



MOLECULAR DETECTION OF *EIMERIA* SPP. IN BUFFALOES IN AL-QADISSIYHA PROVINCE, IRAQ

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Abstract

Eimeria is an apicomplex protozoan which affects many species of wild and domestic animals and birds, including buffaloes. The study was designed for investigation *Eimeria* spp. in buffaloes by Molecular methods for the first time in Iraq. One hundred fecal samples of buffaloes were examined by Conventional PCR from different Al-Qadissiyha province in Iraq during December 2018, to end of August 2019. A total rate of infection were 66% (66/100). In relation to area of study including: AL-Sadeer, AL-Dagarha, Efk and AL-Sanyia with prevalence rate 76%, 68%, 60% and 60% respectively. There was no significant differences ($p > 0.05$). Buffaloes females recorded highest rate 70.1% with *Eimeria* spp infection by PCR while males recorded lowest rate 57.5% without significant (difference $P > 0.05$). The highest infection rate was observed at 1 < year age group 73% (19/26), followed age groups ≥ 3 Years which recorded rate 61, 7% (21/34), while the lowest infection rate recorded in age group 1 to <3 years 56% (26/40). With significant difference ($P > 0.01$). The months of study showed the highest prevalence rate in March 90.9% (10/11). While the lowest rate was recorded in January 45.4% (5/11). Without significant differences ($p \geq 0.05$) among the months of study. Phylogenetic tree analysis of the common five *Eimeria* species (*E. bovis*, *E. zurnii*, *E. cylindrica*, *E. subsphrica* and *candensis*) has been disclosed using PCR technique in this study. The nucleotide sequence of the local *Eimeria* species has been registered by the national gene bank and has been assigned special codes.

Key words: *Eimeria*, infection, buffaloes, provinces, conventional PCR, sequencing.

Introduction

The genus *Eimeria* is a group of Apicomplexa Eimeriidae a the obligatory intracellular protozoan and single-celled parasitic organisms (Shirley et al 2005, Yakhchalil and Rezaei, 2010) *Eimeria* spp. are often found in water buffalo (*Bubalus bubalis*). Here, we report those *Eimeria* species that infect buffalo calves during their foremost year of life (Carlos et al., 2009). The most serious losses are seen in dairy herds where large numbers of calves are kept and older buffaloes act as carriers. They continue to pass oocysts in their faeces (Andrews, 2002). There are 13 known species of *Eimeria*, but not all are pathogenic. The two most common pathogenic species are *Eimeria zuernii* and *Eimeria bovis* causing morbidity or even mortality associated with mucus and blood stained diarrhea in calves (Gulegan and Okursay, 2000; Lucas et al., 2006). All domestic animals are susceptible to coccidial infections. Although, coccidiosis is host specific, every host may be infected with several species of coccidia at the same time (Andrews, 2002).

Transmitted *Eimeria* spp. by fecal-oral, The source of infection is usually by asymptomatic carrier adult animals, These carries act as a source for spreading infection through water and feeding sources (Ocal et al., 2007).

The appearance of clinical signs depends on the number of ingested sporulation oocysts. (Peek, 2010). This disease found probably in all ages of animals and can be a significant problem in the younger one (Urguhart et al., 1996).

Molecular diagnostic methods such as PCR have been proven useful for the species identification and classification of the *Eimeria* and using the sequencers and phylogenetic tree to determination of the parasite species by using (SSU rRNA) gene (Kawwahara et al., 2007).

Material and Methods

Kits

The consistency of the Presto Stool DNA Extraction

Kit (Geneaid Biotech/Taiwan) is tested by isolating genomic DNA from 200 mg stool samples on a lot-to-lot basis. The filtered genomic DNA (A260/A280 ratio between 1.7-2.0) is then analyzed by electrophoresis after the purification process.

Primers

The PCR recognition primers *Eimeria* sp. In this analysis, the small subunit ribosomal gene RNA was developed using the NCBI-Gene bank database and the primers3 plus These primers were given by Macrogen Company, Korea as follows.

Table 1:

Primer		Sequence (5'-3')	Product Size
<i>Eimeria</i> sp.	F	CAGGCTTGTCGCCCTGAATA	426bp
18SrRNA gene	R	CCTCTAAGAAGTGATGCCGG	

Polymerase Chain Reaction (PCR)

The PCR technique was carried out from samples of buffaloes feces for the identification of 18S small subunit ribosomal RNA gene based on *Eimeria*. This method was performed using the method described as the following steps (Kawahara *et al.*, 2010).

Genomic DNA Extraction

Genomic DNA from feces samples was collected using the Presto Stool DNA Extraction Kit (Gene aid Biotech/Taiwan).

Genomic DNA estimation

The genomic DNA collected from fecal samples was tested using a Nanodrop spectrophotometer (THERMO. USA), which checked and measured the purity of DNA by reading the 260/280 nm absorbance as follows:-

1. Choose the appropriate application (nucleic acid, DNA), after opening the Nanodrop program.
2. Several times Dry Chem. wipe was taken and the measuring pedestals cleaned. Then carefully pipet 1 μ l of ddH₂O onto the bottom measuring pedestal sheet.
3. The sampling arm was lowered and the Nanodrop was initialized by clicking Well, then cleaning the pedestals and 1 μ l of the correct.
4. Blanking solution has been applied as black solution which is the same DNA sample elution buffer.
5. The pedestals have been washed and DNA sample pipet 1 μ l for calculation.

PCR Thermo Cycler Conditions

Thermocycler conditions PCR by using traditional thermocycler method PCR.

Table 2:

PCR step	Temp.	Time	Repeat
Initial Denaturation	95°C	5min.	1
Denaturation	95°C	30sec.	35 cycle
Annealing	58°C	30sec	
Extension	72°C	2min.	
Final extension	72°C	5min.	1
Hold	4°C	Forever	-

PCR Product Analysis

The PCR products were analyzed by electrophoresis of the agarose gel as follows:

1. 1.5 percent of the agarose gel was prepared using 1X TBE and dissolved for 15 minutes in a water bath at 100°C, after which it was left to cool (50°C).
2. In the agarose gel solution 3 μ l of ethidium bromide stain was then applied.
3. Agarose gel solution was poured into the tray after fixing the comb in proper position afterwards, left to solidify at room temperature for 15 minutes, then removed the comb gently from the tray and inserted well 10 μ l of PCR product in each comb and 5 μ l of (100bp Ladder) in one well.
4. The gel tray was placed in the electrophoresis chamber and filled by 1X TBE buffer, then a 100 volts and 80 AM electric current was performed for 1 hour.
5. Using UV Trans illuminator, PCR products were visualized.

DNA sequencing method

DNA sequencing method for species typing such positive *Eimeria* sp isolates was performed as follows:

- 1- DHL sent the PCR sample of small subunit ribosomal RNA genes to Macrogen Company in Korea in an ice bag for DNA sequencing by AB DNA sequencing.
- 2- The study of DNA sequencing (Phylogenetic tree research) was carried out using version 6.0 of Molecular Evolutionary Genetics Analytics. (Mega 6.0) and multiple sequence alignment analysis based on Clustal W alignment analysis and evolutionary distances were estimated using the UPGMA phylogenetic tree method Maximum Composite Likelihood.
- 3- Analysis of the typing of *Eimeria* species was carried out by phylogenetic tree analysis between local *Eimeria* sp. isolates and the known *Eimeria* species NCBI-Blast.
- 4- Lastly, reported isolates of *Eimeria* species were submitted to the NCBI-Gene Bank for accession number to Gene bank.

NCBI-Gene bank Submission

The local species of *Eimeria* were submitted in the NCBI-Gene bank database to obtain for the first time in Iraq the Gene bank accession number as figures in appendices for our isolates.

Results

One hundred fecal samples were collected from buffaloes and screened for *Eimeria* infection using molecular technique (PCR) the results showed that the total infection rate of *Eimeria* spp. in buffaloes was 66% (66/100), Table 4-1.

Table 4-1: Total prevalence of *Eimeria* infection by molecular techniques (PCR) in buffaloes.

Host	No. of samples examined	Molecular (PCR)	
buffaloes	Total No.	No. of positive	%
	100	66	66

4-1 Rate of infection with *Eimeria* spp. by Conventional PCR according to sex:-

Based on the results of PCR, 19 buffaloes male were found infected with *Eimeria* out of 33 examined with a prevalence rate 57.5%. While buffaloes female recorded 47 (70.1 %) out of 67. Statistically, there was no significant differences existed ($p > 0.05$) Table

Table 4-1: Infection rate with *Eimeria* spp. by Conventional PCR according to sex of buffaloes.

Sex of buffaloes	No. of samples examined	No. of positive	Percentage (%)
Male	33	19	57.5%
Female	67	47	70.1%
Total	100	66	66%
X ²		1.558	
P value		0.212 (NS)	

chi-square

NS: No significant difference ($P > 0.05$)

4-2 Rate of infection with *Eimeria* spp. in buffaloes by Conventional PCR according to age groups

The highest infection rate was observed at 1<year age group 73% (19/26), followed by age group 1 to <3 years which showed 56% (26/40). In addition the age groups ≥ 3 Years recorded the lowest infection rate which was 61, 7% (21/34), the results showed highly significant difference ($P > 0.01$) in the prevalence rates among different age groups table 4-9.

4-3 Infection rate of *Eimeria* spp. in buffaloes by Conventional PCR relation to areas of study:-

Sixty six samples were showed the presence of

Table 4-2: Rate of infection with *Eimeria* spp. in buffaloes by Conventional PCR according to age.

Age groups	No. of samples examined	No. of positive	Percentage (%)
1<year	26	19	73%
1 to<3years	40	26	65%
e TM 3 Years	34	21	61.7%
Total	100	66	66%
X ²	10.712		
P value	0.005 (HS)		

X² chi-square

HS: Highly significant difference ($P > 0.01$)

Eimeria spp. out of 100 fecal samples. The samples were examined microscopically from four different regions in Al- Qadisiyha city, including: AL-Sadeer, AL-Dagarha, Efaq and AL-Sanyia with prevalence rate 76%, 68%, 60%, 60%, respectively. There was no significant differences existed ($p > 0.05$) table 4-7.

Table 4-3: Infection rate of *Eimeria* spp. by Conventional PCR in buffaloes relation to areas of study.

Age groups	No. of samples examined	No. of positive	Infection Rate %
AL-Sadeer	25	19	76%
AL-Dagarha	25	17	68%
Efaq	25	15	60%
AL-Sanyia	25	15	60%
Total	100	66	66%
X ²	1.961		
P value	0.581 (NS)		

X² chi-square

NS: No significant difference ($P > 0.05$)

4-4 Rate of infection with *Eimeria* spp. by Conventional PCR in buffaloes relation to months of study:-

The results showed that the highest prevalence rate recorded in March 90.9% (10/11). The lowest prevalence rate recorded during January 45.4% (5/11). Statistically, there was no significant differences existed ($p \geq 0.05$) table 4-4.

4-5 Conventional PCR product analysis:

Genomic DNA samples obtained from buffaloes fecal samples were subjected to molecular analysis by PCR using small subunit ribosomal RNA gene specific primers in order to identify the species of *Eimeria*. PCR of all 100 samples employed in the study exhibited distinct band of (426 bp) PCR product size on agarose gel confirming the Fig. 4.5. Agarose gel electrophoresis image that showed PCR product analysis for small subunit ribosomal RNA gene in *Eimeria* sp. Isolates from feces samples

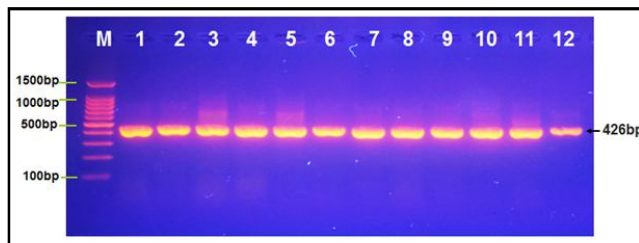
Table 4-4: Rate of infection with *Eimeria* spp. by Conventional PCR in buffaloes relation to months of study.

Month	No. of samples examined	No. of positive	Infection Rate %
December	11	6	54.5%
January	11	5	45.4%
February	11	7	63.6%
March	11	10	90.9%
April	11	9	81.8%
May	12	9	75%
June	11	7	63.6%
July	11	6	54.5%
August	11	7	63.6%
Total	100	66	66%
X ²	8.139		
P value	0.420 (NS)		

X² chi-square

NS: No significant difference (P>0.05)

of buffaloes. M (Marker ladder 1500-100bp). Lane (1-12) some positive *Eimeria* sp. isolates at 426bp product size.



4-6 Phylogenetic confirmative detection

The DNA sequences analysis based on the phylogenetic tree and NCBI–Blast analysis were identical five *Eimeria* species that includes *Eimeria* spp. No. (1, 2, 5, 9, 10, 11, 12, 13, 21, 22, 23, 25, 29, 33, 37, 38 and 39) were closed identical to *Eimeria bovis* genetic Gene bank code MK691697.1), at total homology sequence identity (99.83%, 99.83%, 100.00%, 99.48%, 99.48%, 99.66%, 99.83%, 99.66%, 100.00%, 99.83%, 99.66%, 99.83%, 99.66%, 99.83%, 99.83%, 99.66% and 99.65%) respectively and identified local *Eimeria bovis* isolated were deposited in NCBI Gene bank submission at Gen bank accession number (MN989873).

Eimeria spp. No. (3, 8, 18, 19, 30, 36 and 31) were closed identical to *Eimeria subspherica* genetic Gene bank code AB769640.1, at total homology sequence identity (99.66%, 99.66%, 99.83% 99.83%, 99.66%, 99.47% and 99.83%) respectively and identified local *Eimeria subspherica* isolated were deposited in NCBI Gene bank submission at Gen bank accession number (MN989903).

Eimeria spp.No. (4, 6, 14, 15, 17, 26, 27, 32 and 34) were closed identical to *Eimeria cylinrdica* genetic Gene bank code AB769624.1, at total homology sequence identity (99.66%, 99.66%, 99.66%, 99.83%, 99.83%, 99.83%, 99.66%, 99.66% and 99.83% respectively and identified local *Eimeria cylinrdica* isolated were deposited inNCBI Genbank submission at Gen bank accession number (MN989906).

Eimeria spp. no. (7, 16, 20, 24, 28 and 35) were closed identical to *Eimeria candensis* genetic Gene bank code AB769608.1, at total homology sequence identity (100.00%, 99.83, 99.83%, 99.83%, 99.82% and 99.83%) respectively and identified local *Eimeria candensis* isolated were deposited in NCBI Gene bank submission at Gen bank accession number (MN989907).

Eimeria spp. no. (40) was closed identical to *Eimeria zuernii* genetic Gene bank code KU351735.1, at total homology sequence identity (99.83%) and identified local *Eimeria zuernii* isolated was deposited in NCBI Gene bank submission at Gen bank accession number (MN989912).

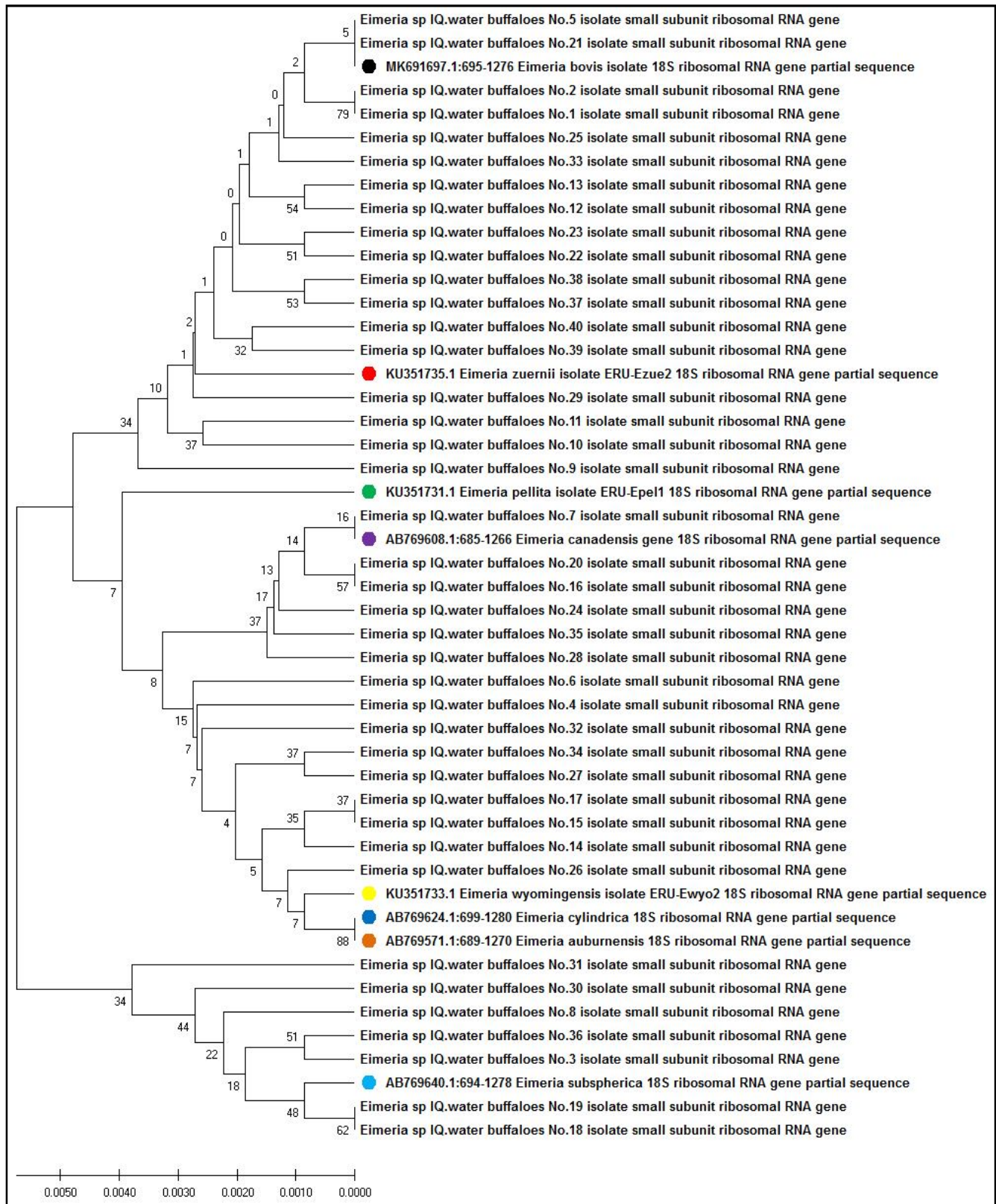
Fig. 4-10: Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence that used for *Eimeria* species typing genetic analysis. The phylogenetic tree was constructed using Maximum Likelihood method (MEGA 6.0 version). The *Eimeria* spp IQ. water buffaloes No.1 -No.40 isolates were showed closed genetic related to NCBI-BLAST *Eimeria* species at total genetic changes (0.001-0.005%).

Discussion

This study revealed an overall prevalence of *Eimeria* spp. in the buffaloes was 66% by using molecular techniques PCR while traditional methods (microscope examination) was 53.8%, this is in agreement with Kawahara *et al.*, (2010) 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 (Muller *et al.*, 1996; Tsuji *et al.*, 1997).

The high prevalence of *Eimeria spp* agrees with most reports recorded by (Kawahara *et al.*, 2010); Nain, 2017).

This result, however, contradicts to the findings of Kanyari *et al.*, (2009) who assertions explained that the grazing habits of ruminants (grazing closer to the earth soil) warrant these animal species to be more infected. These animals did not show any clinical signs of Emeriosis. This might be indicated by the fact that the occurrence of clinical signs in *Eimeria* infection likely depends upon



the balance between the rate of development of resistance and the speed of build-up infection.

This balance may be affected by other factors such as the weather, type of management, hygiene, a method

of feeding, weaning, and the presence of other infections (Vercruysse, 1982).

In relation to area of study including: AL-Sabuffaloes, AL-Dagarha, Efak and AL-Sanyia with prevalence rate

76%, 68%, 60% and 60% respectively. There was no significant differences ($p > 0.05$), this is due to methods of animal husbandry and management, grazing in open farmyard, environmental conditions and animal numbers, similar in these areas.

Buffaloes females recorded highest rate 70.1% with *Eimeria* spp infection by PCR while males recorded lowest rate 57.5% without significant (difference $P > 0.05$), this difference's explained by the more stressful conditions experienced by female especially during pregnancy, delivery and breast feeding and presents large number of female in the livestock for reproductive and economic purposes.

The highest infection rate was observed at 1<year age group 73% (19/26), followed age groups e"3Years which recorded rate 61, 7% (21/34), while the lowest infection rate recorded in age group 1 to < 3years 56% (26/40). with significant difference ($P > 0.01$), the buffaloes calf are more susceptible to infection than older, that attributed many reasons the breeding and overcrowding system observed in the different properties, immature development of the immune system of young ruminants in comparison with older animals. The young animal immune system is still unaware of the invading *Eimeria* parasite because of lack of previous exposure while adult animals had previous multiple exposures to *Eimeria* parasite. Multiple exposures to low dose infection is an important factor that makes the animal more immune to a specific infection (Yu *et al.*, 2011; Dawid *et al.*, 2012).

In this study *Eimeria* infection increasing during spring months, where March recorded 90.9% (10/11). While the lowest rate was recorded in January 45.4% (5/11). Without significant differences ($p \geq 0.05$) among the months of study. The high prevalence of infection during the spring season may be due to climatic conditions which were more suitable for sporulation and survival of coccidian oocysts. The result indicated that the infection rate was high among buffaloes grazing on natural pasture. The reasons for seasonal variation in the rate of infection are thought to be due to variation in temperature, raining, moisture which may facilitate the maturation, shedding and sporulation of oocysts (Kumar *et al.*, 2016).

DNA sequencing results

Forty samples were sequencing 66 positive by Conventional PCR with different shape in microscope examination and sequencing depots in NCBI Genbank data base to get accession number codes for of local *Eimeria* species for the first time in Iraq.

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