Molecular identification and characterization and phylogenetic study of six *Eimeria* species in cattle in Al-Najaf province

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Abstract

The study extended from April 2015 through January 2016 and included 600 cattle with varying age (1 day to 8 years). It was conducted in Al.Najaf province. Direct and flotation methods were used for the diagnosis of *Eimeria* infection. PCR and phylogentic tree was done for all isolates. *Eimeria* infection was reported in 211 out of 600 (35.2 %). PCR permitted the isolation of six *Eimeria* species. Nucleotide sequence was done and phylogenetic tree was established. The percentage of each species was as follows: *E. zuernii* (53.1%), *E. cylindrical* (49.3%), *E. ellipsoidalis* (34.6%), *E. bovis* (28.0%), *E. auburnensis* (22.7%) and *E. alabamensis* (19.4%). The *Eimeria bovis, Eimeria auburnensis, Eimeria cylindrica*, and *Eimeria zuernii* were closely related together. The *Eimeria ellipsoidalis* appeared less related to other *Eimeria* species. Whereas, the *Eimeria alabamensis* appeared significantly different from other *Eimeria* species and out off tree. For our knowledge this is the first molecular study done in Iraq for identification and characterization of *Eimeria* species in cattle. Molecular method can be utilized as a powerful, sensitive tool for the diagnosis of *Eimeria* infection in cattle, and as a superior substitute to the conventional morphologic methods. Phylogenetic tree analysis of the common six *Eimeria* species has been disclosed in this study to add to the original Japanese study by Kawahra in 2010.

Key words: Phylogenetic tree, PCR, *Eimeria*

التشخيص الجزيئي ودراسة الشجرة الوراثية لستة انواع من طفيلي الايميريا في الابقار في محافظة النجف

المستخلص

امتدت فترة العمل منذ شهر نيسان 2015 وحتى شهر كانون الثاني 2016 واجريت هذه الدراسة في محافظة النجف الاشرف. وشملت العينة 600 من الابقار تروحت اعمارها ما بين يوم وثمان سنوات. تم تشخيص الاصابة بطفيلي الايميريا بواسطة الفحص المجهري المباشر وطريقة التطويف. سجلت الاصابة بطفيلي الايميريا في 211 من النماذج الخاضعه للفحص وبنسبة (35,2%). اظهرت طريقة تفاعل البلمرة المتسلسل (PCR) الاصابة بستة انواع من طفيلي الايميريا وهي (*E. zuernii* (53.1%), *E. cylindrical* (49.3%), *E. ellipsoidalis* (34.6%), *E. bovis* (28.0%), *E. auburnensis* (22.7%) and *E. alabamensis* (19.4%).. بعد ذلك اجرى فحص الشجرة الوراثية والذي اظهر ان (*Eimeria bovis, Eimeria auburnensis, Eimeria cylindrica*, and *Eimeria zuernii*) كانت متقاربة جينيا وبصورة كبيرة ولكن ظهر أن (*Eimeria ellipsoidalis*) بعيدة وراثيا وبصورة ملحوظة عن بقية الانواع في حين كانت (*Eimeria alabamensis*) بعيدة جداً عن بقية الانواع. وفقا للمعلومات المتوفرة فان هذه الدراسة تعد فريدة من نوعها في العراق كونها سباقة لاستخدام العزل الجيني والشجرة الوراثية في تشخيص الاصابة بطفيلي الايميريا في المواشي ويعد اضافة للبحث الذي قام به الباحث الياباني كوارا في سنة 2010.

Introduction

Coccidiosis is the result of infection by one of the species of Eimeria or *Isospora*, which infects a long list of mammals, especially the important domestic animals. The number of species of *Eimeria* in cattle is around 13 but they are mostly nonopathogenic. The mostly encountered species in disease causation is *E. zuernii* and to lesser extent is *E. bovis* (Norman and Levine, 1973; Vorste and Mapham, 2012).

It is well documented that this disease causes significant economic drawback through the great loss of domestic livestock especially in areas with limited or restricted environment and areas with great livestock densities. The economic drawback is related

 ISSN 2072-3875

to the substantial mortality caused by infection added to the cost of treatment, on the other hand poor performance of the infected animal in growth and feeding is added to the economic loss. It is worth to mention that even the disease is subclinical, the animal will have poor growth rate due to reduced feed consumption and conversion and ultimately poor rate of growth (Lassen and Ostergaard, 2012).

The standard method for diagnosis and categorization of *Eimeria* is the morphologic examination under light microscopy. Recently attempts were made by researcher to make use of PCR technique for more sensitive detection and categorization of *Eimeria* species and leads to the assortment of these species according to phylogenetic tree which permit highly specific and sensitive detection and more understanding for the virulence (Kawahara *et al*., 2010). The aim of the present work was to apply PCR method for diagnosis of *Eimeria* species and their characterization and study the phylogentic tree among them.

Materials and methods

The primers were used in PCR for detection *Eimeria* spp in feces of cattle and designed by (Kawahara *et al*; 2010). These primers were provided from Bioneer Company, Korea (table 1).

Table (1): Primers used in the current study

Primer		Sequence	PCR product size
Genus	**F**	gcaaaagtcgtaacacggtttccg	348–546bp
	R	ctgcaattcacaatgcgtatcgc	
E.alabamensis	**F**	cattcacacattgttctttcag	184bp
	R	gcttccaaactaatgttctg	
E.auburnensis	**F**	taaattggtgcgatgaggga	295bp
	R	gcaatgagagaaagatttaata	
E.bovis	**F**	tcataaaacatcacctccaa	238bp
	R	ataattgcgataagggagaca	
E.cylindrica	**F**	gacatttaaaaaaccgattggt	304bp
	R	ggctgcaataagatagacata	
E.ellipsoidalis	**F**	caacgtttttccttttcctatca	148bp
	R	actgcgatgagagagagcg	
E.zuernii	**F**	aacatgtttctacccactac	344bp
	R	cgataaggaggaggacaac	

The study was designed to be an observational cross sectional study. The study extended from April 2015 through January 2016. The study included 600 cattle with varying age ranging from 1 day to 8 years. The study was conducted in Al.Najaf province and included four Districts: Najaf, Kufa, Al.Manathera, and Al.Meshkhab.

Ten gm feces samples were collected from rectum of each animal, and placed in sterile plastic containers and labeled for date, region, animal age, and gender according to special form for this study (Appendix I). Then each sample was transferred to the parasitology laboratory in the College of Veterinary Medicine/ Al-Qadissyia University and Al-Qassium Al-khdraa University. In the laboratory, direct wet smear was prepared for individual sample to detect infection under light microscopic examination. Two hundred eleven (211) samples were proved to carry infection by this method, and these were subjected to the subsequent steps of the study while the negative samples were properly discarded Then the samples that showed infection were

 ISSN 2072-3875

examined by flotation method to prove the diagnosis with better morphology. The samples that proved to carry infection by direct smear and flotation methods were stored at -20 ºC for future genetic analysis.

Direct wet method: Adequate fecal sample was taken and then was put on glass slide. A cover slip was applied following addition of one drop of normal saline and was mixed by a wood stick. Examination was done with light microscopy at 10X and 40X Magnification powers (Coles, 1986, and Albakri, 2009).

Flotation method: The flotation method was used in some cases that cannot detection the *Eimeria* oocyst in direct smear.

The PCR technique was performed for detection of *Eimeria* spp in feces samples that were collected from suspected cases of cattle. This method was carried out according to method described by Kawahara *et al*. (2010) as following:

Genomic DNA Extraction

Genomic DNA from feces samples were extracted by using AccuPrep® stool DNA Extraction Kit, Bioneer. Korea, and done according to company instructions as following:

- A 200 mg of the stool sample was transferred to sterile 1.5ml microcentrifuge tube, and then 20µl of proteinase K and 400 µl stool lysis buffer (SL) were added mixed by vortex and incubated at 60 ºC for 10 minutes.
- After 10 mins, the tube was placed in centrifuge at 12,000rpm for 5 mins.
- The supernatant was transferred in to a new tube and 200µl binding buffer was added to each tubes.
- The tubes were incubated again for 10 min at 60 ºC.
- 100 µl isopropanol was added and the samples mixed by lightly vortex for about 5 seconds, then spin down for 10 seconds to down the liquid clinging to the walls and lid of the tube.
- DNA filter column was placed in a 2 ml collection tube and all the mixture was transferred (including any precipitate) to column. Then centrifuged at 8000rpm for 5 minutes, and the 2 ml collection tube containing the flow through was discarded and placed the column in a new 2 ml collection tube.
- 500µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.
- 500µl W2 Buffer (ethanol) was added to each column. Then centrifuged at 8000rpm for 30 seconds. The flow through was discarded and placed the column back in the 2 ml collection tube.
- All the tubes were centrifuged again for 1 minute at 12000 rpm to dry the column matrix.
- The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer were added to the center of the column matrix.
- The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elude the purified DNA.

Genomic DNA estimation

The extracted genomic DNA from feces samples was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at 260 /280 nm as following steps:

- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
- A dry Chem.wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto

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the surface of the lower measurement pedestal.

- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate.
- Blanking solution was added as black solution which is same elution buffer of DNA samples.
- After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

Multiplex PCR master mix preparation

Multiplex PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions (table 2).

Table (2): Multiplex PCR master mix

Multiplex PCR Master mix	Volume
DNA template	5µL
Forward primer (10pmol)	1µL
Reverse primer(10pmol)	1µL
PCR water	39 µL
Total volume	501µL

After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as Taq DNA polymerase, dNTPs, Tris.HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye. Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (THECHNE.USA).

PCR Thermo cycler Conditions

PCR Thermocycler conditions by using convential PCR thermocycler system (3):

Table (3): PCR Thermo cycler Conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	30 cycle
Annealing	55C	30sec	
Extension	72C	45sec	
Final extension	72C	5min	1
Hold	4C	Forever	.

PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

- 1.5% agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool (50°C).
- Then 3µl of ethidium bromide stain were added into agarose gel solution.
- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5µl of (100bp Ladder) in one well.
- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

 ISSN 2072-3875

- PCR products were visualized by using UV Transilluminator.

DNA sequencing method

DNA sequencing method was performed for confirmative detection of some local *Eimeria* species that show positive in PCR technique and submission in NCBI-Genbank data base. The PCR product was purified from agarose gel by using (EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). As the following steps:

- The specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL microcentrifuge tube.
- 400µl Binding Buffer II was added to gel fragment. Then, incubated at 60ºC for 10 minutes and shaked until the agarose gel is completely dissolved.
- Add the above mixture to the EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000rpm for 2 minutes and discard the flow-through in the tube.
- 750µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Then, solution discarded.
- After that, the step 4 was repeated. Then, centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.
- The column was placed in a clean 1.5ml microcentrifuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and store at -20ºC.
- After that, the purified PCR product samples were sent to Macrogen Company in Korea to perform the DNA sequencing by AB DNA sequencing system.

Statistics analysis

Data were summarized, analyzed and presented using Statistical Package for Social Sciences (SPSS version 20) and Microsoft Office Excel 2010. Numeric variables were expressed as mean $\pm$SD (Standard deviation) while nominal variables were expressed as number and percentage. Chi-square test was used to study association between any two nominal variables while t-test was used to study difference in mean of numeric variables between any two groups. P-value was considered significant when it was equal or less than 0.05.

Results

Oocysts of *Eimeria* parasite were identified by direct wet smear and flotation methods using light microscope in a fresh fecal sample as shown in figure (1). The infection was reported in 211 out of 600 animals and hence the infection rate with *Eimeria* parasite was (35.2 %), as shown in figure (2).

 ISSN 2072-3875

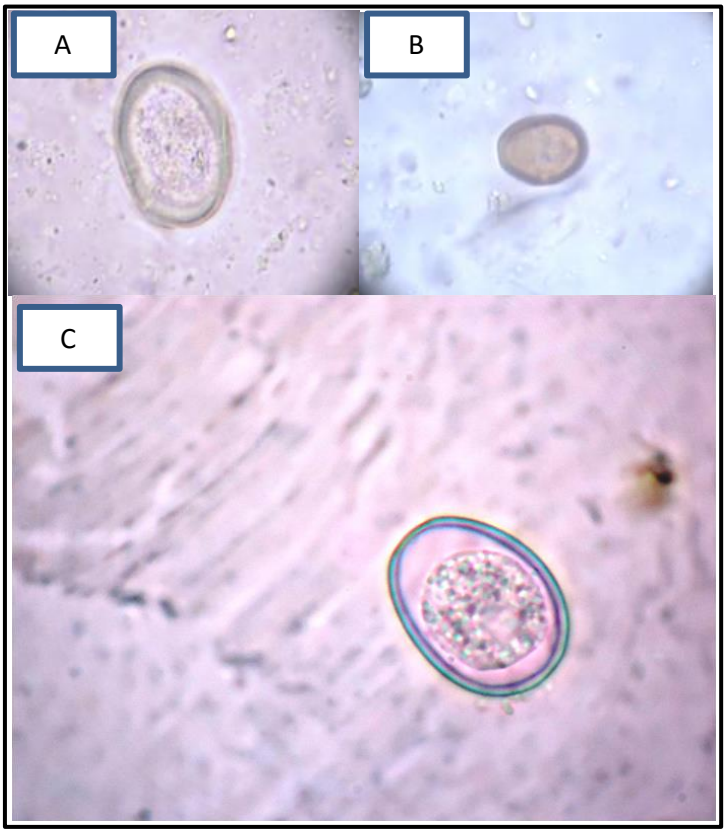

Figure (1): *Eimeria* oocyst detected by light microscopy using direct wet smear (A and B) and flotation (C) method 40X power.

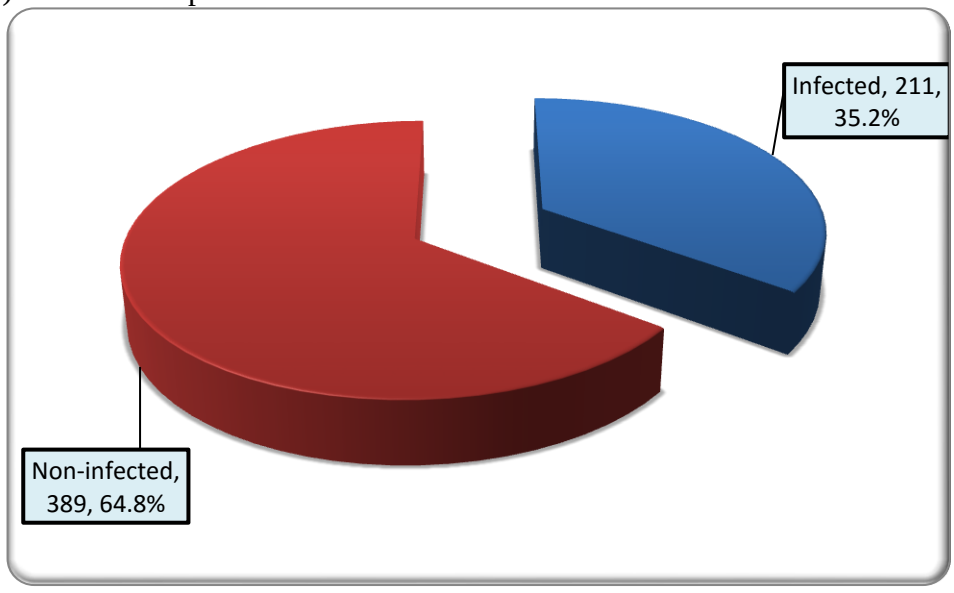

Figure (2): Pie chart showing the rate of *Eimeria* infection among cattle

First of all infection detected by microscopic examination, direct and flotation method, of *Eimeria* was confirmed by PCR using a primer that is general to all *Eimeria* species in cattle, as shown in figure (3).

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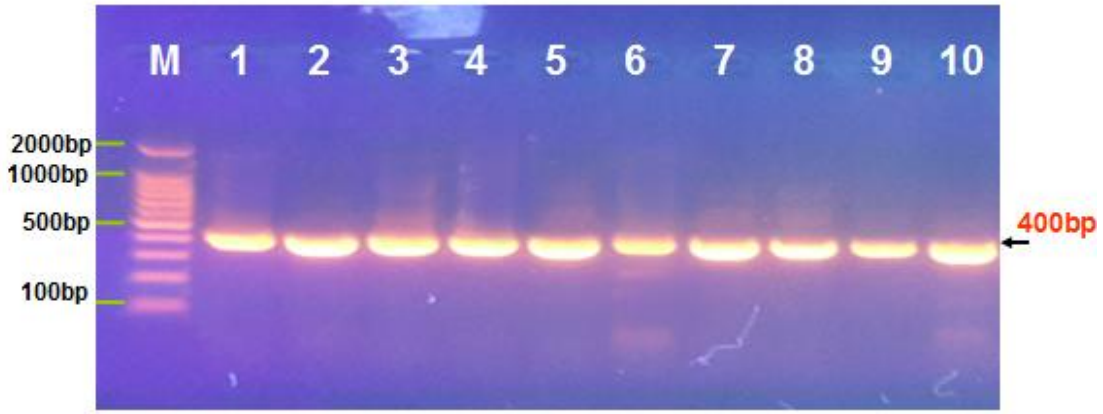

Figure (3): Agarose gel electrophoresis image that show the PCR product analysis of 18S rRNA gene in *Eimeria spp* positive samples. Where M: marker (2000-100bp), lane (1-10) stool positive samples at (~400bp) PCR product

Then a specific primer for each species of *Eimeria* was applied to all infected samples and the results are shown in figures (4 through 9).

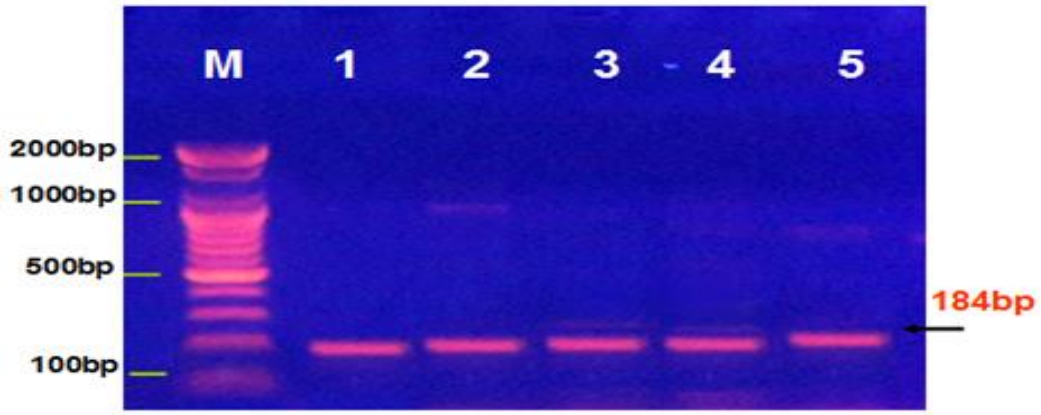

Figure (4): Agarose gel electrophoresis image that show the PCR product analysis of *Eimeria alabamensis* positive samples. Where M: marker (2000-100bp), lane (1-5) positive samples at (184bp) PCR product.

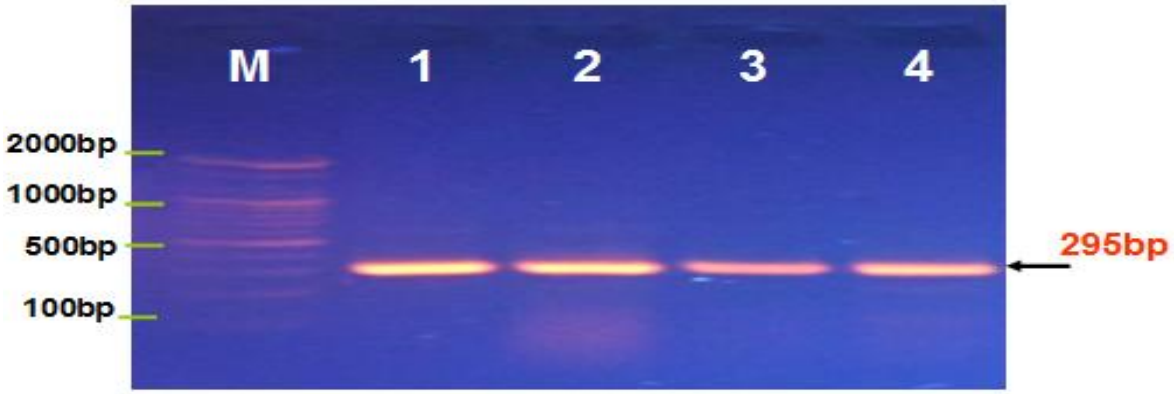

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Figure (5): Agarose gel electrophoresis image that show the PCR product analysis of *Eimeria auburnensis* positive samples. Where M: marker (2000-100bp), lane (1-4) positive samples at (295bp) PCR product.

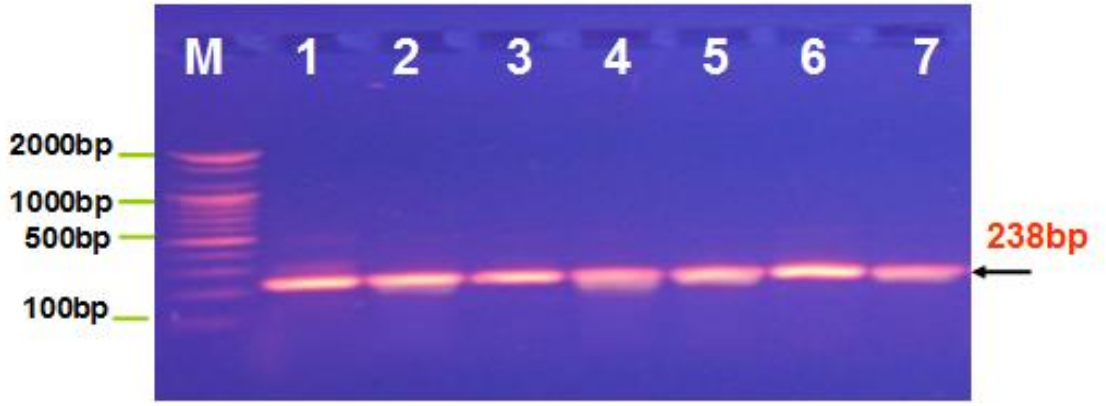

Figure (6): Agarose gel electrophoresis image that show the PCR product analysis of *Eimeria bovis* positive samples. Where M: marker (2000-100bp), lane (1-7) positive samples at (238bp) PCR product.

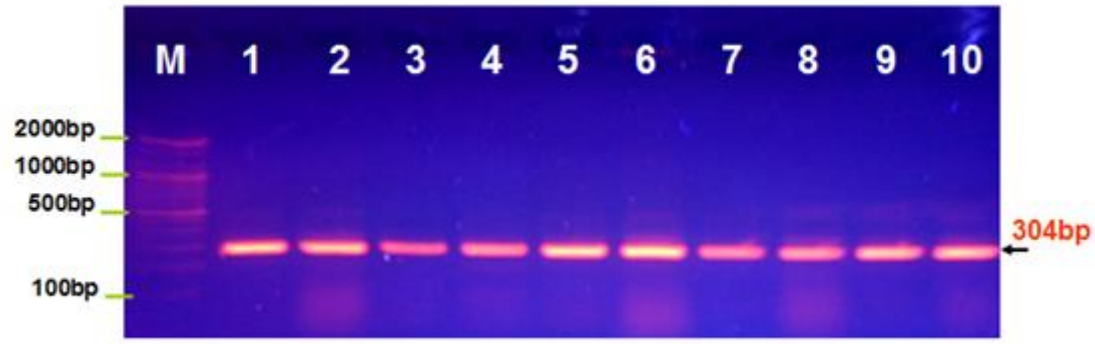

Figure (7): Agarose gel electrophoresis image that show the PCR product analysis of *Eimeria cylindrica* positive samples. Where M: marker (2000-100bp), lane (1-10) positive samples at (304bp) PCR product.

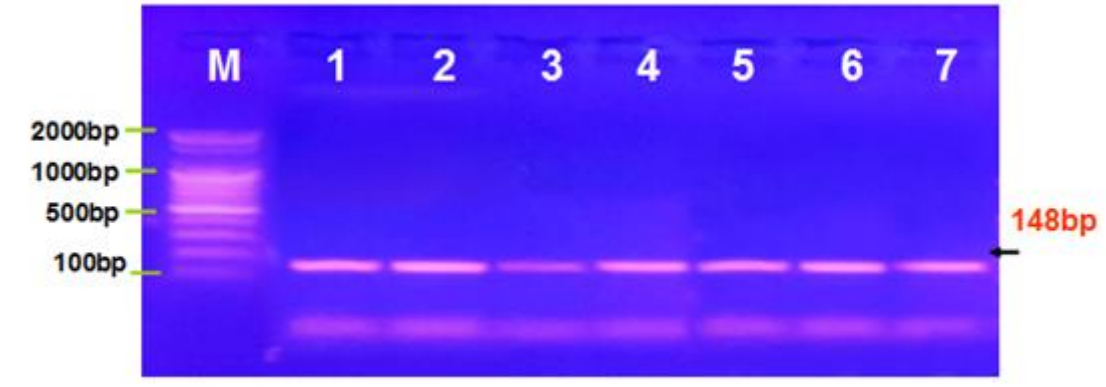

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Figure (8): Agarose gel electrophoresis image that show the PCR product analysis of *Eimeria ellipsoidalis* positive samples. Where M: marker (2000-100bp), lane (1-10) positive samples at (148bp) PCR product.

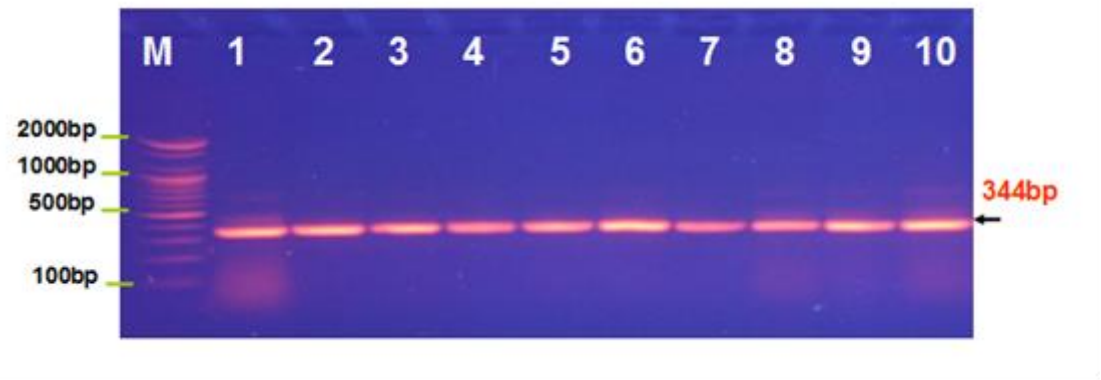

Figure (9): Agarose gel electrophoresis image that show the PCR product analysis of *Eimeria zuernii* positive samples. Where M: marker (2000-100bp), lane (1-10) positive samples at (344bp) PCR product.

The proportion of different *Eimeria* species is shown in table (4-7). The predominat species was *E. zuernii* accounting for 53.1%, followed by *E. cylindrical* accounting for 49.3%. In the third rank comes *E. ellipsoidalis* accounting for 34.6% followed by *E. bovis* accounting for 28.0% and then *E. auburnensis* accounting for 22.7 %. The last species was *E. alabamensis* accounting for 19.4 %.

Table (4): Rate of individual *Eimeria* species

***Eimeria* species**	**N**	**%**
E. zuernii	112	53.1
E. cylindrical	104	49.3
E. ellipsoidalis	73	34.6
E. bovis	59	28.0
E. auburnensis	48	22.7
E. alabamensis	41	19.4

Table (5) showed the detailed account of pure and mixed Eimeria isolates from the cattle enrolled in the present study. This table showed that each Eimeria species may occur as pure infection or mixed infection (figure 10).

Table (5): Detailed account about Rate of pure and mixed *Eimeria* isolates

Rank	*E. zuernii*	*E. cylindrical*	*E. ellipsoidalis*	*E. bovis*	*E. auburnensis*	*E. alabamensis*	Cases N	Isolates N	
1	1	0	0	0	0	0	17	1	
2	0	1	0	0	0	0	11	1	
3	0	0	1	0	0	0	8	1	**Pure**
4	0	0	0	1	0	0	7	1	
5	0	0	0	0	1	0	7	1	
6	0	0	0	0	0	1	6	1	
7	1	0	1	0	0	0	10	2	
8	1	1	0	0	0	0	19	2	
9	1	0	0	1	0	0	10	2	
10	1	0	0	0	0	1	7	2	**Mixed**
11	0	1	1	0	0	0	8	2	
12	0	1	0	1	0	0	6	2	

ISSN 2072-3875

13	0	1	0	0	1	0	7	2
14	0	1	0	0	0	1	4	2
15	0	0	1	1	0	0	9	2
16	0	0	1	0	1	0	6	2
17	0	0	1	0	0	1	7	2
18	0	0	0	1	1	0	13	2
19	1	1	0	0	1	0	9	3
20	1	1	0	0	0	1	7	3
21	1	1	0	1	0	0	8	3
22	1	1	1	0	0	0	9	3
23	1	1	1	0	0	1	10	4
24	1	1	1	1	1	0	6	5
Total							211	

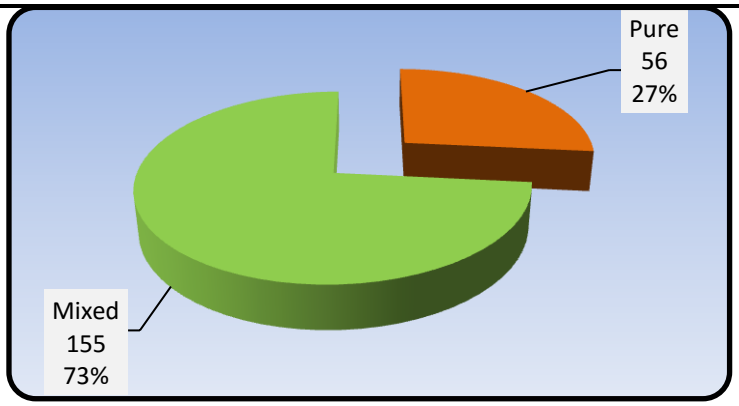

Figure (10): Rate of pure and mixed *Eimeria* isolates

Table (6) showed the number of cases with pure *Eimeria* isolates. The *E. zuernii* was recorded in 17 cases (8.1%), *E. cylindrical* was recorded in 11 cases (5.2%), *E. ellipsoidalis* was recorded in 8 cases (3.8%), *E. bovis* was recorded in 7 cases (3.3 %), *E. auburnensis* was recorded in 7 cases (3.3%) and *E. alabamensis* was recorded in cases 6 (2.8 %).

Table (6): Number of cases with pure *Eimeria* isolates

Rank	*E. zuernii*	*E. cylindrical*	*E. ellipsoidalis*	*E. bovis*	*E. auburnensis*	*E. alabamensis*	N	%
1	1	0	0	0	0	0	17	8.1
2	0	1	0	0	0	0	11	5.2
3	0	0	1	0	0	0	8	3.8
4	0	0	0	1	0	0	7	3.3
5	0	0	0	0	1	0	7	3.3
6	0	0	0	0	0	1	6	2.8
Total							56	26.5

DNA sequencing method were performed for phylogenetic confirmative detection and submitted in NCBI-Genbank data base to get accession number codes for of local *Eimeria* species at first time in Iraq. Specific phylogenetic confirmative detection of local *Eimeria* species was performed by using the phylogenetic tree analysis and compared with NCBI-BLAST *Eimeria* species. Where the local *E. alabamensis* isolate 1 and isolate 2 close related to NCBI-BLAST *E.alabamensis* (AB769700.1) at (99%) and (98%) homological sequence identity respectively, local *E.ellipsoidalis* isolate 1 and isolate 2 close related to NCBI-BLAST *E.ellipsoidalis* (AB769625.1) at (99%) homological sequence identity, local *E.bovis* isolate 1 and isolate 2 close related

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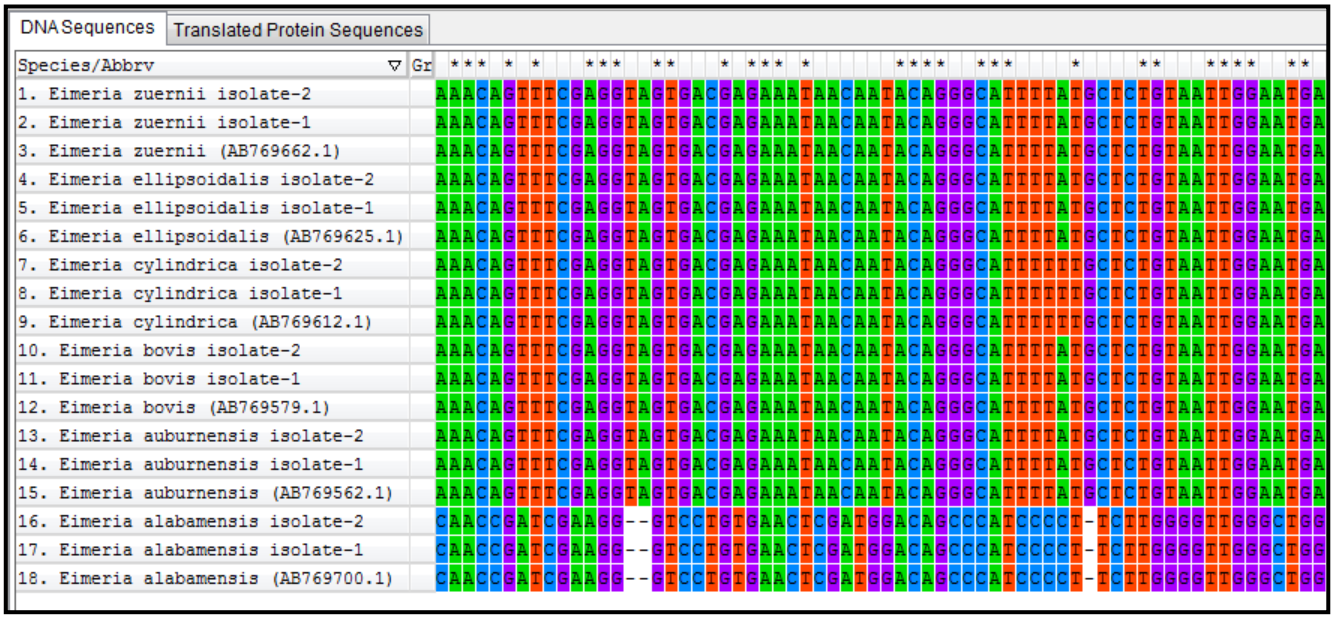
to NCBI-BLAST *E.bovis* (AB769579.1) at (100%) homological sequence identity, local *E.zuernii* isolate 1 and isolate 2 close related to NCBI-BLAST *E.zuernii* (AB769662.1) at (100%) homological sequence identity, local *E.auburnensis* isolate 1 and isolate 2 close related to NCBI-BLAST *E. auburnensis* (AB769562.1) at (100%) homological sequence identity, local *E.cylindrica* isolate 1 and isolate 2 close related to NCBI-BLAST *E.cylindrica* (AB769612.1) at (99%) homological sequence identity. As shown in table (7).

Table (7): NCBI-Blast Homology sequence identity for 18S rRNA gene in local *Eimeria* species isolates and NCBI-Blast *Eimeria* species

Local *Eimeria* sp.	**NCBI-Blast Homology sequence identity**					
	***E. alabamensis* (AB769700.1)**	***E. ellipsoidalis* (AB769625.1)**	***E. bovis* (AB769579.1)**	***E. zuernii* (AB769662.1)**	***E. auburnensis* (AB769562.1)**	***E. cylindrica* (AB769612.1)**
E. alabamensis.1	99%	96%	96%	96%	96%	96%
E. alabamensis.2	98%	95%	95%	95%	95%	95%
E.llipsoidalis.1	NI	99%	99%	99%	98%	98%
E.llipsoidalis.2	NI	99%	99%	99%	98%	98%
E. bovis.1	NI	99%	100%	99%	99%	99%
E. bovis.2	NI	99%	100%	99%	99%	99%
E. zuernii.1	NI	99%	99%	100%	99%	99%
E. zuernii.2	NI	99%	99%	100%	99%	99%
E.auburnensis.1	NI	99%	99%	99%	100%	99%
E.auburnensis.2	NI	99%	99%	99%	100%	99%
E.cylindrica.1	NI	99%	99%	99%	99%	99%
E.cylindrica.2	NI	98%	99%	98%	99%	99%

ISSN 2072-3875



Figure(11): Multiple sequence alignment analysis of the partial 18S rRNA, internal transcribed spacer 1 gene sequence in local Eimeria species and NCBI-Genbank local *Eimeria species* isolates based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) and differences in 18S rRNA gene nucleotide sequences.

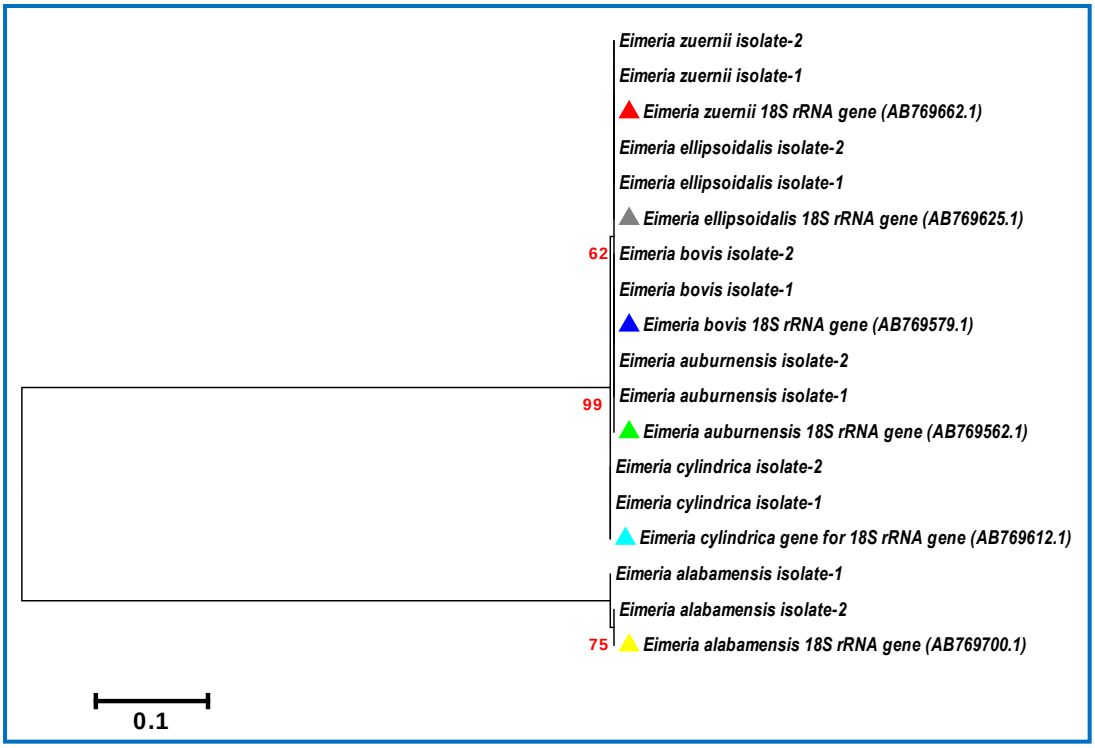


Figure (12): Phylogenetic tree analysis based on the 18S rRNA, internal transcribed spacer 1 gene partial sequence that used for confirmative detection of local *Eimeria* species isolates. The phylogenetic tree was constructed using maximum likelihood tree in (MEGA 6.0 version). The local *Eimeria alabamensis, Eimeria auburnensis, Eimeria bovis, Eimeria zuernii, Eimeria ellipsoidalis and Eimeria cylindrica* isolates were show closed related to NCBI-Blast *Eimeria alabamensis* (AB769700.1), *Eimeria auburnensis* (AB769562.1), *Eimeria bovis* (AB769579.1),

Eimeria zuernii (AB769662.1), *Eimeria ellipsoidalis* (AB769625.1) and *Eimeria cylindrica* (AB769612.1) respectively

The local *Eimeria* species were submitted in NCBI-Genbank data base to get the Gene bank accession number for our isolates at first time in Iraq as following table (8).

Table (8): Genbank accession number codes that given by NCBI-Genbank to local *Eimeria* species isolates

Rank	Locale isolates	Genbank accession number codes
1	*E. alabamensis isolate-1*	*E. alabamensis* (KU641158)
2	*E. alabamensis isolate-2*	*E. alabamensis* (KU641159)
3	*E. ellipsoidalis isolate-1*	*E. ellipsoidalis* (KU641156)
4	*E. ellipsoidalis isolate-2*	*E. ellipsoidalis* (KU641157)
5	*E. bovis isolate-1*	*E. bovis* (KU641152)
6	*E. bovis isolate-2*	*E. bovis* (KU641153)
7	*E. zuernii isolate-1*	*E. zuernii* (KU641162)
8	*E. zuernii isolate-2*	*E. zuernii* (KU641163)
9	*E. auburnensis isolate-1*	*E. auburnensis* (KU641154)
10	*E. auburnensis isolate-2*	*E. auburnensis* (KU641155)
11	*E. cylindrical isolate-1*	*E. cylindrical* (KU641160)
12	*E. cylindrical isolate-2*	*E. cylindrical* (KU641161)

Discussion

Depending on the results obtained by Kawahara *et al* in (2010), who described only six species, the present study was designed to investigate the prevalence of the six same species. The frequency and percentage of each species obtained in PCR analysis was shown in the result chapter. Kawahara *et al*, is the pioneer in the field of *Eimeria* species analysis based on molecular studies and the recorded results in the gene bank were the data about the *Eimeria* species in cattle that have been adopted by the present study.

The molecular result of the present study confirmed the ordinary light microscopy methods, direct and flotation methods, by the use of gene sequence that is general to all *Eimeria* species. The results also showed involvement of all 211 animals by one or more of the six *Eimeria* primers. Mixed infection was realized to affect 155 (73%) of cattle enrolled in the present study, whereas pure infection was seen in 56 (27%) of cattle. This finding agrees with the finding of Alemayehu, *et al*., (2013) who stated that mixed infection with *Eimeria* species is far more common than pure infection. This finding can be attributed to the fact that infection with certain *Eimeria* species will cause the host to acquire immunity for that species only, i.e. there is no cross immunity and subsequently the animal will be exposed to infection with other species and even with mixed infection (Alemayehu, *et al*., 2013).

Pure infection was as follows: The *E. zuernii* was recorded in 17 cases (8.1%), *E. cylindrical* was recorded in 11 cases (5.2%), *E. ellipsoidalis* was recorded in 8 cases (3.8%), *E. bovis* was recorded in 7 cases (3.3 %), *E. auburnensis* was recorded in 7 cases (3.3%) and *E. alabamensis* was recorded in cases 6 (2.8 %).

In the present study it was proved that The local *Eimeria bovis* isolates (1 and 2) were closely related to NCBI-Blast *Eimeria bovis* (AB769579.1), the local *ellipsoidal bovis* isolates (1 and 2) were closely related to NCBI-Blast *Eimeria ellipsoidal*

 ISSN 2072-3875

(AB769625.1), the local *Eimeria zuernii* isolates (1 and 2) were closely related to NCBI-Blast *Eimeria zuernii* (AB769662.1), the local Eimeria cylindrica isolates (1 and 2) were closely related to NCBI-Blast *Eimeria cylindrica* (AB769612.1), the local *Eimeria auburnensis* isolates (1 and 2) were closely related to NCBI-Blast *Eimeria auburnensis* (AB769562.1), the local *Eimeria alabamensis* isolates (1 and 2) were closely related to NCBI-Blast *Eimeria alabamensis* (AB769700.1).

Phylogenetic tree analysis in the present study showed that the *Eimeria bovis, Eimeria auburnensis, Eimeria cylindrica,* and *Eimeria zuernii* were closely related together. The *Eimeria ellipsoidalis* appeared less related to other *Eimeria* species. Whereas, the *Eimeria alabamensis* appeared significantly different from other *Eimeria* species and out off tree. The present study agreed with Kawahara *et al*. in (2010) in that *Eimeria alabamensis* is greatly distant from other species. Also the present study agreed with Kawahara *et al* in (2010) in that *Eimeria bovis* and *Eimeria zuernii* are closely related. Nevertheless, the present study disagree with Kawahara *et al* in (2010) that *Eimeria auburnensis, Eimeria cylindrical* and *Eimeria ellipsoidalis* are closely on the contrary, the present study showed that *Eimeria ellipsoidalis is slightly different and that Eimeria auburnensis, Eimeria cylindrical* are closely related to *E. zuernii* and *E. bovis* and not distant from them.

In the present study, the molecular results showed that *E. zuernii* was the most frequently isolated parasite. This finding is in accordance with (Matijila and Penzhorn, 2002 and Sanchez *et al*., 2008). This confirms the already noticed observation that *E. zuernii* is the most invasive and harmful *Eimeria* parasite, this pathogenesity might be related the strong resistance to environmental factors and the ability to cause infection in small doses and to overcome host immune responses (Daugschies and Najdrowski, 2005).

References

Al-Bakry, H. S. (2009). Diagnosis and classification of Eimeria species in cattle in Mosul. Iraqi J. for Vet. Sci.; 23 (1): 10-13.

Alemayehu, A.; Nuru, M. and Belina, T. (2013). Prevalence of bovine coccidia in Kombolcha district of South Wollo, Ethiopia. J. of Vet. Med. and Animal Health; 5(2): 41-45.

Coles, E. H. (1986). Vet. Clinical Pathology 4th ed., W. B. Saunders Company, Philadelphia. pp:375-379.

Daugschies, A. and Najdrowski, M. (2005). Eimeriosis in cattle current understanding. J. of Vet. Med.; 52: 417-427.

Kawahara, F.; Zhang, G.; Mingala, C. N.; Tamura, Y.; Koiwa, M.; Onuma, M. and Nunoya, T. (2010). Genetic analysis and development of species-specific PCR assays based on ITS-1 region of rRNA in bovine *Eimeria* parasites. Vet. Parasitol.; 174: 49–57.

Lassen B and Ostergaard S. (2012). Estimation of the economical effects of *Eimeria* infections in Estonian dairy herds using a stochastic model. Preventive Vet. Med.; 106(3-4): 258-265.

Matjila, P. T. and Penzhon, B. L. (2002). Occurrence and diversity of bovine coccidian at three localities in South Africa. Vet. Parasitol.; 104: 93-102.

Norman, D. and Levin. N. (1973). The Apicomplexa, and The coccidian proper. In: protozoan Parasites of Domestic Animals and of Man, 9th ed, Burgess Pubilishing Company. pp: 156-185.

Sanchez, R.O; Romero, J.R. and Founroge, R.D. (2008). Dynamics of Eimeriaoocyst excretion indairy calves in the province of Buenos Aires (Argentina), during their first two months of age. Vet. Parasitol.; 151: 133-138.