INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

www.scienceandnature.org

MOLECULAR AND MICROSCOPIC DETECTION OF *CRYPTOSPORIDIUM SPP* IN SHEEP IN AL-TAJI AREA-BAGHDAD/IRAQ

Mohammed T. S. Al-Zubaidi
University of Baghdad/College of Veterinary Medicine
Corresponding author email: mohabood24@gmail.com

ABSTRACT
This study was to detect the *Cryptosporidium spp*. oocysts and its prevalence among naturally infected sheep. Ninety fecal samples were collected in Al- Taji region aged 1-3 year's old sheep from the beginning of February to the end of May 2016. Samples examined by lugol's iodine, modified Ziehl-Neelsen stain and PCR. Results revealed that the total prevalence of *Cryptosporidium spp* infection is 48.88% (44/90) by using PCR technique with highest sensitivity, while the prevalence reach 37.77% (34/90), 27.77% (25/90) by using Ziehl-Neelsen stain and lugol's iodine respectively. Highest prevalence rate of parasite in age group < 6 months 70% (14/20) while the lowest prevalence rate in age group 25-36 months 34.78% (8/23). The study found that 58.13% (25/43) of diarrheic sheep shed *Cryptosporidium* oocysts in feces. The PCR technique was used to determine the presence of *Cryptosporidium* oocysts, particular gene locus in the extracted DNA which were extracted by modified protocol, proteinase K and seven cycles of freezing-thawing in liquid nitrogen was more efficient than only three cycles by deep freeze in facilitate the subsequent steps of DNA extraction. SSU rRNA–based PCR technique by general tools has been used successfully for the detection of *Cryptosporidium* oocysts in stool sample with 100% sensitivity.

KEYWORDS: *Cryptosporidium*, Sheep, Diarrhea, PCR.

INTRODUCTION

Cryptosporidium is an obligate intracellular extra cytoplasmic protozoan parasite belongs to the phylum Apicomplexa. It has an apical compound which helps in the penetration of host cell (Oyibo *et al*., 2011). Many studies had been proved that *Cryptosporidium* is one of important zoonotic pathogens which infect digestive epithelium of a variety of vertebrates and cause gastroenteritis which characterized by diarrhea in animals and human, having a great impact of morbidity and mortality (Fayer and Xiao, 2008; Lange *et al*., 2014). Younger animals appear to be more delicate to cryptosporidiosis and considered as a top cause of diarrhea and death rate in neonatal ruminants (Ozdal *et al*., 2009; Santín, 2013).

The prevalence of *Cryptosporidium* infection was reported 4% to 85% in lambs worldwide (Fasihi-Harandi and Fotohi-Ardakani, 2008). Microscopical confirmation of *Cryptosporidium* differ from 0% in Ethiopia, 2.6% in Australia, 3.7%-47% in Brazil, 13.6%-46.5% in Turkey, 25.7% in Mexico, 29% in Greece, and 42.1% in Serbia (Ozdal *et al*., 2009; Silva-Fiuza *et al*., 2011).There are different methods used to detect cryptosporidiosis, such as modified Ziehl–Neelsen stain (modified acid-fast stain) which detects *Cryptosporidium* oocysts in feces. It is the most commonly used method for the diagnosis of parasite, and Lugol's iodine method which also detect the oocysts of parasite but with less sensitivity (Mehta., 2002; Alles *et al*., 1995). Molecular method like PCR is the most sensitive and specified (97-100%) assay used for detection of Cryptosporidiosis (Morgan *et al*., 1998; Bialek *et al*., 2002). The rRNA (SSU rRNA) gene or the *Cryptosporidium* oocyst wall protein (COWP) gene is used to determine *Cryptosporidium* species by PCR technique (Silva-Fiuza *et al*., 2011). The present study amid to detect the prevalence of Cryptosporidiosis in sheep in AL-Taji area/Baghdad/ Iraq by using conventional staining methods and PCR.

MATERIALS & METHODS

Collection of Fecal Samples

Ninety fecal samples were collected from sheep with and without diarrhea in Al- Taji area-Baghdad from the beginning of February to the end of May 2016 of both genders (55 males and 35 females), aged 1-3 years old. Each sample was placed in a screw capped plastic container, labeled with the number and the date of collection, and transported in the cooling box, to the Department of Parasitology/ Faculty of Veterinary Medicine / University of Baghdad.

Detection of *Cryptosporidium* oocysts

1-Direct Microscopic Examination

This method was done according to the procedure of Coles (1986). One drop of 1% lugol's iodine was placed on a glass slide and small quantity of fecal specimen was added and mixed well by a wooden stick. Forceps was used to adjust the cover slip, and the slides were examined under (40X) and (100X) magnifications.

2-Microscopic Examination by Modified Ziehl–Neelsen

Modified Ziehl–Neelsen stain was done according to the method of Beaver and Jung (1985) and Baxby *et al*. (1984). Smear of sediment of fecal specimen was left to dry and placed in a slides rack for fixation by methanol for

five minutes. Carbol fuchsin stain was applied to the smear for 3 minuts, and the slide was washed with tap water, acid alcohol has been added to the slide for decolorization, the slide was washed with tap water followed by 1% methylene blue for 2 minute. Then after, the slide was rinsed by tap water and dried in air and examined under (40X) and (100X) magnifications.

3- PCR Technique for Detection of *Cryptosporidium*

DNA Extraction from Fecal samples

All fecal samples were used for molecular detection of *Cryptospordium* using PCR technique according to the Ekanayake *et al*. (2006) and Nichols & Smith, 2004. Tube contains stool specimen with potassium dichromate was placed on a vortex and 200 µl of the stool sample was transferred to 1.5ml eppendrof tube. Eight hundred µl of deionized DW was added to the fecal samples. The specimen was centrifuged at 13000 rpm for 10 minutes and the supernatant was removed. One thousand µl of deionized DW was added and the tube was placed on a vortex to separate the pellet. The specimen was centrifuged at 14.000rpm for 3 minutes and the supernatant was collected. Then, the specimens were transferred to a (55°C) in a shaker water bath, proteinase K was added to the specimens at a final concentration of 200 µg/ml.

DNA extraction

Fecal oocysts was subjected to DNA extraction after cycles of freezing and thawing using a DNA purification kit (Wizard Genomic DNA purification kit; Promega). Then, DNA concentration was measured by using NanoDrop spectrophotometer at wave lengths of 260 and 280 nm. DNA quality was assessed by agarose gel electrophoresis (Maniattis *et al*., 1982).

Preparations of the Gel electrophoresis: Tris-borate-EDTA (Ethylen Diamine tetra Acetic-acid) (TBE): To prepare a concentration 10X of Tris-borate buffer (TBE) as a diluant, 100ml of TBE (Biotechnology, con. 1X) was dissolved in 900 ml of DW.

-Loading buffer: by DNA purification kit (Wizard Genomic DNA purification kit; Promega). Ethidium bromide dye: Biotechnology (Korea).

-Preparing the gel for electrophoresis: the 1.5% concentration of the agarose gel was prepared for a separation-PCR product, by dissolving (1.5gm) agarose powder in (100 ml) DW, then it was put in the microwave for one minute.

PCR Technique

The master mixture was prepared by the kit (promega), by adding SSU-F1 primer: 5′-TTC TAG AGC TAA TAC ATG CG 3′and SSU-R1primer: 5′-CCC ATT TCC TTC GAA ACA GGA-3′ for each PCR reaction. DNA bands were visualized by UV transiluminator at a wavelength (302 nm) (Maniattis *et al*., 1982).

Statistical analysis

The Chi-square test was used for the comparison between the results. Differences were considered statistically significant at $P<0.05$ (Snedecor and Cochran, 1989).

RESULTS & DISCUSSION

The results of this study showed significant differences ($p<0.05$) in prevalence of cryptosporidiosis in sheep in AL-Taji area. Prevalence rates 48.88 recorded (44/90), 37.77% (34/90) and 27.77% (25/90) depending on the result of PCR, ZN and Lugol iodine stain, respectively (Table 1; Figure 1, 2). PCR was considered as more sensitive technique used for detection of Cryptosporidiosis, coincided with Morgan *et al.* (1998), Bialek et al. (2002) and Bakheit *et al.* (2008) who argued that PCR-based assays detect one oocyst that could be mis-diagnosed by microscopic investigation.

TABLE 1: Prevalence of *Cryptosporidium* spp according to the Examination methods

Examination methods	No. of fecal samples	
	examined	Positive (%)
Lugol iodine stain	90	25[a] (27.77)
Modified ZN stain	90	34[b] (37.77)
PCR	90	44[c] (48.88)
Total	90	44 (48.88)

Different superscript refers to significant differnces at $p<0.05$

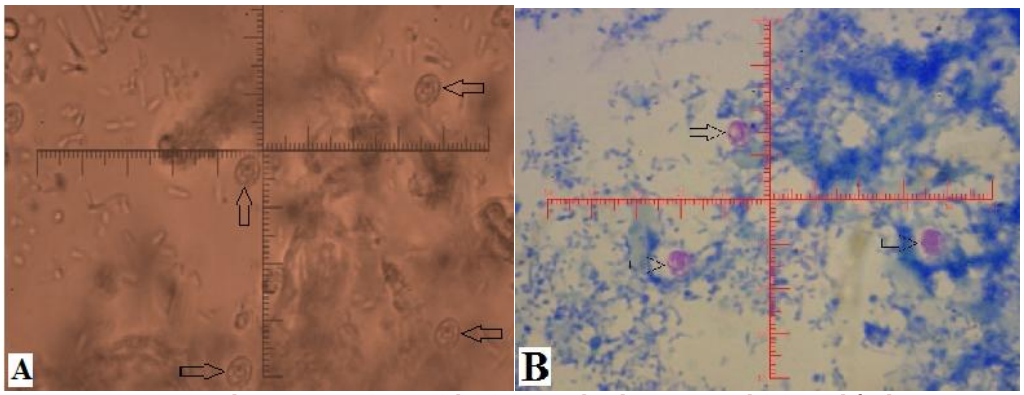

FIGURE 1: Cryptosporidium oocysts (arrows) by (A) Lugol iodine stain and (B) Modified ZN stain 100x

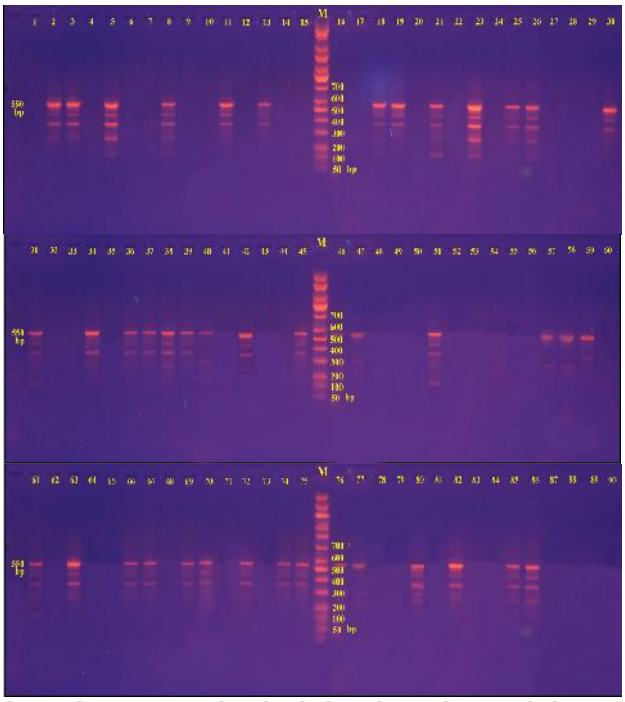

FIGURE 2: An agarose gel electrophoresis stained with ethidium bromide, revealed *Cryptosporidium* SSU rRNA gene sequence (≈ 550 bp) PCR amplification products for the 90 sheep fecal samples.

The study showed a significance difference ($p<0.05$) between age groups and infectivity prevalence of parasite, highest prevalence of cryptosporidiosis was recorded in age group **< 6** months compared with lowest infection rate in age group 25-36 months which reach 70% (14/20), 39.13% (9/23), respectively (Table 2). This may be due to the low immunity status in the newly born lambs and the increased shedding rhythm of oocysts by the infected dam due to hormonal disturbances (Fayer and Xiao, 2008). This result coincided with AL-Zubaidi (1994), AL Azzawi (2003) in small calves, AL-Zubaidi (2009) in goat and Abd Al-Wahab (2003), Kadhim (2009) in small lambs in Baghdad city who found high infection rate in small age groups. Also, this finding agreed with EI-Wahed (1999) in Egypt, Sari et al. (2008) in Turkey who reported high prevalence rate of parasite in small lambs. The highest infection rate in small animal may be due to high shedding rate of *Cryptosporidium* oocysts from dam which contaminate food and water in farm and give chance to infect lambs (Anderson *et al*., 1991).

TABLE 2: Prevalence of *Cryptosporidium* spp according to the age groups

Age group (months)	No. of fecal examined	Samples Positive (%)
< 6	20	14^{c} (70)
6-12	23	12^{b} (52.17)
13-24	24	10^{a} (41.66)
25-36	23	9^{a} (39.13)
Total	90	44 (48.88)

Different superscript refers to significant differnces at $p<0.05$

There was no significance difference between male and female infection rates, 49.09% (27/55) and 48.57% (17/35), respectively (Table 3). This result agreed with Abd Al-Wahab (2003), Kadhim (2009) in small lambs in Baghdad city and Rasheed (1997) in goat kids in Iraq, who found no significance differences in the infection rate between male and female due to equal possibility of exposure to the contaminated environment (Fayer and Xiao, 2008).

TABLE 3: Prevalence of *Cryptosporidium* spp according to gender

Animal gender	No. of fecal examined	Samples Positive (%)
Male	55	27^{a} (49.09)
Female	35	17^{a} (48.57)
Total	90	44 (48.88)

Different superscript refers to significant differnces at $p<0.05$

Significance difference ($p<0.05$) was observed in infection rate of cryptosporidiosis in sheep according to the months of study. The highest infection rate (68.16%; 15/22) was recorded in April, while the lowest infection rate (17.39%; 4/23) in February. This result agrees with Abd Al-Wahab (2003) and Kadhim (2009) who recorded high infection rate of cryptosporidiosis among lambs in March and April. This result may be duo to good environmental condition (temperature and humidity) for the parasite and large number of *Cryptosporidium* oocysts, that shed from pregnant and lactating ewes in the farm which considered as a source of infection to the lambs (Smith et al., 1993; Bulent and Huseyin, 2004; Sari *et al*., 2008; Fayer and Xiao., 2008).

TABLE 4: Prevalence of Cryptosporidium spp according to the Months

Months of study	No. of fecal Samples	
	Examined	Positive (%)
February	23	4^{a} (17.39)
March	24	16^{b} (66.66)
April	22	15^{b} (68.16)
May	21	9^{c} (42.85)
Total	90	44 (48.88)

Different superscript refers to significant differnces at $p<0.05$

The result of this study showed significance difference ($p<0.05$) between infection rate of cryptosporidiosis among diarrheic and non-diarrheic sheep, which reach 58.13% (25/43), 40.42% (19/47), respectively (Table 5). This result explains the role of *Cryptosporidium* as a important cause of diarrhea in sheep and agrees with several researchers, Abd Al-Wahab (2003), Kadhim (2009) in Baghdad, Zorana *et al*. (2006) in Serbia and Sari *et al*. (2008) in Turkey who recorded shedding of *Cryptosporidium* oocysts in 13.7% , 18.61%, 69% and 38.8%, respectively, of the diarrheic lambs. Similar finding was reported by AL-Zubaidi (1994) and AL Azzawi (2003) in diarrheic calves (50.9%), (52.5%) respectively infected with cryptosporidiosis. Table 5 showed that 40.42% of sheep carry infection without signs of diarrhea, rendering them as potent shedders as a source of infection.

TABLE 5: Prevalence of *Cryptosporidium spp* according Type of feces

	No. of fecal Samples	
Types of feces	examined	Positive (%)
Diarrheic	43	25^{a} (58.13)
Non diarrheic	47	19^{b} (40.42)
Total	90	44 (48.88)

Different superscript refers to significant differnces at $p<0.05$

REFERENCES

Abd Al-Wahab, I.H. (2003) Study in the epidemiology of the intestinal protozoa (*Eimeria* spp. *Cryptosporidium* spp. *Giardia* spp.) in the sheep in Baghdad province. MSc Thesis, College of Veterinary Medicine, University of Baghdad.

Al-Azzawi, M.H.K. (2003) Epidemiological study of cryptosporidiosis and the isolation of the parasite antigens, diagnosis and the use of some medicinal plant extracts work therapy. PhD Thesis. College of Vet. Med. Baghdad University, Iraq.

AL-Zubaidi, M.T.S. (1994) Cryptosporidiosis in calves. MSc Thesis. Faculty of Veterinary Medicine, Baghdad University, Iraq.

AL-Zubaidi, M.T.S. (2009) Some epidemiological aspects of Cryptosporidiosis in goats and Ultrastructural study. Ph.D. Thesis, University of Baghdad. pp.133.

Alles, A.J., Waldron, M.A., Sierra, L.S., Mattia, A.R. (1995) Prospective comparison of direct immune fluorescence and conventional staining methods for detection of Giardia and Cryptosporidium spp. in human fecal specimens. J Clin Microbiol. 33:1632–4. [PMC free article].

Anderson, B.C. (1991) Experimental infection in mice of *Cryptosporidium muris* isolated from a camel. J. Protozol.38:165-175.

Bakheit M.A., Torra D., Palomino L.A., Thekisoe O.M., Mbati P.A., Ongerth J., Karanis P. (2008) Sensitive and specific detection of Cryptosporidium species in PCR-negative samples by loop-mediated isothermal DNA amplification and confirmation of generated LAMP products by sequencing, *Vet Parasitol,158*, 11-22.

Baxby, D., Blundell, N. & Hart, C.A. (1984) The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in feces . J. Hyg. 92: 317-323.

Beaver, P.C. and Jung, R.C. (1985) Animal agents and vectors of human disease .(5th ed.) Lea and Febiger .pp 249.

Bialek R., Binder N., Dietz K., Joachim A., Knobloch J., Zelck U.E. (2002) Comparison of fluorescence, antigen and PCR assays to detect Cryptosporidium parvum in

faecal specimens. *Diagn. Microbiol Infect Dis*, 43, 283-288.

Bulent, U and Huseyin, V. (2004) Cryptosporidiosis in Diarrhoeic Lambs on a Sheep Farm. Türkiye Parazitoloji Dergisi 28 (1): 15-17

Coles, E.H. (1986) Veterinary clinical pathology(4th ed.) W.B. Saunders Company Philadelphia. P. 374-453.

El.Wahed,M.A.(1999) *Cryptosporidium* infection among sheep in Qalubia governorate.Egypt.J.Egy. Soc.Parasitol. 29:113-118.

Ekanayake, D., Arulkanthan, A., Horadagoda, N.U., Sanjeevani, G.K.M,; Kieft, R., Gunatilake, S. and Dittus, W.P.J. (2006) Prevelance of *Cryptosporidium* and other Enteric Parasites among Non-Human Primates In Polonnaruwa, Srilanka. Am. J. Fayer, R. and Xiao , L. (2008) *Cryptosporidium* and Cryptosporidiosis . 2nd ed . CRC. Press.

Fasihi-Harandi, M. and Fotohi-Ardakani, R. (2008) Crypto sporidiosis infection ofsheep and goats in Kerman: epidemiology and risk factor analysis. J. Vet. Res., 63: 47-51.

Kadhim, T.A. (2009) Epidemiological and histological study of cryptosporidiosis in sheep of Baghdad province MSc Thesis. Faculty of Veterinary Medicine, Baghdad University, Iraq.

Lange, H., Johansen, O. H., Vold, L., Robertson, L. J., Anthonisen, I.L. and Nygard, K. (2014) Second outbreak of infection with a rare *Cryptosporidium parvum* genotype in schoolchildren associated with contact with lambs/goat kids at a holiday farm in Norway. *Epidemiol. Infect.*, 142: 2105-2113.

Maniattis, T., Fritsh, E.F. and Sambroook, J. (1982) Molecular cloning A laboratory manual. Cold spring Harbor Labratory press.old Spring Harbor.N.Y.

Mehta P. (2002) Laboratory diagnosis of cryptosporidiosis J Postgrad Med. 48:217. [PubMed].

Morgan, U.M., Pallant, L., Dwyer, B.W., Forbes, D.A., Rich, G. & Thompson, R.C.A. (1998) Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. J. Clinic. Microbiol. 36(4): 995-998.

Nichols, R.A.B. & Smith, H.V. (2004) Optimisation of DNA extraction and molecular detection of *Crypto sporidium parvum* oocysts in natural mineral water sources, J. Food Protect, 67: 524–532.

Oyibo, W.A., Okangba, C.C., Nwanebu, F.C. & Ojuromi, T. (2011) Diagnosis of intestinal cryptosporidiosis in Africa: Prospects and Challenges. J. Appl. Bio. 40: 2659 – 2667.

Ozdal, N., Tanritanir, P., Goz, Y., Deger, S. and Kozat, S. (2009) Parasitic protozoans (*Eimeria*, *Giardia*, *and Cryptosporidium*) in lambs with diarrhoea in the Van province, Turkey. Bull. Vet. Inst. Pulawy, 53: 47-51.

Rasheed, R.N.(1997) Cryptosporidiosis in Iraqi goat kids. The Veterinarian,1(6):1-5.

Santín, M. (2013) Clinical and subclinical infections with *Cryptosporidium* in animals. N. Z. Vet. J., 61: 1-10.

Sari, B., Arslan, M.O., Gicik, Y., Kara, M. and Tasci, G.T. (2008) The prevelance of *cryptospordum* species in diarrhoeic lamb in Kars province and potential risk factor. Trop. Anim.Health Prod., 41(5):819-26.

Silva-Fiuza, V.R., Juliboni-Cosendey, R.I., Frazao-Teixeira, E., Santin, M., Pedraza-Diaz. S., Amar, C., Nichols, G.L. & McLauchlin, J. (2001) Nested polymerase chain reaction for amplification of the *Cryptosporidium* oocyst wall protein gene. Emerging infectious diseases. 7: 49–56.

Smith, H.V., Robertson, L. J. and Campbell, A.T. (1993) Diagnosis of *Cryptosporidium* on sheep farm with neonatal diarrhea by immune fluorescense assays.Vet. Parasitol.47:17-23.

Snedecor, G.W. and Cochran, W.G. (1989) Statistical Methods, Eighth Edition, Iowa State University Press.

Zorana, M., Katic-Radivojevic, S. and Kulisic, Z. (2006) *Cryptosporidium* infection in lambs and goat kids in Serbia.Vet.Acta.56(1):49-54.