Indian Journal of Ecology (2025) 52(3): 564-567
DOI: https://doi.org/10.55362/IJE/2025/4537

Manuscript Number: 4537
NAAS Rating: 5.38

Cryptosporidium Genotypes and Subtypes in Sheep in Al-Qadisiyah Province

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***Abstract*:** The accuracy of the sequences was confirmed using *Cryptosporidium* 18SrRNA gene references. The *Cryptosporidium* spp. isolates from sheep faeces included: *C. parvum* (35.29%), *C. hominis* (23.52%), *C. ubiquitum* (11.76%), *C. suis* (11.76%), *C. xiao* (11.76%), and *C. andersoni* (5.88%), with no significant differences . Gene sequence data were recorded in Gene Bank to determine the identity and similarity of *Cryptosporidium* spp. 18SrRNA gene. The 17 samples were compared with global strains in NCBI-Blast. The analysis identified multiple *Cryptosporidium* species, with *C. parvum* being the most prevalent. High sequence homology with global strains, particularly *C. parvum* and *C. hominis*, suggests a significant presence in sheep feces, indicating potential public health risks.

Keywords: Cryptosporidium, Sheep, Genotypes, Subtypes, 18S rRNA gene, GP60 gene

Cryptosporidiosis is a zoonotic parasitic disease caused by *Cryptosporidium*, an intracellular protozoan parasite affecting various vertebrates, including humans. It is particularly significant in domestic ruminants, often causing diarrheal disease in young animals and leading to economic losses (Díaz et al., 2015, Díaz et al., 2018). Transmission occurs through contaminated food and water (Fayer 2000). Diagnosis involves direct, concentration, and staining techniques (Garcia, 2001), while molecular methods like PCR-sequencing provide insights into taxonomy, epidemiology, and genetic diversity. Species classification is based on Gp60 gene analysis, essential for identifying infection sources and assessing public health impact (Stensvold et al., 2015, Leary et al., 2020). The study was conducted on *Cryptosporidium* genotypes and subtypes in sheep in Al-Qadisiyah province

MATERIALS AND METHODS

Sample collection: A total of 200 fecal samples were collected from sheep between October 2020 and March 2021. Samples were obtained from animals of different age groups (<1 and >2 years) and both sexes (155 females and 45 males). The samples were transported in a refrigerated bag to the Parasitology Laboratory, College of Veterinary Medicine, University of Al-Qadisiyah, for further analysis.

Microscopic examination of Cryptosporidium oocysts: The presence of Cryptosporidium oocysts was determined using the Modified Ziehl-Neelsen Staining (MZNS) technique. Each fecal sample was examined through direct smear slides and flotation methods using a NaCl solution.

DNA extraction and molecular analysis: DNA was extracted from all 200 samples using a commercial DNA extraction kit (Add Bio, Korea) following the manufacturer's instructions. The extracted DNA was stored at -20°C until further use in PCR analysis.

Nested PCR: Nested PCR (N-PCR) was used for Cryptosporidium spp. diagnosis based on the 18S rRNA gene. The outer and inner primers used were as follows:

Outer primers:
Forward: 5'-TTCTAGAGCTAATACATGCG-3'
Reverse: 5'-CCCATTTCCTTCGAAACAGGA-3'
Inner primers:
Forward: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3'
Reverse: 5'-AAGGAGTAAGGAACAACCTCCA-3'

For *Cryptosporidium parvum* subtyping, an N-PCR approach was used targeting the GP60 gene with the following primers:

Outer primers:
Forward: 5'-ATAGTCTCCGCTGTATTC-3'
Reverse: 5'-GGAAGGAACGATGTATCT-3'
Inner primers:
Forward: 5'-TCCGCTGTATTCTCAGCC-3'
Reverse: 5'-GCAGAGGAACCAGCATC-3'

For *Cryptosporidium hominis* subtyping, the following primers were used:

Outer primers:
Forward: 5'-TTACTCTCCGTTATAGTCTCC-3'
Reverse: 5'-GGAAGGAACGATGTATCTGA-3'
Inner primers:
Forward: 5'-TCCGCTGTATTCTCAGCC-3'
Reverse: 5'-GCAGAGGAACCAGCATC-3'

Thermal cycling conditions were performed using a thermal cycler (BioRad, USA) and Taq DNA polymerase (AddBio,

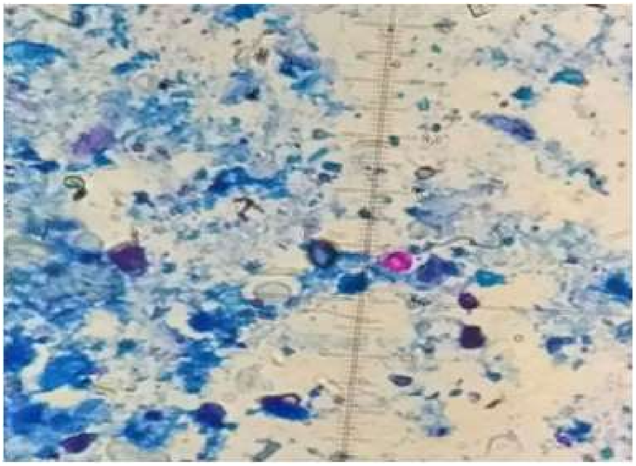

Fig. 1. *Cryptosporidium* spp. oocysts stained with M.Z.N.S at 100x magnification)

Korea). The conditions for the first round included:

Initial denaturation at 95°C for 10 minutes
Followed by 39 cycles of:
95°C for 30 seconds
55°C for 30 seconds
72°C for 60 seconds
Final extension at 72°C for 5 minutes

The second round was performed under similar conditions as the first.

Sequencing: All positive samples were analyzed using molecular methods, and 17 pure DNA samples were sequenced targeting the 18S rRNA gene for genotype identification. The GP60 gene was amplified for *C. parvum* and *C. hominis* subtyping. Ten isolates were sequenced, including six *C. parvum* and four *C. hominis*. The N-PCR

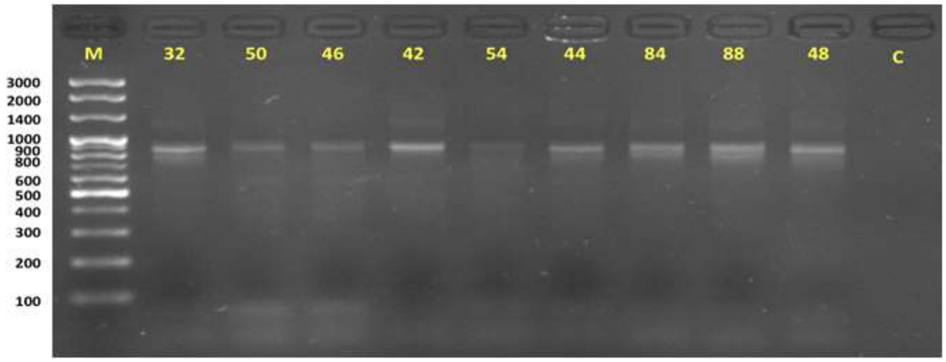

Fig. 2. *Cryptosporidium. spp.* infection is detected by Nested-PCR, targeting 18S ribosomal RNA gene

Table 1. NCBI-BLAST homology sequence identity of *Cryptosporidium* spp. isolates

Accession No.	*Cryptosporidium* spp.	Gen Bank Accession No.	Country	Identity (%)
MW947215	*C. parvum*	AF112570	USA	99.88
MW947216	*C. parvum*	AF112570	USA	99.88
MW947217	*C. parvum*	AH006572	USA	98.24
MW947218	*C. parvum*	KT151531	Iraq	98.12
MW947219	*C. parvum*	KT151531	Iraq	98.24
MW947220	*C. parvum*	AF112570	USA	100
MW947221	*C. hominis*	DQ286403	Chile	100
MW947222	*C. hominis*	AB369994	Saudi Arabia	100
MW947223	*C. hominis*	KF146220	Brazil	99.63
MW947224	*C. hominis*	MK990042	China	100
MW947225	*C. ubiquitum*	MN833282	China	100
MW947226	*C. ubiquitum*	KT027446	USA	99.14
MW947227	*C. suis*	GQ227705	China	99.04
MW947228	*C. suis*	GQ227705	China	98.92
MW947229	*C. xiao*	KM199756	China	99.75
MW947230	*C. xiao*	FJ896050	USA	94.44
MW947231	*C. andersoni*	JN400881	India	100

Table 2. *C. parvum* subtypingwere compared with other NCBI-Blast deposited global strains

Identity (100%)	Country	Gen Bank accession number	Subtype	Accession number
99.87	Spain	KY49903	IIdA17G1a	MW984360
100.00	Spain	KT764969	IIdA17G1	MW984361
99.87	China	KT964799	IIdA17G1a	MW984362
99.62	United Kingdom	GU21438	IId A17G1	MW984363
99.87	Netherlands	MH79636	IIdA17G1a	MW984364
99.61	United Kingdom	HQ14900	IIdA17G1a	MW984365

Table 3. Subtyping of *C. hominis*as compared with other NCBI-Blast deposited global strains

Identity (100%)	Country	GenBank Accession Number	Subtype	Accession number
100.00	China	FJ153239	IbA21G2	MW984366
99.64	China	FJ707313	IbA21G2	MW984367
96.69	Spain	MK105902	IbA21G2	MW984368
99.12	Germany	KM539016	IbA21G2	MW984369

products were sent to Macrogen Co., Korea for direct sequencing, and species and subtypes were identified using BLAST search in the GenBank database.

RESULTS AND DISCUSSION

Diagnostic characterization of *Cryptosporidium* spp.: *Cryptosporidium* spp. oocysts were identified as oval or spherical in shape, appearing dark pink or red on a blue background using Modified Ziehl-Neelsen Stain (M.Z.N.S). The microscopic examination showed that 61 out of 200 samples (30.5%) were positive for *Cryptosporidium* infection. **Molecular examination:** The sequence accuracy was confirmed using *Cryptosporidium* 18SrRNA gene references. The breakdown of *Cryptosporidium*spp. isolates was maximum in *C. parvum* (35.29%) followed by *C. hominis* (23.52%), *C. ubiquitum* (11.76%), *C. suis* (11.76%), *C. xiao* (11.76%) and *C. andersoni* (5.88%). No significant differences were observed at the p-value level of 0.05. Gene sequence data were recorded in the Gene Bank for further comparison

The sequence analysis revealed a variety of *Cryptosporidium* species, with C. parvum being the most prevalent. Sequence homology with other global strains confirmed high identity percentages across the isolates, especially with *C. parvum* and *C. hominis* strains. The results suggest a significant presence of these species in the sheep faeces, which may have implications for public health.

CONCLUSION

This study confirmed the diversity of *Cryptosporidium* species in sheep faeces, with high genetic homology to global strains. Future studies should focus on exploring the epidemiological implications of these findings.

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Received 12 February, 2025; Accepted 02 June, 2025