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Prevalence, Risk Factors Analysis, and Comparative Evaluation of Diagnostic Techniques for *Cryptosporidium* in Calves

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ABSTRACT

Cryptosporidium is a protozoan parasite with a high prevalence in calves, particularly in neonates due to their immature immune systems. Contributing risk factors include poor hygiene, overcrowding, and stress. The current study investigates the prevalence of *Cryptosporidium* infection in calves, assessing two diagnostic methods, microscopy and polymerase chain reaction (PCR), for sensitivity and practicality in rapid diagnosis, while also identifying associated risk factors. A total of 384 diarrheal and semisolid fecal samples were collected from calves across various regions of Mardan. Cryptosporidiosis was diagnosed through microscopy, employing Modified Ziehl-Neelsen (MZN) acid-fast staining, and reconfirmed via PCR. Microscopic examination revealed an overall prevalence of 23.95% (92/384), with 65.21% (60/92) of these cases confirmed positive for *Cryptosporidium* spp. by PCR. Results indicated a statistically significant higher prevalence in diarrheal versus non-diarrheal cases ($p < 0.05$), with a greater occurrence in female calves and a seasonal peak in summer, especially in July. This study concludes that MZN-stained microscopy effectively identifies *Cryptosporidium* oocysts in diarrheal calves and emphasizes PCR's role in confirming infection. Findings underscore the need for targeted control strategies, guided by gene-specific insights, to manage cryptosporidiosis in calves.

INTRODUCTION

Parasites, whether endo-parasites or ecto-parasites, pose serious health issues for both humans and animals and are considered a key global problem. An important clinical and veterinary problem is the protozoan intestinal parasite *Cryptosporidium* (Khan *et al*., 2022). It is recognized as a major foodborne parasite, with the single genus *Cryptosporidium* spp. causing more than 8 million cases of foodborne illness in 2010, ranking it sixth out of 24 possible foodborne parasite (Kotloff *et al*., 2013; Ryan *et al*., 2018; Ryan *et al*., 2021). Microscopic protozoan parasite of the species *Cryptosporidium* target the microvillus epithelial cells lining the digestive and respiratory systems of animals (Díaz *et al*., 2021). Commonly referred to as Crypto, this parasite can lead to malnutrition and cause cryptosporidiosis, an ailment affecting the respiratory and gastrointestinal systems. Symptoms include persistent cough (nasal mucosa, sinuses, larynx, and trachea) and diarrhea (loss of appetite, stomach cramps, vomiting, watery diarrhea, weight loss) in animals and humans (Shirley *et al*., 2012; Korpe *et al*., 2016; Khan *et al*., 2022). More than 170 species of vertebrates can come into touch with *Cryptosporidium*, which is widely dispersed and can be discharged in feces (Daiz *et al*., 2021; Khan *et al*., 2022). Numerous vertebrate hosts, including fish, mammals; including human, marsupials, birds, reptiles, and amphibians, have been identified as hosts for these parasites. While some *Cryptosporidium* species are generalists and can infect a variety of hosts, others exhibit host-specificity in their natural environment (Fayer *et al*., 2010; Xiao, 2010). *Cryptosporidium* can spread through both direct and indirect mechanisms (Elsafi *et al*., 2013).

Young ruminants, especially calves, frequently contract cryptosporidiosis shortly after birth and remain

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infected for several months (Xiao, 2010; Ryan *et al*., 2021). Between eight and fifteen days of age, dairy calves are typically infected, while between one and two months of age, beef calves are frequently affected, mostly through the release of fecal oocysts the goat kids and lambs less than one month (Xiao and Feng, 2008). Cryptosporidiosis can extend through raw unhygienic food/feed and water, posing a risk to both humans and animals. To prevent contracting with this parasite, individuals can take measures such as washing, cooking, boiling, and heating, beverages or food before utilization (Pal *et al*., 2016). Additionally, individuals with diarrhea should avoid community parks and pools to reduce the risk of illness. Separating animals by keeping some in stockyards and others on farms can provide better protection against this fatal disease (Bamaiyi and Redhuan, 2016). Foodborne diseases have significant economic implications, with billions of dollars being spent annually worldwide. Enteric infectious bacteria are a major contributor to these expenditures (Gharekhani *et al*., 2014). *Cryptosporidium* alone causes over 8 million cases of food-borne disease each year, making it well-matched for food-borne disease. Although the whole financial burden of cryptosporidiosis has not yet been fully analyzed, it is estimated between 10 to 83 billion USD yearly (Nyachuba, 2010).

Understanding the prevalence and risk factors associated with this parasite is crucial for implementing effective control measures and minimizing its impact on both animal and human health. Therefore, the current study's objectives were to ascertain the prevalence of cryptosporidiosis in calves, identify numerous risk factors linked to the development of bovine cryptosporidiosis, and assess the sensitivity of two fast diagnostic methods, microscopy and polymerase chain reaction (PCR). Consequently, the findings can contribute to improving disease management strategies and protecting livestock sector..

MATERIALS AND METHODS

Study area

The experiment was conducted in diverse area within Mardan region, located between the latitude of 34.206123 and longitude coordinates 72.029800 in the Khyber Pakhtunkhwa province of Pakistan. Mardan stands out as a significant hub for livestock, with a notable concentration of cattle and sheep farming. To ensure a representative sample, we carefully selected both intensive and semi-intensive farms, taking population size into account. This approach allowed us to collect enough samples, providing a robust basis for our research findings.

Samples collection

A total of 384 samples of semisolid or recently discharged diarrheal feces were taken directly from the rectum. Among these samples, 225 were sourced from dairy farms, while the remaining 159 samples were obtained from household breeds. To acquire valuable insights into potential risk factors for the disease, animal owners were properly interviewed and requested to complete questionnaires. The collected fecal samples were carefully stored in well-sterilized and labeled bottles, and subsequently transferred to the parasitology laboratory, College of Veterinary Sciences, Abdul Wali Khan University, Mardan. There, they were kept in refrigerator temperature (35.6°F to 46.4°F) until further analysis. This meticulous approach ensured the preservation and quality of the samples throughout the research process.

Microscopic identification of Cryptosporidium *species.*

For the microscopic detection of *Cryptosporidium* spp., a thin fecal smear was prepared on a clean slide and fixed in methanol for 2-3 min. The slide was then stained with Carbol fuchsin, covering the entire smear, and left for 15-20 min before being gently washed with tap water. The smear was then treated with acid-alcohol for two to three minutes before being cleaned with sterile water. Methylene blue was then applied, let to sit for 10 to 15 minutes, and then the area was thoroughly cleaned. The slide was air-dried and examined under a microscope at 10X, 400X and 1000X magnification to identify the presence of *Cryptosporidium* oocysts. The *Cryptosporidium* spp. oocysts stained pinkish and appeared as thick-walled, spherical structures measuring about 4-6 µm in diameter. The dimensions of the oocysts were assessed from the margins of their walls. To document the findings, a photograph of the oocysts was captured using a digital camera (30 MP, Model #A-4000 IS, Japan). This standardized microscopic detection by Modified Ziehl-Neelsen acid fast staining technique allows for accurate identification of *Cryptosporidium* oocysts and aids in understanding the prevalence and distribution of this parasite.

The samples for *Cryptosporidium* oocysts were conserved by adding two volumes (1:2) of 75% ethanol and stored at room temperature until further processing.

Molecular identification of Cryptosporidium

Following the manufacturer's instructions, we used the Pure Link-TM Microbiome DNA Purification Kit (Catalog Number A29790) to extract high-quality *Cryptosporidium* DNA from stool samples. DNA extraction was performed on all positive samples. *C. parvum* was then molecularly identified through PCR amplification of an 18S rRNA gene fragment (556 bp) using primers previously described by

Elsafi *et al*. (2013). The forward primer, AWA722F, had the sequence 3'-AGTGCTTAAAGCAGGCAACTG-5', and the reverse primer, AWA1235R, had the sequence 5'-CGTTAACGGAATTAACCAGAC-3'. The PCR reaction, with a total volume of 25 µL, consisted of 12.5 µL of master mix, 1.5 µL of each forward and reverse primer, 2.5 µL of the extracted DNA to be amplified and 7 µL of PCR-grade water. The PCR process involved an initial denatureation step of five minutes at 95°C, followed by 35 cycles with the following thermal cycling protocol: denatureation at 94 °C for 40 s, annealing at 58 °C for 40 s, extension at 72 °C for 1 min, and lastly, amplification at 72 °C for 10 min. The resulting PCR product was then analyzed on a two percent agarose gel using conventional electrophoresis. This PCR-based method enabled the successful identification and detection of *Cryptosporidium* oocysts, feal samples, providing valuable insights for further research and surveillance of this important parasite. The use of appropriate primers and standardized PCR conditions ensures reliable and reproducible results, making this approach a valuable tool in investigating cryptosporidiosis and its prevalence in various populations.

Statistics analysis

Using Exl formulae and IBM SPSS Statistics 20, prevalence was estimated and reported as simple percentages, along with a variety of characteristics and risk factors. The chi-square test (χ^2) was employed, and all values with a p-value of less than 0.05 were considered statistically significant. For data visualization and figure preparation, Adobe Photoshop 8.0 was used to perform editing, cropping, and graphic rendering. This comprehensive approach ensured accurate statistical analysis and aesthetically appealing graphical representation of the findings.

RESULTS

Microscopic examination of Cryptosporidium *oocysts*

The modified Ziehl-Neelsen acid fast staining method (MZN) was utilized to perform morphological identification 92 *Cryptosporidium* oocysts in 384 samples were positive for the presence of *Cryptosporidium* oocysts. Microscopic analysis revealed that the *Cryptosporidium* oocysts appeared as sphere-shaped structures with pink-colored granules against a blue background, as shown in Figure 1. This staining technique provided clear visualization and accurate identification of the oocysts, enabling precise characterization of *Cryptosporidium* in the examined samples.

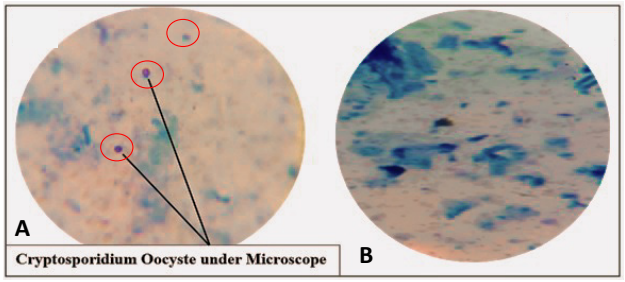

Fig. 1. Microscopic representation of *Cryptosporidium*. A, Positive smear; B, Negative smear.

Prevalence of cryptosporidiosis

The samples collected from farms showed varying prevalence rates of cryptosporidiosis, with the highest prevalence noted at Naway Kaley dairy farm (36.66%), followed by Toro dairy farm (33.33%), New Surkh dairy farm (32.14%), and Khan Dairy farm (26.31%). On the other hand, the lowest prevalence was observed in Iqbal dairy farm, with only (12.50%) of the samples testing positive for cryptosporidiosis (Table I).

Table I. *Cryptosporidium parvum* prevalence in different dairy farms of District Mardan, Khyber Pakhtunkhwa, Pakistan.

Farm name	No. of samples	Positive cases	Prevalence %	p value
Sultan Abad dairy farm	61	14	22.95	0.118
Naway Kalay farm	30	11	36.66	
Iqbal dairy farm	32	4	12.50	
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	
SD	14.92	4.08	8.36	

Table II shows prevalence rate of cryptosporidiosis at different areas of Mardan. The highest prevalence was recorded in Ghareeb Abad Par Hoti (25.58%), followed by Naway Kali (17.94%) and Pir Abad Kaly (26.92%). Conversely, the lowest prevalence was observed in Nee Surkh Dherai, with a rate of (12.00%).

Figure 2 shows month-wise prevalence of *cryptosporidiosis*. The samples were collected from calves spanning from 12, 2021 to 7, 2022. The findings

revealed the highest prevalence of infection in the month of July (37.34%), closely followed by June (26.2% and May (24.52%). On the other hand, the lowest prevalence was observed in the months of December (4.54%) and January (17.39%). The study firmly established a significant correlation between parasitic infection and high temperatures, with the peak prevalence occurring during the warmer months.

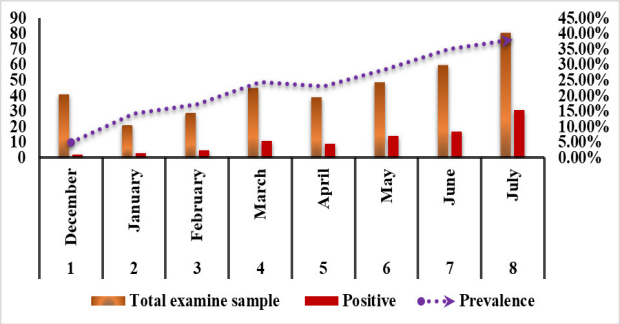

Fig. 2. Month-wise prevalence of *Cryptosporidium* infection.

Table II. Prevalence of *Cryptosporidium* in different areas of District Mardan.

Area	No. of sample	Positive cases	Prevalence (%)	P-value
Ghareeb Abad Par Hoti	43	11	25.58	0.409
Naway Kali	39	7	17.94	
Nee Surkh Dherai	25	3	12.00	
Pir Abad Kaly	52	14	26.92	
Total	159	35	82.45	
Mean	39.75	8.75	32.98	
SD	11.23	4.78	6.97	

Cryptosporidiosis was more common during the summer season (34.75%), followed by the autumn season (21.80%), and less common during the spring season (12.72%) (Fig. 3). Statistical analysis demonstrated a significant difference ($P<0.05$) in the prevalence of cryptosporidiosis between the autumn and summer seasons.

The prevalence of cryptosporidiosis based on the sex of cow calves in shown in Table III. The results indicated a prevalence of (25.12%) for cryptosporidiosis in female calves and (22.75%) in male calves. Interestingly, a higher prevalence of cryptosporidiosis was observed in female calves compared to their male counterparts.

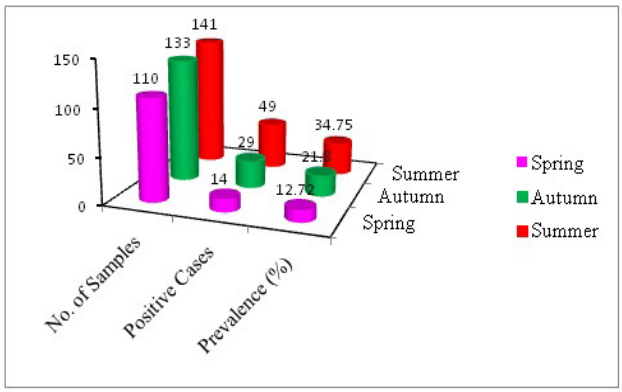

Fig. 3. Seasonal prevalence of *Cryptosporidium parvum*.

Table III. Gender-wise prevalence of *Cryptosporidium* infection.

Gender	No. of samples	Positive cases	Prevalence (%)	p value
Male	189	43	22.75	0.585
Female	195	49	25.12	
Total	384	92	47.87	
Mean	192	46	23.93	
SD	4.24	4.24	1.68	

Table IV. Breed-wise prevalence of *Cryptosporidium* infection.

Breed	No. of samples	Positive cases	Prevalence (%)	p value
Jersey	106	21	19.81	0.205
Cross breed	140	31	22.14	
Holstein Friesians	138	40	28.98	
Total	384	92	70.93	
Mean	128	30.66	35.46	
SD	19.07	9.50	4.76	

Holstein Friesians exhibited the highest prevalence of cryptosporidiosis (28.98%), while Jersey and crossbreeds showed relatively lower rates of (19.81%) and (22.14%), respectively (Table IV).

Molecular detection of Cryptosporidium *infection*

In this study, a total of 384 fecal samples from calves were microscopically examined using the MZN staining technique, and 92 samples were found positive for presence of *Cryptosporidium* oocysts. To confirm these findings, we employed sensitive PCR tools on the 92 positive samples,

resulting in the confirmation of 60 out of 92 samples (53.57%) as positive for cryptosporidiosis (Table V).

Table V. Comparison of two diagnostic techniques: MZN and PCR.

Technique	Total samples	Positive cases	Prevalence (%)	p-value
MZN	384	92	23.99	0.00001
PCR	92	60	53.57	

DISCUSSION

Cryptosporidium, a parasite belonging to the phylum Apicomplexa and the family Cryptosporidiidae, poses a significant threat to both humans and animals in various ecosystems (Votýpka *et al*., 2017). While numerous experiments have been performed on the impact of *Cryptosporidium* on buffalo, chicken, and parrots (Khushdil *et al*., 2016), here the effect of cryptosporidiosis on diarrheal dairy calves in the Mardan district of Khyber Pakhtunkhwa, Pakistan, is the main focus of the current study. Microscopic examination using the MZN staining method confirmed the presence of *Cryptosporidium* oocysts, exhibiting a characteristic round shape and pink color (Khan *et al*., 2022). The prevalence of this oocyst varied across different locations, with the highest prevalence observed at Naway Klay dairy farms compared to other districts of Mardan. Similar findings were also noted in lambs, where the prevalence of cryptosporidiosis was highest compared to other groups. Notably, the prevalence of cryptosporidiosis in sheep was around 11% in district Lakki Marwat, consistent with the results of the current study (Yenene *et al*., 2020).

Cryptosporidiosis has been experimentally proven to cause various abnormalities, posing threats to the gizzards, lungs, liver, intestinal epithelium, and ultimately leading to diarrhea, severe dehydration, anorexia, and even death (Diaz *et al*., 2021). In other countries, such as Sudan, 17% prevalence of the parasite was recorded in small children with diarrhea (Dires, 2021). Similarly, Brazil showed a prevalence rate of 10% for cryptosporidiosis, while Australia recorded over 5% prevalence in sheep (Conceição *et al*., 2021). It is evident from these studies that cryptosporidiosis poses significant health risks to both animals and humans in various regions worldwide (Becher *et al*., 2004). Understanding the prevalence and impact of this parasite is crucial for implementing effective control measures and preventive strategies to safeguard animal and public health (Seyoum *et al*., 2018).

The current study investigated the prevalence of cryptosporidiosis in both semisolid and diarrheic fecal samples from cow calves. The results revealed a higher prevalence of *Cryptosporidium* spp. oocysts in the diarrheic samples (32%) compared to the semisolid samples (9%) among calves under the age of 5 months. Nasir *et al*. (2009), also observed similar patterns in cows and buffalo, confirming that older animals were more prone to infection compared to younger ones. Additionally, they found a higher incidence of the parasite in diarrheic samples compared to non-diarrheic samples. In Iran, Gharekhani *et al*. (2014), reported similar results, showing a higher tolerance to cryptosporidiosis in young animals compared to older ones. However, in buffalo and 30 days aged cow calves, the prevalence of cryptosporidiosis was extensively higher compared to calves aged 3 months, which was consistent with the findings of the current study (Maurya *et al*., 2013). Moreover, Fayer *et al*. (2010) observed a high prevalence of *Cryptosporidium* in young calves (1 year) compared to older animals (over the 1 year), highlighting a clear correlation between age and infection rate. Overall, age was recognized as a critical aspect influencing the prevalence of *Cryptosporidium*, with newborns being particularly susceptible compared to other age groups (Huetink *et al*., 2001). The findings underscore the importance of considering age as a significant factor in understanding and preventing the prevalence of cryptosporidiosis. Implementing appropriate preventive measures and targeted interventions for specific age groups can contribute to effective control and management of this parasite in calves and other animals.

This experiment investigated the month-wise prevalence of cryptosporidiosis in cow calves, and the results revealed the maximum prevalence in July (37.34%) and June (26.28%), while the lowest was recorded in December and January (17.39). These findings suggest that *Cryptosporidium* prevalence is highly influenced by environmental factors, and hot weather appears to be most suitable for the proliferation of this parasite. Our investigation also revealed a similar high incidence of *Cryptosporidium* in sheep during hot and rainy conditions (Jafri *et al*., 2013). The season-based analysis indicated that the summer season was more conducive to compared to the spring or fall seasons, the spread of the cryptosporidiosis. The summer months showed a noticeably higher prevalence, whilst the spring months showed the lowest prevalence. This observation is consistent with similar studies conducted in goats, which showed a high prevalence of cryptosporidiosis during the summer season (Jafri *et al*., 2013; Khan *et al*., 2022). Similarly, Urie *et al*. (2018) reported the highest prevalence of *Cryptosporidium* during the summer season compared to the spring season. Ahmad *et al*. (2020) conducted studies that corroborate

our findings, indicating that summer was the season with the highest occurrence of *Cryptosporidium*, followed by autumn and spring. However, it is essential to consider that other environmental factors may also play a role in the transmission of cryptosporidiosis. For instance, contrary to our findings, indicated that the winter season had the highest prevalence of cryptosporidiosis, followed by autumn and spring (Ranjbar *et al*., 2017). Similarly, Causapé *et al*. (2002) observed the maximum prevalence during the spring season compared to other seasons. In conclusion, the month-wise prevalence of cryptosporidiosis in cow calves indicates a clear association with environmental factors, particularly hot weather, suggesting that seasonal variations significantly impact the spread of this parasite. Understanding these dynamics can aid in implementing targeted control measures during specific seasons to effectively manage and control cryptosporidiosis in livestock (Liu *et al*., 2016).

The prevalence of cryptosporidiosis in calves was found to be non-significantly influenced by the sex, with higher rates observed in female calves (27.17%) compared to male calves (24.70%) in the current study. Similar findings were reported by Liu *et al*. (2016), in goats, where a higher prevalence of cryptosporidiosis was observed in females compared to males. Jafri *et al*. (2013) also reported a higher prevalence of *Cryptosporidium* in female calves compared to males, consistent with our study. This higher rate of cryptosporidiosis in females may be attributed to the presence of different hormones in females. Further supporting our results, Shallangwa *et al*. (2022) recorded a significantly higher prevalence of cryptosporidiosis in female cattle (48%) compared to males (29%). Fan *et al*. (2017) also observed a higher prevalence of *Cryptosporidium* in females (25%) than in males (22%). Similarly, in buffalo, cow calves, sheep, and goats, the prevalence of cryptosporidiosis was significantly higher in females than in males, as reported by Maurya *et al*. (2013). However, contrasting results were observed in some studies. Valentiner *et al*. (2007) found a significantly higher prevalence of Cryptosporidium in male cattle compared to females. In France, Lefay *et al*. (2000) observed a higher prevalence of cryptosporidiosis in male calves compared to female calves. Similarly, in other animals such as pigs, a higher prevalence of cryptosporidiosis was recorded in males compared to females (Paul *et al*., 2008). In conclusion, the sex of the calves appears to play a significant role in the prevalence of cryptosporidiosis, with varying results observed in different studies. The hormonal differences between males and females may be influencing the susceptibility to infection. These findings highlight the importance of considering sex as a factor in understanding and managing cryptosporidiosis in cattle and other animals.

The current study aimed to verify the prevalence of cryptosporidiosis in calves' feces using both PCR and microscopic examination. A total of 384 fecal samples were subjected to microscopic observation through the MZN staining technique, revealing 92 samples with the presence of *Cryptosporidium* oocysts. For confirmation, we employed sensitive PCR tools on these 92 positive samples, of which 60 (53.57%) were confirmed positive for cryptosporidiosis. These findings are in line with a similar study on calves, where a high prevalence of 26% was detected through weekly microscopic observation (Lombardelli *et al*., 2019). Another study following a comparable protocol established 36 positive cases out of 156 through PCR, among which 26 were also established through microscopy (Morgan *et al*., 1998). Wegayehu *et al*. (2016) reported the detection of *Cryptosporidium* from 449 fecal samples of calves in Ethiopia using MZN microscopy, and the prevalence was further confirmed using PCR to identify the glycoprotein gene. Similarly, in clinical studies on patients from India and Africa, a higher prevalence of cryptosporidiosis was observed using PCR reactions, following a protocol similar to the one used in this study (Ali *et al*., 2024). Several studies have reported the detection of *Cryptosporidium* oocysts using various techniques, but the combination of MZN staining followed by PCR appears to be a more widely used and effective approach (Khan *et al*., 2022). Overall, the use of PCR along with microscopic examination using the MZN technique proves to be a valuable and reliable method for detecting cryptosporidiosis in animals. This combination enhances the accuracy and sensitivity of the detection process, making it a preferred approach in various studies.

CONCLUSION

The study offers detailed insights into cryptosporidiosis in calves from various dairy farms in Mardan district. Microscopy with MZN staining confirmed pink, spherical oocysts. *Cryptosporidium* prevalence was significantly higher in diarrheal calves ($p < 0.05$). Seasonal analysis highlighted increased occurrence during summer, especially in July. PCR identified 60 positive cases in fecal samples, emphasizing the need for targeted control measures based on specific genes to manage cryptosporidiosis in animals. Moreover, the PCR overall, the study contributes to understanding cryptosporidiosis in cow calves, underscoring the ongoing necessity for research to implement interventions and control strategies for both livestock and human health.

DECLARATIONS

Funding

The study has not received any external or internal funds for the study.

IRB approval

The study was approved by College of Veterinary Sciences, Abdul Wali Khan University, Mardan and its detected No. Dir/A&R/AWKUM/2022/9396. The Advanced Studies and Research Board meeting was held on 08-June-2022.

Ethics approval

The study was approved by the Ethical Committee of the College of Veterinary Sciences, Abdul Wali Khan University, Mardan and conducted all the experiments in the laboratory of Veterinary Parasitology This research work involved animal (cow calves) experimentation and the clinical trial study detected No. AWKUM-210547932-2021-23. All the fecal samples were collected with the consent of owners. After sampling, free veterinary care and services were also provided such as treatment, deworming, vaccination and extension messages without any fee or medicine charges.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Ahmed, S.A. and Karanis, P., 2020. Cryptosporidium and cryptosporidiosis: the perspective from the Gulf countries. *Int. J. environ. Res. Publ. Hlth.,* **17**: 6824.

Ali, U., Khan, N.U., Ullah, K., Usman, T., Khattak, I. and Karanis, P., 2024. Cryptosporidium from sheep herds around water tributaries of the Malakand region of Pakistan. *J. Water Hlth.,* **22**: 2320-2330.

Atwill, E.R., Johnson, E., Klingborg, D.J., Veserat, G.M., Markegard, G., Jensen, W.A., Pratt, D.W., Delmas, R.E., George, H.A. and Forero, L.C., 1999. Age, geographic, and temporal distribution of fecal shedding of *Cryptosporidium parvum* oocysts in cow-calf herds. *Am. J. Vet. Res.,* **60**: 420-425. https://doi.org/10.2460/ajvr.1999.60.04.420

Bamaiyi, P.H. and Redhuan, N.E.M., 2016. Prevalence and risk factors for cryptosporidiosis: A global, emerging, neglected zoonosis. *Asian Biomed.,* **10**: 309-325. https://doi.org/10.5372/1905-7415.1004.493

Becher, K.A., Robertson, I.D., Fraser, D.M., Palmer, D.G. and Thompson, R.C.A., 2004. Molecular epidemiology of Giardia and Cryptosporidium infections in dairy calves originating from three sources in Western Australia. *Vet. Parasitol.,* **123**: 1-9.

Causapé, A.C., Quılez, J., Sánchez-Acedo, C., Del Cacho, E. and López-Bernad, F., 2002. Prevalence and analysis of potential risk factors for *Cryptosporidium parvum* infection in lambs in Zaragoza (northeastern Spain). *Vet. Parasitol.*, **104**: 287-298. https://doi.org/10.1016/S0304-4017(01)00639-2

Conceição, A.I., Almeida, L.P.S., Macedo, L.O., Mendonça, C.L., Alves, L.C., Ramos, R.A.N. and Carvalho, G.A., 2021. Prevalence of infection by *Cryptosporidium* spp. in calves and associated risk factors in Northeastern Brazil. *Arquivo Brasil. Med. Vet. Zoot.*, **73**: 34-40. https://doi.org/10.1590/1678-4162-12109

Díaz, P., Navarro, E., Remesar, S., García-Dios, D., Martínez-Calabuig, N., Prieto, A., López-Lorenzo, G., López, C.M., Panadero, R. and Fernández, G., 2021. The age-related *Cryptosporidium* species distribution in asymptomatic cattle from north-western Spain. *Animals*, **11**: 256. https://doi.org/10.3390/ani11020256

Dires, T., 2021. *Prevalance and associoted risk factore of in testinal parastice infanctions among praymery school cheldren in deradestancte north Weast Ethiopioa* (Doctoral dissertation).

Elsafi, S.H., Al-Maqati, T.N., Hussein, M.I., Adam, A.A., Hassan, M.M.A. and Al-Zahrani, E.M., 2013. Comparison of microscopy, rapid immunoassay, and molecular techniques for the detection of Giardia lamblia and *Cryptosporidium parvum*. *Parasitol. Res*., **112**: 1641-1646. https://doi.org/10.1007/s00436-013-3319-1

Fan, Y., Wang, T., Koehler, A.V., Hu, M. and Gasser, R.B., 2017. Molecular investigation of Cryptosporidium and Giardia in pre-and post-weaned calves in Hubei Province, China. *Parasit. Vectors,* **10**: 1-7.

Farthing, M.J., 2000. Clinical aspects of human cryptosporidiosis. *Contr. Microbiol.,* **6**: 50-74. https://doi.org/10.1159/000060368

Fayer, R., Santín, M. and Macarisin, D., 2010. *Cryptosporidium ubiquitumn* sp. in animals and humans. *Vet. Parasitol.,* **172**: 23-32. https://doi.org/10.1016/j.vetpar.2010.04.028

Gharekhani, J., 2014. Study on gastrointestinal zoonotic parasites in pet dogs in Western Iran. *Türk. Parazitolojii Derg*., **38**: 172.

Jafri, R.Z., Ali, A., Messonnier, N.E., Tevi-Benissan,

C., Durrheim, D., Eskola, J., Fermon, F., Klugman, K.P., Ramsay, M., Sow, S. and Zhujun, S., 2013. Global epidemiology of invasive meningococcal disease. *Populat. Hlth. Metrics,* **11**: 1-9.

Huetink, R.E.C., Van der Giessen, J.W.B., Noordhuizen, J.P.T.M. and Ploeger, H.W., 2001. Epidemiology of *Cryptosporidium* spp. and *Giardia duodenalis* on a dairy farm. *Vet. Parasitol.*, **102**: 53-67. https://doi.org/10.1016/S0304-4017(01)00514-3

Khan, N.U., Usman, T., Sarwar, M.S., Ali, H., Gohar, A., Asif, M., Rabbani, F., Khan, R.U., Sultana, N., Khan, N.A. and Mobashar, M., 2022. The prevalence, risk factors analysis and evaluation of two diagnostic techniques for the detection of *Cryptosporidium* infection in diarrheic sheep from Pakistan. *PLoS One*, **17**: e0269859. https://doi.org/10.1371/journal.pone.0269859

Khushdil, A., Murtaza, F. and Chattha, M.N., 2016. Cryptosporidiosis among children of district Skardu, Pakistan. *J. Ayub med. Coll. Abbottabad*, **28**: 575-577.

Korpe, P.S., Haque, R., Gilchrist, C., Valencia, C., Niu, F., Lu, M., Ma, J.Z., Petri, S.E., Reichman, D. and Kabir, M., 2016. Natural history of *cryptosporidiosis* in a longitudinal study of slum-dwelling Bangladeshi children: Association with severe malnutrition. *PLoS Negl. Trop. Dis.,* **10**: e0004564. https://doi.org/10.1371/journal.pntd.0004564

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D. and Breiman, R.F., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): A prospective, case-control study. *Lancet,* **382**: 209-222. https://doi.org/10.1016/S0140-6736(13)60844-2

Lechevallier, M.W. and Moser, R.H., 1995. Occurrence of giardia and *Cryptosporidium* in raw and finished drinking water. *J. Am. Water Works Assoc.*, **87**: 54-68. https://doi.org/10.1002/j.1551-8833.1995.tb06422.x

Lefay, D., Naciri, M., Poirier, P. and Chermette, R., 2000. Prevalence of *Cryptosporidium* infection in calves in France. *Vet. Parasitol.*, **89**: 1-9. https://doi.org/10.1016/S0304-4017(99)00230-7

Liu, J., Platts-Mills, J.A., Juma, J., Kabir, F., Nkeze, J., Okoi, C., Operario, D.J., Uddin, J., Ahmed, S., Alonso, P.L. and Antonio, M., 2016. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: A reanalysis of the GEMS case-control study. *Lancet*, **388**: 1291-1301. https://doi.org/10.1016/S0140-6736(16)31529-X

Lombardelli, J.A., Tomazic, M.L., Schnittger, L. and Tiranti, K.I., 2019. Prevalence of *Cryptosporidium parvum* in dairy calves and GP60 subtyping of diarrheic calves in central Argentina. *Parasitol. Res.*, **118**: 2079-2086.

Maurya, P.S., Rakesh, R.L., Pradeep, B., Kumar, S., Kundu, K., Garg, R., Ram, H., Kumar, A. and Banerjee, P.S., 2013. Prevalence and risk factors associated with *Cryptosporidium* spp. infection in young domestic livestock in India. *Trop. Anim. Hlth. Prod.*, **45**: 941-946. https://doi.org/10.1007/s11250-012-0311-1

Monis, P.T. and Thompson, R.C.A., 2003. *Cryptosporidium* and giardia-zoonoses: Fact or fiction? *Infect. Genet. Evol.*, **3**: 233-244. https://doi.org/10.1016/j.meegid.2003.08.003

Morgan, U.M. and Thompson, R.C.A., 1999. Molecular detection of parasitic protozoa. *Parasitology,* **117**: 73-85.

Morris, A.J., Noble, S., Lindo, J.F. and Ong, C.S., 2022. First report of *Cryptosporidium* spp. In: *Cattle from Jamaica.* https://doi.org/10.21203/rs.3.rs-1503219/v1

Nasir, A., Avais, M., Khan, M.S. and Ahmad, N., 2009. Prevalence of *Cryptosporidium* parvum infection in Lahore (Pakistan) and its association with diarrhea in dairy calves. *Int. J. Agric. Biol.*, **11**: 221-224.

Nyachuba, D.G., 2010. Foodborne illness: Is it on the rise? *Nutr. Rev.,* **68**: 257-269. https://doi.org/10.1111/j.1753-4887.2010.00286.x

Pal, M., Tafese, W., Tilahun, G. and Anberber, M., 2016. Cryptosporidiosis: An emerging food and waterborne protozoan disease of global significance. *Fd. Beverage World,* **43**: 43-45.

Paul, S., Chandra, D., Ray, D.D., Tewari, A.K., Rao, J.R., Banerjee, P.S., Baidya, S. and Raina, O.K., 2008. Prevalence and molecular characterization of bovine Cryptosporidium isolates in India. *Vet. Parasitol.,* **153**: 143– 146.

Poulin, R., 2007. Are there general laws in parasite ecology? *Parasitology,* **134**: 763-776. https://doi.org/10.1017/S0031182006002150

Ranjbar, R. and Fattahi, R., 2017. Prevalence of Cryptosporidium spp. in calves under one year old in Ilam county (Iran), from March 2014 to February 2015. *Electron. Physic.,* **9**: 4631.

Ryan, U., Hijjawi, N. and Xiao, L., 2018. Foodborne cryptosporidiosis. *Int. J. Parasitol.,* **48**: 1-12. https://doi.org/10.1016/j.ijpara.2017.09.004

Ryan, U.M., Feng, Y., Fayer, R. and Xiao, L., 2021.

Taxonomy and molecular epidemiology of *Cryptosporidium* and Giardia–A 50 year perspective (1971–2021). *Int. J. Parasitol*., **51**: 1099-1119. https://doi.org/10.1016/j.ijpara.2021.08.007

Seyoum, Z., Getnet, K., Chanie, M., Derso, S. and Fentahun, S., 2018. Morbidity parameters associated with gastrointestinal tract nematodes in sheep in Dabat district, northwest Ethiopia. *BioMed. Res. Int.*, https://doi.org/10.1155/2018/9247439

Shafiq, M.A.B., Maqbool, A., Khan, U.J., Lateef, M. and Ijaz, M., 2015. Prevalence, water borne transmission and chemotherapy of *Cryptosporidiosis* in small ruminants. *Pakistan J. Zool.*, **47**: 1715-1721.

Shallangwa, J.M., Maikai, B.V., Okubanjo, O.O., Tizhe, E.V. and Francis, M.I., 2022. Prevalence of *Cryptosporidium* infection in cattle from selected commercial farms and nomadic settlements in Yola, Adamawa State. *Sokoto J. Vet. Sci.*, **20**: 125-133. https://doi.org/10.4314/sokjvs.v20i2.7

Shirley, D.A., Moonah, S.N. and Kotloff, K.L., 2012. Burden of disease from cryptosporidiosis. *Curr. Opin. Infect. Dis.*, **25**: 555–563. https://doi.org/10.1097/QCO.0b013e328357e569

Urie, N.J., Lombard, J.E., Shivley, C.B., Kopral, C.A., Adams, A.E., Earleywine, T.J., Olson, J.D. and Garry, F.B., 2018. Preweaned heifer management on US dairy operations: Part V. Factors associated with morbidity and mortality in preweaned dairy heifer calves. *J. Dairy Sci*, **101**: 9229-9244. https://doi.org/10.3168/jds.2017-14019

Valentiner-Branth, P., Perch, M., Nielsen, J., Steinsland, H., Garly, M.L., Fischer, T.K., Sommerfelt, H., Mølbak, K. and Aaby, P., 2007. Community cohort study of *Cryptosporidium* parvum infections: sex-differential incidences associated with BCG and diptheria tetanus pertussis vaccinations. *Vaccine*, **25**: 2733-2741. https://doi.org/10.1016/j.vaccine.2006.01.035

Votýpka, J., Modrý, D., Oborník, M., Šlapeta, J. and Lukeš, J., 2017. *Apicomplexa 16*. https://doi.org/10.1007/978-3-319-28149-0_20

Wegayehu, T., Adamu, H. and Petros, B., 2013. Prevalence of *Giardia duodenalis* and *Cryptosporidium* species infections among children and cattle in North Shewa Zone, Ethiopia. *BMC Infect. Dis.*, **13**: 1-7. https://doi.org/10.1186/1471-2334-13-419

Wegayehu, T., Karim, R., Anberber, M., Adamu, H., Erko, B., Zhang, L. and Tilahun, G., 2016. Prevalence and genetic characterization of Cryptosporidium species in dairy calves in Central Ethiopia. *PLoS One,* **11**: e0154647.

Xiao, L. and Feng, Y., 2008. Zoonotic cryptosporidiosis. *FEMS Immunol. Med. Microbiol.,* **52**: 309-323. https://doi.org/10.1111/j.1574-695X.2008.00377.x

Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: An update. *Exp. Parasitol.,* **124**: 80-89. https://doi.org/10.1016/j.exppara.2009.03.018

Yenene, M.W. and Gandile, A.U., 2020. Prevalence of the common intestinal parasitic infections and predisposing factors among the asymptomatic primary school children in Finoteselam town, West Gojam Zone, Amhara region (Ethiopia). *World J. Adv. Res. Rev.*, **8**: 173-188. https://doi.org/10.30574/wjarr.2020.8.2.0210