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CONVENTIONAL AND MOLECULAR DIAGNOSIS OF CRYPTOSPORIDIOSIS IN CALVES

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ABSTRACT

The present study was conducted on 120 newly born calves (37 mixed breed, 66 native breed and 17 buffaloes calves), aged from one day to 6 months, they examined during the period from April 2014 to April 2016, these animals belong to some villages and farms from Assiut governorate. The overall prevalence was15.83 % (20.89% in calves less than two months, 11.36% in calves' from 2-4months with no infection rate from 4-6 months).the prevalence of Cryptosporidiosis in village shad higher rate of infection (19.23%) than farms (9.52%) in Assiut governorate. Mixed breed calves were more susceptible (24.32%) than native breed calves (13.64%) followed by buffalo calves (5.88%). Male calves were more susceptible than female calves to infection {males 16.44% (12/73) – females 14.89% (7/47)}. The clinical findings of cryptosporidiosis in examined calves were showed mild to severe diarrhea with varying degree of dehydration. Some cases were feverish. The state of appetite was different according to the severity of illness. The feces were varied from pasty to watery in consistency, pale yellow, yellow or greenish in color and sometimes contained mucous and blood. Higher infection rate was in non-hot months (22.95%) than hot months (8.47%). The molecular technique used for identification of Cryptosporidium infection in calves was nested PCR which is highly sensitive as a diagnostic tool for cryptosporidiosis and allow a rapid diagnosis in outbreak situations and provide information on genotypes.

Key words: *Cryptosporidiosis, Assiut Governorate, Nested PCR, Modified Ziehl Nelseen stain.*

INTRODUCTION

Cryptosporidiosis is an important protozoan parasitic disease that commonly affects neonatal calves as well as other mammalian hosts including humans. Cryptosporidium species is an important cause of diarrhea in young farm animals. Cattle have been considered to be an important source of zoonotic cryptosporidiosis (Amer *et al.,* 2010 and Maikai *et al.,* 2011). The occurrence of these Cryptosporidium spp. in cattle were shown to be age-related and Cryptosporidium parvum (C. parvum) is the only prevalent zoonotic in cattle, it responsible for 85% of the cryptosporidium infections in pre-weaned calves (Hassanain *et al.,* 2011). Infection of susceptible hosts follow ingestion of Cryptosporidium oocysts which excyst within the intestinal tract under the cell membrane not in the cytoplasm, diarrhea is the result of villous atrophy leading to malabsorption and secondary milk fermentation (DeQuadros *et al.,* 2006; Aynimode and Fagbemi, 2010 and Bhat *et al.,* 2012).

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Most commonly this agent act in concert with other enteropathogenes to produce intestinal damage and diarrhea but significant disease and mortality is recorded with monoinfection, particularly in neonates subjected to concurrent managemental or environmental stress, so Cryptosporidium infection can negatively influence growth rate and feed conversion that leading to a drop in the economic benefits derived from livestock production (Aynimode and Fagbemi, 2010 and Esmail and Ataallah, 2010). Cryptosporidiosis is generally diagnosed by microscopical detection of oocysts in fecal smears by conventional staining methods as Modified Ziehl Neelsen (MZN) staining method, but this method is relatively insensitive and sufficiently sensitive to detect only the clinical cases and cannot detect it in clinically healthy animals. However, polymerase chain reaction (PCR) had been proved to be useful in sensitive and specific diagnosis of cryptosporidiosis. The nested polymerase chain reaction (nPCR) with the utilization of primers specific for the small ribosomal RNA (SSU-rRNA) gene has been found to be highly sensitive as a diagnostic tool for cryptosporidiosis and allow a rapid diagnosis in outbreak situations and provide information on genotypes. (Coupe *et al.,* 2005; Rameriz *et al.,* 2005; Hassanain *et al.,* 2011 and Bhat

et al., 2014). As a result of the economic importance and the less information about the disease in calves at Assuit governorate, the present study was directed to throw light on the epidemiology of the disease by using conventional and molecular techniques in diagnosis.

MATERIALS AND METHODS

1. Animals:

A total number of 120 calves from the farm of faculty of agriculture, the farm of Masr El- Kher, the farm of Abnoub El- Hammam, Veterinary teaching hospital and different villages of Assuit governorate, examined clinically during the period from April 2014 to April 2016 according to (Rosenberger, 1990). These calves aged from 1day to 6 months.

2. Samples:

- Fecal samples were collected directly from the rectum of 120 calves in clean plastic cups on which the date, number, age and sex of the calf in addition to the address of the owner were registered.
- Each sample was divided in to 2 parts:

1) The first part was examined for:

- Gross and microscopical examination according to (Coles, 1989).
- Detection of the oocyte by using MZN stain (EDM, CAT. NO. 2995 Egypt) according to Fayer and Xiae, 2008 and Goldman and Green, 2015.

2) The second part (50 samples from the diarrheic calves) was preserved in potassium dichromate (PD) 2.5% at -20°c for molecular diagnosis, that was done as follow:

Extraction of DNA was made by using QIAamp DNA mini stool kit (QIAGEN, GmbH, Germany. CAT. No. 51504, No. of Preps 50) following the manufacturer's recommendations (Bhat *et al.*, 2012). In brief, approximately 200 mg of the faecal sample was mixed with 1.4 ml ASL buffer in 2.0 ml microcentrifuge tube. The homogenous suspension was heated in water bath at 80º C for 5 min and then centrifuged for 1 min at 14,000 xg to pellet stool particles. Supernatant (1.2 ml) was pipetted out in new 2 ml centrifuge tube, one inhibit EX tablet was added and then vortex. After 1 min incubation, the sample was centrifuged at 14,000 xg for 3 min to pellet out inhibitors bound to inhibit EX. Supernatant (200 µl) was added to new 1.5 ml centrifuge tube containing 30 µl of proteinase K and after vortexing, 200 µl of AL buffer was added. This lysate was incubated at 70º C for 10 min, 200 µl of ethanol was added, and the mixture was applied to QIAamp mini spin column and centrifuged at 8,000 xg for 1 min. Thereafter, 2 washings were given with wash buffers and DNA was eluted in 150 µl of elution buffer and stored at -20º C till use. Concentration of the extracted DNA from samples was measured in Nanodrop instrument. 18S rRNA gene amplification The PCR (primary as well as nested) was optimized to identify the small subunit (18S) ribosomal RNA gene as described by Paul *et al.* (2009). The sequences of the primers were as follows:

	Primers	Sequence(5′ - 3′)	Reference
primary PCR CRP-DIAG1	CRP-fwd	TTCTAGAGCTAATACATGCG	Paul *et al.* (2009)
	CRP-rev	CATTTCCTTCGAAACAGG	
nested PCR CRP-DIAG2	CRP F	GGAAGGGTTGTATTTATTAGATAAAG	
	CRP R	AAGGAGTAAGGAACAACCTCCA	

Two rounds of PCR in a final volume of 25 µl were carried out in a PCR thermal cycler. In the primary PCR assay, the master solution consisted of 2.5 µl of 10X PCR buffer (Bangalore Genei), 0.5 µl of 10 mM dNTP mix (Bangalore Genei), 2.0 µl of 25 mM MgCl2 (Bangalore Genei), 0.5 µl Taq DNA polymerase (Bangalore Genei), 0.5 µl each (20 pmol) of the external forward (CRP-DIAG1 forward) and external reverse (CRP-DIAG1 reverse) primers and 4.0 µl of template DNA isolated from fecalsamples. The volume was made up to 25 µl with nuclease-free water. The cycling conditions were as: initial denaturation at 94º C for 5 min, 34 cycles of denaturation at 94º C for 1 min, annealing at 56º C for 1 min, and extension at 72º C for 1 min, and the final extension was performed at 72º C for 10 min. For nested PCR similar quantities of the PCR mixture constituents except 1.5 µl MgCl2 (25 mM) and 3 µl of template was used. Identical thermocyclic parameters were kept in nested PCR except annealing was done at 57º C. The PCR product was checked for amplification by electrophoresis on a 1.5 % agarose gel and visualized using gel documentation system. The band was read at 834 bp for the nPCR, after PCR amplification of 18S SSU rRNA gene of cryptosporidium spp.

RESULTS

One hundred and twenty calves (69 diarrheic and 51 non diarrheic) were examined for the presence of Cryptosporidium oocysts by using MZN stain, the examination revealed that 18 (26.09) of diarrheic and 1 (1.96%) of non-diarrheic calves were infected by Cryptosporidium and shed its oocysts as shown in (Table1).

The clinical examination of Cryptosporidiosis in examined calves showed mild to severe diarrhea and varying degree of dehydration. Some cases were feverish. The state of appetite was different according to the severity of illness. The feces were varied from pasty to watery in consistency, pale yellow, yellow or greenish in color and sometimes contained mucous and blood as showing in (photos 1, 2, 3).

The parasitological technique used for identification of Cryptosporidium oocysts was MZN as showed in (photo 4). The MZN smears revealed that 15.83 % (19 out of 120) of fecal samples were positive as showed in (Table1).

In the present study, the molecular technique used for identification of Cryptosporidium infection in calves was nPCR. The specific band showed at 834bp after PCR amplification of 18S SSU rRNA gene of Cryptosporidium spp as showed in (photo5).

The overall prevalence of Cryptosporidium infection in examined calves was (15.83%) as shown in (Table 2). The prevalence of Cryptosporidium infection in examined calves in different locality indicated that some villages in Assiut governorate had higher rate of infection (19.23%) than farms (9.52%) in Assiut governorate as shown in (Table6).

The influence of breed on Cryptospordium infection revealed that mixed breed calves were more susceptible (24.32%) than native breed calves (13.64%) followed by buffalo was (5.88%) as showed in (Table3).

The present study revealed that male calves had higher infection rate than female calves to cryptosporidiosis {males 16.44% (12/73)–females 14.89% (7/47)} as showed in (Table4). Calves less than 2 months old had higher infection rate (20.89%) than calves from 2-4 months old (11.36%). Calves from 4-6 months old showed no infection rate as showed in (Table 5). Cryptosporidium infection in calves occurred in non-hot months (22.95%) more than hot months (8.47%) as showed in (Table7).

Table 1: The relationship between cryptosporidiosis and diarrhea:

State of animal	Number of examined calves	Positive	% of infection
Diarrheic	69	18**	26.09%
Non- Diarrheic	51	1	1.96%
Total	120	19	15.83%

** High significant differences ($\chi 2 = 12.809$ and P value <0.01).

Table 2: Comparison between conventional and molecular techniques for diagnosis of Cryptosporidiosis:

Number of Examined cases	Number of positive cases by MZN stain	% of infection	Number of positive cases by PCR	% of infection
50	9	18%	14	28 %

Table 3: Relationship between calvesbreed and Cryptosporidium infection:

Breed	Number of examined calves	Positive	% of infection
Mixed breed Calves	37	9	24.32%
Native breed calves	66	9	13.64%
Buffalo calves	17	1	5.88%
Total	120	19	15.83%

Insignificant differences ($\chi 2 = 3.504$ and P value >0.05).

Table 4: Relationship between sex and Cryptosporidium infection:

Sex	Number of examined calves	Positive	% of infection
Male	73	12	16.44%
Female	47	7	14.89%
Total	120	19	15.83%

Insignificant differences ($\chi 2$ =0.051 and P value >0.05).

Table 5: Relationship between age and Cryptosporidium infection:

Age	Number of examined calves	Positive	% of infection
Less than 2 months	67	14	20.89%
2-4 months	44	5	11.36%
4-6 months	9	0	0%
Total	120	19	15.83%

Insignificant differences ($\chi 2$ =3.641 and P value >0.05).

Table 6: Relationship between locality and Cryptosporidium infection:

Localities	Number of examined calves	Positive	% of infection
Farms	42	4	9.52%
Some villages from Assiut governorate	78	15	19.23%
Total	120	19	15.83%

Insignificant differences ($\chi 2$ =1.93 and P value >0.05).

Table 7: Relationship between seasonal variation and Cryptosporidium infection:

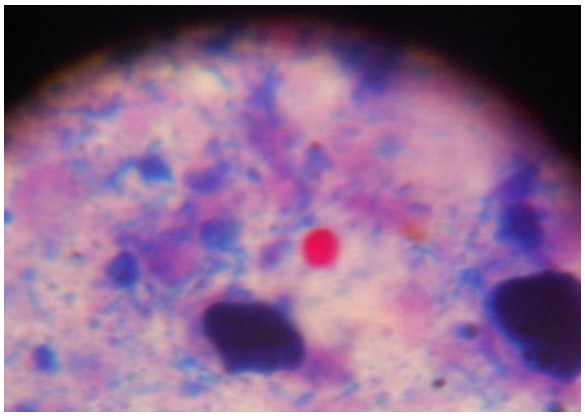
	Number of examined calves	Positive	% of infection
Months			
Hot months	59	5	8.47%
Non-hot months	61	14*	22.95%
Total	120	19	15.83%

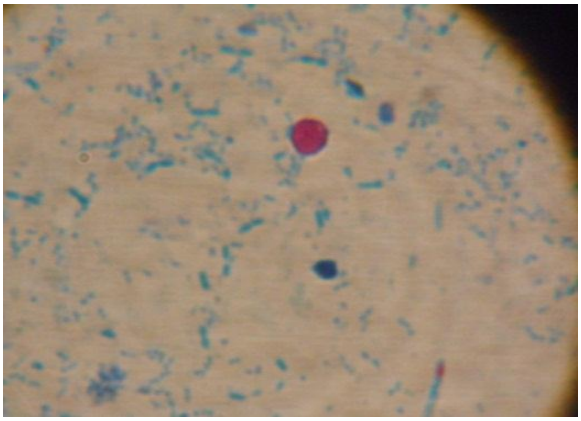
* Significant differences ($\chi 2$ = 4.716 and P value <0.05).

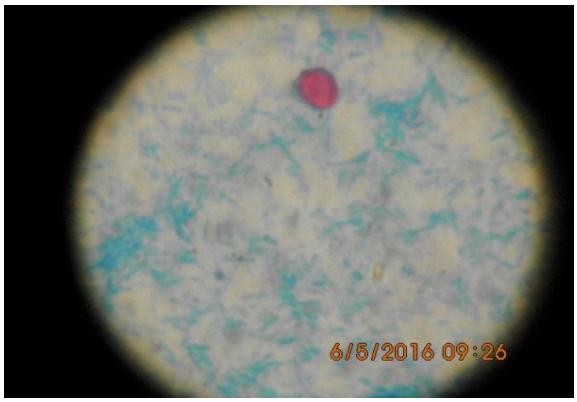
Photo no. (1): Straining and pasty to watery diarrhea.

Photo no. (2): Severe watery diarrhea

Photo no. (3): Severe watery grayish white diarrhea.







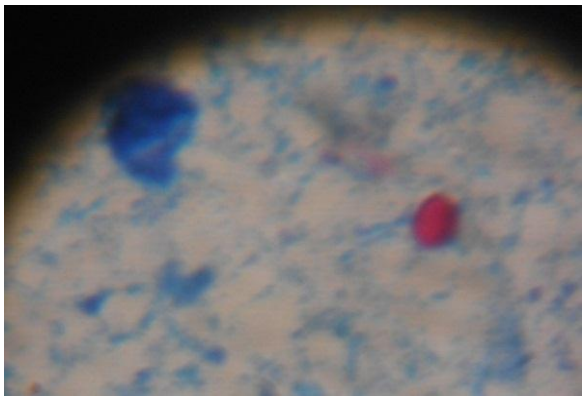


Photo no. (4): Rounded or ovoid pink colored Cryptosporidium oocyst stained by modified- Ziehl Nelseen stain.

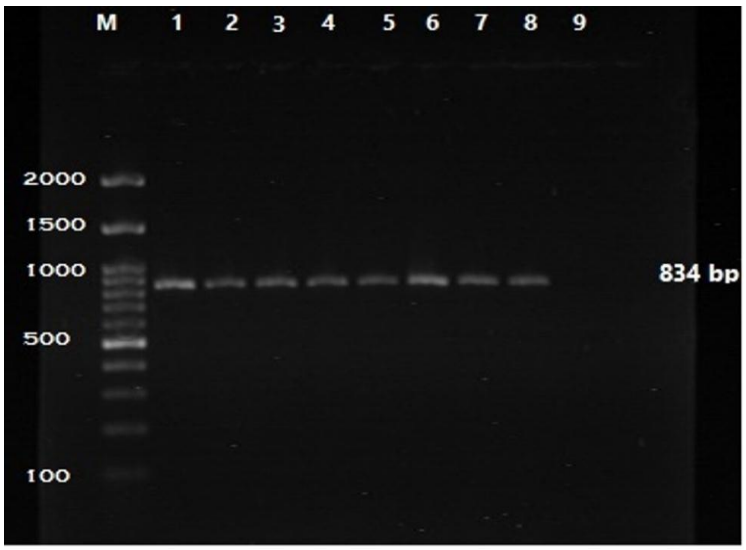


Photo no. (5): Agarose gel Electrophoresis of nPCR amplified DNA from 18S SSU rRNA gene of Cryptosporidium spp M. 100bp plus Marker DNA lanes 1:7 and 8 yielded 834bp PCR product, Lane 9 negative sample.

DISCUSSION

Cryptosporidiosis is an emerging protozoan disease of public health significance, Cryptosporidiosis causes significant morbidity among calves manifested by acute watery or steorrheic diarrhea and colic resulting in weight loss and delayed growth leading to huge economic loss, Cryptosporidium spp. is an apicomplexan, intracellular, extra cytoplasmic protozoan parasite which infect the microvillus epithelium of gastrointestinal tract in animals, birds, reptiles and man (Amer *et al.,* 2010; Bhat *et al.,* 2014 and Kumar *et al.,* 2015).

Clinical findings of cryptosporidiosis in examined calves showed mild to severe diarrhea and varying degree of dehydration. Some cases were feverish. The state of appetite was different according to the severity of illness. The feces were varied from pasty to watery in consistency, pale yellow, and yellow or greenish in color and sometimes contained mucous and blood and these results were in agreement with (Office international des epizooties, 2008; Chako *et al.,* 2010; Mohanty and panda, 2012; Silva *et al.,* 2012 and Masood *et al.,* 2013).

This study revealed that the infection rate of cryptosporidiosis in diarrheic calves was 26.09% which was higher than in non- diarrheic calves 1.96%, these results were similar to (Kumar *et al.,* 2015) that reported higher intensity of infection in diarrheic calves than non-diarrheic calves which is in accordance with our finding. The above observation further explained the fact that generally diarrhea develops when intestinal absorption is impaired or secretion is enhanced. Both of these processes are regulated by the intestinal epithelium cells which are infected by Cryptosporidium spp. In addition to these transport defects, abnormalities in barrier properties of intestinal epithelium mediated by intercellular junction complexes, contribute to Cryptosporidium diarrhea. Also these results were higher than that recorded by (Ayinmode and Fabgemi, 2010) in which the rate of infection was 15.8% in diarrheic calves and this may be attributed to bad sanitary conditions and low body immunity.

In the present study, the parasitological technique used for identification of Cryptosporidium oocysts was MZN. The MZN smears revealed that 15.83 % (19 out of 120) of fecal samples were positive, These results were higher than that reported by (Venue *et al.,* 2013 and Oskouei *et al.,* 2014) but were lower than that recorded by (Aynimode and Fabgemi, 2010; Mohanty and Panda, 2012 and Bhat *et al.,* 2014), the differences between our results and those of the other studies may be attributed to the geographical difference.

In the present study, the molecular technique used for identification of Cryptosporidium infection in calves was nPCR. The specific band showed at 834bp after PCR amplification of 18S SSU rRNA gene of Cryptosporidium spp. and our results were 28% (14 out of 50).

In this study the molecular method (nPCR) was more efficient and reliable than conventional method (MZN smear) in diagnosis of cryptosporidium infection in calves {nPCR 28% (14/50) – MZN smear 18% (9/50)}, the conventional techniques was lower than that recorded by the molecular techniques (n-PCR) and these results were similar to (Mirhashimi *et al.,* 2016) who detected that PCR is the most sensitive test used for diagnosis of bovine cryptosporidiosis compared to other tests especially the traditional

techniques (MZN). Also, (Essa *et al.,* 2014) reported that the molecular techniques (PCR) is the reliable method for identification of Cryptosporidium oocysts and could be used in place of Microscopy methods as (MZN) due to its ability for detection of the DNA genome of the microorganism and this lead to its higher specificity than the conventional methods which was the same reported in our present study. In addition to that, our research was in agreement with (Bhat *et al.,* 2014) and (Asadpour *et al.,* 2013) who detected that the nPCR with the utilization of primers specific for SSU-rRNA gene found to be highly sensitive i.e. about one oocyst in 1ml fecal sample, Hence PCR assay has been considered sensitive more than the conventional techniques (MZN) and can be used as a standard reference test for diagnosis. Similarly, our results were in agreement with (Hassanain *et al.,* 2011; Silva *et al.,* 2012 and Masood *et al.,* 2013) who used the nPCR analysis and the MZN techniques for detection of the prevalence of cryptosporidiosis and they found that using DNA extracted directly with simple modification from Cryptosporidium oocysts in nPCR showed higher sensitivity and specificity in the diagnosis of Cryptosporidium spp. From all the examined Egyptian isolates than the using of the conventional techniques. (Coupe *et al.,* 2005) proposed that the nPCR is a simple, robust, more sensitive and reliable method for detection of Cryptosporidium oocysts more than the traditional methods and this was in agreement with our results (Sakarya *et al.,* 2010) used the nPCR and MZN techniques for diagnosis of Cryptosporidiosis in calves and detected 12 out of 32 calves (37.5%) by nPCR and 7 out of 32 calves (21.88%) by MZN and they concluded that the nPCR can be successfully used for diagnosis and it is more specific and more sensitive especially in cases characterized by excretion of fewer oocysts and this also observed in our present study.

The prevalence of Cryptosporidium infection, the present study revealed that, the disease could be affected by several factors including animal susceptibility, age, sex, breed and seasonal variation. In this study the prevalence was 15.83% (19/120), similar results recorded by (DeQuadros *et al.,* 2006 and Silva *et al.,* 2012). However the prevalence of infection in our study was higher than that recorded in the study of (Mibui and Miriti, 2014) in Dagoretti division, Nairobi Kenya, (Esmail and Ataallah, 2010) in Saghez Iran, (Masood *et al.,* 2013 and Anna-Kjellen, 2007) and this may be attributed to the lower immunity of the calves and the higher shedding rate and bad hygienic management. In addition to that, the present prevalence was lower than the prevalence observed by (Imre *et al.,* 2009 and Guven *et al.,* 2013) and this due to the lower number of the examined cases and also may be due to the vast number of factors suchas breed, age, management, environment, and season as well as diagnostic method. The low prevalence could also be caused by spot fecal sampling instead of serial sampling, which may result in underestimation because of intermittent oocyst excretion. Regarding to the locality, the present study in Assiut governorate was showed that the rate of infection in Assuit farms was (9.52%) and in villages was (19.23%) and these differences may be due to the managemental system, the sanitary conditions and nursing of the calf in farms better than the individual cases in villages.

In relation to the breed of the animals, it was found that infection in our present study was 13.64% in native breed calves and 24.32% in mixed breed calves and in buffaloes were 5.88%, in which the rate of infection in mixed breed calves was higher than in native breed calves and buffaloes and this may be due to the differences in the number of calves, the stress factors to which the animal exposed, the sanitary conditions and also the immune status of calves which is lower in mixed breeds than native breeds and buffaloes. In our study the percent of infection in buffalo calves was 5.88% (1/17), these findings were in agreement with (El- Khodary and Osman, 2008; Amer *et al.,* 2010; Abou El- Ella *et al.,* 2013 and Mahfouz *et al.,* 2014) who stated that the Egyptian buffalo calves had a strong body immunity.

In relation to sex, our study revealed that the rate of infection in male calves was 16.44% higher than female calves which was 14.89% but these results recorded no significance differences similar to (Mohanty and Panda, 2012) and (Malek, 2007) and this may be due to the higher number of male calves than female one and there is no difference in the anatomical, functional and hormonal structures in body system especially in early ages that lead to no particular resistance against cryptosporidium infections in both males and females. While (Aynimode and Fagbemi, 2010 and Guven *et al.,* 2013) noticed that the rate of infection in females was more than males but also recorded no significance differences.

Animal age play a great role in calf susceptibility to cryptosporidium as it has been observed that the calves less than 2 months recorded high infection rate (20.89%), while calves aged from 2-4 months (11.36%), but calves aged from 4-6 months recorded no infection rate (0%), the same results reported by (Esmail and Attallah, 2010) who found that the prevalence of infection of cryptosporidiosis in less than 3 months aged calves was higher than weaned calves and adults, also (Mallinath *et al.,* 2009) showed that the prevalence and intensity of cryptosporidiosis was found more in calves less than 1 month of age compared to adults, In addition to that the present study was also similar to that recorded by (El-Sherif *et al.,* 2000; Bhat *et al.,* 2012; Mohanty and Panda 2012; Guven *et al.,* 2013 and Kumar *et al.,*

2015) and this may be due to the low body immunity during this age, (Ramirez *et al.,* 2004 and Mallinath *et al.,* 2009) reported that the rate of infection with Cryptosporidiosis was lower in the age more than 6 months and this may be attributed to the gradual development of immunity with the advancement of the age, and also the adult animals acts only as a symptomatic carriers of infection for younger calves and the disease is self-limiting in adult animals.

Dealing with seasonal variation of cryptosporidiosis among infected calves, the present study showed that there was a relationship between the rate of cryptosporidium infection and the seasonal changes. The higher rate of infection in non- hot months (22.95%) than hot months (8.47%), this might be attributed to the environmental stress factors which decrease the body immunity such as (increase rate of shedding and increase rate of calving) according to (Malek, 2007; Szonyi *et al.,* 2010; Mohanty and Panda, 2012 and Masood *et al.,* 2013).

CONCLUSION

Cryptosporidiosis is one of the most important enteropathogenic diseases which have the ability to induce diarrhea in neonatal calves and can be affected by some risk factors such as immunity of the calf, hygienic condition, managemental factors, age, sex, breed and seasonal variation. MZN stain is important tool for the detection of this infection. Molecular technique (nPCR) is more reliable method for diagnosis than the conventional techniques (MZN).

Finally, we recommended to pay more attention toward the diarrheic calves and should use more sensitive and specific methods for rapid diagnosis which plays an important role to improve the animal's life.

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التشخيص التقليدى والجزيئي لمرض الكريبتوسبورديوزس فى العجول

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أجريت هذه الدراسة على ١٢٠من العجول حديثة الولادة (٣٧ سلالة مختلطة، ٦٦ سلالة بلدي و ١٧ عجل جاموسي)، تتراوح أعمارهم ما بين يوم واحد و ٦ أشهر، تمت الدراسة خلال الفترة من أبريل ٢٠١٤ إلى أبريل ٢٠١٦، هذه الحيوانات تنتمي إلى بعض القرى والمزارع في محافظة أسيوط.وكانت النسبة العامة للاصابة ١٥.٨٣ ٪ (٢٠.٨٩ ٪ في العجول أقل من شهرين ، ١١.٣٦ ٪ في العجول من ٢-٤شهور مع عدم وجود أي معدل للاصابة من ٤-٦ أشهر). ارتفاع معدل الأصابة في القري ١٩.٢٣% عن المزارع ٩.٥٢% في محافظة أسيوط. وكانت العجول ذات السلالة المختلطة أكثر عرضة (٢٤.٣٢٪) من العجول ذات السلالة البلدي (١٣.٦٤٪)، تليها العجول الجاموسي (٥.٨٨٪). وكانت العجول الذكور أكثر عرضة من الإناث للإصابة {الذكور ١٦.٤٤٪ (٧٣/١٢) – الإناث ١٤.٨٩٪ (٤٧/٧)}. وقد أظهر الفحص الاكلينيكي للعجول المصابة بالكربتوسبوريديوسس انها تعانى من اسهال خفيف إلى شديد وبدرجات متفاوتة من الجفاف. وكانت بعض الحالات محمومة ، ذات شهية مختلفة وفقا لشدة المرض. يختلف قوام البراز من عجينى الى مائى في التناسق ، الأصفر الشاحب والأصفر أو الأخضر في اللون وأحيانا لديه مخاط و دم. وكان أعلى معدل للاصابة بالكريبتوسبوريديوم في العجول في الأشهر غير الساخنة (٢٢.٩٥ ٪) أكثر من الأشهر الحارة (٨.٤٧ ٪). كانت التقنية الجزيئية المستخدمة لتحديد عدوى كربتوسبوريديم في العجول (تفاعل البلمره المتسلسل) وهي حساسة للغاية كأداة تشخيصية لكربتوسبوريديوسس ويسمح بالتشخيص السريع في حالات تفشي وتقديم معلومات عن المورثات.