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RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Analysis of *18S rRNA*, *gp60* and *HSP70* Genes of *Cryptosporidium parvum* Isolated from Cattle Owners and Cattle using Nested PCR

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ABSTRACT

Cryptosporidium (C.) parvum is the most prevalent species in the middle-east countries especially in Iraq and its zoonotic importance is not well documented. Hence, the current work included the study of the genetic profile of *Cryptosporidium* species from the fecal samples of infected cattle owners and cattle. Two hundred stool specimens were collected from cattle owners complaining of watery diarrhea and 100 fecal samples from diarrheic cattle in several rural areas of Zakho district. All stool specimens were analyzed microscopically using modified Ziehl Neelsen stain technique and genetically using traditional PCR approach targeting *18S rRNA*, *HSP70* and *gp60* genes. The prevalence of Cryptosporidiosis in humans was 70% (140/200) and among infected cattle was 62% (62/100) by using modified Ziehl-Neelsen stain. On other hand, the molecular analysis (PCR) identified Cryptosporidiosis in 74.5% (149/200) among human samples and 65% (65/100) among cattle samples. The similarity in the analysis of Targeting genes presented that all isolates were identified as *C. parvum* which have 82–99.8 % similarity to other isolates from Iraq, China and USA. The phylogenetic analysis based on *gp60* sequences of *Cryptosporidium parvum* revealed two subtypes belonging to family subtypes IIa (IIdA21G1 and IIdA19G1). This study confirms the prevalence of *C. parvum* among humans and cattle in this area. Furthermore, the *C. parvum* isolated from cattle are genetically identical to those found in humans.

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INTRODUCTION

Cryptosporidiosis is a neglected zoonotic disease caused by the obligate intracellular apicomplexan, the *Cryptosporidium* (C.) species. This parasite invades the microvillus boundary of the gastrointestinal epithelium of the host (Ryan *et al*., 2014; Wang *et al*., 2018). Cryptosporidiosis is a zoonotic waterborne diarrheal disease prevalent among communities with limited resources and poor socioeconomic status (Parghi *et al*., 2014; Checkley *et al*., 2015). It is transmitted via drinking water contaminated with oocysts, that causes gastrointestinal symptoms (Pielok *et al*., 2019). Children and immunocompromised patients are more likely to be infected by *Cryptosporidium* that causes acute diarrhea (Hijjawi *et al*., 2017; Mohaghegh *et al*., 2017; Daniels *et al*., 2018; Zueter *et al*., 2019). The prevalence of the disease varies among different communities depending on the environmental, climatic and sanitary conditions (El-Kady *et al*., 2018). C. parvum naturally infects cattle, goats and sheep in addition to humans and occasionally can infect other mammals such as mice and dogs and most

of these animals were infected with host-specific *Cryptosporidium* spp. (Al- Zubaidei and Kawan, 2020). Close contact with infected dogs can result in the spread of the Cryptosporidiosis to humans and facilitate the transmission of zoonotic diseases (Hunter and Thompson, 2005). *C. parvum* is reported in canines and humans from both developed and under-developed nations (AL-Yasary and Faraj, 2021). Humans acquire Cryptosporidiosis by several routes, including the drinking of water or ingesting food contaminated with oocyst as well as direct contact with animals (Xiao, 2010).

Detecting oocysts in a stool smear is the most common method for diagnosing *Cryptosporidium* (Casimiro et al., 2009). The diagnosis relies on samples stained with modified acid-fast stain especially prepared for *Cryptosporidium* oocyst (Adam et al., 2007). Nawa days, molecular methods were developed for precise specific identification of the parasite by using Nested PCR techniques for targeting different DNA markers (Calderaro *et al*., 2011; Martín-Ampudia *et al*., 2012). The *Cryptosporidium* epidemiological role in humans and animals can be efficiently determined and supported by a molecular approach using several coding genes such as *18S rRNA*, *HSP70* and *gp60*. The gene for the heat shock protein (*HSP*) is a member of a multigene family that is extremely conserved throughout eukaryotes as well as under a variety of stressful circumstances, *HSP70* shields and maintains cells (Sulaiman *et al*., 2000). The most popular marker for subtyping *C. parvum*, is the 60-kDa glycoprotein gene (*gp60*), which has proven to be a valuable indicator for detecting infection origins, genetic variability, and host adaptation (Yan *et al*., 2017).

The microscopic diagnosis of Cryptosporidiosis is considered by many researches to be suboptimal method due to its poor sensitivity, time consuming and required skilled microscopists. This feature has prompted a shift towards alternative diagnostic techniques such as molecular techniques by using *18S rRNA* for identing distinct species of *Cryptosporidium* and using both *gp60* and *HSP70* for demonstrating a high polymorphism in different species. However, in Duhok province, no attempts have been made for using molecular analysis to detect Cryptosporidiosis among human and cattle by using both *gp60* and *HSP70* genes. Thus, the current study aimed to confirm the genotypes of *C. parvum* in samples collected from humans and cattle in this province.

MATERIALS AND METHODS

Specimens' collection: The study was carried out during the period from August 2021 to January 2022 in Zakho district, Duhok governorate. A total of 300 fecal specimens were included, 200 were collected from cattle owners aged 30 to 50 years who were in close contacts with their animals and suffering from acute or persistent diarrhea and 100 fecal samples were collected from their cattle in multiple fields in Zakho districts, Duhok governorate, Iraq. All fecal samples were aseptically collected and transferred within one hour for processing to the microbiology laboratory at Zakho technical institute, Kurdistan region, Iraq.

Direct stool examination: Each stool sample was examined microscopically using the modified Ziehl-Neelsen technique, then all specimens were kept at -80˚C for molecular analysis (Morgan *et al.*, 1998).

Genomic DNA extraction: The Presto Stool DNA extraction Kit (Geneaid, Taiwan) was used for DNA extraction depending on the manufacturer's guideline. The purity and concentration of the extracted DNA were estimated using NanoDrop (Thermoscientific, U.S.A, 2000), then the extracted DNA was stored at -80°C in a deep freezer until used (Morgan *et al.*, 1998).

Molecular identification using Nested PCR: Nested-PCR technique was used for the identification of *Cryptosporidium* spp. by targeting *18S Rrna*, *HSP70* and *gp60* genes (Zhou *et al.*, 2004; Kuzehkanan *et al.*, 2011; Ghaffari *et al.*, 2014). The Nested polymerase chain reaction was performed using a Gene Amp Thermocycler under the appropriate setting for both first and second PCR primers for each gene used as shown in Table 1.

Amplification of polymerase chain reaction was made for each of the primers in a total reaction volume of 40µl reaction tube containing a mixture of Crystal Hot Start DNA Master (20µl), 8µl of primers (forward and reverse primer) at a concentration of 10pmol, DNA samples (4µl) and Nuclease free water (8µl). Then the PCR products were examined electrophoretically in an agarose gel (1%) stained with RedSafe DNA Dye.

Sequence analysis and phylogenic tree: For DNA sequencing, the obtained PCR products were submitted to Macrogen company (South Korea) using sanger sequenced method and the obtained sequences were cleaned and trimmed using BioEdit software. Then these sequences were submitted to NCBI-GenBank for registration. The phylogenic tree was conducted using MEGA software using the Maximum-Likelihood Method and Jukes-Cantor model for comparing the obtained sequences with the available corresponding nucleotide sequences (Jukes and Cantor, 1969).

Statistical analysis: All collected data were analyzed using SPSS version 25 software, represented as numbers and percentages, calculated with a confidence interval of 95%. Chi-squared (X^2) test was used to determine whether or not there is a statistically significant difference in the prevalence of *Cryptosporidium* with the independent variables. *P*-value ≤ 0.05 was considered significant, and more than this value was considered non-significant.

RESULTS

The total prevalence of Cryptosporidiosis by using conventional microscopic examination was 67.33% (202/300). The rate of Cryptosporidiosis. in humans was 70% (140/200) and among infected cattle was 62% (62/100). On the other hand, molecular analysis (Nested-PCR) of Cryptosporidiosis was 74.5% (149/200) among human samples and 65% (65/100) among cattle samples (Table 2). Samples found positive with Nested-PCR were also positive with microscopic examination, in addition twelve microscopically negative samples (9 from humans and 3 from cattle) were found positive by Nested-PCR. Hence Nested-PCR displayed higher sensitivities than

microscopic analyses. The difference between the prevalence of *Cryptosporidium* using both methods were non-significant ($P>0.05$).

Nested-PCR confirmed the presence of *Cryptosporidium* spp. with an amplicon size of 347 bps (*18S rRNA*); 1400 bps (*gp60*) and 448 bps (*HSP70*) on 1% agarose gel in the first round. On the second round, all PCR products showed specific bands of 240 bps for *18S rRNA* gene, 800 bps for *gp60* gene and 325 bps for *HSP70* gene (Figure 1-3). *C. parvum* were analyzed using BLAST program on NCBI based on *18S rRNA* and *HSP70* genes. The obtained sequences of *18S rRNA* (four from human and three from cattle) were recorded in under the following accession numbers: OM246566, OM089648, OM085741, MZ893463, MZ893462, MZ890125 and MZ889674, while sequences of *HSP70* were registered under accession numbers OR542559 (Human) and OR532267 (cattle). The remaining isolates could not be sequenced due to insufficient DNA in template. *C. parvum* was subtyped by *gp60* gene sequence analysis and only two samples were successfully amplified and registered under accession numbers OR532268 (human) and OR532269 (Cattle). However, the remaining isolates could not be sequenced due to insufficient DNA in template. The alignments of these sequences with reference sequences showed that both isolates belong to two different subtypes IIdA21G1 (Human) and IIdA19G1 (cattle).

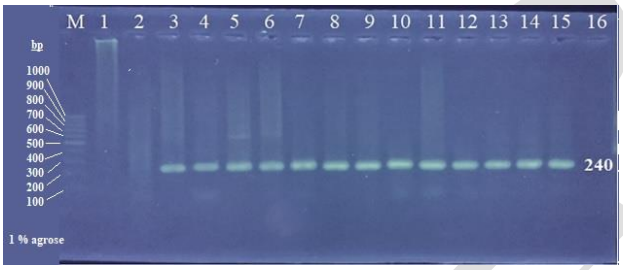

Fig. 1: Agarose gel electrophoresis (1%) of Nested-PCR products. Lane (M) DNA ladder (100-1000 bp), Lanes from 4-15: *Cryptosporidium* samples with amplicon size 240 bps using primers *18S rRNA* (second round), Lane 1: negative control; Lane 3: Positive control.

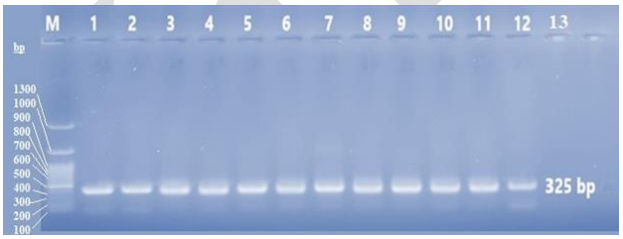

Fig. 2: Agarose gel electrophoresis (1%) of Nested-PCR products. Lane (M) DNA ladder (100-1300 bp), Lanes from 1-11: *Cryptosporidium* samples with amplicon size 325 bps using primers *HSP70* (second round), Lane 12: Positive control; Lane 13: negative control.

Phylogenetic analysis using *18S rRNA* gene revealed that *C. parvum* isolated from human and cattle are genetically different, three isolates (MZ889674, OM246566 and MZ890125) are sister group and form a well-supported clade with other sequences from China (MT757967, MT229421 and MT757969) and Iraq (MT329019 and MT476894) with 76% of the bootstraps. The sequence (MZ893463) form clade with other sequences. Both (OM089648) and (OM85741) form sister taxa with bootstrap 71% and the isolate (MZ893462) form clade with other isolates from China (MT648441 and MT229411) and USA (MT524967) with bootstraps 95% (Figure 4).

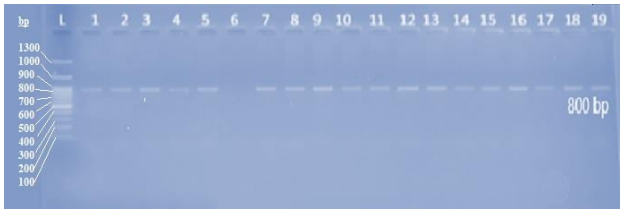

Fig. 3: Agarose gel electrophoresis (1%) of Nested-PCR products. Lane (M) DNA ladder (100-1300 bp), Lanes from 2-5,7-19: *Cryptosporidium* samples with amplicon size 800 bps using primers *gp60* gene (second round), Lane 1: Positive control; Lane 6: negative control.

Phylogenetic investigation based on *HSP70* gene showed that *C. parvum* isolated from human (OR542559) form clade with other isolates from Sweden (KU892574), Czech (KF612325), South Korea (KX342050) and UK (KM085024) with bootstrap 78% and isolate from cattle (OR532267) form clade with other isolates from Spain (MK609845), China (KC885897) and Netherlands (DQ388384) with bootstrap 24% as shown in Figure 5.

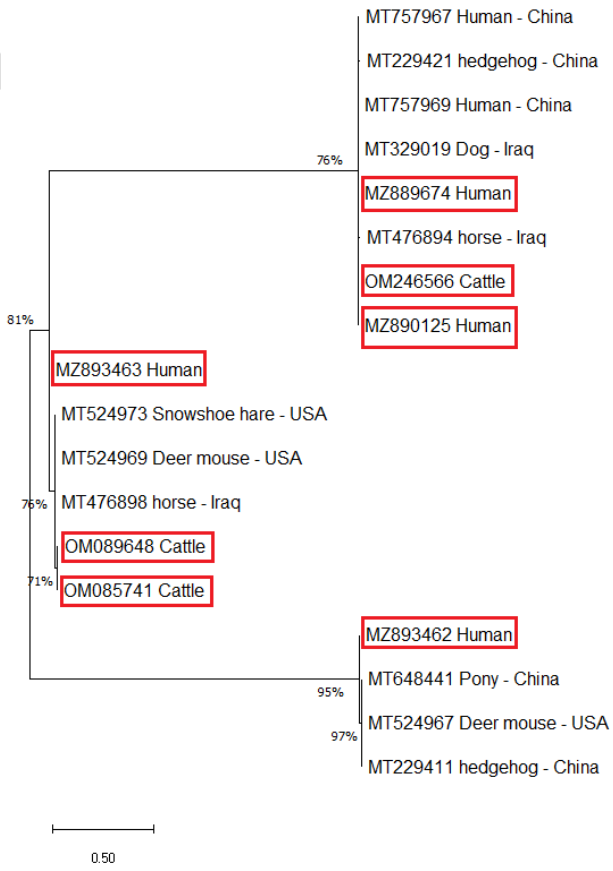

Fig. 4: Phylogenetic tree based on the *18S RNA* among *Cryptosporidium* species isolated from human and cattle. The analysis of the nucleotide sequences was conducted by a Maximum Likehood tree using MEGA-11 software. Red rectangles represent sequences generated in the present study.

DISCUSSION

This study showed that *C. parvum* has been identified the only species of *Cryptosporidium* isolated from human

Table 1: Primers used for amplification of *Cryptosporidium parvum* using Nested PCR.

Gene	Primer 5'-3'	Size (bp)	°C	Reference
18S rRNA	F: GGTGACTCATAATAACTTTACGG	347	59	Kuzehkanan *et al.,* 2011
	R: ACGCTATTGGAGCTGGAATTAC			
	F: TAAACGGTAGGGTATTGGCCT	240	60	
	R: CAGACTTGCCCTCCAATTGATA			
gp60	F: ATAGTCTCCGCTGTATTC	1400	50	Ghaffari *et al.,* 2014
	R: GGAAGGAACGATGTATCT			
	F: TCCGCTGTATTCTCAGCC	800	51	
	R: GCAGAGGAACCAGCATC			
HSP70	F: GGTGGTGGTACTTTTGATGTAT	448	52	Zhou *et al.,* 2004
	R: GCCTGAACCTTTGGAATACG			
	F: GCTGSTGATACTCACTTGGGTGG	325	52	
	R: CTCTTGTCCATACCAGCATCC			

Table 2: The infection rate of Cryptosporidiosis by conventional microscopic method and molecular technique in human and cattle.

Host	No. of fecal samples examined	Microscopic examination No. infected (%)	Nested PCR No. Infected (%)
Human	200	140 (70)	149 (74.5)
Cattle	100	62 (62)	65 (65)
Total	300	202 (67.33)	214 (71.33)
		P value = 0.944	

and cattle may be due to the close physical contact between cattle and human particularly among rural area, which is consistent with the results recorded in earlier studies performed among children in Diwaniyah city and among domestic dogs and humans in Karbala province (Chalmers *et al.*, 2019; Al-Difaie *et al.*, 2020). In the Middle East countries, *C. parvum* is considered as the most prevalent species detected in humans (Caccio and Putignani, 2014). Although, microscopic examination of stool using modified Ziehl-Neelsen techniques remains traditional standard test for detection of *Cryptosporidium* oocyst, nucleic acid amplification using nested-PCR may also play an important role in the detection of the *Cryptosporidium*. The small number of oocysts present in fecal samples can potentially make the microscopic analysis results erratic as well as microscopic examination based on morphological features alone , so this method is

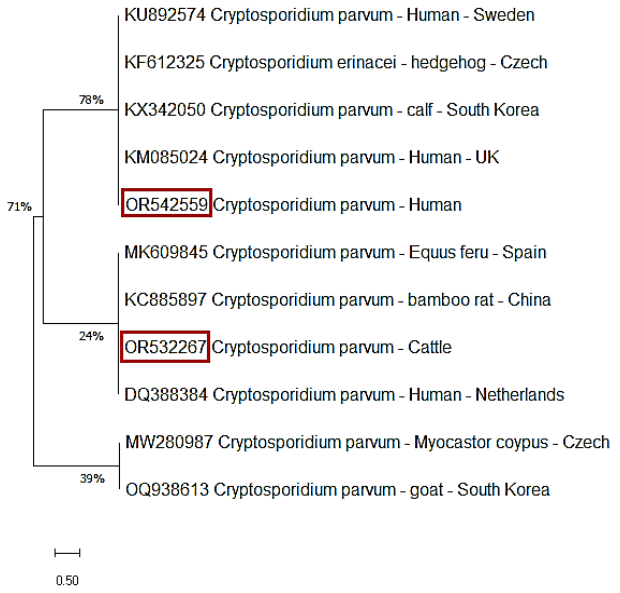

Fig. 5: Phylogenetic tree based on the *HSP70* among *Cryptosporidium* species isolated from human and cattle. The analysis of the nucleotide sequences was conducted by a Maximum Likelihood tree using MEGA-11 software. Red rectangles represent sequences generated in the present study.

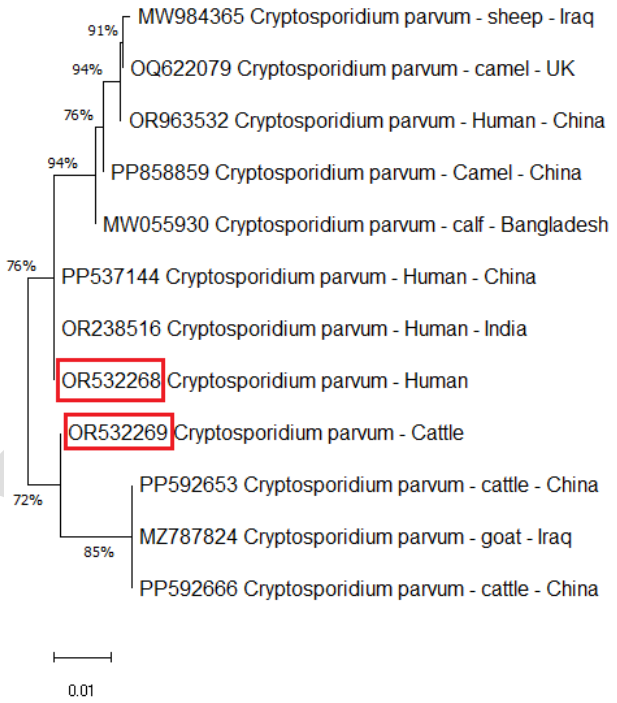

Fig. 6: Phylogenetic tree based on the *gp60* among *Cryptosporidium* species isolated from human and cattle. The analysis of the nucleotide sequences was conducted by a Maximum Likelihood tree using MEGA-11 software. Red rectangles represent sequences generated in the present study.

not considered as a precise tool for differentiation between *Cryptosporidium* spp. since, the parasite oocysts are small in size and lack distinctive morphological characters (Ryan *et al.*, 2014). Therefore, molecular genotyping is more confirmative, and have been used most frequently for the identification and differentiation of species. Few studies on the molecular identification of different species of *Cryptosporidium* from animals and humans have been conducted in Iraq and the identified species included: *C. hominis* and *C. parvum* (Abdul-Sada, 2015; Jawad, 2015; Merdaw *et al.*, 2018; Ahmed *et al.*, 2023).

Cryptosporidiosis is a waterborne disease transmitted through the oral route, due to the contamination of tap water with animals' feces containing the parasite oocysts. This condition is regarded as a public health problem, particularly among people in close contact with domestic animals living in rural area, as indicated from the high infection rate (67.33% microscopically and 71.33%, by Nested PCR) among both humans and their livestock. A higher rate than the present rate, which was 87.57% was reported among the population of Zakho city (Ahmed *et al*, 2022). On the other hand, the infection rates in the

current study are higher than the rates reported in other studies conducted in different cities of Iraq; In Baghdad, the rate of infection among children was 2.2% by using modified Ziehl-Neelsen stain and 3.9% by using Real time PCR (Shakir and Hussein, 2015). In Al-Diwaniyah, the rate of Cryptosporidiosis was 24% among infected humans and 14% among infected camel by using PCR assay technique (Ahmed *et al.,* 2016). Another study conducted in AL-Muthanna reported total rates of infection among children as 21% and 18 % by using modified Ziehl-Neelsen stain and Real time PCR, respectively (Jomah and Mallah, 2016). In Al-Najaf, a study reported that the rate of the Cryptosporidiosis among children was 12.8% by using modified Ziehl-Neelsen stain (Tairsh *et al.,* 2017). In Erbil, a study revealed a rate of 100% among children by using PCR (Azeez and Alsakee, 2017). In Duhok, a rate of 26.15% was reported among cattle using Modified Ziehl-Neelsen Method (Al-Saeed *et al.,* 2019).

The variation in the rates of these studies may be due to many reasons, such as the differences in the studied population; diagnostic methods used, seasonal variations, source of the infection and environmental conditions (Huang *et al.,* 2004; Roy *et al.,* 2004). Another risk factor which could influence the prevalence of Cryptosporidiosis in Iraq is socioeconomic status, since the rates of the infection are high among overcrowded families and in areas with poor hygiene (Al-Warid *et al.,* 2012; Salman, 2014; Korpe *et al.,* 2018). Rural dwellers had higher rates of infection as compared to urban inhabitance, due to increased chances of contact with domestic animals (Ghenghesh *et al.,* 2012).

Recently nested PCR was used as the most modern molecular technique for the detection of the parasites as the morphological characterization of the genus is not useful and create a difficulty in identifying the oocysts of the parasite. In our study, twelve PCR positive samples were negative by microscopic technique this might be attributed to insufficient number of oocysts that passed with the stool which could be below the threshold for detection (Helmy *et al.,* 2013).

Hence Nested PCR is more reliable as it prevents the nonspecific bands and increases the chance to detect low concentration of the parasites.

The phylogenetic studies of *Cryptosporidium* using *HSP70* gene have numerous advantages over the *18S rRNA* gene due to the fact that the *HSP70* gene is more tolerant to deletion and insertion mutations and aligning sequences from extremely diverse organisms is substantially easier (Al-Musawi *et al.,* 2022). Therefore, the sequences of *HSP70* gene showed more heterogeneity than the sequences of *18S rRNA* gene, making it a better target for genetic analysis. In our study, both *HSP70* and *18S rRNA* were used for phylogenetic analysis and there were minor differences in bootstrap values in phylogenetic tree of both genes. This study's sequences of the *HSP70* gene will help in improving molecular analysis. The sequences analysis of *gp60* gene revealed that the unique IId subtype family is present in human and cattle, which were achieved by the GenBank database, and these subtype families are IIdA21G1and IIdA19G1. Both of these subtypes (IIdA21G1 and IIdA19G1) were recorded previously in goats in Iraq (Al-Musawi *et al.,* 2022). According to our results, these subtype families are considered as zoonotic subtypes and were recorded for the first time among cattle in the current study in Iraq. The present findings are in line with other results recorded in Portugal, China and Sudan (Alves *et al.,* 2006*;* Wang *et al.,* 2013; Peng *et al.,* 2016; Taha *et al.,* 2017).

The IId subtype family have a widespread distribution causing infection in human and animal. Probably this subtype family is transmitted from cattle to humans and this is consistent with other studies which stated that animals might be the source of *C. parvum* (Razakandrainibe *et al.,* 2018). The assessed three genes; *18S rRNA*, *gp60* and *HSP70*, showed a satisfactory sensitivity and can be used in studies for identifying *Cryptosporidium* spp. and verifying its genetic diversity. Hence, using several genes enable for more complete understanding of the genetic variability of *C. parvum.*

Conclusions: It can be concluded from the current study that Cryptosporidiosis has a high prevalence among cattle and their owners as indicted by microscopic and molecular analysis of the tested fecal samples. Furthermore, molecular analysis using Nested PCR technique revealed the presence of IId subtype family of *C. parvum* in the tested animal and human samples, thus it is recommended to take preventive measuring for controlling this zoonotic parasitic infection.

Authors contributions: Mohammad I. Alberfkani, Wijdan M.S. Mero: Conceptualization of idea and original draft writing, Mohammad I. Alberfkani: Data collection and Methodology, Sara Omar Swar, Layla A. Almutairi, Haliz Khalid Hasan, Ahmed Ezzat Ahmed, Haval Mohammed Khalid, Wijdan M.S. Mero: Formal analysis, Visualization and language improvement. All authors approved the final draft.

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