



# *Cryptosporidium parvum* infection and management-based risk factors of dairy calves in Taiwan

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**ABSTRACT.** Cryptosporidiosis is one of the major causes of diarrhea in calves. *Cryptosporidium parvum* is considered the most important calf diarrhea pathogen in the *Cryptosporidium* species. Not only could infected calves spread *C. parvum*, but infected adult cattle could also shed oocysts. The objectives of this study were (1) to investigate the prevalence of *C. parvum* in dairy herds in Taiwan, including calves, the dams in delivery enclosure, the floor, and the drinking water; (2) to clarify the relationship of diarrhea, management, and *C. parvum* infection. Twenty dairy herds in Taiwan were selected by random sampling, including 226 calves and 198 dams, and other environmental samples were collected. A questionnaire was filled out by the farm owners to collect information regarding the management of calves and the delivery enclosure. Hierarchical logistic regression was used to analyze the risk factors for *C. parvum* infection. The prevalence of *C. parvum* infection in calves was 26.5% (60/226), while in dams, it was 19.7% (39/198). The *C. parvum* infection in calves increased with environmental contamination of *C. parvum* and clinical signs of diarrhea, while it decreased with a yard provided in the delivery enclosure. In conclusion, the management of the delivery enclosure appears to be more important for preventing cryptosporidiosis in calves in Taiwan.

**KEY WORDS:** *Cryptosporidium parvum*, dairy calf, delivery enclosure, diarrhea, management

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Dairy farms in Taiwan are mostly of small field size, with an open housing system. According to the year-end statistics in 2017, dairy farms in Taiwan had reached a total number of 553, raising 130,413 cattle, or 236 cows per farm. The average size of a dairy farm was 6,607 m<sup>2</sup>. Most of farms have the single calf pen for every calf and others farms adopt group housing in calf-pen. Diarrhea in the calves of a dairy herd is a common disease and induces large economic losses [7]. Cryptosporidiosis is one of the major causes of calf diarrhea [5]. The genus *Cryptosporidium* includes several protozoan parasites that mainly infect the epithelial cells of the gastrointestinal, respiratory, and biliary tracts in animals [12]. The most common species in dairy herds are *Cryptosporidium parvum*, *Cryptosporidium andersoni*, *Cryptosporidium bovis*, and *Cryptosporidium ryanae* [10, 11], with *C. parvum* being considered the most important calf diarrhea pathogen in the *Cryptosporidium* species [23].

The main transmission route of *C. parvum* is fecal–oral infection, with only 5.8 oocysts needed for a median infectious dose for fecal oocyst shedding, or 9.7 for diarrhea [26]. Calves infected by *C. parvum* shed a large number of oocysts in their feces within three days, and infected calves can excrete oocysts for 4–16 days [12]. The characteristics of *C. parvum*, such as only needing a few oocysts to cause infection, shedding a large number in feces, and short lifecycle, lead to large-scale infection in dairy herds.

*C. parvum* can be transmitted by shedding oocysts from infected individuals in the enclosure [22]. This indicates that neonatal calves have a high risk of infection from infected dams with oocysts shedding in the delivery enclosure. Investigation of the relationship between *C. parvum* infection and the environment, such as the drinking tank and the floor of the delivery enclosure, is rare in Taiwan. The most recent research of the prevalence of *Cryptosporidium* in livestock in Taiwan was in 2005, which

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comprised a fecal survey via the modified Ziehl–Neelsen method and an immunofluorescence assay in cattle and goats, showing a prevalence of 37.6% (173/460) and 35.8% (44/123), respectively [25]. The prevalence in the abovementioned research included all species of *Cryptosporidium*, and thus the real status of pathogenic *Cryptosporidium*, which is *C. parvum* in Taiwan, could have been overestimated. Therefore, the prevalence of *C. parvum* in dairy cattle in Taiwan is still unknown. The objectives of this study were (1) to investigate the prevalence of *C. parvum* in dairy herds in Taiwan, including calves and the dams in the delivery enclosure; (2) to clarify the relationship of diarrhea, management, and *C. parvum* infection.

## MATERIALS AND METHODS

### Animal population and sampling

From April 2017 to February 2018, the feces of 226 calves from 20 dairy herds in North, Middle, South, and East Taiwan. Farms were selected by random sampling, the average cow number was  $366 \pm 201$  with 6 to 40 under 1.5 months old calves from each farm (Supplementary Table). All of the calves under 1.5 months old in the selected farms were sampled. The calves' feces samples were defined as being either normal or diarrhea according to Uga's research [10]. Next, 179 and 206 swab samples were taken from the calves' drinking water and rearing enclosure, respectively. From dams in the delivery enclosure, 198 feces samples were taken, while 32 and 30 samples were taken from the drinking water and floor in the delivery enclosure, respectively. All owners agreed samples collection in this study. All sampling procedures were executed by veterinarians under the Animal Protection Act from the Council of Agriculture in Taiwan. No extra animals were intentionally used or purchased in this study.

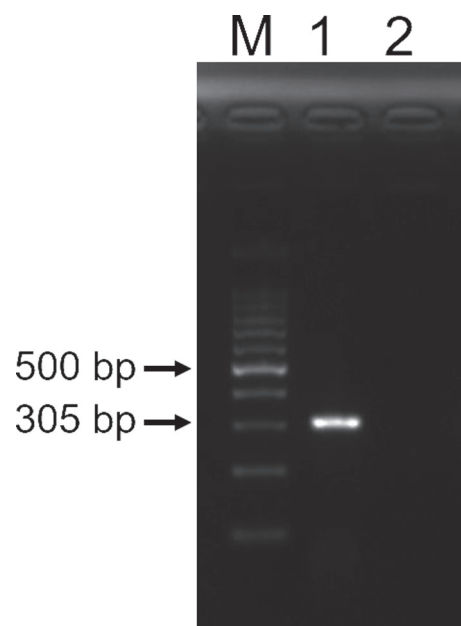
Feces were collected from calves via the hand (with latex gloves) to stimulate the rectum to induce defecation. The drinking water of the calves was collected from the tank used to provide water to the calves. The swab samples of the rearing enclosure were collected using plastic spoons. If the calves were fed in independent cages, samples were collected from the upper, middle, and lower sites of the front and rear fences. If the calves were fed in groups, the sampling sites were taken in duplicate from the front, middle, and rear of all the feeding area. Samples from different sites of the same cages or feeding areas were mixed as the rearing enclosure swab sample for all of those calves that stay in that cage or feeding area. Feces from dams were collected via rectal palpation using a long plastic glove. The collected samples were stored at 0°C in an ice box and transferred to a -20°C refrigerator within 24 hr.

### Oocyst concentration

All solid samples were filtered by gauze to remove most of the fiber. Then, the oocysts were concentrated by salt flotation after filtration. Depending on the samples having been collected from calves or dams, a 10 or 20 g sample was mixed with 30 or 50 ml of reverse osmosis water, and then filtered by gauze to remove most of the fiber. Thirty milliliters of the mixed sample were moved to a centrifuge tube and centrifuged at 3,500 g for 10 min. The supernate was removed and then reverse osmosis water was added to reach 30 ml. Centrifugation was repeated three times; for the third centrifugation, saturated salt solution was added to reach 30 ml, then mixed and rested for 15 min. Following this, 3 ml of the supernate was collected and then reverse osmosis water was added to reach 30 ml in a new centrifuge tube. The tube was centrifuged at 3,500 g for 10 min, and then the supernate was removed; this process was repeated three times to remove the salt. After the third centrifugation, 5 ml of ultrapure water was added to the tube with precipitate. The tube was shaken to mix it completely, ready for DNA extraction. The FastDNA® SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA) was used to extract DNA from the samples. For the water samples without visible fiber, salt flotation after filtration was used to concentrate the oocytes without filtration, and the same DNA extraction step as for the solid samples was used.

### Nested PCR

The extracted genomic DNA sample was used as a template to amplify the 18S rRNA of *C. parvum* by nested PCR [21]. The first-step primers for nested PCR were the forward primer EF:5'-TTCTAGAGCTAATACATGCG-3' and the reverse primer ER:5'-CCCATTTCTTCG AAACAGGA-3'. The second-step primers were the forward primer CphF:5'-AGAGTGCTTAAAGCAGGCATA-3' and the reverse primer IR:5'- AAGGAGTAAGGAACAACCTCCA-3'. The first step of nested PCR was performed using 3 µl of extracted DNA, 10 µl of 2X Taq PCR MasterMix (Genomics Bioscience and Technology Co., Ltd., New Taipei City, Taiwan), 0.5 µl (10 µM) of each forward (EF) and reverse (ER) primer, and 6 µl of pure water in a 0.2 ml Eppendorf. The PCR conditions were as follows: Denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 49°C for 45 sec, 72°C for 1 min, and extension at 72°C for 7 min. The second step of nested PCR was performed using 3 µl of the PCR product from the first reaction rather than the extracted DNA and the same cycling conditions as the first step. The final PCR products were analyzed by gel electrophoresis on a 2% agarose gel and visualized under blue light. The final target was a 305 bp fragment of 18S rRNA of *C. parvum*, as shown in Fig. 1.



**Fig. 1.** The specific fragment of the 18S rRNA of *Cryptosporidium parvum*. M: 100 bp DNA ladder marker; Lane 1: Positive; Lane 2: Negative.

The percentage of diarrhea calves	<input type="checkbox"/>	<10%	<input type="checkbox"/>	10~30%	<input type="checkbox"/>	30~50%	<input type="checkbox"/>	>50%
The distance between calves' enclosure and lactating cows' enclosure	<input type="checkbox"/>	Yes	<input type="checkbox"/>	No				
Frequency of floor cleaning in calves' enclosure	<input type="checkbox"/>	once per day	<input type="checkbox"/>	once every two–three days	<input type="checkbox"/>	once every four–five days	<input type="checkbox"/>	once in more than five days
Frequency of delivery enclosure cleaning	<input type="checkbox"/>	once per day	<input type="checkbox"/>	once every two–three days	<input type="checkbox"/>	once every four–five days	<input type="checkbox"/>	once in more than five days
Frequency of water tank cleaning in calves' enclosure	<input type="checkbox"/>	once per day	<input type="checkbox"/>	once every two–three days	<input type="checkbox"/>	once every four–five days	<input type="checkbox"/>	once in more than five days
Frequency of water tank in the delivery enclosure cleaning	<input type="checkbox"/>	once per day	<input type="checkbox"/>	once every two–three days	<input type="checkbox"/>	once every four–five days	<input type="checkbox"/>	once in more than five days
Disinfection after cleaning	<input type="checkbox"/>	Yes in calves' enclosure	<input type="checkbox"/>	Yes in delivery enclosure	<input type="checkbox"/>	non of both		
Kinds of disinfectant	<input type="checkbox"/>	Benzalkonium chloride	<input type="checkbox"/>	VIRKON S®	<input type="checkbox"/>	Sodium hypochlorite	<input type="checkbox"/>	Others( )
The dry period enclosure and delivery enclosure were separated	<input type="checkbox"/>	Yes	<input type="checkbox"/>	No				

**Fig. 2.** Questionnaire regarding the management of the calves and the delivery enclosure.

### Questionnaire and records

A questionnaire was filled out by the farm owners to collect information regarding the management of calves and the delivery enclosure (Fig. 2). The diarrhea episodes of the calves were recorded at the time of sample collection. Whether a yard for prepartum cows were provided or not and the bedding materials in their delivery enclosure were also recorded.

### Statistical analysis

We first estimated the prevalence of *C. parvum* infection and its 95% confidence interval (CI) in both calves and cows. Furthermore, we adopted hierarchical logistic regression to analyze the relationship between *C. parvum* infection in calves and other environmental explanatory variables (Table 1). We transformed all of the categorical explanatory variables into dummy variables, and centered and standardized the numerical variables. In addition, the farms were designed as a random effect for the model to evaluate the variance of intercept between the different farms. The hierarchical logistic regression was fit by maximum likelihood with Laplace approximation [16]. The computing environment R with the package lme4 [3, 19] was used for model construction.

For model construction, we first fitted the model with each univariable analysis and retained the variables with *P*-values of less than 0.1 for further multivariable model construction.

Multicollinearity between explanatory variables was evaluated using the variance inflation factor (VIF) [13]. The VIF threshold was set as 10 to avoid problematic effects of multicollinearity on the parameter estimations [1]. Variables were discarded from model construction if their VIF value was larger than 10.

The explanatory variables selected in the models were based on the Wald test with a *P*-value threshold set at 0.05 [4, 8]. The backward stepwise method was adopted for variable selection. Values of the Akaike information criterion (AIC) and the AIC weights were used to assess the models' fit and to select the best-fitted model [4, 8]. Based on the fitted model, the receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) was calculated to evaluate the discriminate ability of the model.

## RESULTS

### The prevalence of *C. parvum* in Taiwan and the proportion of positives of *C. parvum* of different environmental factors

The prevalence of *C. parvum* infection in calves was 26.5% (60/226; 95% CI: 20.7–32.3%) and 19.7% (39/198; 95% CI: 14.2–25.2%) in dams. The percentage of *C. parvum*-positive herds was 90.0% (18/20; 95% CI: 76.9–100%). The prevalence of *C. parvum* in North, Middle, South, and East Taiwan is shown in Table 2.

The proportion of positives in the rearing enclosure swab samples was 18% (38/206; 95% CI: 12.8–23.2%), while it was 2.2% (4/178; 95% CI: 0–4.1%) in the drinking water of the calves. The samples from the floor and drinking water in the delivery enclosure had a positive proportion of 16.7% (5/30; 95% CI: 3.6–30%) and 6.3% (2/32; 95% CI: 0–14.7%), respectively. The *C. parvum*-positive proportion of calves based on different environmental explanatory variables is shown in Table 3.

**Table 1.** The definitions of calves and other environmental explanatory variables

Variable	Data type	Description
<i>C. parvum</i>	Binary	The result of PCR detection for <i>Cryptosporidium parvum</i> . This variable was treated as a response variable in the hierarchical logistic regression model for understanding the association between explanatory variables and <i>C. parvum</i> infection.
Farm	Categorical	Farms where samples and questionnaire data were collected. In total, 20 farms were included in this study. This variable was used as the random effect in the model construction.
Farm size	Continuous	The number of calves in each farm was used to represent the farm size.
Diarrheal	Binary	If calves presented with diarrheal symptoms at the time that the sample was collected.
Tank	Categorical	Results of the PCR screening for <i>Cryptosporidium parvum</i> with samples of tank water. The variable was categorized into negative, positive, and no water tank in the calf rearing enclosure.
Rearing enclosure	Binary	<i>C. parvum</i> screening results for the swab samples collected from the calf rearing enclosure.
Floor-Clean	Order	Frequency of floor cleaning in the calves' enclosure. 1, once per day; 2, once every two–three days; 3, once every four–five days; 4, once in more than five days.
Tank-Clean	Order	Frequency of water tank cleaning in the calves' enclosure. 1, once per day; 2, once every two–three days; 3, once every four–five days; 4, once in more than five days.
BirthF-Clean	Order	Frequency of delivery enclosure cleaning. 1, once per day; 2, once every two–three days; 3, once every four–five days; 4, once in more than five days.
BirthT-Clean	Order	Frequency of cleaning of the water tank in the delivery enclosure. 1, once per day; 2, once every two–three days; 3, once every four–five days; 4, once in more than five days.
Calf-dis	Binary	Disinfection of the calves' enclosure. 1, yes; 0, no.
Birth-dis	Binary	Disinfection of the delivery enclosure. 1, yes; 0, no.
Close-lac	Binary	The distance between the calves' enclosure and the lactating cows' enclosure. 1, nearby; 0, not close to one another.
Yard	Binary	Yard provided in the delivery enclosure. 1, yes; 0, no.
Bedding	Binary	Bedding materials provided in the delivery enclosure. 1, yes; 0, no.
Dry-lac	Binary	The dry period enclosure and the delivery enclosure are separate. 1, yes; 0, no.
Cow-p	Continuous	The prevalence of <i>C. parvum</i> in the cows.

**Table 2.** Prevalence of *Cryptosporidium parvum* in North, Middle, South, and East Taiwan

Regions	Prevalence of <i>C. parvum</i> in calves (%; 95%CI)	Prevalence of <i>C. parvum</i> in dams (%; 95%CI)	Areas included in this region
North	1/10 (10.0, 8.6–28.6)	3/10 (30, 1.6–58.4)	Yilan, New Taipei, Keelung, Taipei, Taoyuan, and Hsinchu
Middle	17/64 (26.6, 15.8–37.4)	9/57 (15.8, 6.3–25.3)	Miaoli, Taichung, Nantou, Changhua, and Yunlin
South	36/107 (33.6, 24.7–42.5)	23/90 (25.6, 16.6–34.6)	Chiayi, Tainan, Kaohsiung, Penghu, and Pingtung
East	6/45 (13.3, 3.4–23.2)	4/41 (9.8, 0.7–18.9)	Hualien and Taitung

95% CI: 95% confidence interval.

### Hierarchical logistic regression

The explanatory variables chosen for model construction with a *P*-value below 0.1 in the univariable analysis were Diarrheal=Yes, Rearing enclosure=Positive, BirthF-Clean=2 (once every two–three days), and Yard=Yes (Table 4).

The final fitted model included the explanatory variables of Diarrheal=Yes, Rearing enclosure=Positive, and yard provided had the lowest AIC value and AIC weight of 0.59 (Table 5). Furthermore, the AIC value indicated that the null model of hierarchical logistic regression fit better than that of logistic regression (Table 5).

Based on the final fitted model, the probability of *C. parvum* infection in calves increased with proportion of positives in the rearing enclosure of *C. parvum* and clinical signs of diarrhea, while it decreased with a yard provided in the delivery enclosure (Table 4). The estimated odds ratios of each univariable analysis and the final fitted variables in the hierarchical logistic regression model are shown in Table 4. We created a ROC curve (Fig. 3) and estimated the AUC based on the final fitted model. The computed value of AUC was 77.62% (95%CI: 70.45–84.88%), which indicated that the discriminate ability of fitted model was considered acceptable to excellent [17, 18].

## DISCUSSION

This research comprised a survey of *C. parvum* infection in calves younger than 1.5 months and prepartum cows in dairy herds in Taiwan, which were analyzed using nested PCR. This is the first research of the prevalence of *C. parvum* in dairy cattle in Taiwan. The results show that the prevalence of *C. parvum* infection was 26.5% and 19.7% in calves and dams, respectively, and positivity rate in herds was 90.0%. The prevalence of *C. parvum* in pre-weaned calves was reported from 3.4 to 96.6% in different

**Table 3.** The *Cryptosporidium parvum*-positive rate of calves based on different environmental explanatory variables

Variables	Options n (%)			
Flood-Clean	Once per day 48/188 (25.5)	Once every 2–3 days 4/11 (36.4)	Once every 4–5 days 7/17 (41.2)	Once in >5 days 0
Tank-Clean	Once per day 49/195 (25.1)	Once every 2–3 days 5/21 (23.8)	Once every 4–5 days 6/10 (60)	Once in >5 days 0
BirthF-Clean	Once per day 26/98 (26.5)	Once every 2–3 days 3/32 (9.3)	Once every 4–5 days 13/50 (26)	Once in >5 days 18/46 (39.1)
BirthT-Clean	Once per day 39/162 (24.1)	Once every 2–3 days 12/38 (31.6)	Once every 4–5 days 1/7 (14.3)	Once in >5 days 8/19 (42.1)
Calf-dis	Yes 29/94 (29.8)	No 32/132 (24.2)		
Birth-dis	Yes 5/28 (17.9)	No 55/198 (27.8)		
Close-lac	Yes 42/177 (23.7)	No 18/49 (36.7)		
Yard	Yes 3/33 (9)	No 57/193 (29.5)		
Bedding	Yes 19/60 (31.7)	No 41/166 (24.7)		
Dry-lac	Yes 45/162 (27.8)	No 15/64 (23.4)		

**Table 4.** Explanatory variables statistics and estimated odds ratios of univariable analysis and final fitted model

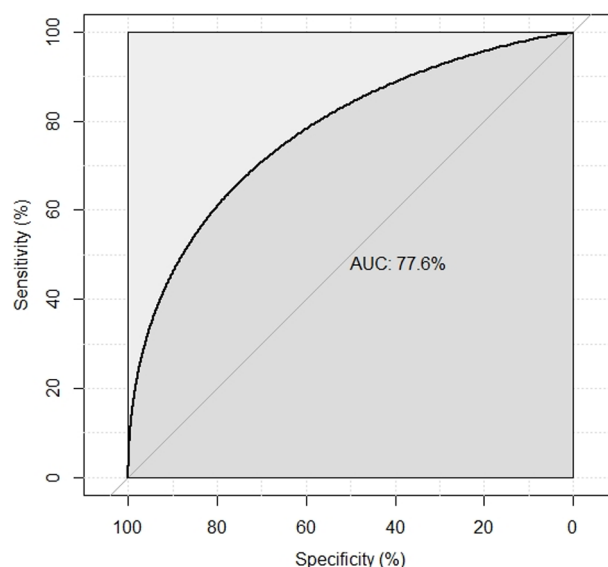
Variables	Univariable analysis					Final fitted model				
	C	SE	Odds ratio	95%CI	P-value	C	SE	Odds ratio	95%CI	P-value
Intercept						-1.420	0.308	0.347	0.344–0.349	<0.001
Diarrheal=Yes	0.878	0.375	2.406	1.153–5.019	0.019	0.795	0.382	2.215	1.047–4.683	0.038
Rearing enclosure=Positive	0.993	0.412	2.700	1.204–6.056	0.016	0.901	0.422	2.462	1.076–5.634	0.033
Yard=Yes	-1.463	0.815	0.231	0.047–1.144	0.073	-1.586	0.800	0.204	0.043–0.983	0.048
BirthF-Clean=2	-1.516	0.841	0.220	0.04–1.140	0.071					

C: coefficient; SE: standard error; 95%CI: 95% confidence interval.

**Table 5.** Comparison of the Akaike information criterions (AIC) and the Akaike weights between the different models of *Cryptosporidium parvum* infection in calves

Model	Variables included	AIC	DAIC <sup>2</sup>	Akaike weight
Hierarchical <sup>1</sup>	Final fitted model	245.4	0	0.586
	Diarrheal=Yes			
	Rearing enclosure=Positive			
	Yard=Provided			
Hierarchical	Reduced model	247.5	2.1	0.205
	Diarrheal=Yes			
	Rearing enclosure=Positive			
Hierarchical	Full model	245.7	0.3	0.504
	Diarrheal=Yes			
	Rearing enclosure=Positive			
	BirthF-Clean=2			
	Yard=Yes			
Hierarchical	Null model	253.1	7.7	0.012
Logistic regression	Null model	263.6	18.2	<0.001

<sup>1</sup> Hierarchical logistic regression. <sup>2</sup> DAIC, difference in AIC value from final fitted model.



**Fig. 3.** Operating characteristic curve (ROC) and estimated the area under the ROC curve (AUC) value of the final fitting model.



areas [23]. The differences mainly based on their different experiment designs and the detection methods of these studies, which resulted in diverse prevalence of *C. parvum*. Longitudinal study and sampling diarrheic calves only presented higher prevalence in the past studies. This study present point-prevalence and collect both diarrheic and non-diarrheic calves under 1.5 months old. The prevalence of this study was 26.5%, which showed the infection of *C. parvum* is severe in calves in Taiwan. In addition, hierarchical logistic regression showed that diarrhea in calves was positively related to *C. parvum*, indicating that the diarrhea in calves caused by *C. parvum* infection in Taiwan dairy herds is an important issue. *C. parvum* has been confirmed as not only a harmful pathogen in calves, but also being able to possibly infect humans—especially children [6]. Therefore, understanding the risk factors of *C. parvum* infection is very important for human and herd health. The rearing enclosure, which was investigated for *C. parvum*, was positively related to *C. parvum* infection in calves, showing that infected calves shed oocytes into environment. Even cleaning the cage every day or using disinfectant could not decrease the *C. parvum* infection rate in calves. This suggests that prevention of infection in dams and decontamination of delivery enclosures are more important for preventing cryptosporidiosis in calves in Taiwan.

This study also indicated that 19.7% of dams had been infected, with the floor and drinking water in the delivery enclosure having a *C. parvum* positive proportion of 16.7% and 6.3%, respectively. Even concrete flooring, which is commonly used in delivery enclosures in Taiwan and is easy to clean, the delivery enclosures were contaminated with *C. parvum*. A previous study indicated that the floor and drinking water in the delivery enclosure having a *Cryptosporidium* oocyte proportion of 80% and 64% in a high contaminated farm [9]. These results support the insight that *C. parvum* can infected neonatal calve soon after delivery [26], suggesting that famers should consider more effective cleaning methods in delivery enclosures to decrease *C. parvum* infection in neonatal calves in Taiwan. However, the frequency of cleaning and the use of disinfectant were not related to *C. parvum* infection, perhaps because most disinfectants have limited efficacy against *C. parvum*, including those recorded in this study [2]. Cleaning the environment and feeding tools by hot water or heating was more efficient for killing oocyte of *C. parvum* [14], but this kind of cleaning procedure was rarely performed in Taiwan dairy herds. That might explained no relationship between the frequency of cleaning (in delivery and calves' enclosure) and *C. parvum* infection in this study. Although there were several studies indicated that large size farm has higher risk of *C. parvum* infection, the farm size did not relate to *C. parvum* infection in this study [20, 24]. It might because of few large farms (>500 adult cows) in Taiwan and only one farm had more than 500 adult cows in this study. So the farm size was not a significant factor of *C. parvum* infection in Taiwan.

On the contrary, provided yard for prepartum cows showed a lower infection rate for *C. parvum* in calves and tended to be negatively related to *C. parvum* infection. Even there is no study describing the relationship between providing yard for prepartum cows and *C. parvum* infection directly, minimizing the exposure of calves to *C. parvum* was indicated as an effective strategy for preventing infection of *C. parvum* [14]. A yard for prepartum cows could provide more space in calving area and decrease the opportunity to contact oocyte of *C. parvum* as the calves born. There was another report indicated the industrial farming system, compared to grazing farming system has higher risk for *C. parvum* infection [15]. That result also suggested large space in calving area may decrease the risk for *C. parvum* infection.

In conclusion, the relationship between diarrhea and *C. parvum* infection in calves is very high in Taiwanese dairy herds, and the management of the delivery enclosure such as providing a yard appears to be more important for preventing cryptosporidiosis in calves in Taiwan.

CONFLICTS OF INTEREST. The authors declare that there is no conflict of interest.

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