

Detection and Identification of *Eimeria* species in Naturally Infected Calves at Assiut Governorate

Safaa S. Malek¹ and Huda M. Kuraa^{2*}

¹Animal Medicine Department (Infectious Diseases), Faculty of Veterinary Medicine, Assiut University

²Animal Health Research Institute, Assiut Laboratory

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Abstract

The current study was carried out to investigate the prevalence of *Eimeria* species among 75 diarrheic calves in Assiut Governorate. Oocysts of *Eimeria* spp. were found in 46.7% (35/75) of the examined fecal samples from diarrheic calves using light microscope and 88% (22/25) by using PCR. Very high significant difference of *Eimeria* infection was recorded in calves 3-6 months of age, 73.3% (22/30) and 1 week-3 months of age 28.9% (13/45). The prevalence of *Eimeria* spp. in calves with diarrhea showed the highest rate in summer (69.2%), followed by winter (36.4%), autumn (25%) and spring (7.7%). Eight species of *Eimeria* were isolated by parasitological examination. The prevalence of *Eimeria* spp. was *E. zuernii* (51.4%) followed by *E. bovis* (31.4%), *E. alabamensis* (31.4%), *E. cylindrica* (25.7%), *E. subspherica* (14.3%), *E. canadensis* (11.4%), *E. ellipsoidalis* (5.7%) and *E. auburnensis* (2.9%). Single infection of *Eimeria* spp. was found in 48.6% of the infected calves, whereas mixed infection involved two, three or four *Eimeria* spp. was observed in 51.4% of the infected calves. In conclusion, season and age of the calves were the most significant aspects connected with the possibility of infection with coccidiosis. The PCR is a more reliable, sensitive and less time-consuming approach for diagnosis of *Eimeria*.

Keywords: *Eimeria* species, PCR, Naturally Infected, Calves.

Introduction

Bovines are one of the main source of meat production in Egypt, they are generally reared in small owner farms and suffer from malnutrition and parasitism [1]. The most important cause of calf morbidity and mortality is diarrhea [2]. Neonatal calf diarrhea (NCD) continues to be the first reason of calf mortality in Egypt; with an estimated 27.4-55% of the total deaths in young calves [3].

Neonatal calf diarrhea is caused by various infectious agents such as viruses, bacteria and protozoa [4]. The disease leads to economic losses in cattle flocks all over the world [5]. These losses are attributable to decreased growth rates, treatment costs and time spent caring for the affected calves [6,7]. Infectious agents may cause initial damage to the intestine, while death from scours usually results from dehydration, acidosis and loss of electrolytes. Determination of enteropathogens causing scours is necessary for performance of effective prevention and treatment [8]. Coccidia is as an important cause of diarrhea

in calves, and is associated with other enteropathogens [9,10].

Bovine coccidiosis is an important disease of apicomplexan parasites of genus *Eimeria* and is one of the main vital and common diseases of cattle worldwide [11]. It is considered one of the five most economically important diseases in the cattle industry [12]. The greatest economic losses are usually caused by acute diarrhea which accounts for approximately 75% of the mortality losses [13]. The highest prevalence of the disease takes place in calves less than one year of age [14]. All calves reared in conventional systems are exposed to coccidia and can be infected early in life [15].

Eimeria spp. are strictly host specific, and more than 20 species of *Eimeria* are defined in cattle [14,16]. *Eimeria bovis* and *Eimeria zuernii* are most commonly pathogenic species in calves worldwide causing morbidity and mortality by disturbing intestinal absorption and often associated with diarrheic feces which contain blood, fibrin and intestinal tissues [14,17,18].

The progress of clinical coccidiosis in cattle mainly depends on many factors such as the species of *Eimeria*, age of infected animal, number of ingested oocysts and breeding system; besides the management practices [14,19]. Relying on temperature, moisture and other ecological factors, sporulation of oocysts occurs within a week and the sporulated oocysts become infective and sustain their infectivity for several months under favorable environmental conditions such as temperature and moisture. The sporulated oocyst has four sporocysts each one contains two sporozoites [20].

The only practical way to recognize bovine *Eimeria* spp. is the detection of oocysts' morphology [14]. Nevertheless, the morphology of oocysts is not completely efficient as numerous *Eimeria* spp. have confusing features beside its intraspecies dissimilarity [21]. In addition, fecal inspection in conjunction with morphological identification is very intensive work which requires skilled method. Detection and differentiation of *Eimeria* by PCR showed higher sensitivity than the conventional identification of oocysts and is considered a useful technique for diagnosis of bovine coccidial infection [22].

Therefore, the present study aimed to determine the prevalence of *Eimeria* species infecting calves and its identification using oocysts morphological features and PCR assay in Assiut Governorate.

Materials and Methods

Animals and sample collection

A total of 75 fecal samples were collected from diarrheic calves from May 2016 to July 2017 in Assiut Governorate. The calves were categorized according to age into two groups: 1 week - 3 months and 3-6 months [23]. The date of sampling, the age and season were recorded for each calf. Thirty grams of feces were collected directly from the rectum using sterile gloves in dry and clean plastic bottles. The fecal samples were transferred immediately to the laboratory and were kept at 4°C in a refrigerator until processing within 48 h of arrival.

Parasitological examination

Microscopic fecal examinations were done for the detection of oocysts by direct smear and concentration flotation technique using saturated salt solution [24].

Sporulation of Eimeria spp. oocysts

The oocysts in positive fecal samples were sporulated using 2.5% potassium dichromate solution, aired frequently by using a pipette and left at room temperature before investigating by light microscope [24,25]. The size of non sporulated oocysts and sporulated oocysts was measured using light microscope with a calibrated eye piece micrometer and the *Eimeria* species were identified according to their size, shape, color and other morphological features such as micropyle, micropyle cap, shape of sporocyst, steida body and residual bodies [24].

Polymerase Chain Reaction (PCR)

Twenty-five fecal samples from calves (20 positive and 5 negative for *Eimeria* spp. by microscopic examination) were tested with PCR assay.

DNA extraction procedures

DNA was extracted using Bioline ISOLATE Fecal DNA Kit 50 Preps Cat No. BIO-52082, Lot No. IS674-114B according to the instructions of manufacturer. DNA was stored at -20°C till used.

PCR assays with Eimeria-common primers

Primers were manufactured by Metabion international (Germany). The up-and downstream primer sequences of *Eimeria*-common sequence in internal transcribed spacer 1 (ITS-1) region were: F: 5'- GCA AAA GTC GTA ACA CGG TTT CCG -3', R: 5'- CTG CAA TTC ACA ATG CGT ATC GC-3' with expected product sizes of 348-546 bp. A volume of 20 µL of reaction mixture comprised of 10 µL MyTaq™ HS Red Mix (Bioline, lot no. MTHRX-516201), 1 µL of the 10 µM primer (0.5 µM each) and 1 µL of extracted DNA. Reaction conditions included an initial denaturing phase at 94°C for 30 sec followed by 35 cycles at 94°C for 10 sec, 55°C for 20 sec, 72°C for 20 sec with final extension at 72°C for 2 min by Applied Biosystems Veriti Thermal Cycler 9902 (Singapore) [22].

PCR assays with species-specific primer

Primers were manufactured by Metabion international (Germany). The up-and downstream primer sequences of *Eimeria bovis* were: F: 5'-TCA TAA AAC ATC ACC TCC AA-3', R: 5'-ATA ATT GCG ATA AGG GAG ACA-3' with expected product size 238 (bp). Primer sequences of *Eimeria zuernii* were: F: 5'-AAC ATG TTT CTA CCC ACT AC-3', R: 5'-CGA TAA GGA GGA GGA CAA C-3' with expected product size 344 bp [22]. The reaction conditions of both *E. bovis* and *E. zuernii* are similar to ITS-1 PCR. The PCR conditions for *E. bovis* included an initial denaturing phase at 94°C for 30 sec followed by 35 cycles which at 94°C for 10 sec, 55°C for 20 sec, 72°C for 20 sec with final extension at 72°C for 2 min. While for *E. zuernii* for the reaction conditions included an initial denaturing phase at 94°C for 30 sec followed by 35 cycles which at 94°C for 10 sec, 52°C for 20 sec, 72°C for 20 sec and final extension at 72°C for 2 min. Then, 10 µL of PCR products were electrophoresed in 1.5% agarose gel (Bioshop ® Canada Inc., Burlington, ON. L7L 6A4) with 100 bp DNA ladder (Biomatik, code: M7123) and the amplified products were visualized using UV transilluminator.

Statistical analysis

Chi- square test was used to compare the prevalence of *Eimeria* spp. among investigated calves according to age and season [26].

Results

Oocysts of *Eimeria* spp. were found in 46.7% (35/75) of the examined fecal samples from diarrheic calves using light microscope and found in 88% (22/25) of the examined fecal samples by PCR. The overall prevalence of *Eimeria* spp. according to age was higher in calves of 3 - 6 months age (73.3%) than that in calves of 1 week -3 months age (28.9%). Very high significant difference of *Eimeria* spp. was recorded between the two groups ($P<0.001$). Concerning the season, very high significant difference in *Eimeria* spp. prevalence of diarrheic calves in summer (69.2%) followed by winter (36.4%), autumn (25%) and spring (7.7%) (Table 1).

Parasitological examination revealed that the isolated eight species of *Eimeria* in calves were *E. zuernii*, *E. bovis*, *E. alabamensis*, *E. cylindrica*, *E. subspherica*, *E. canadensis*, *E. ellipsoidalis* and *E. auburnensis*. The percentages of *Eimeria* spp. infecting calves were *E. zuernii* (51.4%) followed by *E. bovis* (31.4%), *E. alabamensis* (31.4%), *E. cylindrica* (25.7%), *E. subspherica* (14.3%), *E. canadensis* (11.4%), *E. ellipsoidalis* (5.7%) and *E. auburnensis* (2.9%), (Figures 1, 2 and Table 2). Single infection of *Eimeria* spp. was found in 48.6% (17/35) of the infected animals, whereas mixed infection involved two, three or four *Eimeria* spp. was observed in 51.4% (18/35) of the infected animals.

Table 1: Effect of age and season on the prevalence of infection in the examined calves (%) using light microscope

	Age/Season	No. of examined calves	No. of positive samples	%
Age	1 week – 3 month	45	13	28.9
	3 – 6 month	30	22*	73.3
Season	Winter	11	4	36.4
	Summer	39	27*	69.2
	Spring	13	1	7.7
	Autumn	12	3	25

* High significant differences ($P<0.001$)

Table 2: Morphological features of different *Eimeria* spp. isolated from naturally infected calves (n=35)

<i>Eimeria</i> species	No. of infected calves	%	Shape	Size um	Micropyle
<i>E. subspherica</i>	5	14.3	Subspherical	11.7 X11μ	No
<i>E. zuernii</i>	18	51.4	Spherical	18.8 X17.9μ	No
<i>E. bovis</i>	11	31.4	Ovoidal	25.4X17.2μm	Present
<i>E. cylindrical</i>	9	25.7	Cylindrical	22.3 X12.6μ	No
<i>E. alabamensis</i>	11	31.4	Subcylindrical	20.8X13.6μ	No
<i>E. elipsoidalis</i>	2	5.7	Ellipsoidal	17.2 X12.4μ	No
<i>E. canadensis</i>	4	11.4	Ellipsoidal	29.4X20.3μ	Present
<i>E. auburnensis</i>	1	2.9	Ellipsoidal to tapering	33.9 X20μ	Present

Molecular examination of 25 samples (20 positive and 5 negative by microscopical examination) by ITS-1 PCR revealed that all 20 positive samples by microscopical examination were positive by PCR, while 2 negative samples by microscopical examination were positive by PCR (Figure 3A). Further, the samples were examined by species-specific primers for *E. zuernii* and *E. bovis*. Out of the twenty samples positive by microscopy, five samples were identified as *E. bovis* by microscopical examination, while, 6 were positive by PCR (Figure 3B). Moreover, 8 were identified as *E. zuernii*, while PCR identifies 9 samples using the specific primers of *E. zuernii* (Figure 3C).

Discussion

Coccidiosis causes great economic losses for cattle as a result of decrease in feed efficiency which leads to slow weight gain and increased predisposition to other diseases [27]. There are no sufficient records about coccidiosis in calves in Assiut; therefore, our study was planned to throw light on calves' coccidiosis.

Our results indicated that 35 out of 75 (46.7%) examined fecal samples from diarrheic calves using light microscope were positive for *Eimeria* species. The obtained results are higher than 12.1% [28] 40.4% [29] and 27% [10] reported in Egypt. However, the obtained percentage was lower than 100% reported in calves [30] and 64.9% reported in calves by light microscope in villages of Lower Egypt [23].

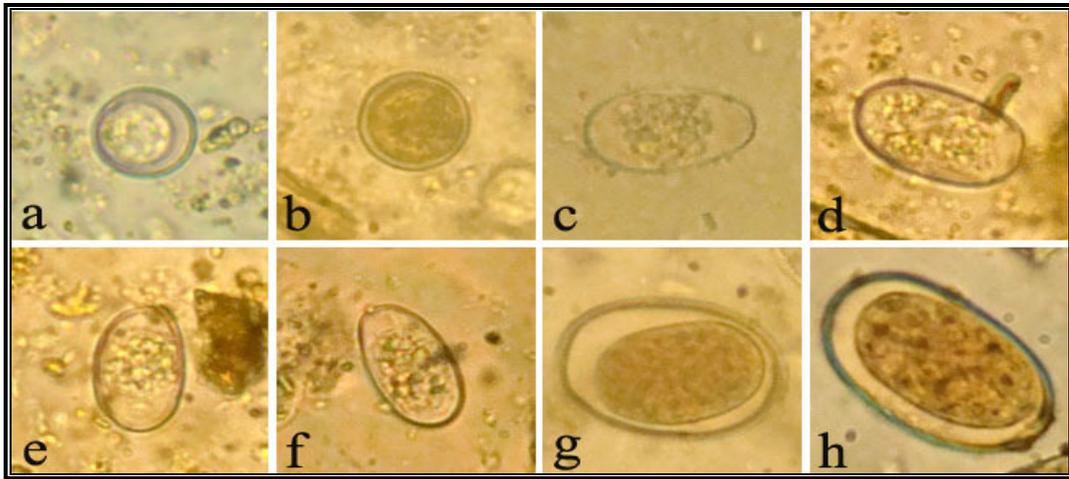


Figure 1: Unsporulated oocysts of *Eimeria* species in naturally infected calves a. *E. subspherica* , b. *E. zuernii*, c. *E. bovis*, d. *E. cylindrica*, e. *E. alabamensis*, f. *E. elipsoidalis*, g. *E. canadensis*, h. *E. auburnensis* X 40.

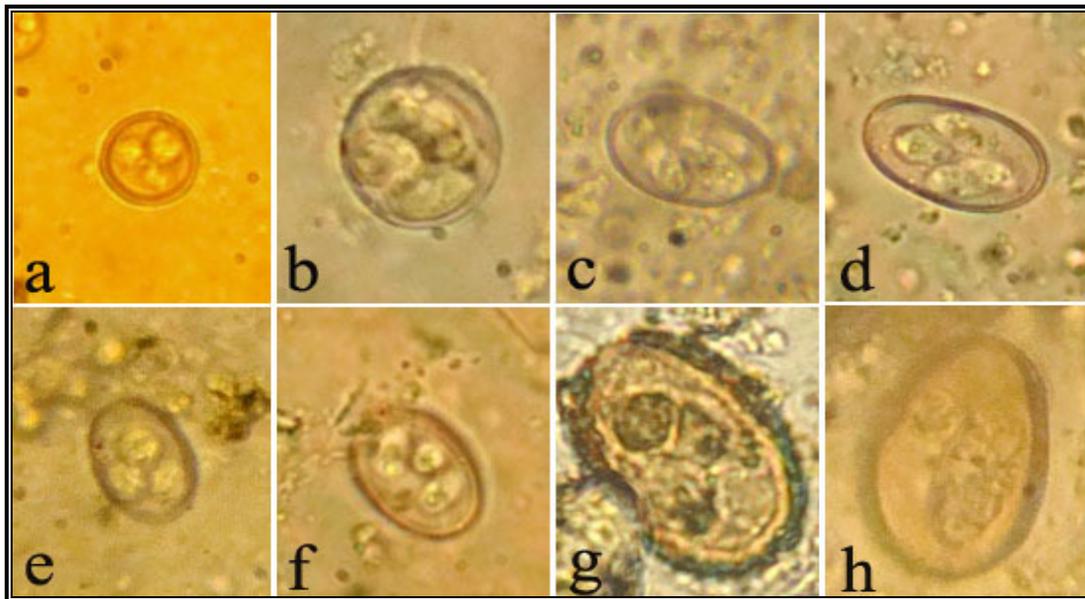


Figure 2: Sporulated oocysts of *Eimeria* species in naturally infected calves a. *E. subspherica*, b. *E. zuernii*, c. *E. bovis*, d. *E. cylindrica*, e. *E. alabamensis*, f. *E. elipsoidalis*, g. *E. canadensis*, h. *E. auburnensis* X 40.

The prevalence of *Eimeria* infection in the existing study is comparable to that recorded in different countries 47.59% in Sudan [31] and 47.1% in Shanghai, China [32]. Our results were higher than 28.3% in Iraq [33], 31.9% in Ethiopia [15], 33.2% of calves in India [34] and 42.7% in calves in Kenya [35], while, was lower than 60.9% in Denmark [36], 51.4% in Ethiopia [37], 54.6% in India [38] and 96% in the central Appalachian region of the United States [39]. This variation may be attributed to the changes in environment,

feeding strategies in addition to husbandry practice of the examined animals in different countries [10,23,29]. Concerning the age, the prevalence of *Eimeria* spp. was lower 28.9% (13/45) in calves 1 week – 3 month of age, while it was higher 73.3% (22/30) in calves 3 –6 months of age. These results were comparable to that obtained by Ahmed and Hassan [23]. While calves less than 3 months age showed lower rate as reported by El-Seify *et al.* [29] who found that the most susceptible age was 3-6 months with the percentage of 37.1%. Moreover, Fadly [10] mentioned that

the prevalence of *Eimeria* was significantly higher (46.6%) in 4-5 months old calves in Behera Governorate, this was also consistent with other studies [14,25,40]. Higher infection rate was detected in calves aged from 3 to 6 months as they discontinue a milk diet and passive immunity drops, while calves of 1 week to 3 months of age has good nursing of the colostrum feeding providing them with sufficient immunity [25,29]. Concerning the season, the prevalence of *Eimeria* spp. in diarrheic calves showed the highest rate in summer (69.2%), followed by winter (36.4%), autumn (25%) and spring (7.7%). These results disagreed with that of El-Seify *et al.* [29] who reported that winter season was the most suitable season for *Eimeria* spp. infection

as the infection rate of *Eimeria* reached to 33.3% and it was followed by spring, summer and autumn where the infection rates were 29.1%, 27.1% and 26.6% respectively. Another study also documented higher prevalence of coccidiosis in winter (45.3%) followed by autumn (33.3%), spring (16%) and summer (13.3%) [10]. Also, higher prevalence rate of *Eimeria* spp. was reported in Egypt during the months of rain [41,42]. No seasonal fluctuation in the prevalence of *Eimeria* infection was reported [43], while higher incidence in spring and autumn than winter and summer was documented [44]. This difference may be due to the variation of samples number, localities, management system, climate variations [10,11].

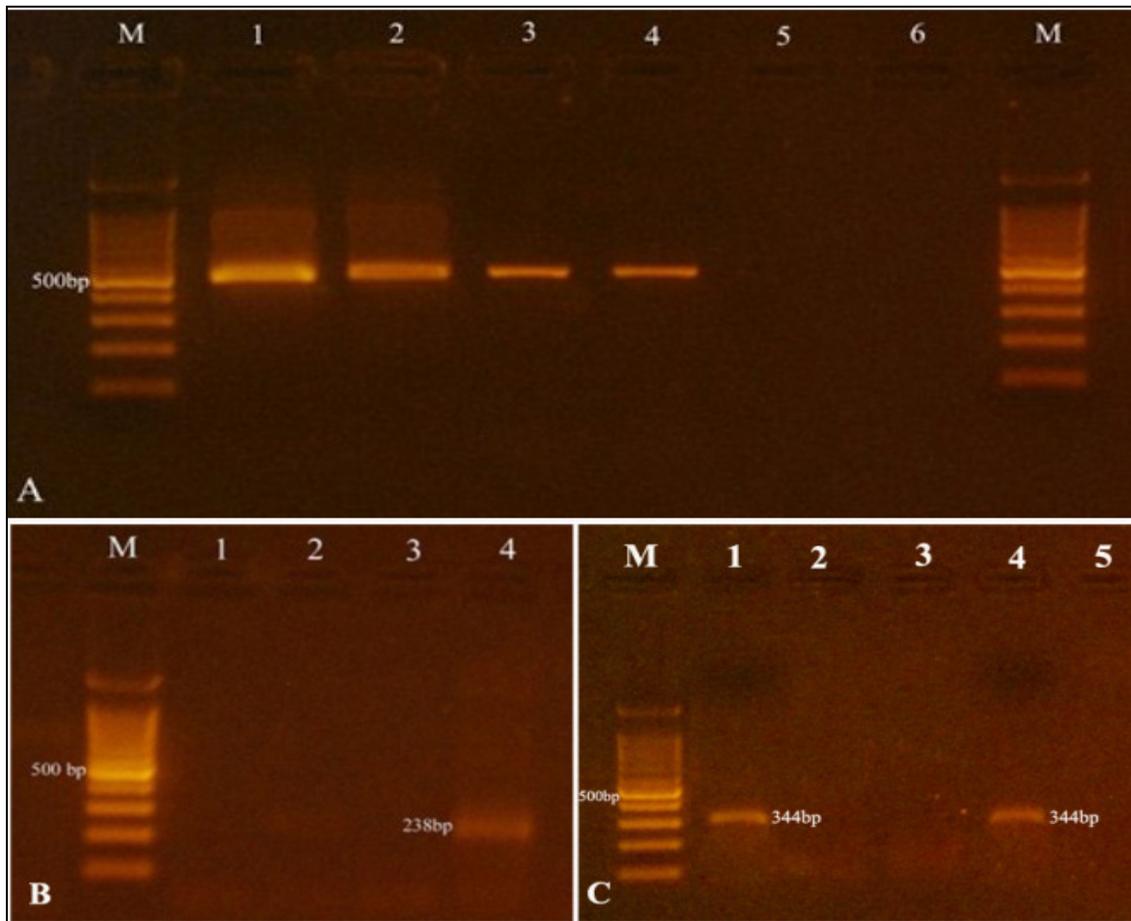


Figure 3: The electrophoresis pattern of PCR amplicon on calves fecal samples (A): using ITS, genus-common of *Eimeria* primer (546 bp PCR product). M: 100bp DNA ladder; Lanes 1, 2, 3, 4: Positive samples for *Eimeria*; Lanes 5, 6: Negative samples. (B): using *Eimeria bovis* primer (238 bp PCR product). M: 100bp DNA ladder; Lane 4: Positive sample; Lanes 1, 2, 3: Negative samples for *Eimeria bovis*. (C): using *Eimeria zuernii* primers (344 bp PCR product). M: 100bp DNA ladder; Lanes 1, 4: Positive samples for *Eimeria zuernii*; Lanes 2, 3, 5: Negative samples.

Parasitological examination revealed that eight species of *Eimeria* were identified in calves. This is within the same range as reported for calves in surveys in different countries: 11 *Eimeria* species in cattle reported in Kafr El-Sheikh Governorate, Egypt [29], 5 species in *Behera Governorate* in Egypt [10], 8 species in South-Western Ethiopia [37], 7 species in India [34] and 8 species in Al-Baha Area, Saudi Arabia [11]. This difference may be due to the different localities and management system [10,11].

In the present study, the most prevalent species were *E. zuernii* (51.4%) followed by *E. bovis* (31.4%) and *E. alabamensis* (31.4%). Similar findings were recorded by Bangoura *et al.* [45] who investigated that *E. zuernii* had a greater effect on the occurrence of diarrhea than *E. bovis*. Moreover, Mundt *et al.* [46] recorded that the level and duration of excretion was considerably higher for—*E. zuernii* than for *E. bovis*. On contrary, many authors found that *E. bovis* was the most frequently identified species followed by *E. zuernii* [10,11,15,37,39].

Single infection of *Eimeria spp.* was found in 48.6% of the infected animals, whereas mixed infection involved two, three or four *Eimeria spp.* was observed in 51.4%. This is in agreement with Ernst *et al.* [47] who recorded that mixed infections were found much more common than mono species infection under natural conditions. Also, similar results were recorded by El-Seify *et al.* [29] who reported single infection in 47.5% and mixed infection in 52.5% of the examined animals. Moreover, Yadessa *et al.* [37] identified single infection in 30.8% and mixed infection in 69.2% of the examined animals. On contrary, Fadly [10] found that mixed infection was observed in 11.11% of the samples.

In the present study, two negative samples by microscopical examination were positive by PCR. Also, two negative samples by microscopical examination of *E. zuernii* were positive by PCR and one negative sample by microscopical examination of *E. bovis* were positive by PCR. So, PCR appeared to be more sensitive than conventional fecal inspection of oocysts. This is in agreement with Kawahara *et al.* [22] who recorded that PCR was effective in detection of *Eimeria* from feces of

diarrheic calves. Information of apicomplexa genomic level has been developing constantly and species determination have been displayed using PCR [48,49].

E. bovis and *E. zuernii* are the most common causes of clinical coccidiosis and highly pathogenic [18]. The DNA sample was used with mixed species, *E. zuernii* and *E. bovis* primer as species-specific primer amplified and produced single bands with the expected sizes. These results agree with Kawahara *et al.* [22] who stated that the ITS-1 regions are flexible corresponding with species variation, showing a pattern of low intra-specific and high inter-specific variations in the DNA sequence, thus reduces the risk of cross-reactions with different species.

Conclusion

In conclusion, the season and age of the calves were the most significant aspects connected with the possibility of infection with coccidiosis, which is a common and important cause of economical loss in calves in Assiut Governorate. PCR technique is more rapid, convenient in detection of *Eimeria* in calves than microscopical examination which is very labor-intensive and require skillful technique.

Conflict of interest

The author declares no conflict of interest.

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الملخص الشري

كشف وتحديد أنواع الأيميريا في الشجول المصابة طبيشياً بمحافظة أسيوط

صفاء سيد حسن حسنين مالك^١ و هدى محمد محمد قراعه^{٢*}
^١ قسم طب الحيوان بكلية الطب البيطري جامعة أسيوط
^٢ قسم الطفيليات بمشهد بحوث صحة الحيوان بأسيوط

أجريت هذه الدراسة لمشرفة مدى انتشار طفيل الأيميريا في عدد ٧٥ من الشجول المصابة بالإسهال في محافظة أسيوط. وجدت نسبة الإصابة بحويصلات الأيميريا ٤٦,٧% (٧٥/٣٥) باستخدام المجهر الضوئي و وجدت نسبة الإصابة ٨٨% (٢٥/٢٢) باستخدام اختبار تفاعل البلمرة المتسلسل في عينات البراز التي تم فحصها للشجول المصابة بالإسهال. وقد اوضحت النتائج وجود فروق مشنوية كبيرة جدا بين نسبة الإصابة بالأيميريا في الشجول التي يتراوح اعمارها من ٣ الى ٦ أشهر ٧٣,٣% (٣٠/٢٢) عن الشجول التي يتراوح اعمارها من اسبوع الى ٣ أشهر ٢٨,٩% (٤٥/١٣). وقد وجد أعلى مشدل للإصابة بالأيميريا في الشجول المصابة بالإسهال في فصل الصيف (٦٩,٢%) يليه الشتاء (٣٦,٤%) يليه الخريف (٢٥%) يليه الربيع (٧,٧%). تم عزل ثمانية أنواع من الأيميريا من الشجول المصابة باستخدام الفحص الطفيلي. كانت الأيميريا الأكثر انتشارا هي الأيميريا زورنى بنسبة (٥١,٤%) يليها الأيميريا بوفيس (٣١,٤%)، الأيميريا ألبامنسيس (٣١,٤%)، الأيميريا سيلينديكا (٢٥,٧%)، الأيميريا سويسفيركا (١٤,٣%)، الأيميريا كانادنسيس (١١,٤%)، الأيميريا إليسويداليس (٥,٧%) و الأيميريا أوبورننسيس (٢,٩%). حيث وجد ان نسبة الإصابة بنوع واحد من الأيميريا في الشجول المصابة هي ٤٨,٦%. كما وجدت نسبة الإصابة المختلطة باثنين أو ثلاثة أو أربعة أنواع من الأيميريا في الشجول المصابة هي ٥١,٤%. ونخلص من هذه الدراسة الى ان المواسم وعمر الشجول أهم الجوانب المرتبطة بإمكانية الشدوى بالكوكسيديا كما أظهر استخدام اختبار تفاعل البلمرة المتسلسل حساسية اعلى بالمقارنة مع فحص البراز التقليدي عن حويصلات الأيميريا، و لذلك فانه يمكنه تحديد الأنواع الهامة من الأيميريا التي تصاب بها الابقار.