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Cryptosporidium parvum infection and associated risk factors in dairy calves in western France

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ABSTRACT

This study was conducted to determine the prevalence and risk factors for *Cryptosporidium* infection in calf neonates on dairy farms in Normandy.

Fecal samples were randomly collected between July 2010 and September 2011 from 968 calves (7–21 days old) on 97 farms. Up to 10 calves were selected and sampled per farm, and feces examined for oocysts by microscopy. *C. parvum* oocyst shedding was scored semi-quantitatively (0–5). A questionnaire about calf-level care and management was completed, and mortality rates were obtained from the French national registration database (BDNI). Bivariable and multivariable analyses of potential risk factors for *C. parvum* oocyst shedding were conducted using generalized estimating equation (GEE) models (family = Binomial).Overall, 402 out of 968 calves (41.5%) were positive for oocysts, and 25.1% of animals had a shedding score >2. Seven of the 97 farms (7%) were negative for oocysts in all fecal samples. At the time of collection, 375 calves (39%) had diarrhea, and its prevalence strongly correlated with the score for *C. parvum* oocyst shedding ($p < 0.0001$). The mortality rate at 90 days was significantly greater for calves with high combined scores of diarrhea and shedding. Factors associated with the shedding of *C. parvum* were the Normande breed (odds ratio = 1.49; 95% confidence interval (CI): 0.93–2.37), dispensing of colostrum using a bucket (odds ratio = 1.37; 95% CI: 1.00–1.89), treatment with halofuginone (odds ratio = 0.46; 95% CI: 0.19–1.15) and feeding with fermented milk (odds ratio = 0.32; 95% CI: 0.17–0.63).

C. parvum is widespread among calves under 21 days old in dairy herds of western France. Shedding of *C. parvum* is associated with a high incidence of diarrhea and increased risk of mortality in young calves. This study identified some associated calf-level factors, although further investigations are necessary to determine appropriate measures that farmers and veterinary practitioners should take to reduce the prevalence of *C. parvum*.

1. Introduction

Cryptosporidium is one of the most common enteropathogens present in calves during their first two weeks of age (Thompson et al., 2007). Young calves mainly shed *Cryptosporidium parvum*, which has a wide host range and is potentially zoonotic. Clinical infection in calves

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is characterized by non-specific diarrhea, dehydration, depression, anorexia and abdominal pain. In most calves, diarrhea starts 3–5 days postinfection and lasts from 4 to 17 days (de Graaf et al., 1999). Oocyst shedding begins at 4 days after birth and peaks at 7–18 days, decreasing after 3 weeks (Nydam et al., 2001; Trotz-Williams et al., 2007). Higher oocyst shedding is frequently seen during episodes of diarrhea (Xiao and Herd, 1994; McCluskey et al., 1995). Despite much endeavor in the development of effective drugs, halofuginone lactate (HL) is the only drug registered in Europe to treat cryptosporidiosis in cattle. HL can reduce, but not completely prevent, oocyst shedding and diarrhea in calves (Joachim et al., 2003), suggesting that additional hygiene measures must be adopted to provide better control of *Cryptosporidium* infections (de Waele et al., 2010). Other enteric viral and bacterial pathogens such as rotaviruses, coronaviruses and *Escherichia coli* can also cause diarrhea in bovine neonates to the same degree of severity as cryptosporidiosis (de Graaf et al., 1999).

Cryptosporidiosis is a multifactorial disease in which many parameters associated with calves, and environment and production practices, may be of significance. Previous studies have indicated that cryptosporidiosis may occur more frequently in dairy calves than in beef calves because the former are born throughout the year and are confined to pens or hutches, which can facilitate a high level of year-round transmission (Olson et al., 2004). Other factors linked to passive immunity of the calf and management of large herds may also be relevant (O'Handley, 2007). Several factors have been identified as significant in increasing the risk of shedding *Cryptosporidium* oocysts by dairy calves. Most studies have focused on factors associated with management of the herd, and attempted to resolve these issues by presenting a questionnaire to farmers (*e.g.*, Garber et al., 1994; Mohammed et al., 1999; Trotz-Williams et al., 2007; Silverlås et al., 2009b). Significant risk factors may differ from survey to survey, indicating that different epidemiological practices may need to be adopted on different farms. Epidemiological data for dairy calf cryptosporidiosis in France is scarce, and there are no risk factor analysis. The first surveys in France underlined the importance of *Cryptosporidium* in neonatal diarrhea and reported that the prevalence of oocyst shedding ranged from 17.9% to 43.4%, depending on the sampling regime (Naciri et al., 1999; Lefay et al., 2000). Molecular characterization of *Cryptosporidium* isolates shed by dairy calves identified the zoonotic *C. parvum* as the dominant species, followed by *C. bovis* and *C. ryanae* (Follet et al., 2011; Rieux et al., 2013).

The aim of this study was to determine the prevalence and risk factors for *Cryptosporidium* infection in bovine neonates in an area of intensive dairy farming in western France.

2. Materials and methods

2.1. Sampling strategy and data collection

The sampling pool consisted of 1676 herds located in the Orne Department of Normandy (France), from which a computer-generated list of 120 farms was randomly selected (Survey Toolbox software). Farms were visited every two weeks during the calving period between July 2010 and September 2011. Ten calves that were 7–21 days old were sampled on each farm during biweekly visits. Calves were randomly selected if there were more than 10 available.

Individual fecal samples were taken directly from the rectum of each calf using sterile plastic gloves and stored at 4 °C before transfer to the laboratory.

At the time of collection, the consistency of each fecal sample and general condition of the calf were scored according to Naciri et al. (1999) in the following manner: for diarrhea, absence (0), mild (1), heavy (2); and for general condition, normal vitality (0), low vitality (1), very low vitality (2), recumbency (3).

Sampling was done in compliance with animal welfare and did not cause distress according to the French Ethics Committee for Animal Experimentation no. 16.

Information on the calf mortality rate was obtained from the French national registration database (BDNI). Mortality was estimated from the number of calves that died before they reached 90 days. Calves that were sold before 90 days were excluded from this calculation.

2.2. Sample analysis

Screening for oocysts was done by staining fecal smears with Ziehl fuchsin and observing at 100× magnification under a phase-contrast microscope according to Heine (1982). The intensity of shedding was evaluated semi-quantitatively according to the average number of oocysts in 10 randomly selected observation fields. Six categories were defined as follows: 0 (absence of oocysts), 1 (<1 oocyst per field), 2 (1–10 oocysts), 3 (11–20 oocysts), 4 (21–30 oocysts) and 5 (>30 oocysts). One person conducted all tests. Considering direct IFAT as the gold standard, Heine staining of fecal smears has a sensitivity of 76.7% and was used because the scoring (1–5) correlates with the number of oocysts in feces (Chartier et al., 2013).

2.3. Questionnaire

A questionnaire was used to collect information about the management of each calf sampled. Questions were designed to gather information about potential predictors of risk for shedding of *C. parvum*. Data were collected in the field during an interview with the farmer or farm manager.

2.4. Statistical analysis

The positive or negative status of each fecal specimen was scored as a dichotomous variable (Heine technique score ≥1 *vs* control: score = 0). All factors investigated were categorical variables collected at the individual level as follows: age, sex, breed, period of birth, type of housing in which the calf was kept, ease of birth, care given to the calf, vaccines and drugs dispensed to the calf, maternal vaccination against neonatal diarrhea, length of time calf stayed with the dam after birth, calving area hygiene, type of colostrum/milk fed, and equipment used to feed calves (Table 1).

Table 1
Potential factors associated with the shedding of *C. parvum* by young dairy calves ($n = 968$) in Normandy, France.

Covariate	Definition	Levels
Age at sampling	Age of calf on day of collection (in days)	≤10 >10 and ≤15 >15
Sex	Sex of the calf	Male *vs* female
Breed	Breed of the calf	Holstein Normande Other breeds
Period	Period of calving	November–January February–April May–July August–October
Calf housing	Type of calf housing after birth	Individual box Collective box With stabling cows Pasture
Assisted birth	Assistance required at birth	Yes/No
Care	Care of the calf at birth	Yes/No
Coccidiostat	Calf was treated with a coccidiostat before sampling	Yes/No
Halofuginone	Calf was treated with halofuginone before sampling	Yes/No
Antibiotic	Calf was treated with an antibiotic before sampling	Yes/No
Anti-inflammatory drug	Calf was treated with an AI drug before sampling	Yes/No
Vaccination of the calf	Calf was vaccinated (respiratory diseases) before sampling	Yes/No
Dietary	Calf received a dietary supplement before sampling	Yes/No
Navel	Navel of the calf was disinfected	Yes/No
Vaccination of the dam	Dam of calf was vaccinated (neonate diarrhea)	Yes/No
Time spent with the dam	Time calf spent with the dam (in hours)	≤2 >2 and ≤6 >6 and ≤12 >12
Pooled colostrum	Calf was fed pooled or commercial colostrum	Yes *vs* No
Bottle	Colostrum was distributed using bottle	Yes *vs* No
Bucket	Colostrum was distributed using bucket	Yes *vs* No
Bucket with teat	Colostrum was distributed using bucket with teat	Yes *vs* No
Colostrum quantity	Quantity of colostrum fed to calf (liters)	Unmeasured (calf with the dam) ≤2 l >2 l
Whole milk	Calf was fed whole milk after colostrum	Yes *vs* No
Milk replacer	Calf was fed milk replacer after colostrum	Yes *vs* No
Medicated milk	Calf was fed medicated milk after colostrum	Yes *vs* No
Fermented milk	Calf was fed fermented milk after colostrum	Yes *vs* No

AI, anti-inflammatory drug.

Factors associated ($p < 0.20$) with the outcome variable in the bivariable analysis were included in a multivariable analysis. The choice of a screening criterion of 0.20 was designed to limit multicollinearity and ensure that potentially important variables were included in the multivariable analysis (Dohoo et al., 1996).

Cramer's V Coefficient (V) was used to investigate collinearity between categorical predictor variables (Cramer, 1946). This measure is defined as: $V = \sqrt{(X^2/nt)}$ where X^2 is the Pearson's Chi-squared statistic, n is the grand total of observations, and t is the smaller of the number of rows minus one or the number of columns minus one. When variables were significantly associated ($V > 0.3$), only one of the two variables was included in the initial model; the variable was chosen based on the strength of association with the outcome variable and biological plausibility.

Bivariable and multivariable analyses were conducted using generalized estimating equation (GEE) logistic models (Liang and Zeger, 1986) to account for within-herd correlation (McDermott et al., 1994; Kadohira et al., 1996). The structure of correlation among observations of the same cluster (herd) was assumed to be constant (option "exchangeable"), which is acceptable when the cluster size is large (>4).

Table 2
Mortality before 90 days according to *C. parvum* shedding and diarrhea scores in 7–21-day-old calves ($n = 766$) in Normandy, France.

	C. parvum shedding score/diarrhea score			
	0 to 2/0–1	0 to 2/2	>2/0–1	>2/2
Calves not sold	498	72	110	86
Calves died before 90 days	19 (3.8%)[a]	4 (5.6%)[a,b]	5 (4.6%)[a]	12 (14.0%)[b]

Percentages in the same row with different superscripts[a,b] are significantly different (Chi-squared test, $p < 0.05$).

Table 3
Univariable analyses for factors associated with the shedding of *C. parvum* by young dairy calves ($n = 968$) in Normandy France using generalized estimating equations (GEE).

Risk factors	Levels	No. of calves	Prevalence (%)						p-Value (GEE)
			Score of shedding						
			0	1	2	3	4	5	
Age at sampling[a]	≤10 days	323	51	8	5	4	4	28	
	>10 and ≤15 days	330	46	8	12	4	2	27	<0.001 (>15 *vs* ≤10)
	>15 days	315	70	10	5	1	2	11	
Breed[a]	Holstein	469	58	8	7	3	3	22	0.03 (Normande *vs* Others)
	Normande	428	53	10	8	4	3	23	
	Others	71	61	10	13	1	1	14	
Halofuginone[a]	No	898	55	9	7	3	3	23	0.12
	Yes	70	61	11	11	3	0	13	
Antibiotic[b]	No	732	58	9	8	2	3	20	0.02
	Yes	236	48	8	6	6	3	30	
Anti-inflammatory[b]	No	948	56	9	8	3	3	22	0.05
	Yes	20	40	10	0	5	0	45	
Dietary[b]	No	816	57	9	8	3	2	21	0.03
	Yes	152	46	8	5	5	7	30	
Bucket[a]	No	564	61	9	8	3	2	16	0.07
	Yes	404	48	8	8	2	4	30	
Bucket with teat[c]	No	926	55	9	8	3	3	22	0.14
	Yes	42	76	5	0	0	0	19	
Calf fed fermented milk[a]	No	941	55	9	8	3	3	23	0.02
	Yes	27	85	4	7	0	0	4	

[a] Kept in the multivariable model.
[b] Variable removed (nonexplanatory).
[c] Variable removed (strongly associated with the variable "Bucket", $V = 0.33$).

All analyses were performed using the statistical software R 3.0.0 (STATS and GEEPACK packages).

3. Results

3.1. Descriptive data

Fecal samples were collected from 968 calves on 97 farms. Of the 120 farms originally selected, 23 refused to participate due to time constraints. On one farm, only eight calves could be sampled due to an insufficient number of newborn animals. To sample 10 calves on each farm, several visits were made as follows: 3 visits for 46 farms, 4 for 33 farms, 5 for 11 farms, 6 for 2 farms, 7 for 4 farms and 8 for 1 farm.

In total, 402 of the 968 calves (41.5%) were positive for oocysts according to Heine staining. Positive fecal samples were distributed as follows: 85 calves (8.8%) with a score of 1, 74 (7.6%) with a score of 2, 29 (3.0%) with a score of 3 or 4, and 214 (22.1%) with a score of 5. Within-herd prevalence of oocyst shedding ranged from 0% to 90% (mean, 44%; first quartile, 10%; third quartile, 80%). Seven of the 97 farms (7%) were negative for all samples, and, by contrast, 10 farms (10%) had high shedding calves (scores of >3) in more than half of the samples.

At the time of sample collection, 375 calves (39%) were diarrheic, and 181 calves (19%) were scored as 2 (heavy diarrhea). Within-herd prevalence of diarrhea ranged from 0% to 80% (mean, 39%; first quartile, 20%; third quartile, 50%). No calves with diarrhea were found on three (3%) farms, and three (3%) farms had a heavy diarrhea score (2) in more than half of the samples.

3.2. Bivariable analysis

Calves aged 16–21 days were least likely to be shedding *C. parvum* oocysts (prevalence = 30% with 15% having scores >3), while the group of calves aged 7–15 days had a prevalence of 51% (with 32% having scores >3).

The highest prevalence for diarrhea (65%) was detected in calves with Heine scores >4, and lower percentages for score 3–4 (47%), score 2 (45%), score 1 (28%) and score 0 (28%) ($\chi^2 = 103$, $p < 0.0001$).

From the pool of calves with combined high scores of diarrhea (heavy) and shedding (>2), 20% were in poor general condition (>0) compared to 7% for calves that had a high score for diarrhea but a low shedding score (0–2) ($\chi^2 = 5.3$, $p = 0.02$). The mortality rate at 90 days was higher in calves with high combined scores of shedding (>2) and diarrhea (heavy) compared with other classes (Table 2).

No significant difference was observed in shedding for breeds, sex, season of birth (maximum in August–October, 48% prevalence; minimum in November–January, 42% prevalence).

3.3. Multivariable analysis

Candidate factors were age, breed and covariates related to care of the calf, type of milk fed and equipment used to feed calves (Table 3).

Treatment with antibiotics, anti-inflammatory drugs and nutritional products were removed because they were administered as a consequence of diarrhea associated with *C. parvum* and not as a plausible cause of infection.

Table 4
Final multivariable GEE logistic model outcomes for the shedding of *C. parvum* by young dairy calves ($n = 968$) in Normandy, France.

Covariate	Levels	Adjusted OR [95% CI]
Age at sampling	≤10	Ref.
	>10 and ≤15	1.24 [0.91–1.70][NS]
	>15	0.45 [0.31–0.65][***]
Breed	Holstein	1.22 [0.74–1.98][NS]
	Normande	1.49 [0.93–2.37][NS]
	Others	Ref.
Halofuginone	Yes *vs* No	0.46 [0.19–1.15][NS]
Bucket	Yes *vs* No	1.37 [1.00–1.89][*]
Calf fed fermented milk	Yes *vs* No	0.32 [0.17–0.63][***]

OR, odds ratio; CI, confidence interval; GEE, generalized estimating equation
Estimated scale parameters (SE): 1.02 (0.093).
[*] $p < 0.05$.
[**] $p < 0.01$.
[***] $p < 0.001$.
[NS] $p > 0.05$.

The final multivariable GEE model included five potential explanatory variables (Table 4). Risk factors significantly associated with the shedding of *C. parvum* were a young age (≤15 days) at sampling and the distribution of colostrum using a bucket. A negatively associated factor was feeding the calf fermented milk (Table 4).

The breed (Normande *vs* others, $p = 0.097$) and treatment with halofuginone ($p = 0.096$) were associated with the shedding of *C. parvum* (Table 4).

4. Discussion

The aim of this study was to determine the prevalence and risk factors for *Cryptosporidium* infection in bovine neonates in an area of intensive dairy farming in western France.

Bivariable analysis showed that the prevalence of diarrhea among young calves was correlated with the degree of *C. parvum* shedding. The general condition of calves was poorer, and the probability of mortality higher, in animals with high scores for diarrhea and shedding compared to those with only a high score for diarrhea. Torsein et al. (2011) reported that the prevalence of calves infected by *Cryptosporidium* is different between herds with high mortality (calves between 1 and 90 days) and herds with low mortality (nonsignificant difference, $p = 0.062$). These results confirm that *C. parvum* is a primary enteropathogen.

Multivariable analysis of our data identified several risk factors. Among them, the use of buckets without teats to feed young calves increases the risk of oocyst shedding. A plausible explanation could be that the walls of the buckets are highly contaminated by oocysts, which could occur when farmers stack them for storage. These oocysts are probably not removed by standard washing procedures and may contaminate subsequent food placed in the buckets. An alternative explanation could be the failure to satisfy the calves' need for suckling in those that are quickly separated from their dams and fed using buckets without teats. This separation may lead calves to suckle another calf (cross-suckling), or other points in their housing area or environment, thereby ingesting oocysts that cause early and repeated infection. A study investigating the effect of teat feeding on cross-suckling in dairy calves reported that teat feeding reduces cross-suckling compared to calves fed from buckets (Jensen and Budde, 2006). Finally, teat feeding allows a more effective reflex closure of the esophageal groove than feeding from the bucket, which probably prevents fermentation and limits colonization by enteropathogens such as *C. parvum* (Ferran and Bouquet, 2013). This notion is supported by the findings of a German study of 249 calves (<14 days old) suffering from acute enteritis, where the esophageal groove did not close in 11.2% of cases (Dirr and Dirksen, 1989).

Other studies indicate that hygiene measures are important during calf feeding. The use of detergent or soap for cleaning calf feeding utensils was a protective factor against the shedding of *Cryptosporidium* in Ontario (Trotz-Williams et al., 2008). Similarly, feeding starter grain as well as milk (compared to milk replacers) are factors associated with an increased risk of shedding (Maldonado-Camargo et al., 1998; Mohammed et al., 1999) and could be interpreted as practices that increase potential contamination of calf feed.

We also identified that Normande calves shed more oocysts than other breeds. Breed type was previously proposed as a factor associated with infection of *C. parvum*. In a study of 391 experimentally infected dairy calves, the Jersey breed was identified as a risk factor for infection by *C. bovis* (Szonyi et al., 2012). In a cross-sectional study of 115 positive fecal samples from dairy cattle in New York State, Holstein calves were identified as more likely to be infected with *C. parvum* (Starkey et al., 2007).

In our study, treatment with HL had a slight protective effect on oocyst shedding. A review of the effects of HL on calf cryptosporidiosis using a meta-analysis (Silverlås et al., 2009a) found that for prophylactic treatment, HL has a slight protective effect on infection and diarrhea prevalence on days 4 and 7 post administration. In a recent randomized controlled field trial, the halofuginone group showed a longer prepatent period than the control, although there was no difference in the number of oocysts shed (Almawly et al., 2013). Other studies have reported a significant reduction in the shedding of oocysts and diarrhea by HL-treated newborn calves (Klein, 2008; de Waele et al., 2010; Trotz-Williams et al., 2011; Keidel and Daugschies, 2013).

Supplementation with fermented milk appeared to be a protective factor against the shedding of *C. parvum*. Fermented milk contains more probiotics (*e.g.*, *Lactobacillus* sp.) than unfermented milk, and, through their modifying activities, these beneficial bacteria are able to prevent pathogens from colonizing and multiplying. Supplementation of feed with *Lactobacillus acidophilus* and *Lactobacillus reuteri* in mice and rats reduces fecal shedding of *C. parvum* oocysts (Alak et al., 1999; Guitard et al., 2006). Another study reported that the oral administration of fermented milk supplemented with two probiotic strains significantly increased delayed-type hypersensitivity, plaque-forming cells and half-hemolysis values in healthy adult mice. Synbiotic fermented milk may help improve intestinal health and have a positive effect on the humoral and cell-mediated immunity of host animals (Wang et al., 2012). Similarly, lactic acid bacteria isolated from the calf gut inhibit those causing diarrhea in calves (Maldonado et al., 2012). However, no differences were reported in the incidence of diarrhea and *Cryptosporidium* oocyst shedding in calves receiving probiotic treatment with lactic acid-producing bacteria under field conditions (Harp et al., 1996).

In our study there was no significant association between the risk of shedding and the season of birth, which contrasts with the findings of Trotz-Williams et al. (2007) who reported that oocyte shedding and the incidence of calf diarrhea was higher during the summer months. Contrastingly, others have reported a higher incidence of calf diarrhea during the winter in North America (Waltner-Toews et al., 1986; Curtis et al., 1988; Frank and Kaneene, 1993). Absence of a seasonal effect in our study may be due to the mild oceanic climate of Normandy characterized by low thermal amplitude, allowing oocysts to survive and overwinter in a more conducive environment that allows them to challenge calves all year round.

Our findings indicated that the type of calf housing used after birth was not a significant risk factor. Sischo et al. (2000) observed that contact between calves in the nursery increased the risk of shedding. The lack of association in our study may be explained by the lack of robustness of the small sample size that was unable to detect differences in important cofactors.

Finally, calves fed with pooled or commercial colostrum did not have a lower risk of shedding. Passive immunity does not impart resistance to this parasite in calves (Trotz-Williams et al., 2007), and the lack of association with *C. parvum* shedding was anticipated.

5. Conclusion

The results of this study show that *C. parvum* is widespread among calves under 21 days old in dairy herds in the Normandy region of France. Shedding of *C. parvum* is associated with a high incidence of diarrhea and increased risk of mortality in young calves. Our study identified some risk calf-level factors, such as the use of buckets without teats to feed (causal) or the supplementation with fermented milk (protective). Further investigation is necessary to define appropriate measures that farmers and veterinary practitioners can take to control *C. parvum* infection.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence the content of this paper.

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