706
>46	2/5 (40.0)	0.6 (0.1, 3.7)	484	193
Type of hutch				
Hutch on ground	152/309 (49.2)	1.0	105,795	52,042
Raised hutch	99/154 (64.3)	1.8 (1.2, 2.7)	24,104	15,496
Mixture of hutch types	90/148 (60.8)	1.6 (1.0, 2.3)	57,385	34,896
Type of hutch floor				
Concrete	34/79 (43.0)	1.0	28,709	12,356
Wood or dirt	307/532 (57.7)	1.9 (1.2, 3.1)	73,797	42,586
Is bedding used in the hutch?				
Yes	152/309 (49.2)	1.0	105,795	52,042
No	189/302 (62.6)	1.7 (1.2, 2.3)	39,952	25,003
How many days are hutches left empty between calves?				
Never empty	31/83 (37.3)	1.0	60,172	22,474
7 days	28/75 (37.3)	1.1 (0.56, 2.0)	80,081	29,897
More than 7 days	282/453 (62.3)	2.8 (1.7, 4.5)	69,235	43,100
How frequently is milk or milk replacer fed daily?				
Twice	186/307 (60.6)	1.0	54,161	32,814
More than twice	155/304 (50.9)	0.7 (0.5, 0.9)	87,470	44,598
How often are the calf milk containers cleaned?				
Every feeding	276/536 (51.5)	1.0	80,513	41,458
2-3 times/week	65/75 (86.7)	6.7 (3.3, 13.8)	21,696	18,803
How are the calf milk containers cleaned?				
Rinsed with water & disinfectant	208/376 (55.3)	1.0	51,295	28,376
Rinsed only with hot water	31/83 (37.3)	0.48 (0.3, 0.8)	60,172	22,474
Rinsed only with disinfectant	102/152 (67.1)	1.7 (1.1, 2.5)	108,796	73,008

How is grain starter fed?				
Metal bucket	99/154 (64.3)	1.0	24,104	15,496
Plastic bucket	242/457 (53.0)	0.64 (0.4, 0.9)	87,791	46,489
What is the dairy's source of water?				
Well	101/236 (42.8)	1.0	76,796	32,866
Pond	240/375 (64.0)	2.4 (1.7, 3.3)	66,148	42,335
At what age are calves moved to the hutch?				
Less than 6 hours old	28/75 (37.3)	1.0	80,081	29,897
6-24 hours old	197/301 (65.4)	3.0 (1.8, 5.1)	51,897	33,966
> 24 hours old	116/235 (49.4)	1.5 (0.9, 2.6)	96,258	47,515
Do workers enter the hutch to treat or evaluate calves?				
Yes	34/79 (41.0)	1.0	28,709	12,356
No	307/532 (57.7)	1.9 (1.2, 3.1)	73,797	42,586

[a]Shedding intensity is the arithmetic mean for the number of oocysts shed per gram for positive fecal samples (Positive column), or the arithmetic mean for the number of oocysts shed per gram for all fecal samples (Total column).

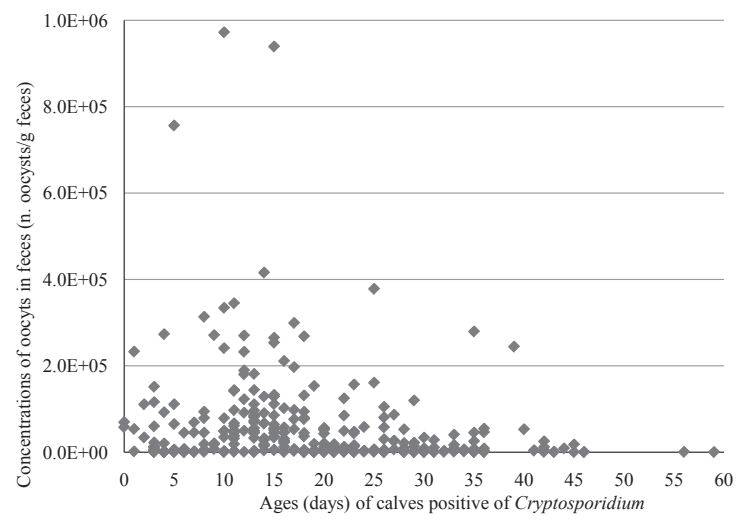

Figure 2 Quantitative shedding of *Cryptosporidium* spp. oocysts in preweaned calves.

12.4% [13/105] isolates were identified as *C. bovis*, and 0.9% [1/105] isolate was identified as *C. ryanae* [Table 3]. *C. parvum* was the predominant species prevalent in calves in all dairies. The 13 isolates of *C. bovis* were detected in Dairy B [3 isolates], Dairy D [2 isolates], and Dairy H [8 isolates]. Ages of calves that shed *C. bovis* were 15, 32, and 34 days old on Dairy B; 5 and 32 days old on Dairy D, and ranged between 20-46 days old on Dairy H. The isolate of *C. ryanae* was detected from an 18-day old calf on Dairy E.

DISCUSSION

Previous works have identified management risk factors associated with *Cryptosporidium* spp. infection in dairy calves in other states and other countries[1, 22-31]. These include calf age, calves housed in a cow barn, herd size, and hay bedding in dairies in New York state [1]; higher prevalence in summer than winter in dairies in New York city [22]; calf age of ≤ 30 days in dairies in New York state [23]; the use of calf diarrhea prophylaxis in pregnant cows and the type of maternity facilities in dairies in Ontario, Canada [24]; age, placing of young stock, routines for moving young stock and time calf stays with the cow in dairies in Sweden [25]; calf age of ≤ 20 days in dairies in Argentina [26]; age [15-21 days old] in dairies in Italy[27]; calf age of ≤ 2 months and poor sanitation in dairies in Kenya [28]; dispensing of colostrum using a bucket and feeding with fermented milk in dairies in France [29]; the use of milking equipment and milking cooler in dairies in Brazil [30];and types of flooring and methods and frequency of cleaning in dairies in Spain[31]. The current study populations comprised calves housed in hutches on all participating dairies on the day of sampling. The point prevalence of *Cryptosporidium* spp. calculated for this study [56%] was higher than that reported in New York city [11-26%] [22]and in Mexico [25%][32] but similar to high prevalence [78%] reported in dairies in Ontario, Canada[24] and52% in dairies in Sweden [25]. An earlier study found that the *C. parvum*point prevalence was highest in 15 day old calves [32]and other studies have reported similar results[1-3].Our study focused on preweaned calves aged 0-60 days which is an age group more susceptibleto *Cryptosporidium* spp. infections compared to older calves. Age as a continuous variable was found not statistically associated with *Cryptosporidium* spp. shedding, but when age was divided into four categories, the calves in the 16-31 days old category

Table 2: Final multivariable fixed effects logistic regression model, including robust SE for management factors associated with shedding *Cryptosporidium* spp. oocysts by calves.

Factor	Adjusted OR	95% C.I.	S.E.	P-value for factor	Robust S.E.	P-value for factor (robust)
Age (Days)						
0-15	1.0	-	-	-	-	-
16-31	1.6	(1.06, 2.3)	0.1970	0.024	0.1828	0.014
32-46	0.99	(0.60, 1.6)	0.2578	0.983	0.3293	0.987
> 46	0.37	(0.06, 2.3)	0.9390	0.289	0.1157	<0.001
How often are the calf milk containers cleaned?						
Every feeding	1.0	-	-	-	-	-
2-3 times/week	8.3	(3.98, 17.4)	0.3755	<0.0001	0.1516	<0.0001
How are the calf milk containers cleaned?						
Rinsed with water and disinfectant	1.0	-	-	-	-	-
Rinsed only with hot water	0.7	(0.41, 1.1)	0.2636	0.1436	0.1402	0.006
Rinsed only with disinfectant	2.4	(1.5, 3.7)	0.2208	<0.0001	0.1182	<0.0001

Table 3: Genotypes of *Cryptosporidium* spp. in preweaned dairy calves in Central Valley, California

Dairy ID	Prevalence % (positive/total)	No. of isolates sequenced	Species determined by BLAST [a]
A	37.3 (31/83)	12	*C. parvum* (12/12)
B	71.2 (52/73)	19	*C. parvum* (16/19) *C. bovis*[b] (3/19)
C	41.8 (33/79)	8	*C. parvum* (8/8)
D	56.5 (39/69)	7	*C. parvum* (5/7) *C. bovis* (2/7)
E	38.7 (29/75)	6	*C. parvum* (5/6) *C. ryanae* [b] (1/6)
F	87.8 (65/74)	8	*C. parvum* (8/8)
G	53.8 (42/78)	14	*C. parvum* (14/14)
H	64.5 (51/79)	31	*C. parvum* (23/31) *C. bovis* (8/31)
Total	56.0 (342/610)	105	*C. parvum* (91/105=86.7%) *C. bovis*(13/105=12.4%) *C. ryanae* (1/105=0.9%)

[a] Species determination based on comparison of DNA sequences of 18S rRNA gene in the GenBank by BLAST analysis
[b] These species are considered to be minimally infective for humans.

had statistically significantly higher odds of oocysts shedding. These findings agree with previous studies mentioned above.The lack of a statistically significant association between calves aged 32 days and above and oocysts shedding is likely due to lower environmental risk and a more developed immune system. We observed that younger calves tended to shed higher numbers of oocysts in feces [Figure 2], compared to older calves. Future studies with a larger sample size in both the number of enrolled dairies and sampled calves as well as more age groups will further characterize the prevalence and intensities of *Cryptosporidium* spp. infection in dairy calves.

Two management factors involving calf feeding were found associated with oocysts shedding in the current study. Specifically, the highest odds of shedding oocysts were associated with cleaning of the milk replacer/whole milk bottles 2 to 3 times per week on a dairyversusdairies with daily cleaning. Infected calves had 8.3 times the odds of coming from a dairy that cleaned the milk bottles only 2-3 times per week compared to those that cleaned them at every feeding. These results are similarto other studies that have shown management factors such as housing [wood vs. plastic hutches], frequency of cleaning, and use of bedding to be associated with risk of *C. parvum* shedding [1, 4,32,33].This could be due to a combination of factors related to *C. parvum* oocysts survival under different conditions on a dairy. Oocysts are known to be robust and to persist in the environment [34], especially moist environments without UV exposure. Our results seem to suggest that oocysts survive on contaminated milk bottles and may promote oocysts transmission among calves. In addition, infrequent cleaning means that fecal material which may be transferred from a calf's mouth or muzzle onto the bottle during feeding is not being removed daily. A worker may notice when a bottle is strongly soiled with dirt/feces and choose to clean it, but may not consider cleaning if it is not obviously soiled. Oocysts can be highly concentrated in fecal material, and easily transferred in high concentrations even when fecal contamination is minimal [35]. A lack of cleanliness in-regards-

to cleaning milk bottles could reflect overall cleanliness of calf hutches on some dairies. In addition to the frequency of cleaning milk bottles, the method for cleaning milk bottles was also significantly associated with oocysts shedding. Rinsing only with hot water was protective [OR 0.7] against *C. parvum* shedding compared to the referent [rinsing with water and a disinfectant]. However, infected calves had 2.4 times the odds of exposure to milk bottles that were cleaned with only disinfectant and this may be related to the thoroughness of cleaning using each of the methods. Perhaps farms that use high temperature water as their sole form of cleaning [OR 0.7] do a more thorough job of cleaning since disinfectant is not used compared to farms using only disinfectant [OR 2.4] or a combination of water and disinfectant [OR 1.0], which assume disinfectant will be adequate in killing pathogens even when debris is not entirely removed. Although several farm-level variables were associated with oocysts shedding in univariate analysis, only the three variables mentioned above were significant in the final model. The latter may be partially explained by residual collinearity between management variables and a lack of power in the study to detect other significant associations due to the small number of farms and animal-level variables. It has been reported that more enrolled farms and larger sample size per farmmay facilitate the detection of associations between variables and *C. parvum* oocysts shedding by reducing clustering at the farm-level [4,32]. In this study farm-level explanatory variables were used to predict an animal-level outcome [except for age of calf], andimportant details regarding risk may have been missed by not measuring more animal-level management variables. However, a major strength of this study was the comprehensive design of the survey questionnaire, which covered the predominant management practices pertaining to calf feeding and husbandry on dairies. We analyzed this dataset usingbinomial logistic regression to build a multivariate fixed-effects model and accounted for the strong influence of clustering by calculating robust standard errors [Table 2]. The robust standard errors were smaller than those calculated in the fixed effects model. The statistical significance of the categorical variables in the final model remained the same after robust standard errors were calculated, except for >46 age level [changed from p-value of 0.289 to <0.001] and one level pertaining to milk bottle hygiene [changed from p-value of 0.143 to 0.006]. There were very few calves aged >46 days on any of the dairies sampled, and they may have been clustered at one dairy, leading to a change in significant with the robust p-value. The milk bottle hygiene level may have become significant because there were few farms that responded with this answer, indicating significant clustering at the farm level.

According to a recent review, species and genotypes of *Cryptosporidium*spp. that are considered zoonotic or potentially zoonotic include [major hosts in parenthesis]: *C. parvum* [cattle], *C. erinacei* [squirrels], *C. scrofarum* [pigs], *C. tyzzeri* [mice], *C. cuniculus* [rabbits], *C. ubiquitum* [cattle], *C. xiaoi* [sheep and goats],

Table 4: Summary of reported species of *Cryptosporidium*spp. infection in preweaned calves

Location	*Cryptosporidium* species	% of the species	Reference
Northern Ireland	*C. parvum*	95.1	Thompson et al., 2007(42)
	C. bovis	3.6	
	C. sp. deer-like genotype	1.3	
Belgium	*C. parvum*	91.8	Geurden et al., 2007(43)
	C. bovis	8.2	
Spain	*C. parvum*	98.7	Quilez et al., 2008(44)
	C. bovis	1.3	
England	*C. parvum*	92.6	Brook et al., 2009(45)
	C. bovis	5.5	
	C. sp. deer-like genotype	1.9	
Sweden	*C. parvum*	20.5	Silverlas et al., 2010(46)
	C. bovis	74.0	
	C. ryanae	5.5	
Japan	*C. parvum*	97.0	Karanis et al., 2010(47)
	C. bovis	3.0	
Czech Republic	*C. parvum*	85.1	Kvac et al., 2011(48)
	C. bovis	1.9	
	C. andersoni	13.0	
	C. ryane	0	
Xinjiang, China	*C. parvum*	59.5	Qi et al., 2015(50)
	C. bovis	24.3	
	C. ryanae	2.7	
	C. andersoni	5.4	
Shaanxi Province, China	*C. parvum*	0	
	C. bovis	50.0	Qi et al., 2015(51)
	C. ryanae	23.1	
	C. andersoni	26.9	

C. fayeri [kangaroo], *C. bovis* [cattle], *C. suis* [pigs], *C. canis* [dogs], *C. andersoni* [cattle], *C. meleagridis* [turkeys], *C. felis* [cats], *C. muris* [mice], chipmunk genotype I [chipmunks], horse genotype [horses], mink genotype [minks], and skunk genotype [skunks] [36]. *Cryptosporidium*spp. infections in cattle are primarily associated with *C. parvum*, *C. andersoni*, *C. ryanae*, and *C. bovis,* although *C. suis, C. hominis, C. serpentis, C. xiaoi, C. ubiquitum, C. meleagridis, C. muris, and C. felis* have also been reported in cattle [37]. Distribution of the four major species in cattle are associated with cattle ages: *C. parvum* is mostly found in preweaned calves with diarrhea, *C. andersoni* is mostly identified in asymptomatic adults, and *C. bovis* and *C. ryanae* are frequently found in post-weaned calves and yearlings. Based on DNA finger printing of 105 isolates of *Cryptosporidium* spp. in our study, approximately 87% of *Cryptosporidium*spp. isolates was identified as *C. parvum*, 12% as *C. bovis*, and 1% as *C. ryanae* in these California dairies during the study period [Table 3]. The dominant species, *C. parvum*, is a species that mainly infects calves and other vertebrate species including humans[10].Both *C. bovis* and *C. ryanae* are species mainly infect cattle [16, 38]. Although few cases of asymptotic *C. bovis* infections in humans has been reported [39,40], its major host is cattle and its impacts on public health are relatively low compared to *C. parvum* [41].

Notably, among 30% [105/341] of *Cryptosporidium* spp. positive samples that were successfully genotyped in this study, the dominant species in preweaned dairy calves is still *C. parvum*, a species infectious to humans and of public health significance. Reports of *Cryptosporidium* spp. species in preweaned calves from literature available in the PubMed database [42-50] are reported in Table 4. According to these studies, *C. parvum* continues to be the major species isolated in feces from preweaned calves, except for reports from Sweden [46] and the Shaanxi province China [51], although infection with *C. bovis*, *C. ryanae*, and *C. andersoni* also occur [Table 4]. One of these studies identified that contact with adult cattle was the primary risk factor for *C. bovis* and *C. andersoni* infection in preweaned calves [48]. Results of thepresent study of *Cryptosporidium* spp. infection in preweaned calves in California dairies are consistent with the overall distributions of *Cryptosporidium*spp. species reported worldwide. *C. parvum* is reported as the dominant species in fecal samples from preweaned calves, although mixed infections primarily with *C. bovis* and *C. ryanae* also occur in this susceptible age group.

CONCLUSION

This cross-sectional study provides information on the prevalence of *Cryptosporidium* spp. in preweaned calves in California dairies with the typical western style of confinement dairy production. Our results revealed that *Cryptosporidium* spp. species in preweaned dairy calves [between 0 and 2 months old] in the region continue to be of zoonotic concern and public health significance based on *Cryptosporidium* spp. species [*C. parvum*] determined in this age group. Continuous and enhanced facility and equipment sanitation practices including thorough cleaning of milking bottles are recommend in order to reduce the risk of *Cryptosporidium*spp. infection in young dairy calves.

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