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Molecular Detection of *Cryptosporidium* spp among Cattle and Sheep in Wasit Province, Iraq: Prevalence and Some Virulence Factors

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Abstract

Cryptosporidium is a protozoan parasite found in animals or humans. The present study was designed to assess the prevalence of *Cryptosporidium* infection in the cattle and sheep population of Wasit Province, Iraq and to explore two virulence genes, *Cryptosporidium* oocyst wall protein (COWP) and surface protein of sporozoite (CP15). So, total of 200 faecal sample was collected from animals such as 100 cattle and 100 sheep from November 2025 to April 2026. The samples were studied microscopically by modified Ziehl – Neelsen staining and molecularly by nested polymerase chain reaction (nPCR). *Cryptosporidium* oocysts were found by microscopic examination in 55/100 (55.0%) of cattle and 26/100 (26.0%) of sheep. Nested PCR detected Cryptosporidium DNA in 56/100 (56.0%) cattle and 26/100 (26.0%) sheep. These COWP and CP15 virulence-associated genes were then examined using conventional PCR. Among the selected positive samples, COWP was detected in 8/11 (72.73%) cattle and 7/7 (100%) sheep, whereas CP15 was detected in 6/11 (54.55%) cattle and 5/7 (71.43%) sheep. The results confirm the presence of *Cryptosporidium* in cattle and sheep in Wasit Province and show the presence of COWP and CP15 genes in the examined samples that were found positive for *Cryptosporidium*.

Keywords

Cryptosporidium spp,
Nested PCR,
Virulence factors,
Cattle, Sheep.

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1. Introduction

Cryptosporidium is a typical intracellular parasite, infecting the microvillus cells of the intestine in humans and various animals around the world, causing cryptosporidiosis, characterized by abdominal pain, severe dehydration and profuse watery diarrhea in humans and various animals (Liu et al., 2020; Innes et al., 2020). This is estimated to contribute approximately 30-50% to the deaths of young people and second highest cause of diarrhea and deaths in children after rotavirus (Gharpure et al., 2019). *Cryptosporidium* life cycle occurs in a single host and the infective stage is the oocytes (Butt et al., 2018). Oocytes can resistant to many disinfectants, and is responsible for causing severe infection in a host.

A *Cryptosporidium* infection occurs through contaminated water or food ingestion as well as direct contact with an infected human and animals (Gunasekera et al., 2020). For decades, the microscopic examination methods were used to make the diagnosis in the laboratory of *Cryptosporidium*. This approach was regarded as the gold standard method, but had low sensitivity and specificity (Haque et al, 2005). The detection of *Cryptosporidium* oocyte is done by using modified Ziehl- Nelseen stain (Shakir & Areej, 2015). Other diagnostic methods such as molecular techniques used to detect Cryptosporidium at the species level and the genotype (Xiao et al.2004).

There are many virulence factors that contributes in the infection severity, *Cryptosporidium* oocyst wall protein (COWP) and Surface Protein of sporozoite (CP15) genes were used and obtained from positive samples. *Cryptosporidium* virulence factors have been identified as the genes involved in the initial interaction between parasite oocytes and sporozoites with host cells such as excystation, attachment, invasion of host cells, gliding motility and damage to host cells due to a build-up of inflammatory cells and cytokines in the infection site (Mohammad, 2018).Surface protein of sporozoite (CP15) is characterized via the infective sporozoite stage and helps in the invasion of the host to cause infection , and it's an essential role in the mammalian cells invasion by *Cryptosporidium* sporozoites (Mohammad, 2018). Cysteine protease virulence factors (CPs) are contributed in a main role in hemoglobin degradation for obtaining of nutrients, parasite invasion, antigen presentation, and surface protein processing (Abaza, 2020).

2. Materials and Methods

2.1 Fecal Sample Collection

During November 2025-April 2026, two hundred of cattle and sheep animals with diarrhea were sampled in a clean screwcapped cup in Al-Karamma hospital in Wasit Province, Iraq. Each sample was split into two; the first sample for parasite diagnosis using M.Z.N stain and the second sample was kept in the (-20 °C) lab freezer for parasite detection by PCR.

2.2 Microscopic Examination

A small amount of fecal sample was placed in a drop of normal saline (0.9%) on a glass slide and stained with M.Z.N and examined under a microscope, looking for the presence of oocystes (Al-Zubaidi, 2015).

2.2.1 DNA Extraction

DNA extraction was achieved from feces samples directly by kit Presto TM stool DNA Extraction, Geneaid company, made in Taiwan) depended on directions of manufacture by

stool lysis methods and Proteinase K. DNA extracted was checked using Nanodrop spectrophotometer and stored at -20 C.

2.2.2 Molecular Detection of Cryptosporidium spp. using PCR.

Fecal DNA was used for molecular detection of Cryptosporidium spp. using the 18S rRNA gene. The PCR assay was carried out with the forward and reverse primers 5'-TGGCACCAGAATCAGCTGAA-3' and 5'-GACAGGTTGAGTTGGAGCAGA-3', respectively. GoTaq® Green PCR Master Mix (Promega, USA) was used to prepare the reaction mixture following the manufacturer's instructions. Final volume of reaction was 25 µL. A T100™ Thermal Cycler (Bio-Rad, USA) was used for PCR amplification. The amplification process was denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 5 min.

PCR products were analysed by agarose-gel electrophoresis and then visualised on a UV transilluminator. The presence of a single band signalled at ~654 bp was regarded as evidence of Cryptosporidium spp. DNA. Figure 2 shows representative amplification products from cattle whereas Figure 3 shows representative amplification products of sheep samples.

2.2.3 Conventional PCR Detection of the COWP Gene

Samples that tested positive for Cryptosporidium by molecular detection methods were then tested for the Cryptosporidium oocyst wall protein (COWP) gene by conventional PCR. Validation laboratory protocol was used to identify gene specific primers targeting the COWP gene. GoTaq® Green PCR Master Mix (Promega, USA) was used to prepare the PCR reaction which was amplified in a T100™ Thermal Cycler (Bio-Rad, USA).

Products were amplified by electrophoresis on 1.5% Agarose and then detected under UV light. In the present study, a specific product of about 227 bp was detected, and it was assumed that this product was positive for COWP gene. The electrophoretic profile of the cattle samples are shown in Figure 5 and of the sheep samples in Figure 6. Note for Methodologists: The COWP primer sequences have been inserted here only after verification with the original laboratory primer record or the source of the actual primer used in the experiment. The 18S rRNA primers found in Section 2.2.2 are NOT to be repeated as COWP primers.

2.2.4 Conventional PCR Detection of the CP15 Gene

Conventional PCR with gene-specific primers was performed for C. parvum on the samples that were positive for Cryptosporidium, following the laboratory protocol, for the gene Surface Protein of Sporozoite (CP15). The PCR amplification was carried out in a final reaction volume of 25 µL with GoTaq® Green PCR Master Mix (Promega, USA). DNA extracted, gene specific primers, nuclease free water and PCR master mix were all components of the reaction mixture.

After amplification, the PCR products were run on a 1.5% agarose gel and then visualized with a UV transilluminator. In the present study, amplification of a specific product of about 517 bp was observed and was considered as a positive amplification for the CP15 gene. Electrophoretic results for cattle and sheep

samples are represented as typical results in Figures 4 and 7, respectively.

Table 1. PCR targets, primers, and expected amplification products used for molecular detection of *Cryptosporidium* spp. and virulence-associated genes

Target	**Gene**	**PCR assay**	**Primer information**	**Product size in the present study**
Cryptosporidium detection	18S rRNA	PCR/nPCR	F: 5′-TGGCACCAGAATCAGCTGAA-3′; R: 5′-GACAGGTTGAGTTGGAGCAGA-3′	654 bp
Virulence-associated gene	COWP	Conventional PCR	Gene-specific primers; sequences to be verified from the laboratory primer record	227 bp
Virulence-associated gene	CP15	Conventional PCR	Gene-specific primers; sequences to be verified from the laboratory primer record	517 bp

Note that the 18S rRNA primer pair was only used for molecular detection of *Cryptosporium* spp. and not amplification of COWP or CP15. The COWP and CP15 were carried out separately by their specific gene-specific primer pairs.

3. Results and Discussion

3.1 Microscopic Examination

Of the 200 samples of cattle faeces analyzed, 55/100 (55.0%) were positive for *Cryptosporidium* oocysts with modified Ziehl–Neelsen (M.Z.N.) staining, whilst 26/100 (26.0%) of the sheep samples were positive for *Cryptosporidium* oocysts. The overall microscopic prevalence was 40.5% (81/200). There was a significantly ($p < 0.05$) greater difference between cattle and sheep (Table 2).

Table 2. Prevalence of *Cryptosporidium* in cattle and sheep according to modified Ziehl–Neelsen (M.Z.N.) staining

Animals	**Number of samples examined**	**M.Z.N. positive (+)**	**Prevalence (%)**
Cattle	100	55	55.0
Sheep	100	26	26.0
Total	200	81	40.5
P-value	0.00049		

Oocytes of *Cryptosporidium* were found to be spherical or oval in shape with pink color, as seen in Figure 1.

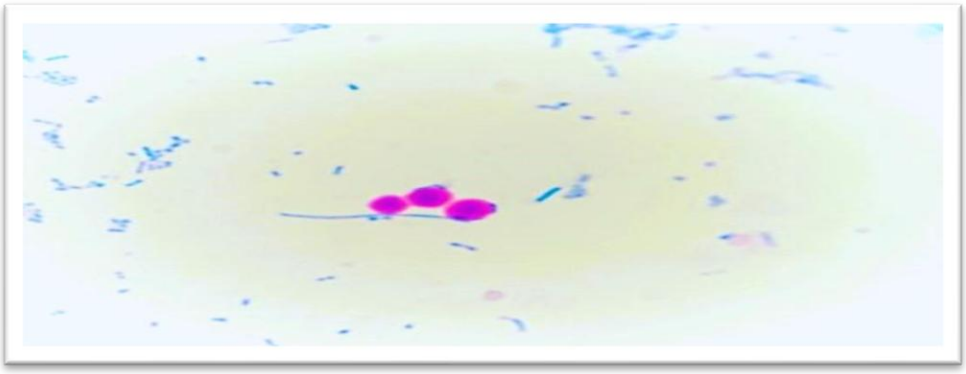

Figure 1. Microscopic detection of *Cryptosporidium* oocysts in fecal samples using modified Ziehl-Neelsen staining.

From Table 2, the present results nearly similar to finding by (Al-Saeed et al., 2020) in Duhok city which registered the rate of *Cryptosporidium* prevalence in sheep and cattle respectively 11.07%, 26.15%. The prevalence variations of sheep and cattle due to many factors including; the host age, sanitary conditions, breeding and management system, these factors may increase the factor of risk linked with transmission and prevalence of Cryptosporidium between these animals (El-Gohary et al.,2013).

3.2 Molecular Analysis by PCR

The nested PCR (nPCR) examination showed that *Cryptosporidium* DNA was detected in 56/100 (56.0%) cattle and 26/100 (26.0%) sheep. The overall prevalence of the molecular was 41.0% (82/200), there was a statistically significant difference between cattle and sheep (p = 0.0002) (Table 3).

Table 3. Prevalence of *Cryptosporidium* in cattle and sheep according to nested PCR (nPCR)

Animals	**Number of samples examined**	**PCR positive (+)**	**Prevalence (%)**
Cattle	100	56	56.0
Sheep	100	26	26.0
Total	200	82	41.0
P value	0.0002		

In the present study, the prevalence of cattle was higher than reported by Alseady and Kawan 2019 who reported 38% (38/100) using nPCR in Baghdad Province. It was also higher than the prevalence of Wang et al. (2020) in dairy cattle of China, 59/1414 (4.2%) with nPCR. The prevalence was higher than in the central southern part of Iraq reported by Al-Ardi (2025) (8/50, 16%) and Iran (22/1300, 1.69%) reported by Dalimi et al. (2017) by nPCR/RFLP methods.

Cryptosporidium was molecularly analyzed in the 200 cattle and sheep fecal samples using primers specific for 18S rRNA gene. Nested PCR was used to amplify a 654-bp product which was then analyzed by agarose gel electrophoresis (Figures 2 and 3).

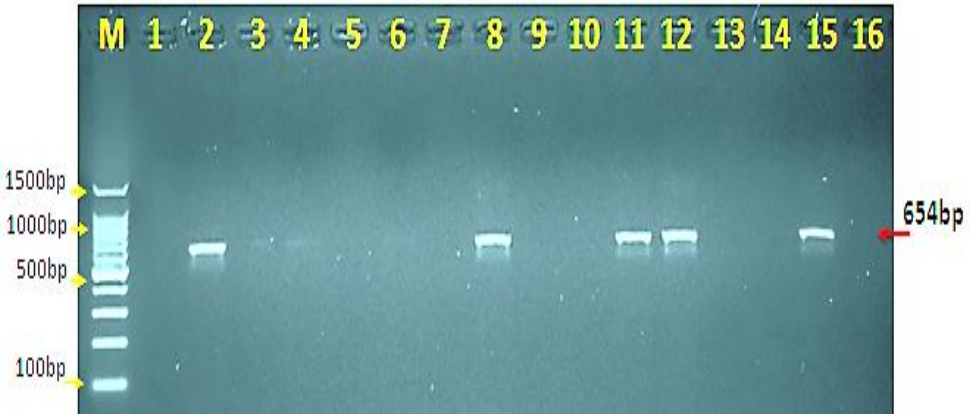

Figure 2: Agarose gel electrophoresis of the 18S rRNA PCR products for molecular detection of *Cryptosporidium* in cattle fecal samples. M: DNA marker (100–1500 bp); lanes 1–16 represent examined samples; the expected positive amplification product was approximately 654 bp.

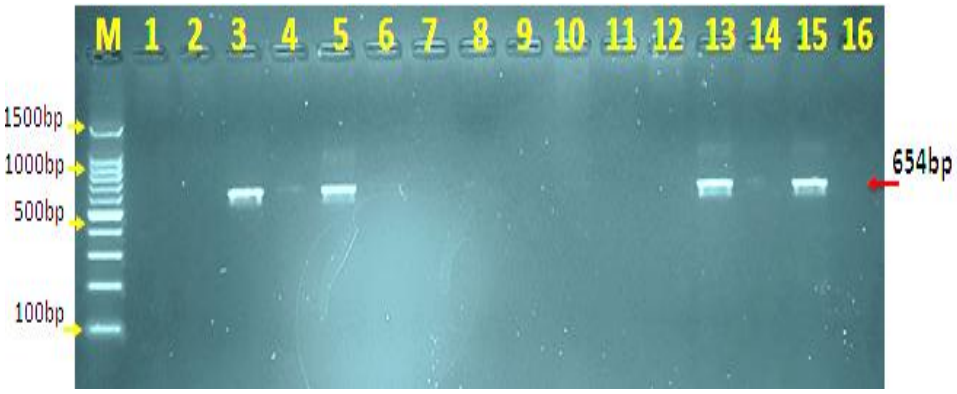

Figure 3. Agarose gel electrophoresis of the 18S rRNA PCR products for molecular detection of *Cryptosporidium* in sheep fecal samples. M: DNA marker (100–1500 bp); lanes 1–16 represent examined samples; the expected positive amplification product was approximately 654 bp.

3.3 Molecular detection of Virulence Associated Genes

Cryptosporidium is a significant protozoan parasite and a significant cause of diarrheal disease in man and animals, especially in the neonatal host. Diarrhea and abdominal pain are common symptoms of cryptosporidiosis (Hasheminasab et al., 2022).

The present study aimed to analyze two virulence associated genes of *Cryptosporidium* namely Cryptosporidium oocyst wall protein (COWP) gene and surface protein of sporozoite (CP15) gene in *Cryptosporidium* positive cattle and sheep samples using conventional PCR. Molecular analysis revealed that both genes were present in >85% of samples selected as positive.

The frequency of the COWP gene was 8/11 (72.73%) and of the CP15 gene was 6/11 (54.55%) of the examined cattle samples. In sheep, the COWP gene was detected in 7 of 7 samples (100%), while the CP15 gene was detected in 5 of 7 samples (71.43%). The results of these are represented in Figures 4–7.

The PCR products were separated on the 1.5% agarose gel and stained with ethidium bromide and examined under UV light. The COWP gene, on the other hand, amplified a 227 bp product, and the CP15 gene amplified a 517 bp product. Electrophoretic profiles of these are shown in Figures 4–7.

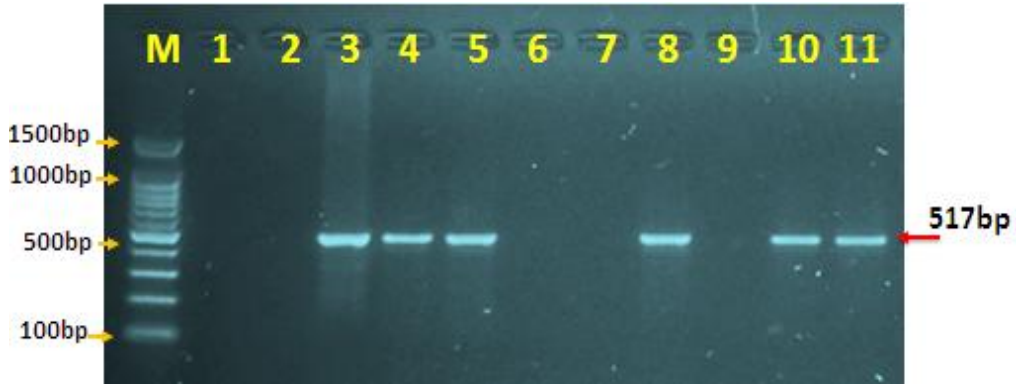

Figure 4. Agarose gel electrophoresis of the CP15 gene PCR products in *Cryptosporidium*-positive cattle samples. M: DNA marker (100–1500 bp); lanes 1–11 represent examined samples; the expected CP15 amplification product was approximately 517 bp.

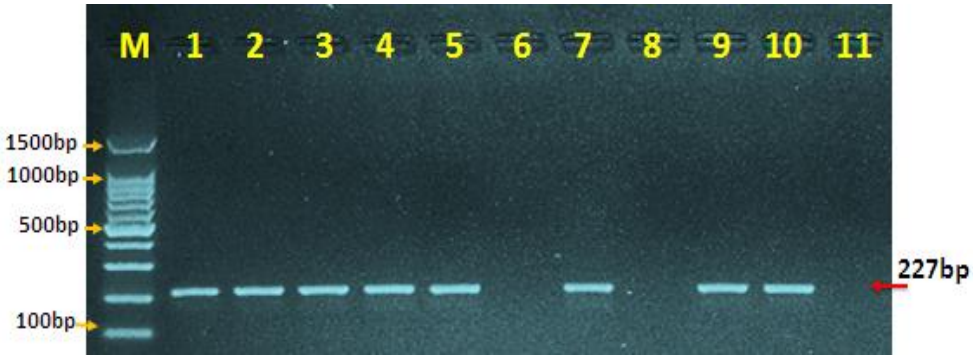

Figure 5. Agarose gel electrophoresis of the COWP gene PCR products in *Cryptosporidium*-positive cattle samples. M: DNA marker (100–1500 bp); lanes 1–11 represent examined samples; the expected COWP amplification product was approximately 227 bp.

Conventional PCR amplification identified the presence of COWP gene in sheep samples, as indicated by a specific PCR product of an approximate 227 bp (Figure 6). The bands that were observed in the positive lanes, matched the expected size of the COWP PCR product.

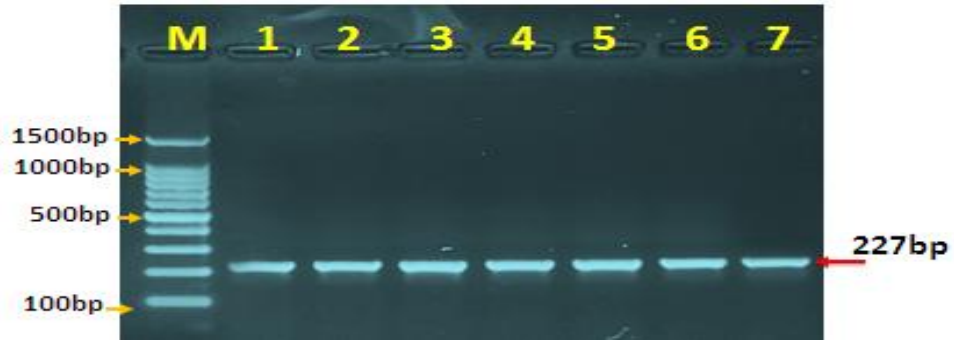

Figure 6. Agarose gel electrophoresis of the COWP gene PCR products in *Cryptosporidium*-positive sheep samples. M: DNA marker (100–1500 bp); lanes 1–7 represent examined samples; the expected COWP amplification product was approximately 227 bp.

In the sheep samples Figure 7 shows the amplification of the CP15 gene using conventional PCR. The positive amplification bands were detected at the expected size (about 517 bp), thus confirming the presence of the CP15 virulence associated gene in the tested samples.

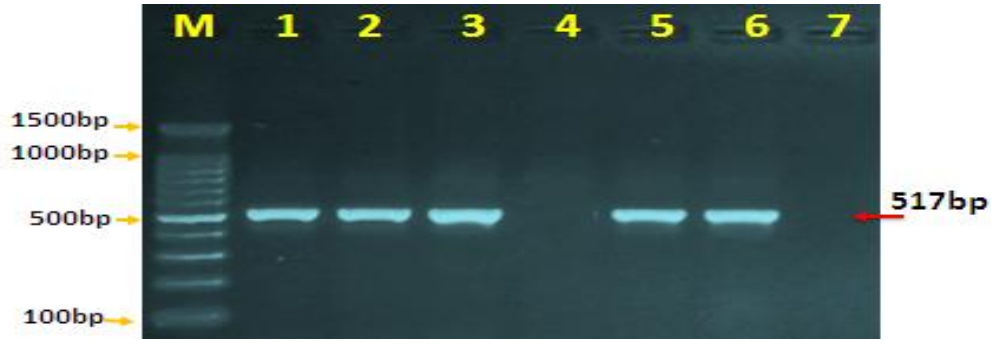

Figure 7. Agarose gel electrophoresis of the CP15 gene PCR products in *Cryptosporidium*-positive sheep samples. M: DNA marker (100–1500 bp); lanes 1–7 represent examined samples; the expected CP15 amplification product was approximately 517 bp

In the present study, Conventional PCR, it was found that the COWP gene and CP15 gene exist in most of the samples with percentage ranging from (54.55 - 100%). CP15 is a glycoprotein which plays a role in parasite attachment, motility and invasion epithelial cells of host (Mohammad, 2018).

4. Conclusion

Cattle and sheep were found to be infected with *Cryptosporidium* in Wasit Province, Iraq. The microscopic examination of the cattle and sheep using modified Ziehl–Neelsen staining revealed 55.0% (55/100) and 26.0% (26/100) oocysts, respectively, of Cryptosporidium. Nested PCR detected Cryptosporidium DNA in 56.0% (56/100) of cattle and 26.0% (26/100) of sheep. The molecular investigation also showed the presence of the COWP and CP15 virulence associated genes in the tested positive samples. The results suggest that cryptosporidiosis was a significant problem among the cattle and sheep examined and molecular techniques should be used in addition to microscopy for detection.

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