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RESEARCH ARTICLE

Prevalence and Diagnostic Methods for Cryptosporidium in Different Dairy Farm Environments

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ABSTRACT

The prevalence of Cryptosporidium infection varied among different dairy farms, with Iqbal Dairy Farm recording the lowest prevalence (12.5%), while Nawan Kalay Farm (36.66%) and Toru Dairy Farm (33.33%) had the highest. Sex-based prevalence analysis indicated a slightly higher infection rate in female calves (25.12%) compared to males (22.75%). Among breeds, Holstein Friesians had the highest prevalence (30.53%), whereas Jersey (22.58%) and crossbreeds (21.98%) exhibited lower infection rates. A total of 384 samples were examined using modified Ziehl–Neelsen (MZN) staining, of which 92 were identified as positive for Cryptosporidium. PCR analysis was performed for confirmation, revealing that 60 of the 92 samples (53.57%) were true positives, while 52 (46.42%) tested negative. The molecular detection method (PCR) demonstrated a statistically significant difference in sensitivity compared to microscopy, confirming Cryptosporidium infection in 60 out of 112 samples (53%). These findings highlight the variability in Cryptosporidium prevalence across farms, breeds, and sex, as well as the importance of molecular techniques for accurate diagnosis.

1. Introduction

Cryptosporidium is a protozoan parasite that poses a significant challenge to dairy farm environments due to its ability to cause cryptosporidiosis, a zoonotic disease affecting both humans and livestock [1]. The parasite is primarily transmitted through the fecal-oral route, with contamination occurring via infected animal feces, water sources, and farm equipment [2]. Cryptosporidium infections in dairy cattle can lead to severe economic losses due to reduced milk production, increased veterinary costs, and high morbidity rates, especially in neonatal calves [3]. The prevalence of Cryptosporidium varies across dairy farms, influenced by environmental conditions, management practices, and geographical location [4]. Identifying and quantifying Cryptosporidium infections in dairy farm environments require accurate diagnostic methods, as effective detection is crucial for implementing control and mitigation strategies.

Several studies have reported a high prevalence of Cryptosporidium in dairy farms worldwide, particularly among young calves, which are more susceptible to infection due to their underdeveloped immune systems [1]. Cryptosporidium parvum is the most commonly detected species in dairy cattle, though other species such as C. bovis and C. ryanae have also been identified [3]. The prevalence rates vary depending on the region and farm management practices. For instance, a study in the United States found Cryptosporidium infection rates ranging from 20% to 50% in pre-weaned calves [4]. Similar findings have been reported in European and Asian dairy farms, where poor hygiene and high stocking densities contribute to increased transmission rates [2,3].

Environmental factors play a critical role in the persistence and spread of Cryptosporidium in dairy farms. The parasite's oocysts are highly resistant to environmental stressors, allowing them to survive for extended periods in soil, water, and manure [3]. Contaminated water sources, particularly those shared between animals and humans, are major reservoirs for Cryptosporidium transmission [4]. Additionally, intensive farming systems with inadequate waste management practices have been linked to higher prevalence rates of infection [1].
Detecting Cryptosporidium in dairy farm environments requires robust diagnostic techniques to ensure accurate identification and effective control measures. The available diagnostic methods include microscopic, molecular, and immunological approaches, each with varying levels of sensitivity and specificity [2]. Microscopic Techniques is a traditional microscopy, particularly acid-fast staining and direct fluorescent antibody (DFA) tests, has been widely used for Cryptosporidium detection in fecal samples [3]. These methods are cost-effective and provide rapid results; however, they may lack the sensitivity to detect low-level infections [4]. Molecular Techniques is a polymerase chain reaction (PCR)-based methods have revolutionized Cryptosporidium detection by offering high specificity and sensitivity. PCR can differentiate between Cryptosporidium species and genotypes, making it a valuable tool for epidemiological studies (Ryan et al., 2021). Real-time PCR (qPCR) has further enhanced detection capabilities by allowing for quantification of oocyst loads in environmental and clinical samples [1, 2, 3].

2. Materials and methods

2.1. Study Area and Sample Collection

This study was conducted across multiple dairy farms in different geographical regions to assess the prevalence of Cryptosporidium (Figure 1). A total of eight (8) dairy farms were selected based on herd size, management practices, and environmental conditions. A total of 384 fecal samples fecal samples were collected from calves all farm, ensuring representation from different age groups (Table 1). In addition, water and environmental samples (bedding, feed, and manure) were collected to evaluate potential sources of contamination.

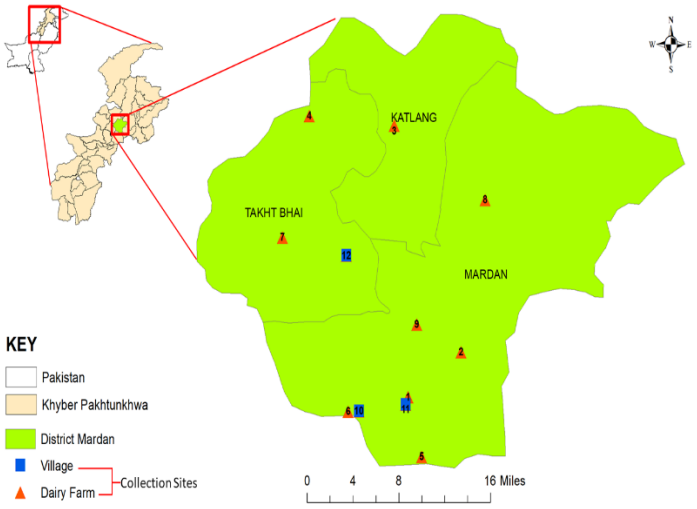


Figure 1: Study area map; different colors indicate different regions where collect the various samples.

Table 1: shown calves age ≤5 M means less than ≥ 5 M more than five months.

S.No	Area of sampling	Age group ≤5 M	≥ 5 M	Total sampling
1	Nawkaly Dairy farm	17	13	30
2	Harichand dairy farm	40	21	61
3	Iqbal dairy farm	21	11	32
4	Laly dairy farm	15	10	25
5	Toro dairy farm	14	13	27
6	Dairy farm	13	15	28
7	Dairy farm	10	11	21
8	Dairy farm	10	10	20
9	House hold	78	62	140
Total collected sample		218 166		384

2.2 Ethical Consideration

The owners of domestic and dairy farm calves, as well as the head of the veterinary department, gave their consent. The study's goal, the characteristics, symptoms, and socioeconomic status of each individual were verbally explained to their owners. The samples came from dairy calves raised by government and private dairy farmers in and around Mardan, respectively.

2.3 Processing and Preserving Samples

All of the samples were gathered in sterile, clean white bottles, moved to Abdul Wali Khan University's parasitology lab in Mardan, and kept in a refrigerator pending additional examination.

2.4 Microscopic Analysis

Acid-fast staining and direct fluorescent antibody (DFA) tests were employed to identify Cryptosporidium oocysts in fecal samples. Smears were prepared from fecal suspensions, stained using the modified Ziehl-Neelsen technique, and examined under a light microscope at 1000x magnification. DFA assays were performed using commercially available kits to enhance detection specificity.

2.5 Molecular Detection

Stool samples were treated with the PureLinkTM Microbiome DNA Purification Kit (Catalog Number A29790) to extract high-quality microbial and host DNA. DNA extraction was performed on fecal and environmental samples using a standardized protocol. PCR amplification targeting the 18S rRNA gene of Cryptosporidium was conducted to confirm species identification. Real-time PCR (qPCR) assays were used for quantification and differentiation of Cryptosporidium species, with positive and negative controls included in each run.

2.6 Prevalence

The following formula was used to determine the prevalence rate of cryptosporidiosis in the research area. Prevalence (%) = Total number of positive samples divided by the total number of examinees × 100

2.7 Data Analysis

Prevalence rates were calculated based on the proportion of positive samples in each category (age group, farm type, and environmental source). Statistical analyses were conducted using (XL formulas and IBM, SPSS Statistics 20) to compare detection rates between different diagnostic methods. Sensitivity and specificity analyses were performed to evaluate the reliability of each technique.

3. Results

The prevalence ratio of cryptosporidiosis differs among dairy types. Iqbal Dairy Farm 4/32 (12.5%) had the lowest prevalence percentage of cryptosporidiosis infection, whereas Nawan Kalay Farm 11/30 (36.66%) and Toru Dairy Farm 8/24 (33.33%) had the highest prevalence ratio (Table 2). The total denotes the sum of the samples, the mean value denotes the average, the standard deviation denotes the standard deviation, and the percentage indicated a distinct prevalence ratio.

Table 2: Cryptosporidium Prevalence in Various Dairy Farms

Farm Name	No. of samples	Positive Cases	Prevalence %	p-value
Sultan Abad Dairy Farm	61	14	22.95	
Nawan Kalay Farm	30	11	36.66	
Iqbal Dairy Farm	32	4	12.5	
Laly Dairy Farm	20	4	20	
Toru Dairy Farm	24	8	33.33	
New Surkh Dairy Farm	28	9	32.14	
Raees Dairy Farm	11	2	18.18	
Khan Dairy Farm	19	5	26.31	
Total	225	57	202.09	
Mean	28.12	7.12	25.26	0.1181
SD	14.92	4.08	8.36	

Additionally, the prevalence of Cryptosporidium was evaluated based on sex. According to the study's findings, the prevalence of women was 25.12%, while that of men was 22.75%. Table 3, show the prevalence of cryptosporidiosis in cow calves by sex.

According to cow calves' breeds, Holstein Friesians had the greatest risk of cryptosporidiosis (30.53%), while Jersey and crossbreeds had the lowest rates (22.58% and 21.98%, respectively) (Table 4).

Table 3: Cryptosporidium Prevalence by Sex

Gender	No. of Sample	Positive Cases	Prevalence (%)	p-value
Male	189	43	22.75	
Female	195	49	25.12	0.585
Total	384	92	47.87952788	
Mean	192	46	23.93976394	
SD	4.242640687	4.2426	1.680709647	

Table 4: Table showing percentage of *Cryptosporidium* based on breeds of cow calves

S NO	Bread of calves	Total examine sample	Positive	Prevalence	P-value
1	Jersey	106	21	19.8	
2	Cross Breed	140	31	22.14	
3	Holstein Friesians	138	40	28.98	0.005
	Totals	384	92	75.09	
Mean		182.5	46	37.545	

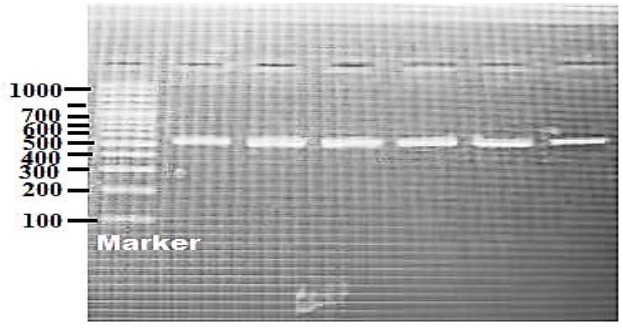
SD	18.82374387	9.504385	4.77257792

Twenty samples of cow calves that were expected to have a Cryptosporidium infection were clinically observed during the current study, while 384 samples were observed microscopically using the MZN staining technique were 92 samples showed a positive prevalence of Cryptosporidium using the straightforward Polymerase Chain Reaction (PCR). We employed sensitive PCR instruments for 92 positives as confirmation. Out of 92 samples, 60 (53.57%) were verified to be positive and 52 (46.42%) to be negative by PCR. The 92 samples that were positive under a microscope were all PCR-confirmed. Consequently, 60 of the 92 samples tested positive, and there was a statistically significant difference between the two diagnostic methods (PCR vs. microscopy). Through the use of molecular technology (PCR), it was determined that 60/112 (53%) of the 112 samples contained Cryptosporidium in cow calves (Table 5 and Fig 2).

Table 5 Compare of two diagnostic technique MZN and PCR

Methods	**Total sample**	**Positive**	**Prevalence**	**P-value**	**Sensitivity**	**specificity**
MZN	384	92	23.99%	0.0001		**value**
PCR	92	60	53.57%			

Figure 2. View of *C. parvam* ribosomal RNA gene after amplification (556bp)



4. Discussion

The month-by-month prevalence of Cryptosporidium in cows and calves was also examined in this study; the results indicated that the highest percentage prevalence was observed in July and August of 2022 (43%) and the lowest was recorded in December and January of 2021 (4.8%, 14.28%). This finding indicated that Cryptosporidium will have an impact on the environment and that hot temperatures will be conducive to the parasite's growth. Similar to our research, a high prevalence of the Cryptosporidium impact was seen in sheep during hot and wet weather [5]. In line with our research, Nagamani et al. [6], discovered that cooler months in Kuwait had the highest frequency. The month's base result was confirmed by the season base experiment conducted on calves in the area of Mardan.

According to the season-based study's findings, the summer months were more suited for the spread of the Cryptosporidium infection than the spring or fall months; a noticeably greater percentage of prevalence was noted in the summer, while the lowest was in the spring. In a related study, the goats showed a higher prevalence over the summer [7]. According to Urie et al. [8], the summer months in the US have a greater frequency of Cryptosporidium than the spring months. Causapé et al. [9], showed that the summer months had the highest prevalence of Cryptosporidium, followed by the fall and spring seasons. Their findings were similar to ours. The prevalence of Cryptosporidium infection was also reported by other researchers [10]. The transmission of Cryptosporidium was also influenced by environmental factors, meaning that summer was the ideal time of year for animals to harbor this parasite type as opposed to fall. Several researchers reported varying levels of Cryptosporidium prevalence. For example, Fayer, et al. [3], found that the highest prevalence was in the winter, followed by autumn and spring, which differed from our findings. Causapé et al. [9], found that the highest prevalence was in the spring.

Compared to males, females had a greater impact on the prevalence of Cryptosporidium infection. According to the results of the current investigation, females had a higher % prevalence than males. When compared to male dairy cattle, the prevalence of females is 27% higher. A similar finding revealed that the prevalence of Cryptosporidium was higher in female goats than in male goats [7]. This will occur because ladies have a variety of hormones. Contrary to our study, the male sex had a considerably higher prevalence of Cryptosporidium than the female sex [5].

The prevalence of Cryptosporidium was higher in the female sex than in the male sex, male calves were shown to have a higher prevalence of Cryptosporidium than female calves [7]. In France, which differed from our study, similar outcomes were also noted in male calves, who had a greater percentage prevalence of Cryptosporidium than female calves [11]. In line with this study, Cryptosporidium was shown to be much more common in females (about 48%) than in males (29%), with a significantly larger $P<.05$.

According to a similar study, the prevalence of Cryptosporidium was significantly greater in females (25%) compared to males (22%). Our results showed that compared

to male cattle, buffalo, sheep, and pigs, the prevalence of Cryptosporidium was significantly greater in female buffalo, cow calves, sheep, and goats [12].

The findings of were comparable to those of our investigation. Male animals were shown to have a higher prevalence of Cryptosporidium than females, however this difference is not statistically significant [7]. In order to determine the prevalence of Cryptosporidium in the feces of cow calves, the current study used PCR and microscopic analysis. Fecal samples from 384 cow calves were analyzed under a microscope using the Modified ZheilNeelson (MZN) staining method. 60 samples were positively impacted by molecular mechanisms. Weekly microscopic observations of calves with high prevalence revealed a similar outcome of 26% [13]. Out of 156 samples, 36 were verified to be positive by PCR, and 26 of them were confirmed by microscopy, indicating that the current investigation followed a comparable technique [14]. Formal-ether was used to isolate Cryptosporidium from the fecal samples, and the MZN technique which is more prevalent in diarrhea samples was used to determine the positive prevalence. PCR was also effectively used to identify the glycoprotein gene [7,13] The current thesis's findings were comparable to those of Wegayehu et al. [15], who reported 449 calves' feces samples from Ethiopia that were verified by MZN microscopy. The polymerase chain reaction revealed a considerable increase in the prevalence of Cryptosporidium. A clinical investigation using PCR reactions revealed a greater incidence of Cryptosporidium in patients from India and Africa; the current study used a similar approach [16]. Furthermore, additional research has been published on the detection of oocytes using various methods; nevertheless, PCR has confirmed that there are more practical methods [15]. As demonstrated experimentally in the current investigation, the polymerase chain reaction methodology is a successful molecular biology method for detecting 100% oocyte in an animal stool sample [17]. Helmy et al. [18], used Zheil Nelson modified microscopy to identify Cryptosporidium infection in calves, and they received considerable approval using contemporary biological procedures, such as polymerase chain reaction.

5. Conclusions

These findings highlight the variability in Cryptosporidium prevalence across farms, breeds, and sex, as well as the importance of molecular techniques for accurate diagnosis.

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The study was not received any internal or external funds.

Conflicts of interest

There are no conflicts of interest.

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