Plant Archives Vol. 20, Supplement 2, 2020 pp. 508-512 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

MOLECULAR DETECTION OF *EIMERIA* SPP. IN DEER AT MIDDLE PARTS OF IRAQ

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Abstract

Eimeria is an apicomplexan protozoa which affect many species of wild and domestic animals and birds including deer. The study was designed for investigation *Eimeria* spp. in deer by Molecular methods for the first time in Iraq and Globally**.** One hundred fecal samples of deer were examined by Conventional PCR from different provinces in the middle part of Iraq during December 2018, to end of September 2019. A total rate of infection were 77% (77/100). Male and female recorded 63.6 % (21/33) and 83.5 % (56/67) rate of infection respectively significant differences existed (p≥ 0.05). There were significant differences in the rate of infection between age groups of deer were recorded in Fawn deer (<3-11 months) which 82.6% (19/14), while the lowest recorded in adult deer at age group (11-20 months) which 72.2% (26/36). The rate of infection by Conventional PCR were recorded at four provinces. Highest rate was recorded in Baghdad 80.5% (30/36) and lowest rate was in Babylon 72% (8/11). The rate of infection with *Eimeria* spp. deer relation to months of study. The results showed that the highest infection rate recorded during March 90.9% (10/11) and the lowest rate recorded during December 64 %(7/11) with the significant differences. Nine species of *Eimeria (E. abenovi, E. crandalis, E. elegans, E. gazella, E. idmii, E. intricate, E. rheemi, E. parva* and *E. sordida*) by sequencing of 40 deer fecal samples isolate for the first time in Iraq and global.
Keywords: *Eimeria*, infection, deer, 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; Yakhchali and Rezaei, 2010). These parasites are common in most domestic and wild ruminants, such as cattle (Bangoura *et al.*, 2011), sheep (Fthenakis *et al.*, 2012) and deer (Davidson *et al.*, 2014). 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 (Urquhart *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 (Ogedengbe *et al.*, 2015).

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-Genbank database and the primers3 plus These primers were given by Macrogen Company, Korea as follows.

Table -1

Primer		Sequence (5'-3')	Product Size
Eimeria sp. 18SrRNA gene	F	CAGGCTTGTCGCCCTGAATA	426bp
	R	CCTCTAAGAAGTGATGCGGG	

Polymerase Chain Reaction (PCR

The PCR technique was carried out from samples of deer 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.*, 2007)

Genomic DNA Extraction

Genomic DNA from feces samples was collected using the Presto Stool DNA Extraction Kit (Geneaid 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:-

- Choose the appropriate application (nucleic acid, DNA), after opening the Nanodrop program.
- Several times Dry Chem. wipe was taken and the measuring pedestals cleaned. Then carefully pipet 1µl of ddH2O onto the bottom measuring pedestal sheet.

- The sampling arm was lowered, and the Nanodrop was initialized by clicking Well, then cleaning the pedestals and 1µl of the correct.
- Blanking solution has been applied as black solution which is the same DNA sample elution buffer.
- The pedestals have been washed and DNA sample pipet 1µl for calculation.

PCR Thermo Cycler Conditions

Thermocycler conditions PCR by using traditional thermocycler method PCR (Table 2):

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

PCR Product Analysis

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

- 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).
- In the agarose gel solution 3µ of ethidium bromide stain was then applied.
- 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 10µl of PCR product in each comb and 5µl of (100bp Ladder) in one well.
- 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.
- 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:

- 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.
- 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 ClustalW alignment analysis and evolutionary distances were estimated using the UPGMA phylogenetic tree method Maximum Composite Likelihood.
- 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.
- Lastly, reported isolates of *Eimeria* species were submitted to the NCBI-GenBank for accession number to Genbank.

NCBI-Genbank Submission

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

Results

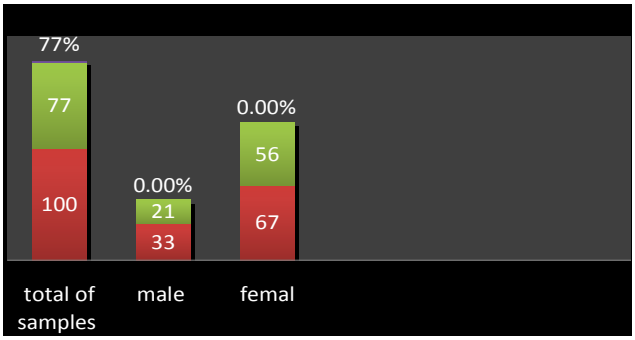
One hundred fecal samples were collected from deer and screened for Eimeria infection using molecular technique (PCR) the results showed that the total infection rate of *Eimeria* spp. in deer was 77% (77/100), (Table 1).

Table 1 : Total prevalence of *Eimeria* infection by molecular techniques (PCR) in deer

Host	No. of samples examined	Molecular (PCR)	
Deer	Total No.	No. of positive	%
	100	77	77

(1) Rate of infection with *Eimeria* spp. by Conventional PCR according to sex

Based on the results of Conventional PCR, male were recording the lowest rate 63.6% (21/33), While the highest infection rate female was recorded 83.5 % (56/ 67). Statistically, with significant differences (p≥ 0.05) (Fig. 1).

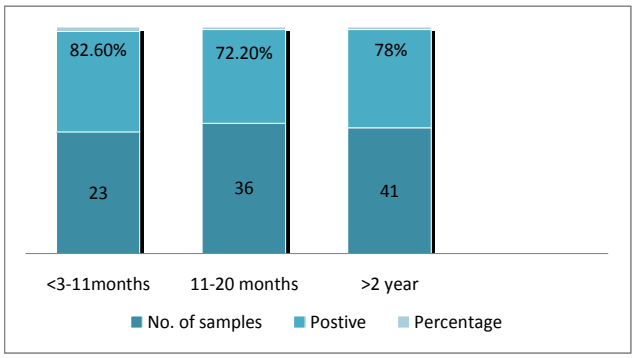


significant differences at a level of P≤0.05

Fig. 1 : Infection rate with *Eimeria* spp by Conventional PCR according to sex of deer

(2) Rate of infection with *Eimeria* spp. in deer by Conventional PCR according to age groups

The results showed that the highest infection rate of *Eimeria* spp. 82.6% (19/140) was recorded in Fawn deer at age group (<3-11 months), while the lowest 72.2% (26/36) recorded in adult deer at age group (11-20 months) which with significant differences (p≤0.05) (Fig. 2).

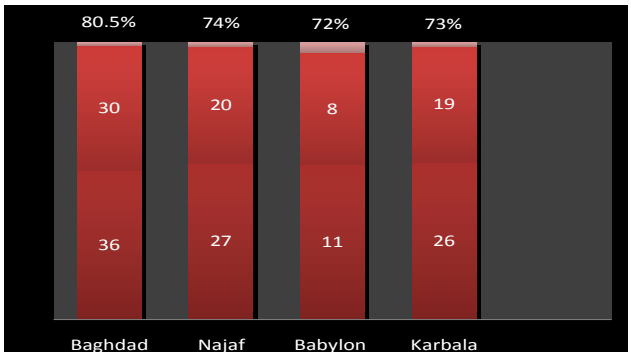


significant differences at a level of P≤0.05.

Fig. 2 : Rate of infection with *Eimeria* spp. in deer by Conventional PCR according to age.

(3) Infection rate of *Eimeria* spp. in deer by Conventional PCR relation to areas of study

The rate of infection by Conventional PCR were recorded at four provinces Baghdad, Al- Najaf, Babylon and Karbala Highest rate was recorded in Baghdad 80.5% (30/36) and lowest rate in Babylon 72% (8/11) with significant differences (p≤0.05). (Fig. 3).

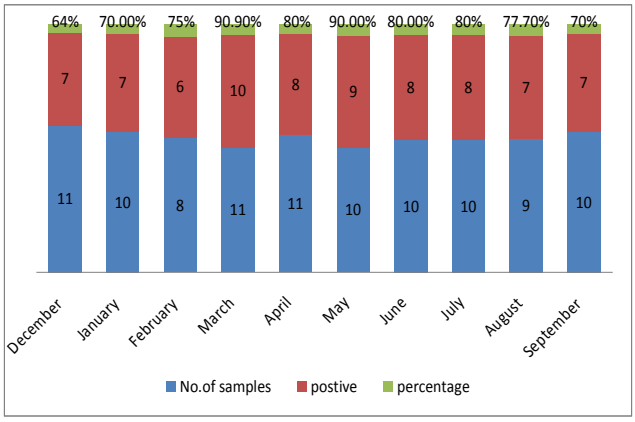


Significant differences at a level of P≤0.05.

Fig. 3 : Infection rate of *Eimeria* spp. by Conventional PCR in deer relation to areas of study.

(4) Rate of infection with *Eimeria* spp. by Conventional PCR in deer relation to months of study

The results showed that the highest infection rate recorded during March90.9% (9/10), and the lowest rate recorded during December 64 % (7/11) with the significant differences (p≤0.05) (Fig. 4).

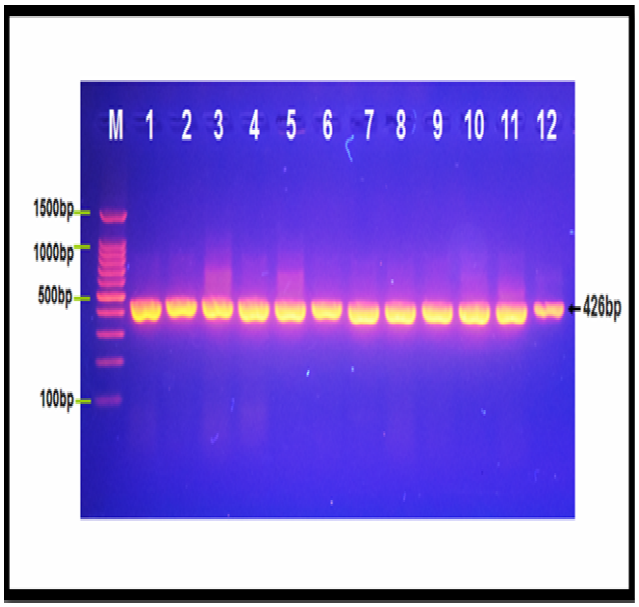


Significant differences at a level of P≤0.05.

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

(5) Conventional PCR product analysis:

Genomic DNA samples obtained from deer 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 Figure (5): Agarose gel electrophoresis image that showed PCR product analysis for small subunit ribosomal RNA gene in *Eimeria* sp. Isolates from feces samples of deer. M (Marker ladder 1500-100bp). Lane (1-12) some positive *Eimeria* sp. isolates at 426bp product size.



(6) Phylogenetic confirmative detection

Specific phylogenetic confirmative detection of local *Eimeria* species were preformed by using The phylogenetic tree analysis and compared with NCBI-BLAST *Eimeria* species. Where the local *Eimeria idmii* accession numbers (MN473472 MN473473, MN473474, MN473475, MN473492 and MN473493) close related to NCBI-BLAST *Eimeria* spp. (JX8392861) at (96%), *Eimeria sordida* accession numbers (MN473476, MN473477, MN473484 and MN473506) sequence identity with *Eimeria* spp. (JX8392861) was (98%), *Eimeria parva* accession numbers (MN473478, MN473485 and MN473507) was (96%) with *Eimeria* spp. (JX8392861), *Eimeria gazella* (MN473479, MN473480, MN473481, MN473482, MN473483, MN473486, MN473487, MN473488, MN473497, MN473498 MN473499, MN473500, MN473502, MN473503, MN473504,MN473508, MN473510 and MN473511) close related to *Eimeria* spp. (JX8392861) at (98%), *Eimeria* accession numbers (MN473489 and MN473501) was(97%) with *Eimeria* spp. (JX8392861), *Eimeria intricate* (MN473490) recorded identity (97%) to *Eimeria* spp, *Eimeria rheemi* (MN473491) was (97%) with *Eimeria* spp. (JX8392861), *Eimeria crandallis* accession numbers (MN473494 and MN473495) close related to *Eimeria* spp. (JX8392861) at (98%) and *Eimeria elegans* (MN473496, MN473505 and MN473509) was (97%) with *Eimeria* spp. (JX8392861).

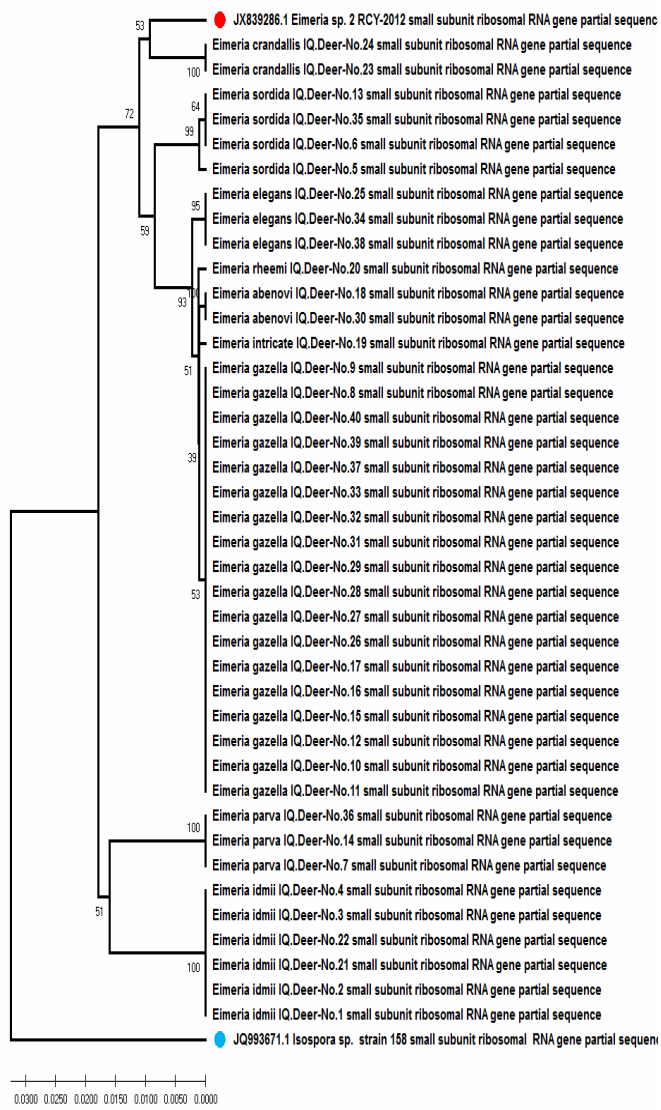


Fig. 6 : Phylogenetic tree analysis based on small subunit ribosomal RNA gene, partial sequence in local deer *Eimeria* sp. isolates that used for genetic relationship identification. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA. X version). The local deer *Eimeria* sp. were showed closed genetic related to NCBI-BLAST *Eimeria* sp. 2 RCY-2012 (JX839286.1) NCBI BLAST *Isospora* sp. Was showed different and out of tree at total genetic changes ((0.005-0.03%).

Discussion

The current study revealed an overall prevalence of *Eimeria spp.* in the deer 77% using molecular techniques PCR. These results are in line with the findings of (Biu *et al.*, 2009; Zeid and Haider, 2018). The high prevalence of *Eimeria spp.* in small ruminants agrees with most reports recorded by (Wang *et al.*, 2010; Mulugete *et al.*, 2011)

This result, however, contradicts to the findings of (Kanyari *et al.*, 2016) 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 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 (Al-Jubory, 2012).

Conventional PCR recorded significant difference between male and female with Eimerial infection, 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 (Dawid *et al.*, 2012). The study recorded higher infection rate of *Eimeria* spp. in Fawn deer at age groups (<3-11 months).

The fawn deer 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 ruminants immune system is still unaware about the invading *Eimeria* parasite because of lack of previous exposure while adult animals had previous multiple exposure to *Eimeria* parasite. Multiple exposures to low dose infection is an important factor that make the animal more immune to a specific infection (Yu *et al.*, 2011).

Higher rate of infection at Baghdad province 80.5% and lower rate at Babylon 72%, the captive living condition is major factor for making the animal more susceptible to contract parasitic infestation as compared to the range condition. Another possibility of parasite transmission by contamination of food and water by disseminate oocysts and where animals are moved from one enclosure to another without proper parasites treatment zoologists and garden among animal in zoos , though tier shoes clothes hands , food or with working tools shoes (Adetunji, 2014).

In the current study Eimerial infection increasing during spring months, where March recorded highest rate of infection 90.9%, while lowest recorded in December 64 % with the significant differences, highly 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 deer grazing on natural pasture). The reasons for seasonal variation in 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 77 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 globally and Iraq.

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