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MOLECULAR CHARACTERIZATION AND PHYLOGENIC ANALYSIS OF *CRYPTOSPORIDIUM* SPECIES ISOLATED FROM CATTLE IN SULAYMANIYAH, IRAQ

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Received: 18 March 2024; **Accepted:** 20 April 2024

ABSTRACT

Diarrhea is globally reported to be a common and major disease in newborn calves. According to the World Health Organization, it can cause a massive economic loss in the cattle industry. Thirty preweaning calves were collected. Calveswere taken from four different places in Sulaimaniyah province (Kalar, Halbjai Shahid, Khurmal, and Sharazor) between September 2022 and March 2023. The animals were divided into 3 age groups: (5–30 days), (31–60 days), and (61–90 days). Rectal swabs were being collected directly from diarrheal calves into plastic specimens for PCR testing. A fragment of the SSU rRNA gene was amplified using the Taq DNA master mix (Addbio, Republic of Korea). Phylogenetic trees were constructed based on a partial fragment of the SSU rRNA gene. The results showed that the higher infection rate (25%) appeared in calves less than one month old, while the lower infection rate (10%) appeared in the two months old and (12%) in three months old calves. In addition, the two sequences (BA/1/2022 and BA/2/2022) were *C. ryanae*, while the other sequence (BA/3/2022) was identified as *C. parvum*. This study reported the occurrence of Cryptosporidium species for the first time in four different places in Sulaimaniyah province, northern Iraq, using molecular techniques.

Keywords: *Cryptosporidium parvum*, *Cryptosporidium ryanae*, Diarrhea, phylogenic analysis.

INTRODUCTION

Calf diarrhea is a commonly reported and major disease affecting the newborn calf worldwide (Cho & Yoon, 2014; Fayer & Xiao, 2008; Lihua Xiao & Cama, 2018). It remains the most important reason for economic loss to the cattle industry, according to the World Health Organization (WHO) (Smith, 2009). In addition to its public health effects because of its zoonotic characteristic (do Couto, Lima Mde, & do Bomfim, 2014; Zhang *et al.*, 2022).

Depending on recent reports, digestive diseases (diarrhea) are measured as the leading cause of disease and death in the pre-weaning period. Calf diarrhea (Carter, Renaud, Steele, Fischer-Tlustos, & Costa, 2021). In addition to the common fact that dairy production depends on the successful raising of calf and heifers, it is estimated that

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about 75% of mortality in calf in dairy production is caused by acute diarrhea. (Muktar, Mamo, Tesfaye, & Belina, 2015). It has a different etiology and numerous infectious agents, including the newest reported agents such as viruses (*Rotavirus, coronavirus, bovine norovirus, Nebo virus and Bovine viral diarrhea virus*), bacteria (*Escherichia coli, Salmonella spp.* and *Clostridium perfringens*) and parasite (*Cryptosporidium parvum*) (Cho *et al.*, 2013). *Cryptosporidium spp.* is a protozoan parasite that is commonly associated with disease and resistance to the environment. (Silverlås, de Verdier, Emanuelson, Mattsson, & Björkman, 2010). Cryptosporidium species are presently documented: *C. muris, C. andersoni, C. parvum, C. hominis, C. wrairi, C. felis, and C. cannis* in mammals; *C. baïleyi, C. meleagridis, and C. galli in birds; C. serpentis and C. saurophilum* in reptiles; *and C. molnari* in fish (L. Xiao, Fayer, Ryan, & Upton, 2004). The common way for cryptosporidiosis diagnosis is by observing the oocysts in the feces using

specialized techniques as an initial screening method. (Azami, 2007). Followed by detection of endogenous developmental stages by histopathology and electron microscopy, or immunological assays and molecular identification techniques are described.

Sulaymaniyah is one of the biggest cities in Iraq and is located in the North-East. The city includes several districts and many villages, and the cattle population in the city is about 40,000. Different outbreaks of diarrhea in calves have recently occurred. However, a definitive molecular characterization of the causative agent has not been followed by molecular techniques. In this study, we detect and characterized the gene and conduct a phylogenetic analysis of the sequences with different isolates that were published in GenBank databases.

MATERIALS AND METHODS

Animal Material

Fecal swab samples were collected into plastic specimens for PCR testing from 30 preweaning calves with diarrhea in four different locations around the Sulaimaniyah province (Kalar, Halbjai Shahid, Khurmal, and Sharazor) between September 2022 and March 2023. The animals were divided into 3 age groups: the first group (5–30 days), the second group (31–60 days), and the third group (61–90 days). Samples were being collected directly from diarrheal calves by rectal swab. For complete data collection, an index card was filled out for each animal, indicating the following data: sampling date, address, breed of animal, clinical signs of diarrhea and dehydration (age, weight, body temperature, heart rate, and respiratory rate), and number of animal identifications. Rectal swabs were then transported to the Sulaimaniyah Veterinary Laboratory in a cold pack within hours of collection and stored in a -20°C freezer until analyzed.

Sample preparation

DNA extraction

Fecal samples were vortexed in phosphate-buffered saline (1 mL 0.1 M PBS, pH 7) before genomic DNA was extracted from feces using a DNA extraction kit (Genet Bio Co., Korea) according to the manufacturer's instructions.

Oligonucleotide primers

In this study, for amplification of a fragment of the SSU rRNA gene of Cryptosporidium, two sets of primers are used: the first set of outer primers (F1-f and F1-r) and the inner primer (F2-f and F2-r).

Table 1: Primers used in the current study.

Primer Name	Sequences 5-----3	Amplicon Size	Reference
F1-f	5'- TTCTAGAGCTAATACATGCG-3	1325 bp	(1)
F1-r	5'-CCCATTTCCTTCGAAA CAGGA-3		
F2-f	5'-GGAAGGGTTGTATTTATTAGATAAAG-3'	826-86 bp	(1)
F2-r	5'-AAGGAGTAAGGAACAACCTCCA-3		

Amplification of DNA extracts.
In detecting Cryptosporidium from feces samples, the most accurate diagnostic method is thought to be polymerase chain reaction (PCR), which has a high degree of sensitivity and specificity. A fragment of the SSU rRNA gene was amplified by using Taq DNA master mix (addbio, Republic of Korea). The reactions were carried out in a 0.2 mL PCR tube based on the following specifications: 10 l master mix, 5 l DNA, 1 l forward (10 pmol), 1 l reverse primers (10 pmol), and 3 l ultra-pure water to make up a final volume of 20 L. The conventional PCR machine (Hercuvan, USA) was programmed as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 min. Annealing at 50 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min A second step for nestet PCR was then performed to amplify 826–864 bp from 1 l of the primary PCR mixture using primers forward (F2-f) and reverse (F2-r). The PCR and cycling conditions were identical to the primary PCR, except the extension time was changed to 40 s. Ten ml of the amplified PCR products were run on a 1% agarose gel and stained with ethidium bromide in Tris-acetate-EDTA (TAE) buffer (1). The PCR product was run via gel electrophoresis in 150 volts for 50 minutes and visualized under a UV transilluminator (UVETIC, UK).

Phylogenetic tree and sequence analysis
Phylogenetic trees were constructed based on a partial fragment of the SSU rRNA gene. The sequence homology and multiple sequence alignment at the nucleotide and amino acid levels were performed by the CLUSTALW program (2), and the phylogenetic tree was constructed by the MEGA.X program employing the neighbor-joining (NJ) method (3).

Nucleotides Sequencing and Registration
In this research, three field partial nucleotide sequences of the fragment SSU rRNA gene were determined by Macrogen Co., Republic of Korea, and these sequences were registered under certain accession numbers within the GenBank databases, which belong to the National Center for Biotechnology Information (NCBI).

Statistical analysis
Statistical analysis was done by SPSS (version 25.0) and the Significance level: 0.05 was determined. Fisher exact test used to determine the associations between variables according to the infectious status. odds ratios were used to determine the risk of infection concerning gender, age, location, and breed. Multiple Correspondence Analysis (MCA) was used to investigate the links between categorical variables.

RESULTS

The prevalence of Cryptosporidium species in relation to age (Table 1) showed that 30 diarrheal calves were divided into three groups (5–30, 31–60, and 61–90) days old. The higher infection rate (16.7%) appeared in calves less than one month old, while the lower infection rate (2 positive in 10 samples, 20%) appeared in the age group of 31–60 days; the infection rate of the last group (61–90 days) 12.5%.

Regarding the breed, as shown in (Table 2), the results were 18.2%, 20%, and 11.1% in Friesian, Local, and Simmental breed

respectively. Moreover, the results for males and females were 7.7% and 23.5%, although the results regarding the geographical location showed that the highest result was in Khurmal (33.3%), and the lowest was in Sharazor (0%).

The outcome of the present study showed that the infection of the animals with cryptosporidium differed according to sex, breed, geographical location and age of the animals, so cryptosporidium was slightly higher in female animals 23.5% (OR: 3.7) than in male animals at 7.7%. Local breed had the highest prevalence of cryptosporidiosis which was 20% (OR: 2) compare to other breeds Friesian and Simmental 18.2% and 11.1% respectively. Regarding the geographical location, Khurmal had the highest prevalence which was 33.3% (OR: 3) while Halabja and Kalar had 18.2%, 14.3% respectively. The lowest infection rate in geographical locations was Sharazor. Cryptosporidiosis had different infectious status regarding the ages of the animals. Animlas with the range of 31-60 days had the highest prevalence which was 16.7% (OR:1.75) whereas 5-30 and 61-90 days of age had the prevalence of 16.7% and 12.5% respectively. (Table 2).

Table 2: Prevalence rate of *Cryptosporidium* species related to sex, breed, geographical locations, and age with the odd ratio.

variable		number	Infected [%]	Odd ratio
sex				
	male	13	7.7	Reference
	female	17	23.5	3.7
breed				
	Simmental	9	11.1	Reference
	Friesian	11	18.2	1.78
	Local	10	20	2
Location				
	Sharazor	6	00	-
	Kalar	7	14.3	Reference
	Halabja	11	18.2	1.33
	Khurmal	6	33.3	3
Age (Days)				
	61 – 90	8	12.5	Reference
	60 – 31	10	20	1.75
	5 – 30	12	16.7	1.4

Regarding the distributions of infections according to sex, breeds, geographical location and age, this study recorded that an association has been found between sex, breeds and geographical location with the infection status. Similarly, among all three groups of ages. Meaning that all three breeds, two sexes, four locations and three age groups had a relation with the infection. Moreover, we can say infection with cryptosporidiosis may be recorded in different levels with regards to sex, breed, age and geographical locations. (Figure 1).

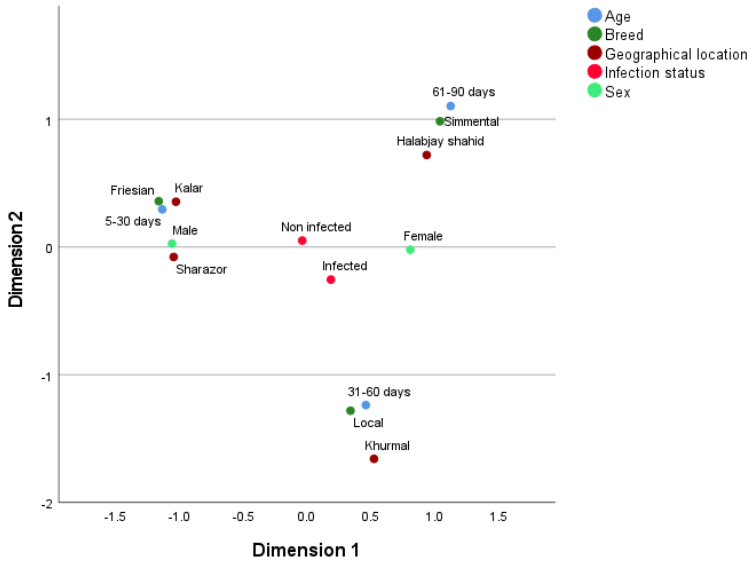

Figure 1: Illustrates the distributions of sex, breed, geographical location and age and associated with infection status.

Sequencing results

Three samples of the PCR products were subjected to sequencing: BA/1/2022 (OQ732749), BA/2/2022 (OQ732750), and BA/3/2022 (OQ732751). These are the accession numbers for the field isolates in GenBank. By using online BLAST to compare each of the two sequences to the published Cryptosporidium spp. sequences in Genbank, the results revealed that two sequences (BA/1/2022 and BA/2/2022) were *C. ryanae*, while the other sequence (BA/3/2022) was identified as *C. parvum*.

Analyzing DNA sequence and phylogenetic tree

By using online BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) to compare each of the two sequences to the published Cryptosporidium spp. sequences in Genbank, the results revealed that two sequences (BA/1/2022 and BA/2/2022) were *C. ryanae*, while the other sequence (BA/3/2022) was identified as *C. parvum*. Based on phylogenetic study, it was determined that the field strains belong to different spp. two strains (BA/1/2022 and BA/2/2022) were clustered with *C. ryanae*; however, another strain (BA/3/2022) was clustered with *C. parvum* (figure 4). The primary genetic nucleotide change (insertion and deletion) in a specific location fragment of the SSU rRNA gene is what differentiates *C. ryanae* from *Parvum*, as shown in (figure 4). The nucleotide sequences of the two *C. ryanae* SSU rRNA genes had limited diversity and were closely related to one another, with 99.70% DNA sequence identity. Compare *C. ryanae* with reference strains that share the highest levels of identity with Bangladesh and China strains (99.70%) and other strains (99.60%) in different countries (table 3). However, nucleotide sequences of the one field isolate (*C. parvum*-BA/3/2022) closely related to Iran (MK426795) and China (MT002720) isolated around 98.82% homology (table 4).

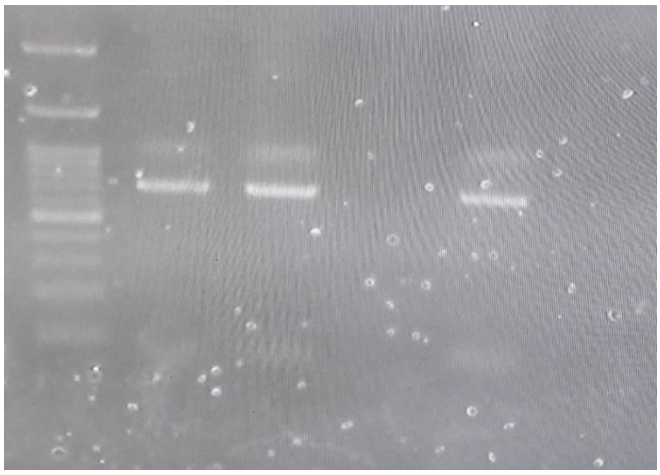

Figure: 2. Gel electrophoresis shown PCR result positive for amplification of a fragment of SSU rRNA gene of Cryptosporidium spp. around 800 bp.

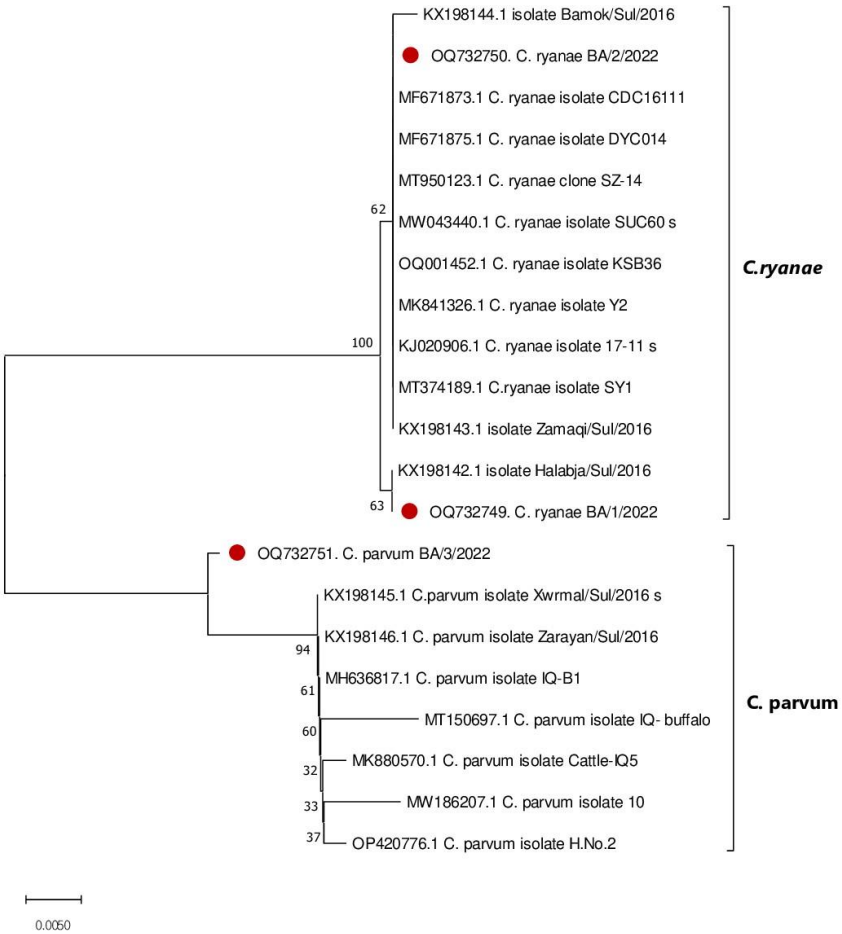

Figure 3: Neighbor-Joining method SSU rRNA gene sequences were used to generate a phylogenetic tree for Cryptosporidium spp. (*C. parvum, and c. ryanae*). The black circle indicates the Iraqi sequences that have been identified in the present study.

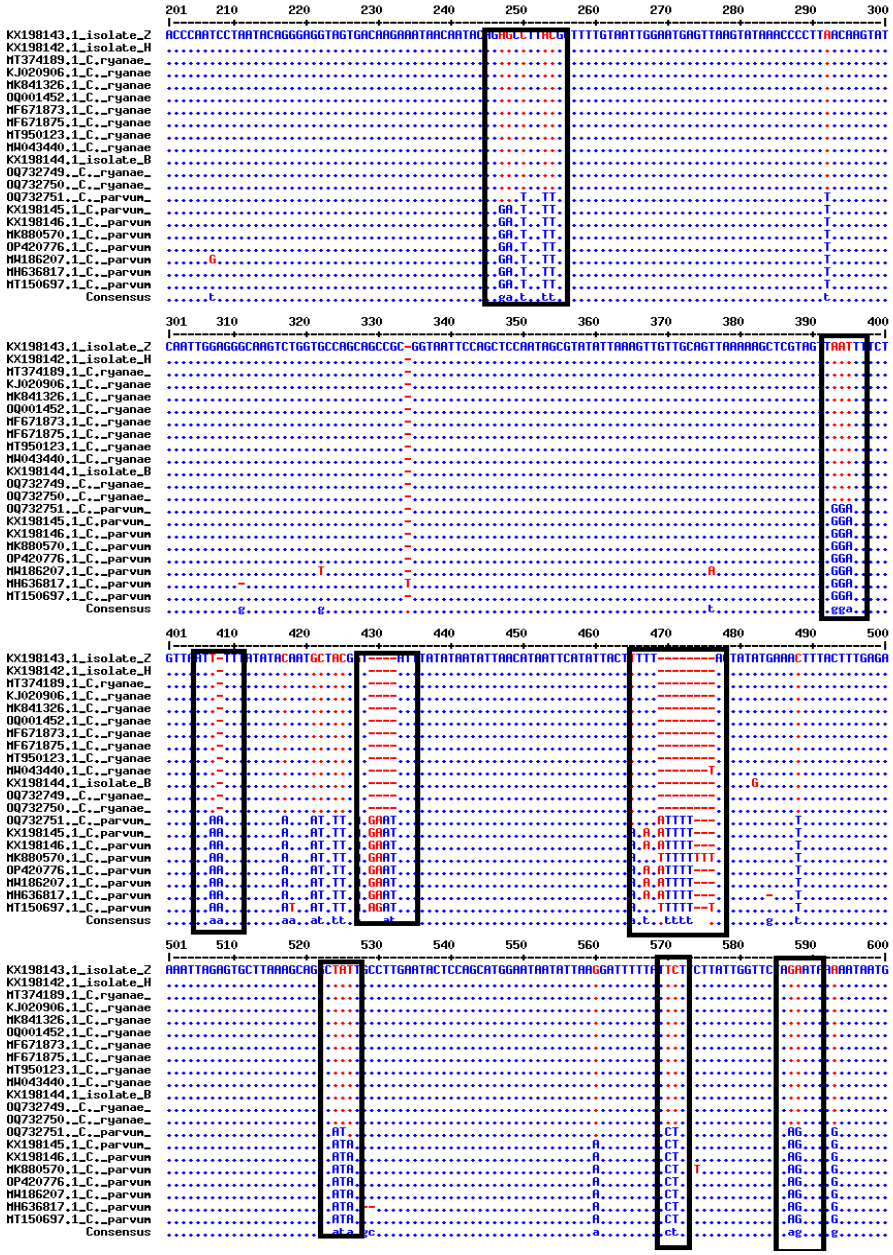

Figure 4: Multiple sequence alignment of fragment SSU rRNA gene for showing variation and genetic differentiation of Cryptosporidium, spp. (*C. parvum, and C. ryanae*) black box indicator for divergency.

Table 3: DNA sequence of field strain *C. ryanae* (BA/2/2022) with other reference strains.

Accession No.	Strain/isolate	Countries	DNA identity %
MF671876	MYC116	China	99.70
MW043439	PIC89	Bangladesh	99.70
OQ001439	KSB23	South Korea	99.70
LC73869	Saraburi138	Thailand	99.70
KX198143.1	Sul/2016	Iraq	99.60
AB777176	37257	Egypt	99.60
MW788446	Mo501	Thailand	99.60
KC582862	555c	Poland	99.60

Table 4: DNA sequence of field strain *C. parvum* (BA/3/2022) with other reference strains

Accession No.	Strain/isolate	countries	DNA identity %
MT002720	LJ4	China	98.82
MN803325	ERU-KyCpar1	TURKEY	98.82
MK982463	SH25	Bangladesh	98.82
MK426795	CPUA4	IRAN	98.82
MK014780	B24	FRANCE	98.82
ON515728	Barandozchai1	IRAN	98.82
MN914084	ISS-C_L3197	GERMANY	98.82
KX198146.1	Zarayan/Sul/2016	IRAQ	98.43

DISCUSSION

In relation to zoonotic cryptosporidiosis, calves have been regarded as the main source (Baroudi *et al.*, 2017). The dairy calves play a crucial role in *C. parvum* transmission (calf to calf or calf to human being). Age appeared to be a significant factor influencing the incidence of Cryptosporidium, according to (Huetink, van der Giessen, Noordhuizen, & Ploeger, 2001) and (Nguyen *et al.*, 2007). Our results support the finding from the previous study that calves under three months of age are more susceptible to infection than older calves. According to (Kvac, Kouba, & Vitovec, 2006). The animal develops resistance with age as a result of immune development over time. In this regard, according to (Brook, Hart, French, & Christley, 2008), Calves under 4 months old had a 13-fold higher risk of getting a Cryptosporidium infection than older calves did. Additionally, (L. Xiao *et al.*, 2004) concurred that while Cryptosporidium was found in all age groups, younger calves are substantially more likely to contract the disease than older cattle.

The infection rate varied between the different animal breeds that were examined (Table 2). Local breeds had the largest prevalence (20%), followed by Friesian (18%) and Simmental (11%), which had the lowest prevalence. In this investigation, Cryptosporidium was recorded more frequently in females (23%) than males (7%). The current findings, which were higher than those previously reported in Iran by (Ranjbar, 2017) showed that 14.8% of male calves were infected with *C. parvum* as opposed to 16.9% of female calves. This result agrees with (Fikre, 2017), who found that females were more likely to have a cryptosporidium infection (26.7%) than male calves (20.8%).

Mallinath (2009) reported that in India, female calves had a 6% infection rate, whereas male calves had a 3.67% infection rate. According to (Ayinmode, 2010) There was a 38.1% infection rate among female calves in south-western Nigeria, as opposed to a 17.1% infection rate among male calves. DNA analysis of field sequences revealed the presence of two Cryptosporidium species in calves: *C. ryanae* and *C. parvum*.

Based on phylogenetic study, it was determined that the field strains belong to different spp. Two strains (BA/1/2022 and BA/2/2022) were clustered with *C. ryanae*; however, another strain (BA/3/2022) was clustered with *C. parvum* (figure 3). In addition, the field sequence identities found in the current study are closely related to sequences from Turkey, China, and Bangladesh strains (tables 3 and 4). These findings support earlier molecular research on European and North American cattle, which discovered that the primary Cryptosporidium species impacting both diarrheal and healthy suckling calves is *C. parvum* (Santin, Trout, Xiao, Zhou, Greiner, & Fayer, 2004), (Trotz-Williams *et al.*, 2006). These results are consistent with

earlier research and show that the shedding of *C. parvum* oocysts was substantially related to watery diarrhea, especially in calves under 2 weeks old, who had a higher likelihood of being positive than other animals. (Duranti *et al.*, 2009), (Garro, Morici, Utges, Tomazic, & Schnittger, 2016).

CONCLUSIONS

In spite of several outbreaks of diarrhea in Sulaymaniyah province, no reports have been conducted to confirm the causative agents. This may cause a failure in the diagnosis of diarrheal calves. Economic losses will then come due to improper treatment. The results confirmed that Cryptosporidium spp. is one of the causes of diarrhea in newborn calves in Sulaymniyah province and showed that two sequences (BA/1/2022 and BA/2/2022) were *C. ryanae*, while the other sequence (BA/3/2022) was identified as *C. parvum.*

ACKNOWLEDGMENTS

We would like to thank the Veterinary Teaching Hospital belonging to the College of Veterinary Medicine, University of Sulaimani, Sulaymaniyah, for their support in preforming all skin tests and processing all blood samples. This project is funded by the University of Sulaimani, Ministry of Higher education and scientific research, Kurdistan Regional Government, Kurdistan, Iraq.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

STATEMENT OF HUMAN AND ANIMAL RIGHTS

All samples taken of this study were conducted in conceit with the owners according to the approved principles of the ethics by the college of the veterinary medicine research committee, University of Sulaimani, Kurdistan Regional Government, Kurdistan/ Iraq.

DATA AVAILABILITY

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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