Research Journal of Parasitology

ISSN 1816-4943

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Parasitology

ISSN 1816-4943
DOI: 10.3923/jp.2017.19.26

Research Article
Prevalence and Molecular Epidemiology of *Cryptosporidium* Infection in Calves and Hospitalized Children in Egypt

[1]Nahed H. Ghoneim, [2]Mohey A. Hassanain, [1]Dalia A. Hamza, [2]Raafat M. Shaapan and [2]Sara H. Draz

[1]Department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[2]Veterinary Research Division, Department of Zoonotic Diseases, National Research Centre, Dokki, Giza, Egypt

Abstract

Background and Objective: *Cryptosporidium* species are important zoonotic protozoan parasites that infect the gastrointestinal tract of most vertebrate animals and man. Cryptosporidiosis infection is responsible for numerous outbreaks of diarrheal disease worldwide. This study was planned for prevalence and molecular detection of *Cryptosporidium* spp., in calves and hospitalized children from different Egyptian governorates (Cairo, Giza and Al-Bahira). **Materials and Methods:** A total of 253 fecal samples from cattle and buffalo calves <2 months, 2-6 months and <6 months of age and 115 stool samples from children <2 years, 2-6 years and 6-12 years old were screened by modified Ziehl-Neelsen (MZN) staining technique for the detection of *Cryptosporidium* oocysts followed by molecular characterization using complemented DNA Polymerase Chain Reaction (cPCR). **Results:** An overall *Cryptosporidium* spp., infection rates of 30.4 and 33.9% were detected among calves and children, respectively. The highest prevalence (32.7 and 44.4%) was demonstrated in younger calves (<2 months) and children (<2 years), respectively. On the other hand, a lower prevalence (20.0 and 27.0%) was detected in older calves (>6 months) and children (6-12 years), respectively. The prevalence in relation to fecal consistency was higher in diarrheic (39.8 and 41.1%) than in non-diarrheic samples (20.8 and 23.4%) from calves and children, respectively. The PCR analysis of 7 and 6 MZN stain positive calves and children fecal samples, respectively, revealed the expected positive bands at 835 bp for all 6 tested children fecal samples and for only 3 calve fecal samples, while the other 4 were negative PCR for *Cryptosporidium* spp. **Conclusion:** The prevalence of *Cryptosporidium* spp. had a relationship with age and the high infection rate in calves and children can act as a great source of cryptosporidiosis. The obtained data from this study indicates an important public health problem and a potential risk of zoonotic transmission from animal to human beings in Egypt.

Key words: *Cryptosporidium* spp., calves, children, prevalence, molecular epidemiology

Received: September 01, 2016 Accepted: November 07, 2016 Published: December 15, 2016

Citation: Nahed H. Ghoneim, Mohey A. Hassanain, Dalia A. Hamza, Raafat M. Shaapan and Sara H. Draz, 2017. Prevalence and molecular epidemiology of *Cryptosporidium* infection in calves and hospitalized children in Egypt. Res. J. Parasitol., 12: 19-26.

Corresponding Author: Raafat M. Shaapan, Department of Zoonotic Diseases, National Research Centre, Post Box 12622, El-Tahrir Street, Dokki, Giza, Egypt
Tel: 002-0105280571 Fax: 00202-3371362

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cryptosporidiosis is an important zoonotic disease caused by a small apicomplexan protozoan parasites belonging to genus *Cryptosporidium* which infects the gastrointestinal epithelium of most mammalian hosts including human worldwide. Domestic cattle have been considered the major reservoir of *Cryptosporidium* for human infections[1,2]. Although *Cryptosporidium* spp. were subsequently found in a broad range of farm animals, its impact was neglected until the early 1980s when it was found to be a common serious primary cause of outbreaks of diarrhea in calves[3]. The protozoan *Cryptosporidium* parasites have become recognized as a cause of water and food born related outbreaks of diarrhea in humans and animals which certainly given the parasite a more widespread recognition[4,5]. Also the parasite is common etiologic agent associated with self-limited diarrhoea in immune-competent subjects but potentially poses serious public health issues in immune-deficient individuals, such as AIDS patients and may lead to life-threatening chronic diarrhea[6].

Animal husbandry is seen as a threatening source of infection for humans by the release of tremendous numbers of resistant oocysts in surface water and environment and it is difficult to control disease, which result in significant economic losses[7]. The microorganism is ubiquitous in environment including a number of animal species, so there is every possibility of zoonotic transmission of infection from animal to human beings, especially under poor hygienic conditions[8]. Cattle particularly calves have been identified as a major reservoir of zoonotic species and genotypes of *Cryptosporidium*[7]. Several outbreaks in humans have been associated with infected calves, while small outbreaks have occurred in veterinary students[9]. *Cryptosporidium* is increasingly a well-recognized cause of neonatal calf diarrhea which may lead to progressive dehydration, growth retardation and possibly death[10]. *Cryptosporidium* also was responsible for diarrheal illness among American military participated in a military exercise in the Northwestern Egyptian desert[11]. There is a paucity of information about cryptosporidiosis in human[12,13] and animals[14,15] in Egypt.

The diagnosis of *Cryptosporidium* spp., using light microscopy, with haematoxylin and eosin (H and E) staining, is often insufficient to confirm the presence of the organisms[16]. Current routine diagnostic procedures with acid-fast staining of *Cryptosporidium* spp., oocysts in fecal smears remains the largely used conventional methods for diagnosis[17]. Enzyme immunoassays (EIA) that detect parasite copro-antigen are efficient methods but there is a conflict about the sensitivity of this immuno-detection methods[18]. None of the common diagnostic laboratory techniques, such as acid-fast staining and direct or indirect immunofluorescence microscopy are able to distinguish species or subtypes of *Cryptosporidium*, which is important for understanding dynamics and transmission pathways[19]. Now Polymerase Chain Reaction (PCR) is becoming increasingly popular for diagnosis as a tool to detect *Cryptosporidium* DNA in feces[20]. This technique allows the species identification and subtyping of *Cryptosporidium* and also tracing of different transmission ways of the parasite[21].

However, cryptosporidiosis is responsible for considerable part of diarrheal illness among human and livestock, little known data is available about *Cryptosporidium* spp., infection in farm animals, the biological and epidemiological diversity together with its immense zoonotic importance in Egypt, so the objective of this study is to investigate the prevalence and molecular epidemiology of *Cryptosporidium* spp., in calves and hospitalized children from different Egyptian governorates to improve our understanding of interspecies transmission and public health significance of *Cryptosporidium* spp.

MATERIALS AND METHODS

Samples collection and preparation

Calves: A total of 253 calve fecal samples were collected from different Egyptian governorates (Cairo, Giza, Al-Bahira). Three age groups were selected as <2, 2-6 and >6 months of age. Fecal samples were collected from animal's rectum in a separate clean labeled container and tested samples were differentiated into diarrheic and non-diarrheic type.

Children: A total of 115 stool samples were collected from children at age from <2, 2-6 and 6-12 years old. The samples were collected from pediatric clinics and some laboratories in the same previous governorates in addition from Abu-Elriech Hospital, Cairo, Egypt. Each stool sample was collected in clean, dry, disposable plastic container labeled by the age, place of collection and also, diarrheic and non-diarrheic state of sample was differentiated.

Fecal samples preparation: Sheather's sucrose flotation method was used for the concentration of *Cryptosporidium* oocysts, concentrated samples were examined for the presence of oocysts by scanning of fecal smears on slides using the 40X objective lens of a bright-field microscope and the presence of oocysts in the smear was confirmed under the oil immersion objective lens. The positive samples were then stored at 4°C for further molecular analysis[22].

Detection of oocysts

Staining of *Cryptosporidium* oocysts: Fine calves and children fecal smears from of both diarrheic and non-diarrheic types were fixed with methanol spirit and stained with Ziehl-Neelsen stain according to the procedure described by Henriksen and Pohlenz[23]. The acid-fast staining technique has been modified and improved, including: Hot and cold modified acid-fast stains; incorporation of dimethyl sulfoxide (DMSO) and the detergent tergitol (modified Ziehl-Neelsen, mZN). This technique is a simple and effective method for *Cryptosporidium* spp., oocysts stain as bright red against a background of blue-green fecal debris and yeasts[24].

Identification of *Cryptosporidium* spp., oocysts: Higher-magnification objectives must be used to confirm the presence of *Cryptosporidium* spp., oocysts, the measurement can be helpful in distinguishing oocysts from other microscopic objects with help of stage micrometer conjugated with an eyepiece micrometer under the light microscope at the eyepiece 10X and the objective 100X[25]. The standard unit of measurement is the micron (μm = 0.001 mm), use about 20-50 oocysts, with the range in parenthesis to calculate the mean[26].

Molecular identification

Purification of *Cryptosporidium* spp., oocysts: Before DNA extraction and inoculation, *Cryptosporidium* oocysts obtained from calve and children highly positive fecal samples were refined from the potassium dichromate solution by centrifugation at 2000 rpm for 5 min and then the oocysts were washed 4 times with distilled water and then stored at -20°C in PBS solution[27].

DNA extraction: It was carried out using the QIAamp DNA stool mini kit (QIAGEN, Germany) following the manufacturer's instructions with some modifications according to Lalonde and Gajadhar[28] as follows: (i) Oocysts were first suspended in 300 mL of ATL buffer and subjected to 8 cycles of freeze-thaw for 1 min in liquid nitrogen and 1 min in a 95°C water bath, (ii) Lysed suspensions were incubated with 20 mL proteinase K (20 mg mL^{-1}, Qiagen) for 3 h at 56°C, followed by incubation at 70°C for 10 min in 300 mL AL buffer with vortexing for 10 sec every 3 min DNA.

Polymerase Chain Reaction (cPCR): The PCR master mix was prepared according to Emerald Amp GT PCR mastermix (Takara) code No. RR310A kit and the oligonucleotide primers metabion (Germany) was used in cPCR, with specific sequence and amplify a specific product according to procedures described by Adamska *et al.*[29] (Table 1).

The cycling conditions of the primers during cPCR at temperature and time conditions of the two primers during PCR carried out according to Paul *et al.*[30] using emerald Amp GT PCR master mix (Takara) kit. The agarose gel electrophoreses was carried out according to Sambrook *et al.*[31] with modification and the gel was photographed by a gel documentation system and the data was analyzed through computer software.

RESULTS

***Cryptosporidium* spp., detection and prevalence:** The obtained oocysts from calves and children fecal samples were morphologically confirmed as *Cryptosporidium* spp., oocysts which characterized by spherical shaped, smooth wall, acid fast (red-pink) on green back and their mean size was 5.1×4.5 μm with shape index of 1.1 μm (Fig. 1). The overall prevalence of *Cryptosporidium* species among calves was found to be 30.4%, the highest prevalence of 32.7% was recorded in <2 months calves followed by 28.3% in 2-6 months aged while, the lowest prevalence of 20.0% in >6 months age group. The overall *Cryptosporidium* prevalence among children was 33.9%, the highest prevalence of 44.4% demonstrated in younger children (<2 years) followed by 30.9% in 2-6 years old children, on other hand, lower prevalence of 27.0% was detected In older children (6-12 years) (Table 2).

Relation of *Cryptosporidium* spp., prevalence and fecal consistency: *Cryptosporidium* spp., prevalence was higher in total diarrheic (39.8 and 41.1%) than in total non-diarrheic fecal samples (20.8 and 23.4%) from calves and children, respectively (Table 3, 4). The higher extent of prevalence of 42.9% (36 out of 84) in diarrheic than 22.6% (19 out of 84) in non-diarrheic fecal samples from younger calves (<2 months), followed by 35.3% (12 out of 34), higher than 19.2% (5 out of 26) in 2-6 month aged, while 30% (3 out of 10) less than 13.3% (2 out of 15) in >6 month aged calves diarrheic and non-diarrheic fecal samples, respectively (Table 3). The diarrheic children stool samples revealed higher

Table 1: Oligonucleotide primers sequences source

Primer	Sequence	Amplified product (bp)
CX1F	TTCTAGAGCTAATACATGCG	1325
CX1R2	CCCTAATCCTTCGAAACAGGA	
CX2F	GGAAGGGTTGTATTTATTAGATAAAG	840
CX2R	AAGGAGTAAGGAACAACCTCCA	

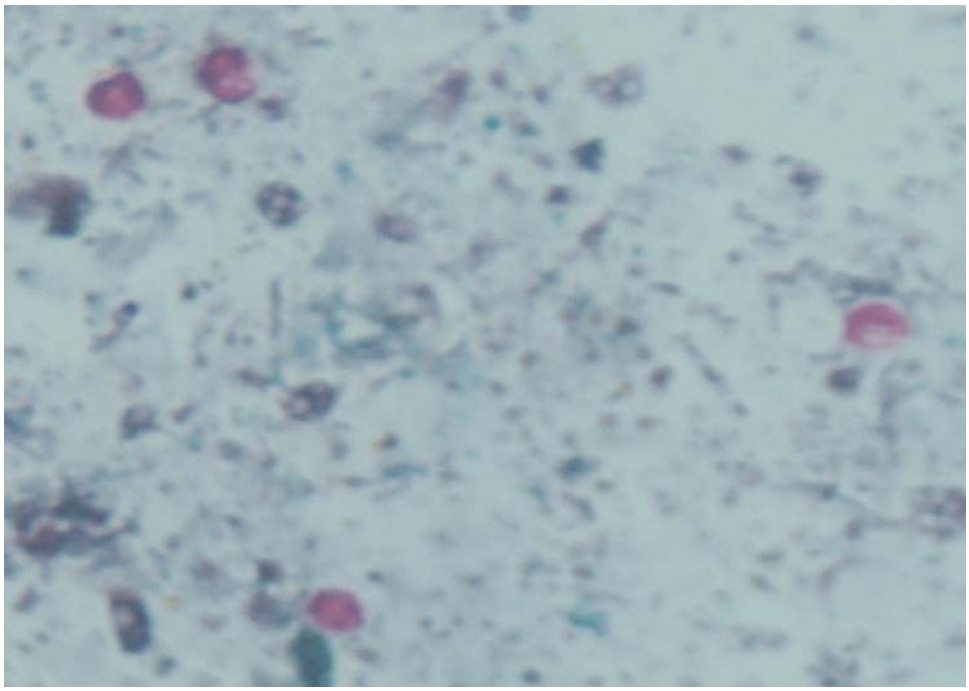

Fig. 1: Modified Ziehl-Neelsen stained fecal smear (X100) showing *Cryptosporidium* spp., oocysts

Table 2: Prevalence of *cryptosporidium* spp., infection in calves and children using modified Ziehl-Neelsen staining technique

			Examined prevalence samples			
Parameters	Total No.	Age	Positive	No.	%	Total+Positive (%)
Calves	253	<2 months	168	55	32.7	77 (30.4)
		2-6 months	60	17	28.5	
		>6 months	25	5	20.0	
Children	115	<2 years	36	16	44.4	39 (33.9)
		2-6 years	42	13	30.9	
		6-12 years	37	10	27.0	

Table 3: Prevalence of *Cryptosporidium* spp., infection in different age group in diarrheic and non-diarrheic calves

	Fecal consistency							
	Total		Diarrheic			Non-diarrheic		
Age	No.	Positive (%)	No.	Positive	%	No.	Positive	%
<2 months	168	55 (32.7)	84	36	42.9	84	19	22.6
2-6 months	60	17 (28.3)	34	12	35.3	26	5	19.2
>6 months	25	5 (20.0)	10	3	30.0	15	2	13.3
Total	253	77 (30.4)	128	51	39.8	125	26	20.8

Cryptosporidium prevalence of 54.5% (12 out of 22) than 28.6% (4 out of 14) in non-diarrheic samples from younger children (<2 years), followed by 37.5% (9 out of 24) higher than 22.2% (4 out of 18) in 2-6 years aged, while 31.8% (7 out of 22) more than 20.0% (3 out of 15) in 6-12 years aged children diarrheic and non-diarrheic fecal samples, respectively (Table 4).

Molecular detection of *Cryptosporidium* spp.: Polymerase Chain Reaction (cPCR) analysis of 6 children and 7 calvae fecal samples which were previously confirmed as positive with the modified Ziehl-Neelsen stain technique for *Cryptosporidium* oocysts, revealed the expected positive bands at 835 bp for all 6 tested children fecal samples (lane 1-6) and for only 3 calves fecal samples

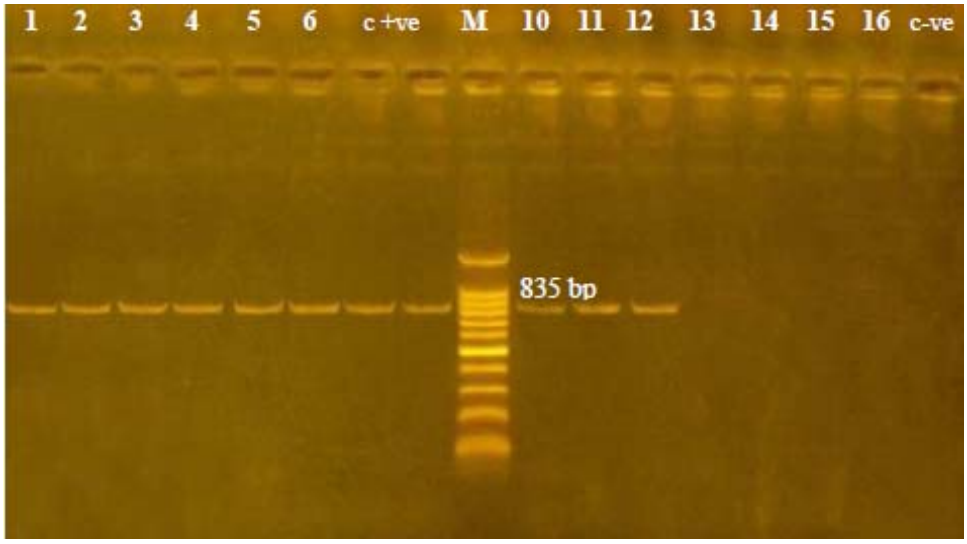

Fig. 2: PCR analysis for *Cryptosporidium* spp., Lane 1-6: Positive children fecal samples, Lane 7 and 8: Control positive samples, Lane 9: DNA markers, Lane 10-12: Positive calve fecal samples, Lanes 13-16: Negative calve fecal samples and Lane 17: Control negative samples

Table 4: Prevalence of *Cryptosporidium* spp., infection in different age group in diarrheic and non-diarrheic children

	Fecal consistency							
	Total		Diarrheic			Non-diarrheic		
Age	No.	Positive (%)	No.	Positive	%	No.	Positive	%
<2 years	36	16 (44.4)	22	12	54.5	14	4	28.6
2-6 years	42	13 (30.9)	24	9	37.5	18	4	22.2
6-12 years	37	10 (27.0)	22	7	31.8	15	3	20.0
Total	115	39 (33.9)	68	28	41.1	47	11	23.4

(lane 10-12), while the other 4 calves samples (lanes 13-16) were negative PCR for *Cryptosporidium* spp. (Fig. 2).

DISCUSSION

The obtained oocysts from calves and children fecal samples in this study were confirmed as *Cryptosporidium* spp., oocysts. They were morphologically similar to those described in human and livestock animals in many previous studies[14,15,26]. The microscopical identification of the *Cryptosporidium* spp., depend upon the standard specifications, such as morphology and measurements of the oocysts. Xiao *et al.*[25] reported that the morphometric measurement of oocysts is the main key factor for *cryptosporidium* spp., taxonomy and is one of the essential requirements for establishing a new species.

The screening of calves and children fecal samples by using MZN technique (Table 2) revealed 30.4 and 33.9% overall prevalence of *Cryptosporidium* species infection, respectively. The present study results was higher than a previous study i.e., 9.5 and 19.2% by Amer *et al.*[15] and Helmy *et al.*[32], respectively in calves, on the contrary, <54.4% reported by Hassanain *et al.*[14] in cattle calves and (47%) detected by Abd El Kader *et al.*[33] in human in Egypt. The result of age wise distribution of the parasite (Table 3, 4) showed that the highest prevalence (32.7 and 44.4%) was demonstrated in younger calves (<2 months) and children (<2 years), respectively. On the other hand, lower prevalence (20.0 and 27.0%) was obtained in older calves (>6 months) and children (6-12 years), respectively. A similar age-related pattern of *Cryptosporidium* spp., distribution was obtained by many researchers who referred the decrease infection rate in correlation with older ages to several factors, such as an age-related resistance due to maturation of the intestinal mucosa[34] and species-specific resistance[35].

Regarding to the relation between infection prevalence and fecal consistency, this study revealed higher *Cryptosporidium* spp., prevalence in total diarrheic (39.8 and 41.1%) than in total non-diarrheic fecal samples (20.8 and 23.4%) from calves and children, respectively, these

finding are in accordance with El-Khodery and Osman[36] and Helmy *et al.*[32] who reported that diarrheic animals and human revealed a higher rate of *Cryptosporidium* infection in compared to non-diarrheic ones. The higher prevalence of cryptosporidiosis in diarrheic calves than non-diarrheic animals means that additional factors are needed to produce clinical disease and the non-diarrheic animals are asymptomatic carrier state epically in adults, representing a potential source of infection[37]. Similarly, it is worth mentioning that the excretion of *Cryptosporidium* oocysts by non-diarrheic children, not complaining from any signs indicated that these individuals can be regarded as carriers with a possibility to spread the infection through improper personal hygiene or bad sanitation to others[38].

The PCR analysis of 7 and 6 MZN stain positive calves and children fecal samples, respectively, revealed the expected positive bands at 835 bp for all 6 tested children fecal samples and for only 3 calve fecal samples while the other 4 were negative PCR for *Cryptosporidium* spp. The confirmatory PCR positive results for 9 out of 13 selected human and animal samples which were highly positive by microscopical examination for *Cryptosporidium* spp. was consistent with previous reports done in Egypt in calves[14] and children[32] who also found that majority of MZN stain identified positive animal and human samples for *Cryptosporidium* spp., oocysts were successfully proved by PCR analysis and recommended the use of extremely important molecular procedures in association with biological techniques to overcome *Cryptosporidium* spp., diagnosis problems.

The estimated *Cryptosporidium* spp., prevalence in the current study could have been affected by intermittent shedding of oocysts, as all subjects were only sampled once and the higher infection rates in human and calves obtained by this results pointing to the cross-transmissible, awareness of cryptosporidiosis as a potential zoonotic infection which emerged as a significant public health concern and indicating that animals especially calves as a source of human infection[39]. The majority of cases diagnosed with *Cryptosporidium* spp., in Egypt may be attributed to lack of hygiene, poor living conditions and direct contact with farm animals where cryptosporidiosis has a high prevalence contribute for the spread of the infection[16]. The variation in *Cryptosporidium* spp., prevalence in this study and the other previously reported infection rates may be attributed to differences in the age and breed of calves, season of specimen collection, environmental settings, management and husbandry regimens as well as tools used for detection of *Cryptosporidium* in fecal samples[13].

CONCLUSION

The high prevalence of *Cryptosporidium* spp., in this study suggested that children, like calves may also serve as an important reservoir of *Cryptosporidium* spp. and the relationship between young ages and the high infection rate in calves and children improved our understanding of interspecies transmission of cryptosporidiosis and public health significance of *Cryptosporidium* in Egypt. Also, we can conclude that the routine diagnostic procedures with acid-fast staining of *Cryptosporidium* spp., oocysts in fecal smears may be of benefit and the molecular detection should also be a good confirmatory option.

SIGNIFICANT STATEMENT

Children, like calves, serve as an important reservoir of *Cryptosporidium* spp., young ages had the high infection rates of cryptosporidiosis and acid-fast staining may be of benefit, while the molecular detection should also be a good confirmatory option.

REFERENCES

1. Xiao, L. and R. Fayer, 2008. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic. Int. J. Parasitol., 38: 1239-1255.
2. Del Coco, V.F., M.A. Cordoba and J.A. Basualdo, 2009. Cryptosporidiosis: An emerging zoonosis. Rev. Argent Microbiol., 41: 185-196.
3. Tzipori, S., I. Campbell, D. Sherwood, D.R. Snodgrass and A. Whitelaw, 1980. An outbreak of calf diarrhoea attributed to cryptosporidial infection. Vet. Rec., 107: 579-580.
4. O'Donoghue, P.J., 1995. *Cryptosporidium* and cryptosporidiosis in man and animals. Int. J. Parasitol., 25: 139-195.
5. Shaapan, R. and F. Khalil, 2014. Zoonotic Significance of Cryptosporidiosis. LAMBERT Academic Publishing, Saarbrucken, Germany, ISBN-13: 978-3-659-53506-2, Pages: 68.
6. Almeida, A., M.J. Moreira, S. Soares, M.D.L. Delgado and J. Figueiredo *et al*., 2010. Presence of *Cryptosporidium* spp. and *Giardia duodenalis* in drinking water samples in the North of Portugal. Korean J. Parasitol., 48: 43-48.
7. Xiao, L. and Y. Feng, 2008. Zoonotic cryptosporidiosis. FEMS Immunol. Med. Microbiol., 52: 309-323.
8. Abu El Ezz, N.M.T., F.A.M. Khalil and R.M. Shaapan, 2011. Therapeutic effect of onion (*Allium cepa*) and cinnamon (*Cinnamomum zeylanicum*) oils on cryptosporidiosis in experimentally infected mice. Global Veterinaria, 7: 179-183.

9. Gait, R., R.H. Soutar, M. Hanson, C. Fraser and R. Chalmers, 2008. Outbreak of cryptosporidiosis among veterinary students. Vet. Rec., 162: 843-845.
10. Silverlas, C., K. de Verdier, U. Emanuelson, J.G. Mattsson and C. Bjorkman, 2010. *Cryptosporidium* infection in herds with and without calf diarrhoeal problems. Parasitol. Res., 107: 1435-1444.
11. Sanders, J.W., S.D. Putnam, P. Gould, J. Kolisnyk and N. Merced *et al*., 2005. Diarrheal illness among deployed U.S. military personnel during Operation Bright Star 2001-Egypt. Diagn. Microbiol. Infect. Dis., 52: 85-90.
12. Youssef, F.G., I. Adib, M. Riddle and C. Schlett, 2008. A review of cryptosporidiosis in Egypt. J. Egypt. Soc. Parasitol., 38: 9-28.
13. Ibrahim, M.A., A.E. Abdel-Ghany, G.K. Abdel-Latif, S.A. Abdel-Aziz and S.M. Aboelhadid, 2016. Epidemiology and public health significance of *Cryptosporidium* isolated from cattle, buffaloes and humans in Egypt. Parasitol. Res., 115: 2439-2448.
14. Hassanain, M.A., F.A.M. Khalil, K.A. AbdEl-Razik and R.M. Shaapan, 2011. Prevalence and molecular discrimination of *Cryptosporidium parvum* in calves in Behira provinces, Egypt. Res. J. Parasitol., 6: 101-108.
15. Amer, S., S. Zidan, Y. Feng, H. Adamu, N. Li and L. Xiao, 2013. Identity and public health potential of *Cryptosporidium* spp. in water buffalo calves in Egypt. Vet. Parasitol., 191: 123-127.
16. Bhaijee, F., C. Subramony, S.J. Tang and D.J. Pepper, 2011. Human immunodeficiency virus-associated gastrointestinal disease: Common endoscopic biopsy diagnoses. Pathol. Res. Int. 10.4061/2011/247923
17. Morgan, U.M., L. Pallant, B.W. Dwyer, D.A. Forbes, G. Rich and R.C. Thompson, 1998. Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: Clinical trial. J. Clin. Microbiol., 36: 995-998.
18. Geurden, T., E. Claerebout, J. Vercruysse and D. Berkvens, 2008. A Bayesian evaluation of four immunological assays for the diagnosis of clinical cryptosporidiosis in calves. Vet. J., 176: 400-402.
19. Amar, C.F.L., P.H. Dear and J. McLauchlin, 2004. Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces. Lett. Applied Microbiol., 38: 217-222.
20. Lupo, P.J., R.C. Langer-Curry, M. Robinson, P.C. Okhuysen and C.L. Chappell, 2008. *Cryptosporidium muris* in a Texas canine population. Am. J. Trop. Med. Hyg., 78: 917-921.
21. Minarovicova, J., E. Kaclikova, K. Krascsenicsova and P. Siekel, 2007. Detection of *Cryptosporidium parvum* by polymerase chain reaction. J. Food Nutr. Res., 46: 58-62.
22. Sangster, L., D.P. Blake, G. Robinson, T.C. Hopkins and R.C.C. Sa *et al*., 2016. Detection and molecular characterization of *Cryptosporidium parvum* in British European hedgehogs (*Erinaceus europaeus*). Vet. Parasitol., 217: 39-44.
23. Henriksen, S.A. and J.F. Pohlenz, 1981. Staining of cryptosporidium by a modified Ziehl-Neelsen technique. Acta Veterinaria Scandinavica, 22: 594-596.
24. Casemore, D.P., M. Armstrong and R.L. Sands, 1985. Laboratory diagnosis of cryptosporidiosis. J. Clin. Pathol., 38: 1337-1341.
25. Xiao, L., C. Bern, J. Limor, I. Sulaiman and J. Roberts *et al*., 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis., 183: 492-497.
26. Fayer, R. and L. Xiao, 2008. *Cryptosporidium* and *Cryptosporidiosis*. 2nd Edn., CRC Press/Taylor and Francis Group, Boca Raton, FL., ISBN-13: 9781420052268, USA, Pages: 560.
27. Arrowood, M.J. and C.R. Sterling, 1987. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J. Parasitol., 73: 314-319.
28. Lalonde, L.F. and A.A. Gajadhar, 2009. Effect of storage media, temperature and time on preservation of *Cryptosporidium parvum* oocysts for PCR analysis. Vet. Parasitol., 160: 185-189.
29. Adamska, M., A. Leonska-Duniec, M. Sawczuk, A. Maciejewska and B. Skotarczak, 2012. Recovery of *Cryptosporidium* from spiked water and stool samples measured by PCR and real time PCR. Vet. Med., 57: 224-232.
30. Paul, S., D. Chandra, D. Ray, A.K. Tewari and J.R. Rao *et al*., 2008. Prevalence and molecular characterization of bovine *Cryptosporidium* isolates in India. Vet. Parasitol., 153: 143-146.
31. Sambrook, J., E.F. Fritsch and T.A. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA., ISBN-13: 9780879695774, Pages: 397.
32. Helmy, Y.A., J. Krucken, K. Nockler, G. von Samson-Himmelstjerna and K.H. Zessin, 2013. Molecular epidemiology of *Cryptosporidium* in livestock animals and humans in the Ismailia province of Egypt. Vet. Parasitol., 193: 15-24.
33. Abd El Kader, N.M., M.A. Blanco, M. Ali-Tammam, A.E.R.B. Abd El Ghaffar and A. Osman *et al*., 2012. Detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* in human patients in Cairo, Egypt. Parasitol. Res., 110: 161-166.
34. Harp, J.A., D.B. Woodmansee and H.M. Moon, 1990. Resistance of calves to *Cryptosporidium parvum*: Effects of age and previous exposure. Infect. Immun., 58: 2237-2240.
35. Fayer, R., M. Santin and L. Xiao, 2005. *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). J. Parasitol., 91: 624-629.

36. El-Khodery, S.A. and S.A. Osman, 2008. Cryptosporidiosis in buffalo calves (*Bubalus bubalis*): Prevalence and potential risk factors. Trop. Anim. Health Prod., 40: 419-426.
37. Azami, M., 2007. Prevalence of *Cryptosporidium* infection in cattle in Isfahan, Iran. J. Eukaryot. Microbiol., 54: 100-102.
38. Ghazy, A.A., S. Abdel-Shafy and R.M. Shaapan, 2015. Cryptosporidiosis in animals and man: 2. Diagnosis. Asian J. Epidemiol., 8: 84-103.
39. Ghazy, A.A., S. Abdel-Shafy and R.M. Shaapan, 2016. Cryptosporidiosis in animals and man: 3. Prevention and control. Asian J. Epidemiol., 9: 1-9.