MOLECULAR DIAGNOSIS OF *CRYPTOSPORIDIUM SSP.* IN WATER BUFFALOES AT BABYLON PROVINCE, IRAQ

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ABSTRACT

This study was aimed recording the prevalence rates of the *Cryptosporidium* spp. and identify its species in buffalo at Babylon province by using microscopic (flotation and staining) and molecular technique to examined (100) buffalo fecal samples collected randomly from different sexes, ages, and regions, during the period from the beginning of October 2019, to end of March 2020. The percentage of infection with the Cryptosporidiosis was 40% by using the microscopic method, while, nested polymerase chain reaction (nPCR) technique showed the infection rate was 61%, and according to age groups, recorded higher infection 77.5% at (≤6) months age group, followed by age group (>6 -12) months and (>1 -2) years which showed 60% and 50%, besides, the age group (>2) years recorded 40%, which, the lowest infection rate. Also, females recorded (67.74%) as a higher prevalence rate than males (50%). Sequence analysis for ten samples that were positive by (n PCR) technique showed that 6 sequences belong to *Cryptosporidium bovis* (MT150692, MT150693, MT150694, MT150698, MT150699, and MT150701), while 3 sequence belongs to *C. parvum* (MT150696, MT150697, and MT150700), as well as, one of sequence belong to *C. andersoni* (MT150695), with a genetic difference of (0.010%) Datasets suggest strong genetic distinctiveness amongst species. The first diagnose of the *Cryptosporidium* and its species in water buffalo in Iraq by using molecular assay (nPCR).

Key words: Cryptosporidiosis, Nested PCR, phylogenetic tree, Iraq.

التشخيص الجزيئي لطفيلي الابواغ الخبيئة في جاموس محافظة بابل/ العراق

علي رحيم عبدالله العامري عامر مرحم عبد العامري
باحث استاذ دكتور
فرع الطفيليات– كلية الطب البيطري– جامعة بغداد– العراق

المستخلص

تهدف دراستنا إلى تسجيل معدلات انتشار طفيلي الابواغ الخبيئة وتحديد أنواعه في الجاموس في محافظة بابل باستخدام الفحص المجهري التقليدي والتقنية الجزيئية, تم فحص 100 عينة من براز الجاموس جمعت عشوائياً من الجنسين ولمختلف الأعمار والمناطق، خلال الفترة من بداية تشرين الاول 2019 حتى نهاية اذار 2020, وكانت نسبة الإصابة بهذا الطفيلي 40٪ باستخدام الفحص المجهري، بينما أظهرت تقنية تفاعل سلسلة البلمرة المتداخلة (nPCR) أن معدل الإصابة كان 61% . كما سجلت الإناث معدل اصابة أعلى من الذكور. أظهر تحليل التسلسل لعشر عينات كانت إيجابية باستخدام تقنية (n PCR) أن (6) عينات تنتمي إلى *C. bovis* بينما ينتمي (3) عينات إلى *C. parvum*, بالإضافة إلى واحدة من العينات ينتمي إلى *C. andersoni* حسب قاعدة بيانات بنك الجينات العالمي، كما اظهرت مع وجود فرق جيني (0.010%) وهذا يشير إلى تميز وراثي قوي بين الأنواع. الدراسة الاولى لتشخيص لطفيلي الابواغ الخبيئة وأنواعه في جاموس الماء في العراق باستخدام طرق الجزيئية (تقنية تفاعل سلسلة البلمرة المتداخلة).

الكلمات المفتاحية: الابواغ الخبيئة, تفاعل سلسلة البلمرة المتداخلة, الشجرة الوراثية,العراق.

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INTRODUCTION

Cryptosporidium is a protozoan parasite that completes its life cycle in the intestinal and respiratory surface epitheliums of mammals, birds, and reptiles, it's an intracellular extracytoplasmic parasite that acts as a coccidial parasite as a major cause of diarrhea in farm animals and humans worldwide (53). Buffalo cryptosporidiosis is often accompanied by watery diarrhea, which is the most important clinical signs and has contributed to high mortality rates (12). Other signs such as metabolic acidosis and electrolyte loss, anorexia, reduced milk consumption, dehydration, growth disorders, slow walking and depression (18,30). The severity of the disease depends on various factors, including that of the host such as the immune system and accompanied by another infection like rotavirus (44). *Cryptosporidium* infection occurs as a result of the ingestion of parasitic oocysts that are excreted in the feces of infected animals. Therefore, autoinfection due to the presence of thin-walled oocysts can occur within the same host (31). Transfer of *Cryptosporidium* spp. can occur in different ways, through direct or indirect contact from animal to animal, from animal to human (zoonotic transmission), It can also occur due to the ingestion of food or water that is contaminated by infected oocysts, or through vectors such as rodents, arthropods or even birds, they can act as mechanical transmission means (7,27). The diagnosis of cryptosporidiosis is based on the identification of *Cryptosporidium* oocysts in the fecal sample using conventional microscopic and immunodiagnostic methods (19), as well as, molecular techniques (more sensitive and specific) such as the polymerase chain reaction (PCR) are often used today for genotyping cryptosporidiosis (40). Several studies agree on the higher sensitivity of PCR targeting the 18S rRNA gene, Nested PCR was introduced to improve detection sensitivity (28). This study aimed to detection of *Cryptospridium spp*. in water buffalo by using microscopic examination and molecular method (n PCR) in Iraq.

MATERIALS AND METHODS

One hundred buffalo fecal samples collected randomly from different sexes, ages, and regions of Babylon province (Al-Hilla, Abi-Gharaq, Al-Musayib, Al-Sadda, Al-Mahaweal, Al-Kafel, and Al-Hashimiyah). during the period from the beginning of October 2019, to end of March 2020. The microscope act as the primary diagnosis method by using sheather's flotation, modified Ziehl-Neelsen staining and calibration by ocular micrometer to investigate and measurements of *Cryptosporidium* oocyst, (29), and finally samples saved in the freeze until DNA extracted to detect *Cryptosporidium* by Molecular diagnosis (nested PCR), the DNA extracts according to the manufacturer's instructions (AccuPower® PCR Premix Kit) from Bioneer company, based on the 18S ribosomal rRNA gene (41). The primers of nPCR are designed according to the instructions of the Korean manufacturer (Macrogen company) table (1), then placed in PCR Thermocycler in a condition that using an n-PCR thermal cycler system according to (41). We constructed a phylogenetic tree for our *Cryptosporidium* versus NCBI-Blast-GenBank. The positive PCR 18S rRNA gene was analyzed for DNA sequencing (Molecular Evolutionary Genetics Analysis version 6.0) and Multiple sequence alignment analysis (ClustalW). Evolutionary distances were computed by the Maximum Composite Likelihood as described by (48). For statistical analysis, using SPSS version 17 and, Chi-square (X2) test was used for comparison between the results, and the differences were considered statistically significant at ($P \leq 0.05$) (37).

Table 1. The primer with their sequence

Primer	Sequence 5'-3'	Amplicon	Company\country
***18SrRNA*gen *Cryptosporidium* spp. PCR**	**F- AGACGGTAGGGTATTGGCCT R- TCCTTGGCAAATGCTTTCGC**	**616bp**	**Macrogen\ Korea**
***18SrRNA*gen *Cryptosporidium* spp. Nested PCR**	**F-AACGGGAATTAGGGTTCGA R- TGCTTTCGCATTAGTTTGTCTT**	**567bp**	

RESULTS AND DISCUSSION

Our results showed the prevalence rate of cryptosporidiosis was 40% by the conventional microscopic examination method (sheather's flotation, modified Ziehl-Neelsen staining and calibration by using ocular micrometer for oocyst measurements), and the recognized of the oocyst of the *Cryptosporidium* depend on the morphologically characteristic, the oocysts of *Cryptosporidium* bovis is similar to *C. parvum*, in Sheather's (sugar) floatation smear, the oocysts appeared as spherical/ rounded in shape with a thin greenish membrane, the four sporozoites looked as black bodies inside the oocysts (Fig. 1), while, in stained with (mZN) stain appeared as spherical/ rounded in shape stained red bodies with a clear halo around the oocyst, against a dark blue background of the methylene blue stain, and the measurement was (4.3 x 4.8 ± 0.8) µm by ocular micrometer (Fig.2).

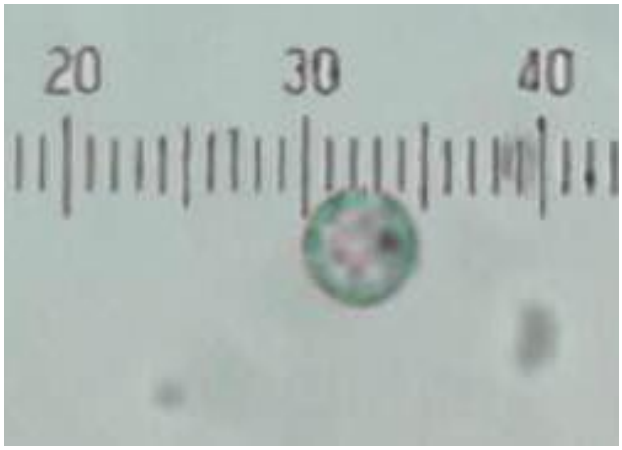

Figure 1. *C. parvum* oocysts floatation with Sheather's solution, magnification (100X)

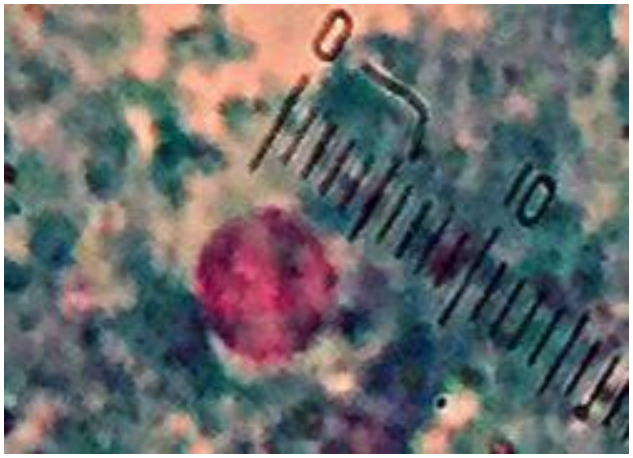

Figure 2. *C. parvum* oocysts, stained with Modified Ziehl Neelsen magnification (100X)

And the morphology of *C. andersoni* oocysts in Sheather's (sugar) floatation, appeared as round or oval in shape with a thin greenish membrane while the four sporozoites looked as black bodies inside the oocysts (Fig. 3), while in mZN stain the oocyst appeared as red ovoid bodies with a clear halo around the oocyst against a dark blue background of the methylene blue stain and the average length and width measurements was (7.0 x 5.6 ± 0.8) µm (Fig. 4).

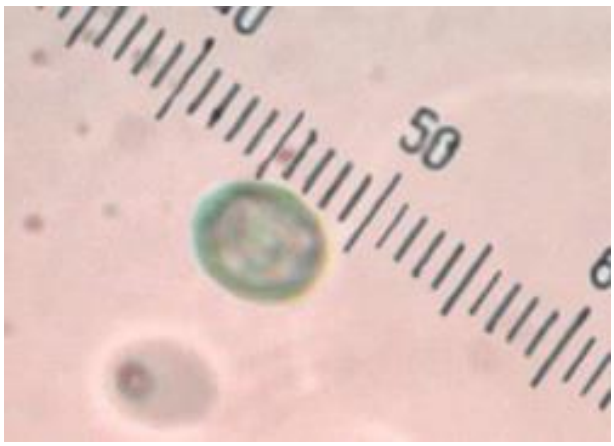

Figure3.*C. andersoni* oocysts, floatation with Sheather's solution magnification (100 X)

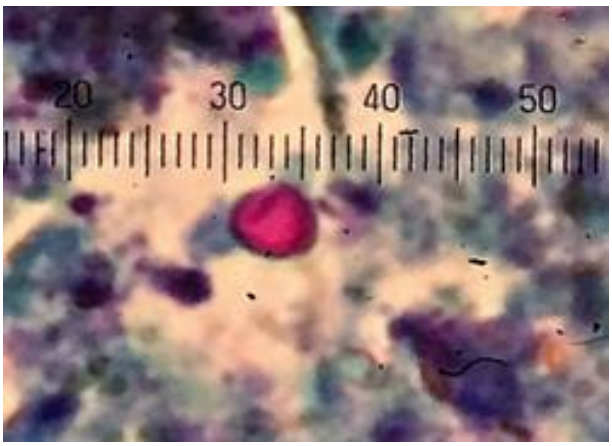

Figure 4. *C. andersoni* oocysts stained with Modified Ziehl Neelsen magnification (100X)

While by using the molecular technique (Nested PCR) the results showed that the total infection rate of *Cryptosporidium* spp. in buffalo was 61% (Table 2), the statistical analysis showed significant differences between these two techniques and its relation with sensitivity and specificity of each diagnostic technique ($p<0.05$). By nPCR, the fecal samples exhibited a distinct band of 567 bp on agarose gel for *Cryptosporidium* spp. as depicted (Fig. 5).

Table 2. Total prevalence of *Cryptosporidium* infection by microscopy and Nested PCR in water buffalo fecal sample

Host	No. of samples examined	Conventional microscopy No. of positive (%)	Molecular-Nested PCR No. of positive (%)
buffalo	100	40 (40%)	61 (61%)

P<0.05

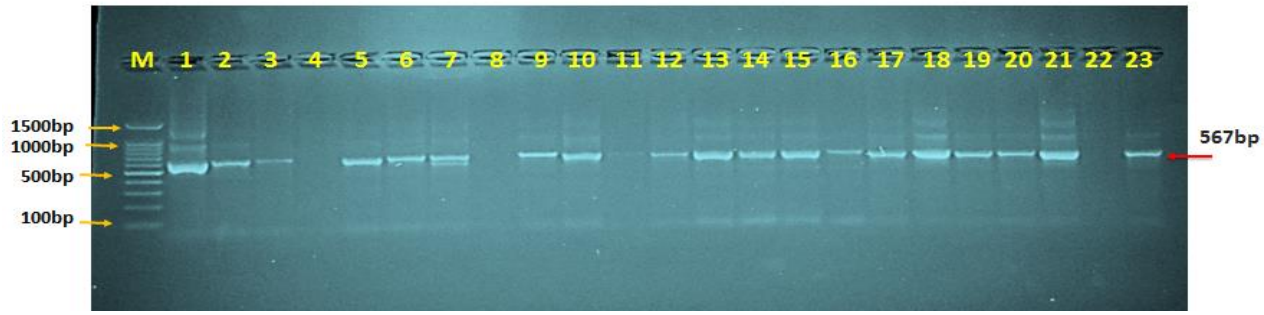

Fig. 5. Agarose gel electrophoresis image that showed the nPCR product analysis of 18S rRNA gene in *Cryptosporidium* spp. from buffalo fecal samples. Where M: marker (1500-100bp) and Lane (1-23) some positive *Cryptosporidium* spp. were showed at (567bp) nested PCR product.

The results showed a significant difference was ($p < 0.05$) in the prevalence rate among different age groups (Table 3) showed higher infection 77.5% (31/40) observed at (≤ 6) months age group, followed by age group (>6 -12) months and (>1-2) years which showed 60% (12/20) and 50% (10/20), in addition, the age group (>2) years recorded the lowest infection rate which was 40% (8/20).

Table 3. Prevalence of *Cryptosporidium* infection by nested PCR in relation to age groups of water buffaloes

Age groups	No. of samples examined	No. of positive	Percentage (%)
≤ 6 Months	40	31	77.5
>6 – 12 Months	20	12	60
>1-2 Years	20	10	50
>2 Years	20	8	40

P<0.05

According to the sex of animal the significant difference was ($p < 0.05$), the female recorded higher prevalence rate than the male, (67.74%) in females and (50%) in males (Table 4).

Table 4. Prevalence of *Cryptosporidium* infection by n PCR in relation to sex of water buffalo

Sex of buffalo	No. of samples examined	No. of positive	Percentage (%)
Male	38	19	50
Female	62	42	67.74

P<0.05

Ten samples randomly selected from water buffalo were positive by nested PCR, the obtained sequences of nested PCR products were analyzed by using the NCBI BLAST tool, and the results showed the presence of three *Cryptosporidium* species in the buffaloes, namely *C. bovis C. parvum*, and *C. andersoni*. and *C. bovis* had the highest incidence in the analysis samples (6/10), followed by *C.parvum* (3/10), and *C. andersoni* (1/10) (Table 5).

Table 5.*Cryptosporidium* strains in water buffalo and NCBI BLAST homology sequences

Cryptosporidium spp. isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)		
		Identical NCBI BLAST *Cryptosporidium* species	Genbank Accession number	Identity (%)
Cryptosporidium spp. IQ-buffalo isolate No.1	MT150692	*Cryptosporidium bovis*	MK982466.1	99.42%
Cryptosporidium spp. IQ-buffalo isolate No.2	MT150693	*Cryptosporidium bovis*	MK982466.1	99.62%
Cryptosporidium spp. IQ-buffalo isolate No.3	MT150694	*Cryptosporidium bovis*	MK982466.1	99.61%
Cryptosporidium spp. IQ-buffalo isolate No.4	MT150695	*Cryptosporidium andersoni*	KF271468.1	99.43%
Cryptosporidium spp. IQ-buffalo isolate No.5	MT150696	*Cryptosporidium parvum*	AY458612.1	99.63%
Cryptosporidium spp. IQ-buffalo isolate No.6	MT150697	*Cryptosporidium parvum*	AY458612.1	99.26%
Cryptosporidium spp. IQ-buffalo isolate No.7	MT150698	*Cryptosporidium bovis*	MK982466.1	99.81%
Cryptosporidium spp. IQ-buffalo isolate No.8	MT150699	*Cryptosporidium bovis*	MK982466.1	99.65%
Cryptosporidium spp. IQ-buffalo isolate No.9	MT150700	*Cryptosporidium parvum*	AY458612.1	99.26%
Cryptosporidium spp. IQ-buffalo isolate No.10	MT150701	*Cryptosporidium bovis*	MK982466.1	99.22%

A Phylogenetic tree of *C. bovis*, *C. parvum*, and *C. andersoni* referenced against those of Gen-Bank which highlight differences by DNA STAR (48). *Cryptosporidium* spp. (1, 2, 3, 7, 8 and 10) were closely related to NCBI-Blast *Cryptosporidium bovis* (MK982466), (5, 6 and 9) were closely related to NCBI-Blast *Cryptosporidium parvum* (MK982466), and No.(4) to NCBI-Blast *Cryptosporidium andersoni* isolates (KF271468) with a genetic difference of (0.010%) Datasets suggest strong genetic distinctiveness amongst species (Fig.6).

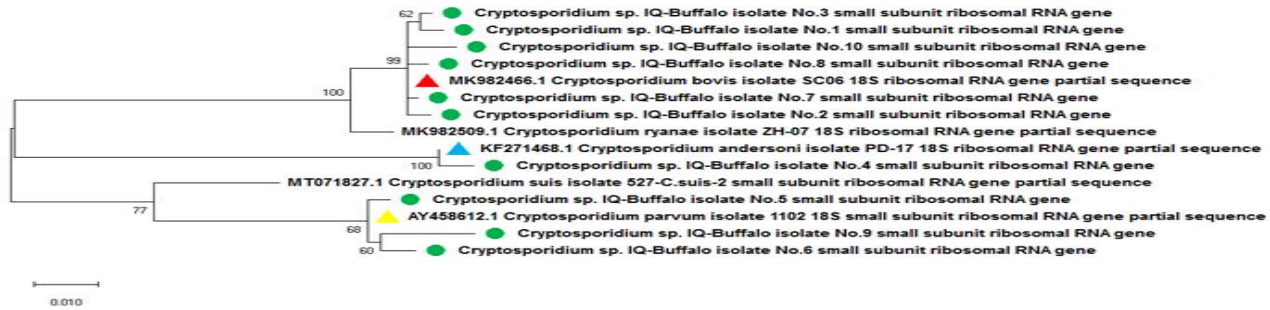

Figure 6. Phylogenetic tree analysis based on the partial sequence Small subunit *rRNA gene* in *Cryptosporidium* spp. isolated from fecal of water buffalo and *Cryptosporidium* species genetic identification.

The morphology of *Cryptosporidium* species oocysts were isolated from the feces of the naturally infected animal that were completely sporulated, spherical, ovoid-shaped to ellipsoidal with a thin greenish membrane. It contains four sporozoites that resemble black bodies in oocysts on a wet smear (floatation) and red ovoid-shaped bodies with a transparent halo around the oocyst on a dark blue background of methylene blue in (mZN), but with slightly different dimensions. The oocysts of the two species: *C. parvum and C. bovis* had almost the same morphological properties and measured (4.4 x 4.8 ± 0.8) µm by ocular micrometer, and the average length and width of *C. andersoni* oocysts were (7.0 x 5.6 ± 0.7) µm. These results were in agreement with the results of (34) reported the *C. parvum* oocyst measurements were (3.2-5.8 x 4.3-4.9) µm in size in Egypt, in the province of Babylon (Iraq), the average length of the *C. andersoni* oocysts was (7.1 ± 0.92) µm and width (5.6 ± 0.61) µm (5), In the United States, reported the mean dimensions of *C. andersoni* was (7.30 x 5.19) µm (50), and in the city of Baghdad recorded the dimensions of *C. parvum* were (4.9 x 4.3 ± 0.9) (5). The overall prevalence of cryptosporidiosis in buffalo in this study was 40% by conventional microscopic examination and 61% by molecular technique (nested PCR), and the agreement or disagreement with the results of other studies depends on the different factors that affected on the result between countries such as the sampling plan, study season, level of hygiene measures applied and the diagnostic methods used, besides, attributed to the system of rearing, water supply, management, and sanitary conditions in and around the farms (21). Microscopically, in Baghdad city, recorded the infection rate was 53.93% in buffalo (3), in Salah al-Din Province, the prevalence rate was 31.5% reported by (2), and reported the prevalence rate of cryptosporidiosis in buffalo was 10% in Babylon Province (39). Also, the prevalence of infection in water buffalo in different regions of the world, such as in Egypt, recorded the prevalence rate of *C. parvum* was 22.5% in water buffalo (45). In the calves, reported the infection rate was 21.8% in diarrheal calves in Turkey (26), in Iran, recorded the infection rate of diarrheal buffalo calves were 45.5%, and 2.5%, respectively by (10,49), and in India, Sudan and Thailand, recorded the infection rate were (21%, 85%, 58.3%, and 51%) respectively, in diarrhoeic bovin calves (14,42,47). By molecular (nPCR) examinatiom, we recorded a higher prevalence rate of Cryptosporidiosis (61%) in this study. The widespread use of the SSU rRNA gene in the detection/genotyping of *Cryptosporidium* is mainly due to the multiple copies of the gene and the presence of semi-conserved and hypervariable regions that facilitate the design of genus-specific primers as well as, the 18S rRNA-based PCR protocol was shown to be more sensitive than the modified Ziehl-Neelsen (mZN) staining technique (17,52,55). The agreement or disagreement with the other study depends on several factors mentioned previously that make it expected that there will be a difference in the results of studies for different countries and regions such as in Pakistan, recorded infection rate was 24% in buffaloes (36), and the prevalence rate was 37.5% in water buffalos in Nepal recorded by (20). However, the prevalence rate of *Cryptosporidium* infection was 73.3% of the herds and 32.2% of the individual buffalo in the positive herds in Egypt (23). In Australia, the prevalence rate of *Cryptosporidium* in water buffaloes was 30%

recorded by (53), and reported the prevalence rate of *Cryptosporidium* infection was 48.2% in Brazil (9). According to age groups the results showed the highest rate in the age group (≤ 6) and the lowest rate in the age group (>2) years. The higher prevalence of infection in newborns is due to lower tolerance levels in young newborns due to the poor development of acquired immunity (46). Our results were consistent with other studies showing that younger age groups have the highest infection rate, which decrease with age of the animal increase, this corresponds to the studies have shown in the, in Egypt by (15) for buffalo calves, in Australia by (1) in water buffalos, and in India by (46) in buffaloes. According to the sex, our study showed the female was higher proportion than male. It has been agreed with other studies that indicate that animals raised in the same place and exposed to the same conditions have close prevalence rates where male or female have no factors that facilitate infection, but the highest proportion found in adult females could be due to the stress during pregnancy, parturition and milking times, which make the females more prone to the infection (6,35,51). In addition to the PCR technique the phylogenic tree analysis act as one of the good ways to detect and identify Genetic variants.(16). The DNA Sequence of the ten samples showed the presence of three species of *Cryptosporidium* in water buffalo, they are *C. bovis, C. parvum,* and *C. andersoni* and the most prevalent species was: *C. bovis*. Our results were consistent with other results that recorded in many studies such as in Egypt reported *Cryptosporidium parvum, C. ryanae, C. bovis* and combinations of *C. parvum* plus *C. andersoni* in buffalos (23), In China, *Cryptosporidium parvum, C. bovis, C. andersoni* and *C. ryanae* were recorded by (32,38,54). In Egypt, the identification of *Cryptosporidium* species / genotypes were *C. ryanae* (~ 59%) and *C. parvum* (~ 41%) in buffalo calves was reported by (33), while, the results were *C. parvum* (88.2%) and *C. ryanae* (11.8%) in buffaloes in Thailand (27). Two species of *Cryptosporidium, C. parvum* and *C. bovis*, have been identified in buffalo fecal samples, *Cryptosporidium parvum* is the most frequently classified as positive(~80%) in farmed buffaloes in Australia (1,53). Other studies from several countries have reported that *C. parvum* is alone or the predominant species in buffalo calves, such as in Egypt, Spain, Italy, and India (8,11,22,46), also in adult buffalo such as the Italian, South Africa, and Egypt (13,25, 43). This study described the diversity of *Cryptosporidium* in buffalo in Babylon province/Iraq. Where, three species of *Cryptosporidium* were detected in this study, *C. bovis, C. parvum* and *C. andersoni*, and the absence of the other *Cryptosporidium* species that infected buffalo in this study can be attributed to the small number of samples collected for sequence analysis that did not fully cover the study area and therefore would not represent the total number of buffaloes present in them or possibly due to the low distribution of these species in our study area.

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