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Prevalence, Electron Microscopy and Molecular Characterization of *Cryptosporidium* species Infecting Sheep in Egypt

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ABSTRACT

Cryptosporidium sp. is predominant universally and sheep are an imperative zoonotic supply of the disease. Owing to the little information presented with respect to *Cryptosporidium* sp. infecting sheep, this study was directed to survey the predominance and molecular characterization of *Cryptosporidium* sp. among sheep of different ages and sexes in Qalyubia governorate, Egypt. The fecal specimens were gathered from 432 sheep of various ages (≤1 to <6, 6-12 and >12 months) and sexes. The samples were microscopically examined after staining by modified Zeihl- Neelsen technique and the intestinal mucosa was scanned by electron microscopy. A nested PCR was connected to amplify a 830 bp of 18S rRNA sequence of *Cryptosporidium*. RFLP (restriction fragment length polymorphism) technique using *SspI* and *VspI* enzymes for digestion of the secondary product of PCR for species identification was applied. The total infection rate was 25.93%. The parasite was more prevalent in males than females of different age groups. Two zoonotic *Cryptosporidium* species were distinguished after RFLP-PCR sequencing: *C. parvum* and *C. ubiquitum* (identified previously as Cervine genotype). The finding recommends that sheep must be considered as a noteworthy potential source of human cryptosporidiosis. A strict reconnaissance of zoonotic cryptosporidiosis must be set up to counteract human infection and to assess forthcoming disease when applying control programs.

Introduction

Cryptosporidiosis is considered as an important enteric diseases causing severe diarrhea and inefficient weight gains with a great hazard among small ruminant population (Foreyt, 1990). Molecular techniques could identify *Cryptosporidium* sp. in sheep feces in different countries (Soltane *et al*., 2007; Geurden *et al*., 2008; Quilez *et al*., 2008; Fayer and Santin, 2009; Féres *et al*., 2009; Paoletti *et al*., 2009; Yang *et al*., 2009; Wang *et al*., 2010;). *C. parvum, C. xiaoi* and *C. ubiquitum* are mainly responsible for *Cryptosporidium* infections in sheep, whereas *C. hominis*, *C. andersoni*, *C. fayeri*, *C. suis* and pig genotype II have already been recognized in a low number of animals (Majewska *et al*., 2000). Sheep can harbor the zoonotic *Cryptosporidium* sp. causing clinical manifestations in human and they are considered as a main source of *Cryptosporidium* infection either by direct infection or by pollution of the environment (Castro-Hermida *et al*., 2007; Geurden *et al*., 2008; Paoletti *et al*., 2009). In Egypt, owing to the incessant human contact with sheep which usually excrete the most number *Cryptosporidium* oocysts during diarrhea (Bukhari and Smith, 1997) and due to the less information about molecular characterization and zoonotic impact of sheep cryptosporidiosis, so the present study was conducted to determine the prevalence of *Cryptosporidium* among different age and sex groups of sheep to identify the species infecting them using RFLP-PCR molecular technique, in addition to electron microscopic study of the parasite

Materials and methods

Samples

The identification of the oocysts of *Cryptosporidium* was done on haphazardly gathered fecal specimens from sheep (124 males 308 females) obtained from different localities in Qalyubia governorate, Egypt. The samples were gathered directly from the rectum into plastic packs that were promptly topped, marked, put in a protected holder and transported to the parasitology laboratory in the Faculty of Veterinary Medicine, Mohstohor, Egypt, to be prepared and examined within

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24 h. of collection. The specimens were categorized into three groups consistent with age: group 1 (≤1 to <6 months of age, 124 animals), group 2 (6-12 months of age, 124 animals) and group 3 (>12 months, 184 animals). Direct microscopic examination of fecal smears prepared from fresh or concentrated fecal samples, followed by modifying Ziehl-Neelsen staining were performed for accurate oocysts detection (Casemore *et al*., 1985). The clinical symptoms and death cases accompanied cryptosporidiosis were registered. Small parts of the intestinal mucosa of dead lambs due to cryptosporidiosis were kept in glutraldehyde (2.5%) at 4°c for scanning electron microscope. The specimens were rinsed in distilled water, dehydrated in acetone alcohol series (25%, 50%, 75%, and 100% for 10 min each). The critical point drying was done using CO_2. to complete the drying process. Finally, the samples were coated by gold using a spraying tool. Finally, the samples were visualized with SEM (Kuo, 2007).

Molecular diagnosis

It was applied on *Cryptosporidium* oocysts obtained from 112 positive animals. The whole technique was done according to Xiao *et al*. (1999) with modification, where a nested PCR- was applied using the following primers (Promega, USA): Primary PCR primers were 5'-TTCTAGAGCTAATACATGCG-3´(SSU-F2) and 5´-CCCATTTCCTTCGAAACAGGA-3´(SSU-R2) and secondary primers were 5´-GGAAGGGTTGTATTTATTA-GATAAAG-3´(SSU-F3) and 5-AAGGAGTAAGGAACAA-CCTCCA -3´ (SSU-R4). The amplification was done in Perkin Elmer Gene Amp, PCR Thermocycler 2400. The primers were used to amplify 830 bp of the 18S rRNA gene. Primary PCR mixture contained 3 mM $MgCl_2$, 1× PCR buffer, 0.2 mM each dNTP, 1U of BSA (0.1 g/10 ml), 2.5 U Taq and 1M for forward and reverse primers. Total of 35 cycles for 45s at 94ºC, for 45s at 55ºC s and for 60s at 72ºC. Initial heating step for 3 min at 95ºC and a final extension for 7 min at 72ºC. The secondary step was done as follow: Total of 40 cycles for 30s at 94ºC, for 90s at 58ºC, and for 2 min at 72ºC. Initial heat for 3 min at 94ºC and a final extension for 7 min at 72ºC. PCR products were investigated on agarose gel electrophoresis (1.5%). A PCR- RFLP assay of the secondary PCR products was applied as follow: a total volume of 50 µl reaction mixture contained 20U of *VspI*, 20U of *SspI* (Promega, USA), and 5 µl of the restriction buffer to digest 20µl of each secondary PCR product for 1hour at 37°C. Digested products were additionally fractionated on agarose gel (1.5%) and envisioned by ethidium bromide staining. *Cryptosporidium* sp. genotypes were identified by their band designs. Gel free PCR products were sequenced in both directions utilizing a big dye terminator cycle (Applied Biosystem), loaded on an AbiPrism 3100 and GeneScan Analysis v3.1 Software (Applied Biosystem, USA) as previously indicated (Bensch *et al*., 2000). The obtained sequence were compared with the accessible sequences in the database of the GenBank of National Center for Biotechnology Information. The alignment of the sequence and the determination of the phylogenic association among various *Cryptosporidium* sp. were done using.Clustal W (ver. 2) and Mega (Ver. 6) programs.

Statistical analysis

It was done utilizing ANOVA with two factors under significance level of 0.05 for the entire results using SPSS (Ver. 19). (Steel *et al*., 1997) Data were dealt with as a complete randomization outline and different examinations were done applying LSD.

Results

Out of 432 fecal speciemens of sheep collected from various localities in Qalyubia governorate, *Cryptosporidium* oocysts could be identified in 25.93% after staining by modified Zeihl- Neelsen technique (Fig.1 A, B). Diarrhea, dehydration were the common symptoms of cryptosporidiosis

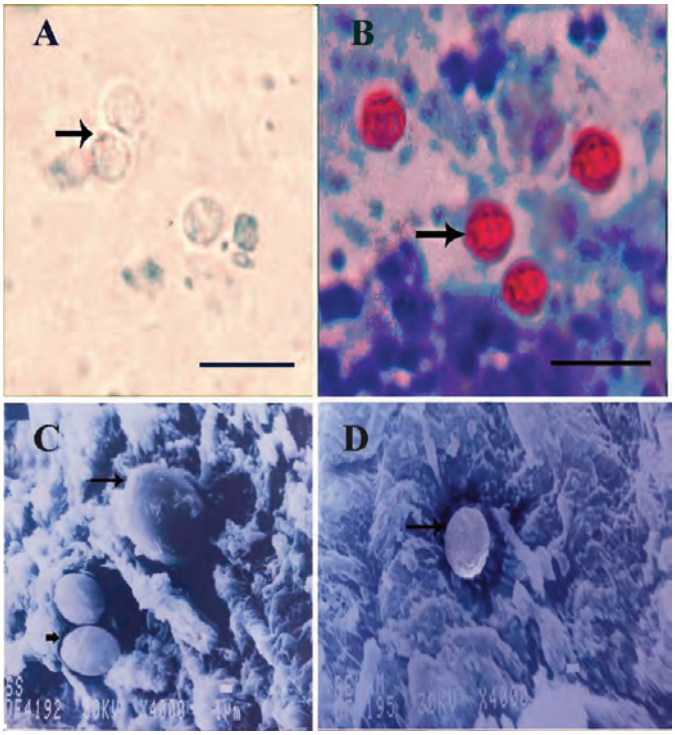

Fig. 1. A: *Cryptosporidium* oocysts in fresh fecal sample X400 (arrow; scale bar= 10um), B: *Cryptosporidium* oocysts stained by modified Zeihl- Neelsen stain X1000 (arrow; Scale bar=10 um).C: Scanning electron microscope showing large gamont of *Cryptosporidium* (long arrow), thin wall *Cryptosporidium* oocysts (short arrow), D: A thick wall oocyst of *Cryptosporidium* (arrow) (Scale bar =1um).

specially in lambs <1 month of age. Four cases were found in a state of hypothermia followed by death. The results were displayed according to different age and sex groups (Table 1). Statistical analysis didn't show any substantial effect of sex on the rate of *Cryptosporidium* infection , where the total infection rates among males and females were not significantly different(P>0.05). Though, the prevalence was significantly higher among males of ≤1-6 month age than females of the same age. Moreover, there was a significant effect of age on the infection rate of *Cryptosporidium*, with the noteworthy prevalence in lambs of ≤1 to <6 months of age (45.16%).

PCR could amplify 830 bp region of 18 SrRNA gene (Fig. 2). PCR-RFLP analysis of 830 bp region using restriction enzymes *SspI* of nested PCR products uncovered three distinct bands at 440, 267 and 106 bp regions proposing that the species was either *C. parvum* or *C. hominis*, while the band patterns of 454, 384 bp were predictable with *C. ubiquitum* (Fig. 3). To discriminate between *C. hominis* and *C. parvum*, the nested PCR products of all species were further digested with *VspI*. The band's profiles were predictable with *C. parvum* (629, 115bp) and PCR products of 461, 169, 115 bp were consistent to *C. ubiquitum* patterns (Fig. 4). The correlation of PCR-RFLP sequence (sequence 1 and 2) with the database in the Gen. Bank and phylogenetic analysis permitted recognizing them as *C. parvum* and *C. ubiquitum* (Fig. 5). The detected sequences were submitted in the gene bank under accession numbers KU715980 and KU715981.

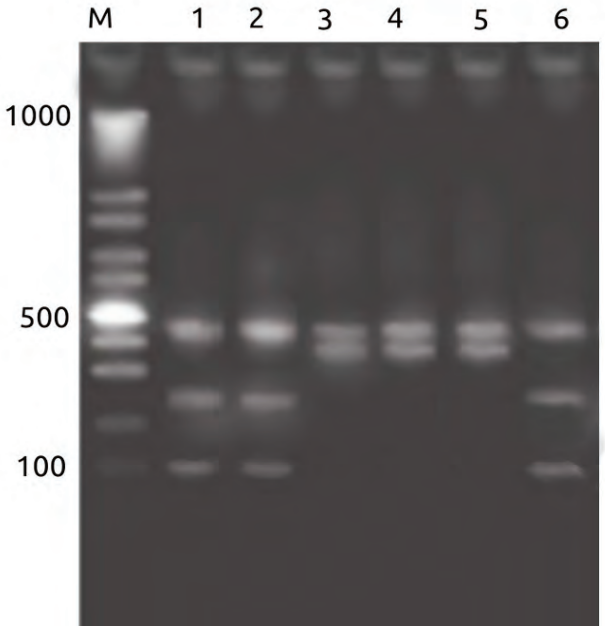

Fig.3. PCR- RFLP of *Cryptosporidium* species using *SspI* enzyme; Lane: 1, 2, 6 *C. parvum* / *C. hominis*, Lane 3,4,5: *C. ubiquitum.*

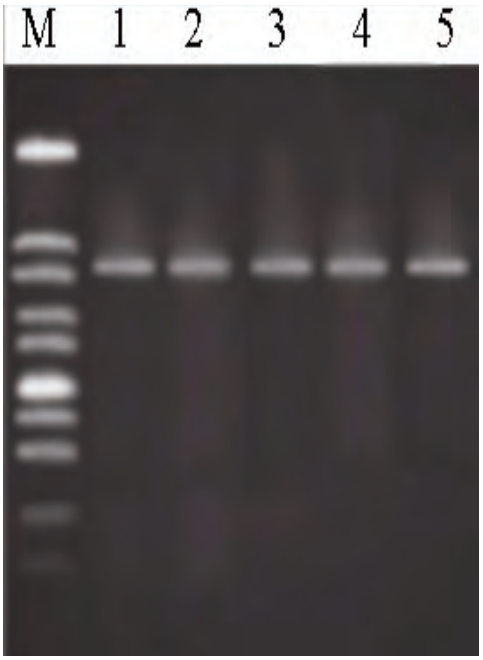

Fig .2. Lane 1-5, Amplified 830 bp of 18s rRNA gene for *Cryptosporidium.*

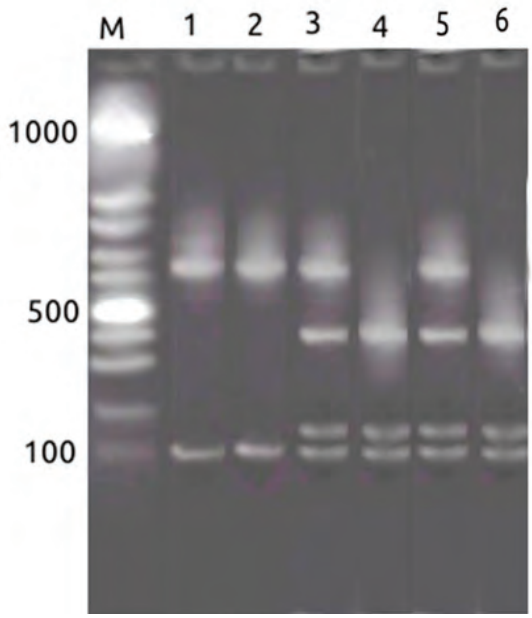

Fig. 4. PCR- RFLP of *Cryptosporidium* species using *VspI* enzyme; Lane 1,2: *C. parvum*, Lane 3,5: *C. parvum* + *C. ubiquituum*, Lane 4,6: *C. ubiquitum.*

Table 1. Frequency of *Cryptosporidium* oocysts detection in feces of sheep of different sex and age

Groups	Age (month)	NO. of examined animals			Positive		Mean (%)
		Total	Male	Female	Male	Female	
Group1	≤1 to <6 months	124	32	92	16 (50.00)^{cB}	40 (43.48)^{cA}	45.16^{c}
Group2	6-12	124	44	80	12 (27.27)^{bA}	20 (25.00)^{bA}	25.81^{b}
Group 3	>12	184	48	136	8 (16.67)^{aA}	16 (11.76)^{aA}	13.04^{a}
Total	-	432	124	308	36 (29.03)^{A}	76 (24.68) ^{A}	-

^{a,b,c} Values containing the same superscript are not significant (P>0.5) within the same column.

^{A,B,C} Values containing the same superscript are not significant (P>0.5) within the same row.

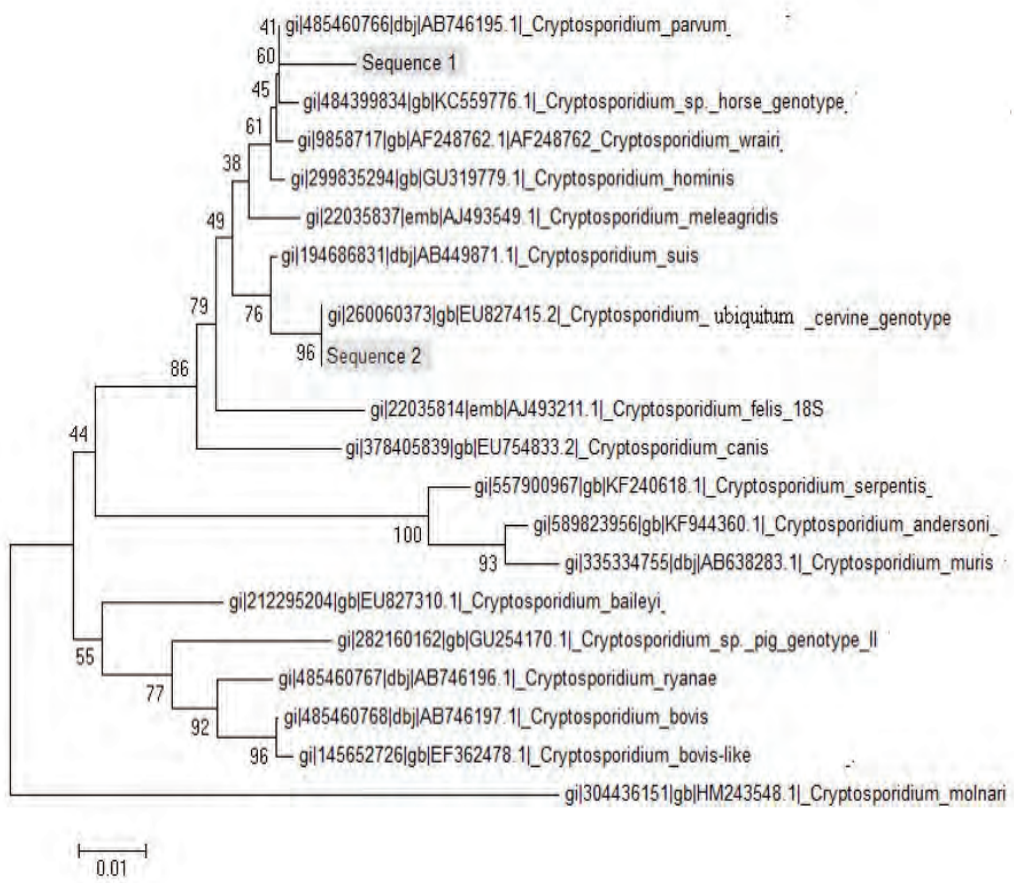

Fig. 5. A Phylogenic relationship estimated by the Maximum Likelihood with Tamura-Nei model shows the phylogenic comparison of the available 18S rRNA sequences from respective *Cryptosporidium* isolates with two species detected in this study (sequence 1 and sequence 2).

Table 2: *Cryptosporidium* species identified in sheep with regard to age

Cryptosporidium sp.	Group1 (≤1 to <6 months)	Group2 (6-12 months)	Group3 (>12 months)	Mean
	Infected /124	infected/124	Infected/184	
C. parvum	20 (16.13)^{bB}	8 (6.45)^{aA}	8 (4.35)^{abA}	8.98^{b}
C. ubiquitum	28 (22.58)^{cC}	20 (16.13)^{bB}	16 (8.70)^{bA}	15.80^{c}
C.parvum+C.ubiquitum	8 (6.45)^{aB}	4 (3.23)^{aAB}	0 (0.00)^{aA}	3.23^{a}
Mean	15.05^{B}	8.60^{A}	4.35^{A}	-

^{a,b,c} Values containing the same superscript are not significant (P>0.5) within the same column.
^{A,B,C} Values containing the same superscript are not significant (P>0.5) within the same row.

The infection rate varied significantly (P<0.05) among the examined groups and *C. ubiquitum* was the most prevailing species. Mostly, *Cryptosporidium* sp. prevailed at age group of ≤1-6 months when contrasted with the other two age groups (6-12 and >12 months). A significant variation was recorded between 1st and 2nd age group and also between the 1st and 3rd age group for the prevalence of *C. parvum* (P<0.05). *C. ubiquitum* was more pervasive at age group of ≤1-6 months (22.58%). Mixed infection with *C. parvum* and *C. ubiquitum* showed no significant variation between 1st and 2nd age group, but an efficient significance was found between 1st and 3rd age groups (P<0.05) (Table 2).

Scanning electron microscopy of the intestine showed the presence of gamont like stage measuring 6.5X5 µm and fully developed thin wall oocysts measuring 2-5 µm in diameter, located in the intestine and attached to the microvillus brush border of the epithelium of the small intestine with velvet appearance of the intestinal microvilli surrounding the parasite (Fig. 1.C). Thick wall oocysts were also seen with increasing of the globule secretion of the goblet cell due to distortion in the intestinal glands and villi (Fig. 1. D).

Discussion

Cryptosporidium infections of sheep is widely distributed in several countries, the prevalence rate ranged from 1.6 to 77.4% using microscopy and molecular assay (Santín *et al.*, 2007; Fiuza *et al.*, 2011). Few studies had been carried out in

Egypt and to date; most of *Cryptosporidium* studies in sheep focused on traditional microscopic examination and lacked the more sensitive technique. In the present study, the overall prevalence of *Cryptosporidium* was 25.93% among the analyzed sheep of different age and sex groups. This result was broadly consistent with other studies (Santin *et al*., 2007 and Connelly *et al*., 2013). The prevalence of *Cryptosporidium* was higher than that were formerly found in Egypt (2.5% and 32.2%) (Mahfouz *et al*., 2014; Helmy *et al*., 2012 respectively) , in Iran (11.3%) (Heidari and Gharakhani, 2012), in Iraq (13.3%) (Mahdi and Ali, 2002), in Tunisia (11.2%) (Soltane *et al*., 2007), in Belgium (13.1%) (Geurden *et al*., 2008). Higher results were recorded in Mexico (67.5%) (Romero-Salas *et al*., 2016), in Spain (31-59%) (Causape *et al*., 2002; Castro-Hermida *et al*., 2007). However, the comparison of prevalence in different geographical locations must be limited by the animal characters, breeds and rearing condition. PCR-RFLP method recognized two types of *Cryptosporidium* infecting sheep: *C. parvum* and *C. ubiquitum* (Cervine genotype). Those were previously encountered by (Mirhashemi *et al*., 2016). Nevertheless, *C. xiaoi* was the only species encountered in sheep in Egypt (Mahfouz *et al*., 2014). This recorded difference may be ascribed to difference in sheep breeds or sample size. In accordance with the herein results, *C. ubiquitum* was the most predominant species among different ages in Iran (Geurden *et al*., 2008), in China (Wang *et al*., 2010; Shen *et al*., 2011). The occurrence of *C. parvum* among sheep population seems to be consistent with those of some reports that review the role of sheep as a zoonotic source of *Cryptosporidium* in human (El-Sherbini and Mohamed, 2006; Paoletti *et al*., 2009; Helmy *et al*., 2012; Yang *et al*., 2014). The zoonotic significance of *C. parvum* and *C. ubiquitum* was in the past perceived, recommending that this animal might assume bigger parts as reservoir species than that already thought, posing a substantial public health danger (Santin and Fayer, 2007). Thus, an important public health control and prevention strategies must be considered for the surveillance of *Cryptosporidium* in sheep. The higher infection rate among males than female sheep recorded in the current study was predictable with that of Heidari and Gharakhani (2012); Jafari *et al.* (2013) and Romero-Salas *et al.* (2016). Yet, it is inverse to an alternate investigation (Regassa *et al*., 2013; Gharekhani *et al*., 2014) in Iran.The purpose behind this slight uniqueness is not known, in spite of the fact that this result could be credited to the standard routine of having an unequal female: male ratio in a herd and the more contact of males to disease than females. It could likewise be related to host genetics, physiology and immunology or environmental and management practices. The actual mechanism of the influence of these factors are not understood and need clarification (Ayinmode *et al*., 2010). Concerning age, the present study demonstrates a statistical difference among different age groups for *Cryptosporidium* infection, where the most noteworthy infection rate was in sheep (≤1 to <6 months of age). This corresponded with that recorded by (Ayinmode *et al*., 2010; Gharekhani *et al*., 2014; Romero-Salas *et al*., 2016). The clinical manifestations of cryptosporidiosis agreed that noticed by Angus *et al.* (1982)

Electron microscopic study showed different stages of *Cryptosporidium* were found mainly attached the border of the intestinal epithelial. The common site the parasite was mainly the small intestine. This was in accordance with Caccio and Widmer (2014), who stated that *C. parvum* and *C. ubiquitum* mainly infecting and producing pathological lesions in the small intestine.

Conclusion

This study is the first to distinguish *Cryptosporidium* sp. infecting sheep in Qalyubia governorate, Egypt. The recorded *Cryptosporidium* sp. are *C. parvum* and *C. ubiquitum,* which have critical zoonotic significance. This finding proposes that sheep must be considered as a huge public health risk and as a potential wellspring of human cryptosporidiosis. The reconnaissance of zoonotic cryptosporidiosis must be set up to prevent human disease and to assess prospective infection when applying control programs.

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