Prevalence and molecular characterization of Cryptosporidium sp. in ruminant livestock in South Sinai, Egypt

Samir A. Taha[1]; Safaa M. Barghash[2]; and Sara S. Serag[1,*]

[1] Zoology Department, Faculty of Science, Zagazig University, Egypt.
[2] Parasitology Unit, Animal and Poultry Health Department, Animal Production and Poultry Division, Desert Research Center, Cairo, Egypt.

Corresponding author: barghash_7@yahoo.com

ABSTRACT : **Cryptosporidium is apicomplexan parasite that can infect a wide range of animal species as well as humans causing life-threatening diarrhoea, especially in young hosts. In Egypt, few studies on the molecular characterization of *Cryptosporidium* have been described in humans, but few data were available in animals has been performed until now. The present study aimed to detect and characterize *Cryptosporidium spp*. for the first time in 195 faecal specimens from ruminant animals of different sites from the South Sinai Governorate. The study was carried out from October 2020 to October 2021 using Ziehl-Neelsen fast stain (ZN) and a nested-PCR (nPCR) that targeted the Cryptosporidium oocyst wall protein *(COWP)* gene. Results showed an overall prevalence rate of infection in 105/195 (53.85%) and 86 /195 (43.59%) using ZN and nPCR, respectively. It was (68.6%, 50%, and 48.2%) by ZN and (49%, 53%, and 39.2%) by nPCR in cattle, goats, and sheep, respectively. Host-species, age, and status of faecal samples were risk factors for cryptosporidiosis, whereas location, sex, and clinical state have no significant difference (p> 0.05). The infections were due to *C. parvum*, which is dominant in Egyptian isolates preserved in GenBank data, and no correlation between the genotype and the geographic origins. We concluded that the presence of potentially zoonotic species of *Cryptosporidium* in ruminants in this region suggest that livestock could potentially contribute to human cryptosporidiosis in the study area. Further molecular studies on local human populations are required to understand the transmission dynamics of *Cryptosporidium* spp. in that region.**

KEYWORDS: Cryptosporidium parvum, nested-PCR, Gene sequencing, Ruminants, Egypt

----

I. INTRODUCTION

Cryptosporidiosis is a global disease caused by apicomplexan parasites belonging to the genus Cryptosporidium. It is a significant source of gastrointestinal illness for a wide range of hosts, including humans (Xiao, 2010; Ryan et al., 2016). Molecular investigations have been identified nearly 40 species and over 50 genotypes. Out of them, 13 Cryptosporidium species are currently accepted, based on host specificity, pathogenesis, morphology and genotyping (Fayer et al., 1997 and Fall et al., 2003): C. hominis, C. parvum, C. wrairi, C. felis, C. canis, C. andersoni, and C. muris as infecting mammals. Of which C. hominis and C. parvum are the most prevalent in humans and ruminants, causing asymptomatic or mild-to-severe gastrointestinal diseases (Ryan et al., 2014; Feng et al., 2018; Firoozi et al., 2019; Roellig and Xiao, 2020).

Among livestock, ruminants are considered important reservoirs of both host-specific and zoonotic Cryptosporidium species as they shed a large number of oocysts that cause environmental contamination (Xiao, 2010 and Santin, 2020). In particular, cattle have been considered a health risk to humans due to the potential source of cryptosporidiosis (Santin, 2020). Cryptosporidium is common in young domestic ruminants (calves, lambs and goat kids). It is associated with economic losses due to the cost of veterinary care, and the clinical symptoms included diarrhoea, dehydration, delayed growth and weight loss, often leading to death (Ye et al., 2013; Hatam-Nahavandi et al., 2019; Mammeri et al., 2019 and Santin, 2020). The present study represents the first record about the infection of such an area with Cryptosporidium in Sinai (south province), where no information was available concerning the infection of ruminants with such parasites. Therefore, the present study aimed to use molecular tools to identify Cryptosporidium spp. in ruminant livestock.

II. MATERIALS AND METHODS

2.1. Study area

Sinai is triangular in northeast Egypt that occupies an area of 61,000 square km between the Mediterranean Sea to the north and the Red Sea to the south and is a land bridge between Asia and Africa. The nomadic Bedouin trips migrated in search of water, and pasturage sharply decreased but increasingly attracted to agriculture recently. Sheep and goats in Sinai were restricted for centuries, but many new farms included these animals and cattle, were modern established. Those animals originated from the north and middle Sinai and other sites of Egypt. Also, many cattle are imported annually from Brazil. There is an irregular use of antiprotozoal drugs. Two main sites (Ras-Sudr and El Tur) were selected for field blood samples due to the increased number of farms and settled animals. Besides, that imported animals from the north (Alarish) and middle Sinai (Alhasna and Nekhel). Climate sharply changes from north to south by the year.

2.2. Sampling and field data

In the present study, *Cryptosporidium* infections were investigated from 15 randomly selected farms found in El tur and Ras-Sudr. The study was carried out from October 2020 to October 2021 on 114 diarrheic and 81 non-diarrheic. The faecal samples were collected from 51 cattle, 112 sheep, and 32 goats and classified into 3- groups: group I aged 0–3 months, group II aged from 3 months to 1 year and group III aged > 1year. The faecal specimens were collected directly from the rectum of the animals to the Parasitology Lab. Modified precipitation technique performed on all samples within 24 hrs. of collection and then divided into two parts; one for microscopic examination and the other was stored at 4°C until used in molecular studies. The scientific team recorded data in questionnaires during the sampling time for further analysis.

2.3. Macroscopical examination

Faecal samples have been examined by the naked eye to establish the faecal consistency as normally (well-formed faeces), pasty (soft, not well-formed) or diarrheic (liquid faeces), besides the presence of mucus or blood before being transported in a cool box.

2.4. Microscopy screening

All samples were concentrated and then screened for the presence of Cryptosporidium oocysts by Modified Ziehl–Neelsen according to the method described by Henriksen and Pohlelenz (1981). Smears of faecal specimens were placed on a slides rack and left to dry at room temperature, and then fixed in methanol for ten minutes. Carbol fuchsin fast-stain was performed on the faecal smears for 3 min, and then the slides were washed with tap water. A concentration of 7% Sulphuric acid has been added to the specimens for de-colourization for 30 seconds and rinsed with tap water for two min., and followed by staining with 5% malachite green for 2-5 min. Finally, rinsed with tap water and left to dry at room temperature to examine at a magnification of objective lens (40X) and (100X) respectively.

2.5. DNA extraction and species-specific primers

Genomic DNA was extracted from 100 mg of each faecal sample using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). It is used according to the manufacturer's recommendations and subsequently frozen at-20°C until use. Cryptosporidium extracted DNA amplification was performed by a nested-PCR that targeted the Cryptosporidium oocyst wall protein (COWP) gene, which included two sequential PCR reactions as followed in Table 1.

2.6. PCR amplification

The first PCR reaction was conducted with the outer primers, COWPF and COWPR in a 25μL total volume containing 12.5μL Premix Taq DNA Polymerase (TaKaRa, Tokyo), 1μM of each primer, and 5μL of genomic DNA. The PCR reaction was started with a one-step initial denaturation at 94°C for 5 min, was followed by 35 cycles of denaturation at 95°C for 30 s, 65°C for 40 s and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. In the second step, the pair of primers Cry9 and Cry15 were, respectively, used as the forward and reverse primers. The reaction mixture had the same composition as the first PCR, except the template was replaced by 1μL of the first PCR product. It started with an initial denaturation step of 94°C for 30 s, followed by 35 cycles of denaturation (94°C for 30 s), primer annealing (55°C for 40 s), and extension (72°C for 45 s). The final extension step was at 72°C for 10 min. Reactions were performed in a gradient thermal cycler after adjusting the thermal profiles. The amplified nPCR products were stained with ethidium bromide and electrophoresed on agarose gel (1.5%) in TAE buffer and then visualized under a UV trans-illuminator.

2.7. DNA sequence and Phylogenetic analyses

The PCR products were purified using a QIA-quick PCR product extraction kit (Qiagen Inc. Valencia CA). They were sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA), Cat. No.4336817. The BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed to establish sequence identity to GenBank accessions. The comparative analysis of sequences was performed using the CLUSTALW multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, designed by (Thompson et al., 1994). Phylogenetic analyses were analyzed using maximum likelihood, neighbour-joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

III. RESULTS AND DISCUSSION

3.1. Parasitological and molecular prevalence of Cryptosporidium

Modified Ziehl–Neelsen acid-fast microscopy for *Cryptosporidium* oocysts screening applied in 195 faecal specimens from animals (cattle, sheep and goats) of different sites, species, ages, sexes, and statuses on 15 farms (Table 2). It was present in the faecal samples collected from 14 studied farms with an overall prevalence of 105/195 (53.85%). The oocysts were round and stained red with a green background (Figure 2). It was higher detected in ruminants in El-Tur (58.8%) compared to Ras-Sudr (47%) and in males (59.2%) than females (50.4%). By age group: the infection rates were 74% in the animal group aged less than three months, 47.8% in the group aged less than one year, and 45.3% in adults over one year. Cryptosporidium was present at much higher frequencies seen in cattle (68.6%), followed by nearly equal frequencies in goats (50%) and sheep (48.2%). Animals with diarrheic faecal specimens' were having a higher percentage of Cryptosporidium oocysts (85%) than soft (60.8%) and normal statuses (29%). Also, the symptomatic animals with no known cause were positively infected with Cryptosporidium (67.6%) more than The clinically asymptomatic animals (46%). Based on smears microscopical examination, significant differences have been found within the age groups (P= 0.001), and within the status of faecal specimens revealed risk factors affected cryptosporidiosis (P <0.0001).

In the present study, PCR analysis gave positive results of Cryptosporidium, which was lower than that reported by using, modified Ziehl–Neelsen acid-fast staining (microscopically examination) (Table 2). However, the identification of the COWP gene was successful and confirmed the presence of Cryptosporidium in 86 (81.9%) out of 105 microscopically positive specimens (Figure 3). It generated bands with 769bp in the first round and 553bp in the second round of PCR. It was sharp in the concentrated samples than that directly frozen. Despite that, significant differences have been estimated between animal species and within sex and age groups. The intercept between all parameters showed highly significant differences (p< .0001). Cryptosporidium prevalence varies in different groups using the selected nested-PCR. The higher-rated recorded in Ras-Sudr (46.9%), goats (53%), and females (45.4%) in contrast to microscopical examination. The correlation between microscopical and PCR detection of Cryptosporidium appeared in the higher results were recorded in the youngest aged group (60%), symptomatic animals (62%), and in diarrheic faecal samples (76.6%). The statistical analyses showed a significant difference between the results obtained by PCR and microscopical examination (Table 3).

Molecular characterization of Cryptosporidium isolates

In the present study, Cryptosporidium parvum has detected in the study area. The sequence analysis of the COWP gene PCR products identified six typical C. parvum that submitted to GenBank compared to the other Cryptosporidium preserved strains. The analysis of the six genetic loci showed no genetic diversity between them, and their accession numbers and data were listed in Table (4). They identified under accession numbers: MW925057, MW925058, MW925059, MW925060, MW925061, and MW925062. They are clustered together with another six Egyptian strains of C. parvum followed by the name Sakha, which were preserved in GenBank data (Figure 4). They are identical, and the sequence identity between them is 100% with no different SNPs or divergent and 0.00 genetic distance. Their identities with other preserved strains range from 75.8 to 100%, and genetic distance from 0.00 to 29.4.

Concerning the prevalence of Cryptosporidium in ruminants, C. parvum infections commonly cause profuse watery diarrhoea, which sometimes contains mucus or blood, dehydration, and weight loss. The disease causes severe illness and death in young ruminants. Therefore, Cryptosporidium infections lead to substantial economic losses (De Graaf et al., 1999). C. parvum is not host-specific; accordingly, an environment contaminated with oocysts during an outbreak in calves can rise to infection in lambs and goat kids that subsequently use the same grazing area (Kabir et al., 2020).

During the present study, Cryptosporidium spp. infected cattle, sheep, and goats are of public health significance in the study area, resulting in diarrhoea and inefficient weight gains and may lead to economic losses due to morbidity and mortality. Out of the examined faecal samples of ruminant livestock (cattle, sheep and goats), the prevalence of Cryptosporidium was higher, based upon microscopic examination (53.85%), than by using nPCR targeting the COWP gene (44.1%). It may be because microscopic techniques could distinguish a microorganism among various species since it shares morphological characteristics and size with other microorganisms causing false-positive results (Thompson et al., 2016 and Yap et al., 2016). The negative nPCR may be due to the presence of inhibitors in faecal samples, cyst quantity and quality, samples storage conditions, DNA extraction method, the gene targeted and the number of copies, and choice of primers and cycling conditions (Wilke and Robertson, 2009). Al-Zubaidi (2017) showed significant differences (p<0.05) in the prevalence of cryptosporidiosis in sheep, depending on the used tool; it was 48.88% and 37.77% by PCR and ZN, respectively.

During the present study, the overall prevalence rate of Cryptosporidium was 44.10 %, of which the prevalence of Cryptosporidium spp. in cattle, sheep and goats were 49%, 53%, 39%, respectively. In sheep, the result is lower than the recorded prevalence in India was more than 50% (Bhat et al., 2019). Some reports showed a considerably higher prevalence of Cryptosporidium oocyst as in Spain (31-59%), which warns with care because matching for characteristics of animals and their raising conditions is a challenging variant (Romero-Salas et al., 2016). In contrast, the lower prevalence in Iraq was 48.88% using PCR (Al-Zubaidi, 2017). Comparable results recorded a lower prevalence of cryptosporidiosis in cattle from Assam, India 16.43% by Das et al. (2015), and 33.2% in calves in Egypt by Amer et al. (2010). It was higher (64%) in cattle in Mexico (López Torres et al., 2020). These variations in the prevalence may be due to sampling size, geographical region, climate, and age of animals, diagnostic methods used, breed, hygiene conditions, and the poor hygienic-sanitary conditions in the agroecosystems in agreement with Santin (2020).

The present study showed a significant difference (p=0.001) between age groups and the infectivity prevalence of parasites. The highest prevalence of cryptosporidiosis recorded in the age group < three months reach 60%. It is similar to Majeed et al. (2018), who reported that the age group was a high prevalence of Cryptosporidium spp. in the pre-weaned lambs and kids less than three months. A similar study in Turkey showed the positivity of Cryptosporidium infection was higher in 3–7 days of age and lower in 16–30 days of age in calves, lambs and goat kids (Kabir et al., 2020). Also, the oocysts presented with higher rates in ≤ 7 day-old age groups and lower rates in > 21-day-old age groups (Sari et al., 2009). It agrees with the study in Iraq by Al-Zubaidi (2017), who recorded a significant difference (p<0.05) between age groups in sheep and infectivity prevalence of parasites in the age group < six months compared with the lowest infection rate in the age group from 25 to 36 months reached 70%, 39.13%, respectively. Also, such finding agreed with Abd-El-Wahed (1999) in Egypt, Sari et al. (2009) in Turkey, who reported a high prevalence rate of the parasite in small lambs. Bovine cryptosporidiosis is a disease of neonates. The low prevalence in adults referred to asymptomatic carriers of infection might be a source of infection for younger animals, as reported by Xiao and Feng (2008). In extensive farming systems, the calves moved to the pasture with age reduced contact (Martins-Vieira et al., 2009).

Lambs are weaned 30–60 days after birth in the present study, and, therefore, a high risk of maternal transmission of the protozoan to lambs may occur. Sharing the same night shelter and the natural suckling reported in the present survey may contribute to transmitting Cryptosporidium infection among different animals, agrees with Dessì et al. (2020). Concerning sex, the present study showed no significant difference between male and female infection rates (p > 0.05). This result agreed with Das et al. (2018), who found no significant variation (p > 0.05) in the sex-wise occurrence of Cryptosporidium in cattle. Similarly, no sex preponderance of Cryptosporidium was noted in sheep and goats in Kuwait (Majeed et al., 2018). In Iran, the examined ruminants (cattle, sheep and goats) were infected with Cryptosporidium with no significant difference due to equal possibility of exposure to the contaminated environment (Abd Al-Wahab, 2003; Fayer and Xiao, 2007; Al-Zubaidi, 2017 and Firoozi et al., 2019).

Also, the study showed a significant difference (p<0.05) between the infection rate of cryptosporidiosis among diarrheic, soft and non-diarrheic animals, which reached 85%, 60.8%, and 29% by Ziehl–Neelsen, and 76.6%, 46.3% and 22.8% by nPCR, respectively. However, the highest percentages of infection have recorded in lambs with diarrhoea coincides with those reported by Papanikolopoulou et al. (2018); Mammeri et al. (2019), and Rabee et al. (2020). Our findings were in agreement with the results of Dessì et al. (2020) in Italy. However, these differences were only statistically significant in pre-weaned animals where the risk was positive to Cryptosporidium spp. in sheep (Quílez et al., 2008; Al-Zubaid, 2017; Das et al., 2018 and Papanikolopoulou et al., 2018).

The molecular characterization of Cryptosporidium DNA where DNA isolation from faecal specimens was not simply due to the inhibitors in stool, like plant and food impurities that can interfere with the PCR reaction. In general, the present study has reported lower infection rates using molecular methods than microscopical examination, especially in samples containing a low number of oocysts. Such findings have agreed with the previous studies (Fayer et al., 2007; Mueller-Doblies et al., 2008 and Diaz et al., 2010).

Genotyping analysis of Cryptosporidium demonstrated the existence of at least three species in Egypt: C. hominins, C. parvum, and C. ubiquitum. Of which, C. hominis is almost exclusively a human parasite. In the present study, sequence analysis of the presently submitted isolates showed 100 % identity with only C. parvum agrees with Amer et al., 2010. The identified Egyptian isolates under accession numbers: AB5114045, AB5114046, AB5114059, AB5114060, AB5114061, and AB5114064 isolated from calf (Amer et al., 2010). Also, 100% identity with accession numbers: BX538351 (Bankier et al., 2003) United Kingdom, AB089292 and DQ388390 from human and Homo sapien (Satoh et al., 2005 and Wielinga et al., 2008) isolated from Japan and accession no. KY706489 isolated from mussel-Italy (Marangi, 2018). Whereas it was closely related to two Egyptian isolates with accession numbers (MK033055 and MK033056) were isolated from water and human stool by El-Badry et al. (2018) and Ghallab et al. (2018). Other studies in Egypt (Mahfouz et al., 2014 and Elmadawy et al., 2017) identified the prevalence of two Cryptosporidium sp. in sheep (C. parum and C. ubiquitum), with the latter being more common.

Globally, there are differences in the distribution of the pathogenic C. parvum, and the smaller ruminant-specific species among different countries were C. ubiquitum and C. xiaoi (Xiao, 2010). In the Middle East: only C. xiaoi or C. parvum was initially recorded from sheep and goats in Tunisia (Soltane et al., 2007). In Jordan, Hijjawi et al. (2016) found C. xiaoi was the most common species in small ruminants, followed by C. parvum and C. andersoni. C. parvum was the predominant species identified in small ruminants in Kuwait, with only a few samples containing C. ubiquitum (n = 5) and C. xiaoi (n = 2) (Majeed et al., 2018). On the other hand, European studies revealed that C. parvum was mostly affected lambs, whereas C. ubiquitum and C. xiaoi were commonly in healthy lambs (Mueller-Doblies et al., 2008 and Quílez et al., 2008).

Table (1): Oligonucleotide primers sequences

Target gene	Primer	Sequence	Amplicon Size	Reference
COWP	COWPF	ACCGCTTCTCAACAACCATCTTGTCCTC	769 bp	Feltus *et al.*, 2006
	COWPR	CGCACCTGTTCCCACTCAATGTAAACCC		
	Cry9	GGACTGAAATACAGGCATTATCTTG	553 bp	
	Cry15	GTAGATAATGGAAGAGATTGTG		

Table (2): Parasitological and molecular detection of *Cryptosporidium* spp. in South Sinai Governorate

Parameter	Symbol	No.	Crypto-MIC +Ve	Crypto-MIC (%)	*P*	Crypto-PCR +Ve	Crypto-PCR (%)	*P*
Location	El-Tur	114	67	58.8	0.587	48	42	0.237
	Ras-Sudr	81	38	47		38	46.9	
Species	Cattle	51	35	68.6	0.213	25	49	0.011
	Goats	32	16	50		17	53	
	Sheep	112	54	48.2		44	39.2	
Sex	Male	76	45	59.2	0.727	32	42	0.056
	Female	119	60	50.4		54	45.4	
Age	<3 months	50	37	74	0.025	30	60	0.001
	< 1 year	92	44	47.8		37	40.2	
	>1 year	53	24	45.3		19	35.8	
Clinical	A symptomatic	124	57	46	0.493	42	33.9	0.232
	symptomatic	71	48	67.6		44	62	
Status	Hard	79	23	29	0.000	18	22.8	0.000
	Soft	69	42	60.8		32	46.3	
	Diarrhea	47	40	85		36	76.6	
Intercept				0.000			0.000	

P= probability, MIC= microscopical

Table (3): Correlation between variables using microscopic examination and molecular investigation

Variable	*Cryptosporidium*-Microscope Sp.	Age	Sex	Site	State	Status	*Cryptosporidium*-PCR Sp.	Age	Sex	Site	State	Status
Species	.	0.000	0.003	0.000	0.336	0.018	.	0.000	0.002	0.000	0.495	0.009
Age	0.000	.	0.053	0.125	0.429	0.425	0.000	.	0.023	0.245	0.367	0.357
Sex	0.003	0.053	.	0.001	0.038	0.032	0.002	0.023	.	0.001	0.094	0.063
Site	0.000	0.125	0.001	.	0.228	0.001	0.000	0.245	0.001	.	0.094	0.003
State	0.336	0.429	0.038	0.228	.	0.005	0.495	0.367	0.094	0.094	.	0.010
Status	0.018	0.425	0.032	0.001	0.005	.	0.009	0.357	0.063	0.003	0.010	.

Significant differences have been recorded between species and each category of age, sex, site (locality), and status of fecal samples whereas, no significant differences between age and each of clinical state, site, and status by the two tests.

Figure 1: Map of Sinai Peninsula shows the source of the collected samples from different ruminants.

Figure 2: Photomicrograph of fecal smear stained with ZN showing cyst of *Cryptosporidium*
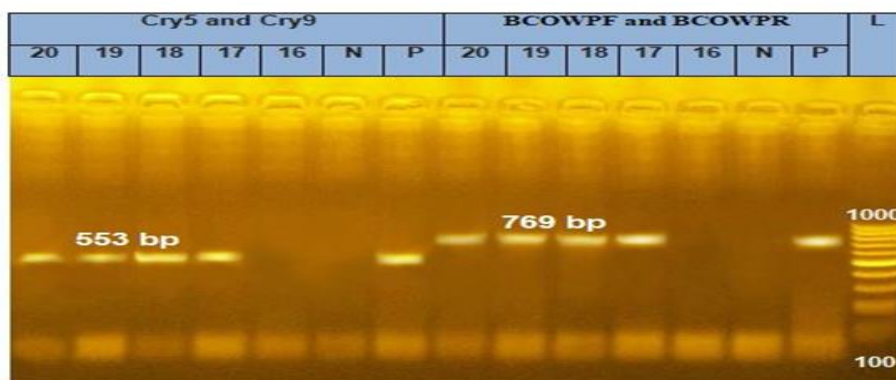

Figure (3): 1.5% Agarose gel electrophoresis shows the positive and negative results using nested PCR targeted *COWP* gene. L=ladder of 100-1000 bp, Lanes P and N correspond to positive and negative controls, respectively. Lane 16 corresponds to a negative result. Lanes: from 17 to 20 correspond to positive results.
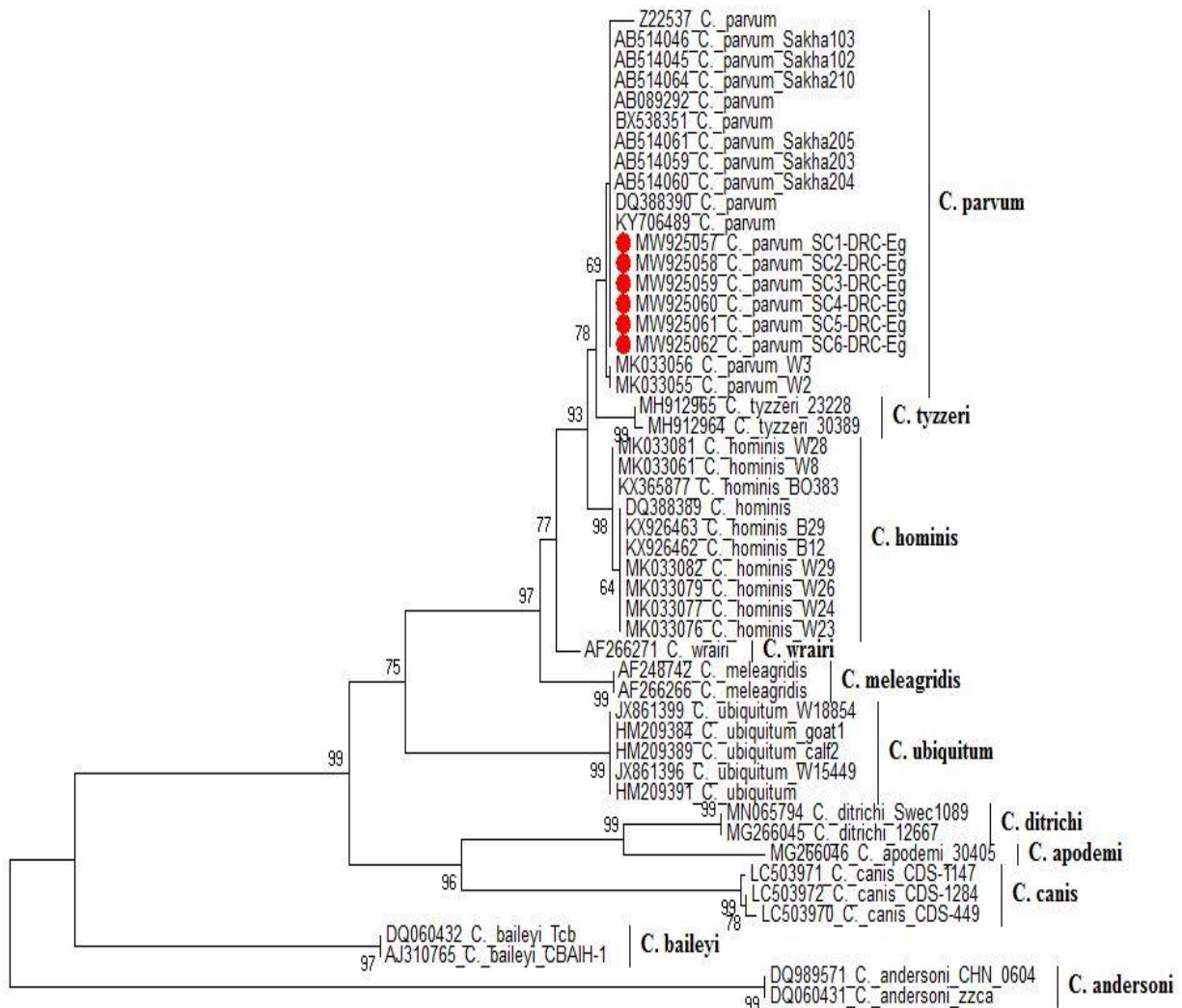
Figure 4: Consensus tree constructed based on the nucleotide sequence data of the *COWP* gene (553 bp) of submitted *Cryptosporidium* isolates and other referenced isolates in GenBank Sequences.

V –CONCLUION

In the present study, the prevalence and genetic diversity of Cryptosporidium in ruminants settled in South Sinai by Ziehl-Neelsen and nested-PCR based assay revealed that microscopical examination was higher than nPCR-to detect the findings of Cryptosporidium. Also, C. parvum is only prevalent with a relatively moderate rate compared to those previously studied in different locations in Egypt. Cattle, goats and sheep were infected, and species, age, and status of faecal samples were risk factors for cryptosporidiosis, whereas location, sex, and clinical state have no significant difference. Our submitted isolates were 100% identical with other Egyptian isolates from cows and closely related to two other isolates from water and human were recorded in GenBank data. These findings suggest that zoonotic transmission of Cryptosporidium is probably occurring frequently in the study area.

REFERENCES

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

Abd Al-Wahab, I., (2003): Study in the epidemiology of the Intestinal Protozoa (*Eimeriaspp. Cryptosporidium spp. Giardia spp.*) in the Sheep in Baghdad Province. M. Sc. Thesis, College of Veterinary Medicine, University of Baghdad. pp: 123,

Al-Zubaidi, M.T. (2017): Molecular and microscopic Detection of *Cryptosporidium spp* in Sheep in AL-Taji area-Baghdad/Iraq. Intl. J. Sci. and Nat., (8):372-376.

Amer, S.; Honma, H.; Ikarashi, M.; Tada, C.; Fukuda, Y.; Suyama, Y. and Nakai, Y. (2010): *Cryptosporidium* genotypes and subtypes in dairy calves in Egypt. Vet., Parasitol., (169):382-386.

Bankier, A.T., Spriggs,H.F., Fartmann,B., Konfortov,B.A., Madera,M., Vogel,C., Teichmann,S.A., Ivens,A. and Dear,P.H.(2003): Integrated mapping, chromosomal sequencing and sequence analysis of *Cryptosporidium parvum.* Genome Res., 13 (8): 1787-1799 .

Bhat, A.M.; Malik, H.; Wani, N.M.; Paul, S.; Gupta, S.; Dolma, T. and Singh, S. (2019): First report of *Cryptosporidium* sp. infection in sheep population of Ladakh, India. J. Parasit. Dis., (43):513-516.

Das, M.; Deka, D.K. and PC, S. (2015): *Cryptosporidium* infection in cattle of sub-tropical region of Assam, India. Intl. J Sci. Res., (4):10-12.

Das, P.; Deka, D.; Borthakur, S.K.; Roychoudhury, P. and Das, M. (2018): Studies on occurrence, molecular detection and genotyping of *Cryptosporidium parvum* along with associated risk factors in cattle and human from Aizawl district, Mizoram, India. Biol. Rhythm Res., (51):238-253.

De Graaf, D.C.; Vanopdenbosch, E.; Ortega-Mora, L.M.; Abbassi, H. and Peeters, J.E. (1999): A review of the importance of cryptosporidiosis in farm animals. Intl. J. Parasitol., (29):1269-1287.

Dessì, G.; Tamponi, C.; Varcasia, A.; Sanna, G.; Pipia, A.P.; Carta, S.; Salis, F.; Díaz, P. and Scala, A. (2020): *Cryptosporidium* infections in sheep farms from Italy. Parasitol. Res., (119):4211-4218.

Diaz, P.; Quilez, J.; Chalmers, R.M.; Panadero, R.; Lopez, C.; Sanchez-Acedo, C.; Morrondo, P. and Diez-Banos, P. (2010): Genotype and subtype analysis of *Cryptosporidium* isolates from calves and lambs in Galicia (NW Spain). Parasitol., (137):1187-1193.

El-Badry, A.A., Hamdy, D.A. and Abd El Wahab, W.M. (2018): *Strongyloides Stercoralis* Larvae Found for the First Time in Tap Water Using a Novel Culture Method. Parasitol Res., 117, 3775-3780.

Elmadawy, R.S.; Diab, M.S. and Elnaker, Y.F. (2017): Prevalence, electron microscopy and molecular characterization of *Cryptosporidium* species infecting sheep in Egypt. J. Advanced Vet. Res., (7):47-52.

Fall, A.; Thompson, R.C.A.; Hobbs, R.P. and Morgan-Ryan, U. (2003): Morphology is not a reliable tool for delineating species within *Cryptosporidium*. J.Parasitol., (89):399-402.

Fayer, R.; Santin, M. and Trout, J.M. (2007): Prevalence of *Cryptosporidium* species and genotypes in mature dairy cattle on farms in eastern United States compared with younger cattle from the same locations. Vet Parasitol., (145):260-266.

Fayer, R. and Xiao, L., (2007): *Cryptosporidium* and cryptosporidiosis. CRC press.

Feltus, D.C.; Giddings, C.W.; Schneck, B.L.; Monson, T.; Warshauer, D. and McEvoy, J.M. (2006): Evidence Supporting Zoonotic Transmission of *Cryptosporidium* spp. in Wisconsin. Clin. Micro., 44(12):4303-4308.

Feng Y, Ryan UM, Xiao L (2018): Genetic Diversity and Population Structure of *Cryptosporidium*. Trends Parasitol., 34(11):997-1011

Firoozi, Z.; Sazmand, A.; Zahedi, A.; Astani, A.; Fattahi-Bafghi, A.; Kiani-Salmi, N.; Ebrahimi, B.; Dehghani-Tafti, A.; Ryan, U. and Akrami-Mohajeri, F. (2019): Parasites &Vectors, 12, 510. https://doi.org/10.1186/s13071-019-3759-2

Ghallab, M.M.I., Khater, M.M., Rubio, J.M. and El-Badry, A.A.(2018): Genotypic variance analysis of *Cryptosporidium* human isolates from stool in Egypt. J. Egypt. Soc. Parasitol., 48(3),: 557 – 562.

Hatam-Nahavandi K, Ahmadpour E, Carmena D, Spotin A, Bangoura B, Xiao L (2019): *Cryptosporidium* infections in terrestrial ungulates with focus on livestock: a systematic review and meta-analysis. Parasite and Vectors, 12(1):453

HENRIKSEN, S.A. AND POHLENZ,J.F.L.(1981): STAINING OF CRYPTOSPORIDIA BY A MODIFIED ZIEHL-NEELSEN TECHNIQUE. ACTA VET SCAND., (22):594.

Hijjawi, N.; Mukbel, R.; Yang, R. and Ryan, U. (2016): Genetic characterization of *Cryptosporidium* in animal and human isolates from Jordan. Vet. parasitol.,(228):116-120.

Kabir, M.H.B.; Ceylan, O.; Ceylan, C.; Shehata, A.A.; Bando, H.; Essa, M.I.; Xuan, X.; Sevinc, F. and Kato, K. (2020): Molecular detection of genotypes and subtypes of *Cryptosporidium* infection in diarrhoeic calves, lambs, and goat kids from Turkey. Parasitol. Intl.,(79):102163.

López Torres, L.; López Cuevas, O.; Vázquez Vázquez, C.; Alvarado Gómez, O.; Vázquez Alvarado, R.; Rodríguez Fuentes, H.; Chaidez Quiroz, C. and Vidales Contreras, J. (2020): Prevalence of *Cryptosporidium* spp. in dairy livestock from the Laguna region, Mexico. Revista bio ciencias.(7), e881 .

Mahfouz, M.E.; Mira, N. and Amer, S. (2014): Prevalence and genotyping of *Cryptosporidium spp* in farm animals in Egypt. J.Vet.Med. Sci., (14), 0272.

Majeed, Q.A.H.; El-Azazy, O.M.E.; Abdou, N.-E.M.I.; Al-Aal, Z.A.; El-Kabbany, A.I.; Tahrani, L.M.A.; AlAzemi, M.S.; Wang, Y.; Feng, Y. and Xiao, L. (2018): Epidemiological observations on cryptosporidiosis and molecular characterization of Cryptosporidium spp. in sheep and goats in Kuwait. Parasitol. Res,(117):1631-1636.

Mammeri, M.; Cartou, L.; Chevillot, A.; Thomas, M.; Julien, C.; Vallée, I.; Polack, B.; Follet, J. and Adjou, K.T. (2019): First identification of *Cryptosporidium parvum* zoonotic subtype IIaA15G2R1 in diarrheal lambs in France. Veterinary Parasitology: Regional Studies and Reports. (18):100355.

Marangi, M.(2018): *Toxoplasma gondii* and other zoonotic protozoans in the Mediterranean mussel *Mytilus galloprovincialis* and the Blue mussel *Mytilus edulis.* Intl. J Food Prot. 82(3):535-542.

Martins-Vieira, M.; Brito, L. and Heller, L. (2009): Oocistos deCryptosporidium parvum em fezes de bezerro infectado experimentalmente. Arquivo Brasileiro Med.Vet Zoo.,(61):1454-1458.

Mueller-Doblies, D.; Giles, M.; Elwin, K.; Smith, R.P.; Clifton-Hadley, F.A. and Chalmers, R.M. (2008): Distribution of Cryptosporidium species in sheep in the UK. Vet. Parasitol.,(154):214-219.

Papanikolopoulou, V.; Baroudi, D.; Guo, Y.; Wang, Y.; Papadopoulos, E.; Lafi, S.Q.; Abd El-Tawab, M.M.; Diakou, A.; Giadinis, N.D. and Feng, Y. (2018): Genotypes and subtypes of *Cryptosporidium* spp. in diarrheic lambs and goat kids in northern Greece. Parasitol. Intl.,(67):472-475.

Quílez, J.; Torres, E.; Chalmers, R.M.; Hadfield, S.J.; Del Cacho, E. and Sánchez-Acedo, C. (2008): *Cryptosporidium* genotypes and subtypes in lambs and goat kids in Spain. Appl. Environ. Microbiol.,(74):6026-6031.

Rabee, A.H.; Merza, A.D. and Al-Hasnawy, M.H. (2020): Detection of parasites causing diarrhoea in lambs of Babylon Governorate, Iraq. Plant Archives.(20):1528-1532.

Roellig DM, Xiao L (2020): *Cryptosporidium* genotyping for epidemiologytracking. Methods Mol Biol 2052:103–116

Romero-Salas, D.; Alvarado-Esquivel, C.; Cruz-Romero, A.; Aguilar-Domínguez, M.; Ibarra-Priego, N.; Merino-Charrez, J.O.; De León, A.A.P. and Hernández-Tinoco, J. (2016): Prevalence of *Cryptosporidium* in small ruminants from Veracruz, Mexico. BMC Vet. Res.,(12):1-6.

Ryan U, Fayer R, Xiao L (2014) *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology 141(13):1667–1685

Ryan U, Zahedi A, Paparini A (2016) *Cryptosporidium* in humans and animals—a one health approach to prophylaxis. Parasitol. Immunol., 38(9):535–547

Santin, M. (2020): *Cryptosporidium* and Giardia in Ruminants. Veterinary Clinics of North America: Food Animal Practice.(36):223-238.

Sari, B.; Arslan, M.Ö.; Gicik, Y.; Kara, M. and Taşçi, G.T. (2009): The prevalence of *Cryptosporidium* species in diarrhoeic lambs in Kars province and potential risk factors. Trop. Anim. health Produc.,(41):819-826.

Satoh,M., Kimata,I., Iseki,M. and Nakai,Y.(2005) Gene analysis of *Cryptosporidium parvum* HNJ-1 strain isolated in Japan. Parasitol. Res. 97, (6), 452-457.

Soltane, R.; Guyot, K.; Dei-Cas, E. and Ayadi, A. (2007): Prevalence of *Cryptosporidium* spp.(Eucoccidiorida: Cryptosporiidae) in seven species of farm animals in Tunisia. Parasitology, (14):335-338.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013): MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thompson, J.D.; Higgins, D.G. and Gibson, T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22(22):4673-4680.

Thompson, R.A.; Koh, W.H. and Clode, P.L. (2016): *Cryptosporidium*-what is it? Food and Waterborne Parasitology.(4):54-61.

Wielinga, P.R., de Vries, A., van der Goot,T.H., Mank,T., Mars,M.H., Kortbeek,L.M. and van der Giessen,J.W.(2008) Molecular epidemiology of *Cryptosporidium* in humans and cattle in The Netherlands. Int. J. Parasitol., 38 (7), 809-817.

Wilke, H. and Robertson, L.J. (2009): Preservation of *Giardia* cysts in stool samples for subsequent PCR analysis. J.Microbiol. Methods.,(78):292-296.

Xiao, L. (2010): Molecular epidemiology of cryptosporidiosis: an update. Exper. Parasitology.(124):80-89.

Xiao, L. and Feng, Y. (2008): Zoonotic cryptosporidiosis. FEMS Immunol. & Medical Microbiol., (52):309-323.

Yap, N.J.; Koehler, A.V.; Ebner, J.; Tan, T.K.; Lim, Y.A. and Gasser, R.B. (2016): Molecular analysis of *Cryptosporidium* from cattle from five states of Peninsular Malaysia. Mol. Cell. probes.(30):39-43.

Ye J, Xiao L, Wang Y, Wang L, Amer S, Roellig DM, Guo Y, Feng Y (2013): Periparturient transmission of *Cryptosporidium xiaoi* from ewes to lambs. Vet.Parasitol. 197(3–4):627–633