



MOLECULAR DETECTION AND DISCRIMINATION OF THREE POULTRY *EIMERIA* SPECIES IN KERBALA AND BABYLON PROVINCES, IRAQ

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ABSTRACT

Coccidiosis is an intestinal disease of chickens in which the Causes include a various species of protozoan parasites that located within the genus *Eimeria*. Diagnosis and genetic characterization of different species of *Eimeria* are central dogma to the preventative, resistance, and control of coccidiosis. The aim of the present study was to identified different avian *Eimeria* species that considered the causal agents of avian coccidiosis in each of the Kerbala and Babylon provinces, Iraq.

The present study observations were revealed increasing and decreasing the percentages of infection with coccidiosis during different months of a year and that due to the large change in temperature and humidity in Iraq, so the results showed that the highest percentage of infection in both of Kerbala and Babylon provinces were in December (23.12%) and the lowest percentage in July (1.87%).

A total of 200 samples of feces and intestines were collected from chickens that suspected to infection with *Eimeria* during the period from August 2013 to July 2014 and the results revealed that 160 samples were positive depending on the macroscopic and microscopic examination which in turn have undergone the second stage of the examination for the purpose of qualitative accurate diagnosis of the species of *Eimeria* that causing coccidia in chickens using of molecular methods for the target region ITS1 nucleic acid DNA encoded ribosomal RNA , these techniques depend on qRT- PCR technique ,Which diagnosed three species of *Eimeria* includes *E. tenella* which formed the largest proportion of the total positive samples (66.8%) then the species *E. necatrix* which accounted for (30%) and finally the species *E. maxima* which form (3.13%).Therefore , Using of the molecular methods such as REAL-TIME PCR which characterized by highly accuracy, but these methods expensive economically compared to routine methods.

Key Words: Avian Coccidiosis, *Eimeria*, Oocysts, RT-PCR, Iraq

INTRODUCTION

Avian coccidiosis is one of the most important diseases affecting the intensive poultry industry worldwide. Coccidia are almost usually found wherever chickens are raised and it is exceedingly rare to find a commercial chicken flock not affected. The infection causes tissue damage in the intestinal tract leading to interruption in digestive processes, blood loss, and increased susceptibility to other diseases, subclinical enteric infection, and sub acute mortality, the disease is treated by used of anticoccidial drugs that added in the feed or vaccination (1, 2, 3, 4). There are nine commonly recognized species of chicken coccidia; *Eimeria acervulina*, *E. brunetti*,

E. maxima , *E. mitis*, *E. necatrix*, *E. praecox* , *E.mevati* , *E. hagani* and *E. tenella*, each *Eimeria* species develops in a particular location within the chicken digestive tract with some interfere seen between these species(5).

The specific identification of *Eimeria* species and strains is important for diagnosis and domination, as well as for epidemiology and biological studies of the population (6).Depended on the routine tests, the *Eimeria* species have been identified by morphology and/or morphometry of their sporocysts and oocysts as well as their modes of development, and assessing the site and extent of the pathological lesions in the intestine of chicken (7, 8). However, these ways are costly, time-consuming, require

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skilled personnel and can be unreliable under the circumstances of mixed field infections, particularly when the overlap in biological and morphological characteristics makes the unambiguously identification and differentiation of *Eimeria* species impossible (9, 10).

Molecular techniques have some advantages over traditional methods in that they rely only on the genomic sequence of the *Eimeria* species, several techniques based on the polymerase chain reaction using primers that specifically targeting different regions of the *Eimeria* genome have been described (5, 11, 12, 13, 14, 15). For molecular detection of *Eimeria* species in chickens, the DNA sequence of the first and second internal transcribed spacers (ITS-1 and ITS-2) of the nuclear DNA, which separate the ribosomal genes, is used most frequently, besides its heterogeneity in both sequence length and base composition of the ITS sequence, the rDNA is a member of a multiple copy gene family and provides large numbers of potential PCR targets (16, 17, 18, 19, 20). Nevertheless, the practical implementation of these techniques in tradition diagnosis and epidemiological studies has been limited (21).

The number of studies related to the presence of chicken *Eimeria* species in Iraq are limited. These studies have been conducted in few locations and species identification was depended on the oocysts morphology, necropsy and/or histopathological findings. And as a result, nine *Eimeria* species (*E.acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox* and *E. tenella*) have been reported to diagnosed in chickens (22, 23).

In this study, we aimed to identify the *Eimeria* species causing coccidiosis in broilers using a molecular technique based on the Quantitative Real-Time PCR and determine the prevalence of coccidial infections in commercial broiler flocks in chickens.

MATERIALS AND METHODS

1- Stool Sample collection

From August 2013 to July 2014 a total of 200 samples of fresh fecal droppings and intestines were collected from suspected infected chickens with coccidiosis attending to the veterinary hospital and veterinary clinics were spread in Kerbala and Babylon provinces , Iraq for the examination and treatment .

The oocysts were isolated from intestines and stool of infected chickens and collected in Eppendorf tubes and stored in freezing until used in DNA extraction . (12)

2- Direct examination method (microscopic examination):

The first step in the present study is identified the positive samples from whole samples that collected from suspected chickens with coccidiosis and examined microscopically to determined the oocysts either by direct methods in cases of acute diarrhea in which the samples examined either freshly or used of a natural formalin buffer solution which concentrated 10% or by staining the samples with Iodin or Giemsa stain to made the internal components more clear.

3-Stool DNA Extraction

Genomic DNA was extracted from stool samples of chicken by using AccuPrep® Stool DNA Extraction Kit (Bioneer, Korea)

4-DNA profile

For detection of DNA that extract from stool samples through the use of a Nanodrop spectrophotometer (THERMO. USA) for detection and measurement the concentration of nuclear acids (DNA and RNA) ,Where is detected DNA concentration (ng / μ l) and measuring the purity of the DNA by reading the absorbance at a wavelength of between (280-260 nm) (21).

5-qRT-PCR protocols

Real-Time PCR was preformed for detection of poultry *Eimeria* species by using the primers and TaqMan probe specific for ITS1 region In the DNA that code for Ribosomal RNA, technique was carried out according to method described by(13).

Real-Time PCR master mix preparation

Real-Time PCR master mix was prepared by one step Reverse Transcription and Real-Time PCR detection kit (AccuPower RocketScript RT-qPCR PreMix, Bioneer. Korea), and done according to company instructions as following Table (1):

Table 1: Explained the main components of the mix for qRT-PCR technique.

qRT-PCR Master mix	Volume
2X Green star master mix	25 μ L
DNA template	5 μ L
ITS1 forward primer 10pmol	1 μ L
ITS1 reverse primer 10pmol	1 μ L
DEPC water	18 μ L
Total	50 μ L

Primers:

The primers were designed in this study by using the complete sequence of ITS1 region in the rDNA using NCBI Gene-Bank and Primer3 plus online and provided by (Bioneer company, Korea) as showed in Table (2):

Table 2: Explained forward and reverse primers used in the qRT - PCR technique with nucleotide sequence and the size of the resulting DNA.

Real-Time Primer	Sequence	PCR SIZE
E.tenella	F TGTTCCTGTTGTGT-GCTCTG	81bp
	R AGTTCCAAGCAGCAT-GTAACGG	
E.maxima	F TCCCAAAGCG-GTTTCATCATCC	72bp
	R ACAGTCCCACAAAT-GCAGAACG	
E.necatrix	F ATTGGTCTTGCGAG-GCATGTG	96bp
	R ACAAGCCCCACACAT-GCAAG	

The qRT-PCR master mix reaction components that mentioned in (table 2) were added into standard qPCR tube containing (8 wells strips tubes which containing Rocket Script reverse transcriptase and TaqMan probe premix). Then all strips tubes vortexing for mixed the components and centrifuge for 3000 rpm for 3 minutes in Exispin centrifuge, after that transferred into Exicycler Real-Time PCR thermocycler.

6-Real-Time PCR Thermocycler conditions

Real-Time PCR thermocycler conditions was set up according to primer annealing temperature and RT-qPCR TaqMan kit instructions as following Table (3):

Table 3: Explained Thermal cycler program or qRT-PCR technique.

Step	Condition	Cycle
Reverse transcriptase	95 °C 15 min	1
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		

Thermal cycles were applied to inspect the Real-Time PCR and relying on instructions AccuPower® 2X Green-Star™ qPCR Master Mix as well as by calculating the degree Tm prefixes using the device MiniOpticon Real-Time PCR system BioRad / USA as in Figure (1) below:

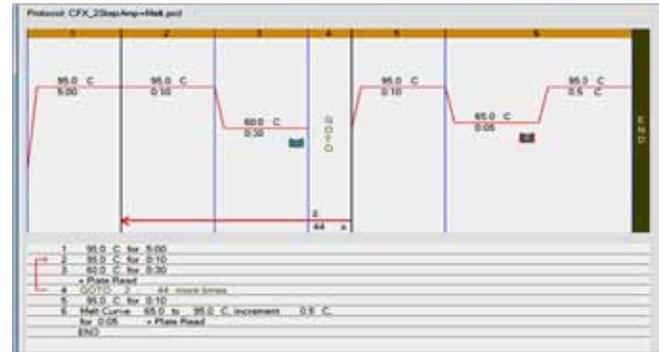


Figure 1: Explained situations of Thermo cycler for REAL-TIME PCR.

7-Real-Time PCR Data analysis

qRT-PCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification of gene in Real-time cycle number.

Statistical analysis

The results of present study analyzed statistically by Program The Statistical Analysis (SAS) by using of the Lest Significant Difference (LSD) test and Duncan test depending on the level of probability P <0.05 to find the significant differences (24).

RESULTS

Results of molecular examination by qRT-PCR

The results of molecular examination by using of qRT-PCR revealed that from 200 collected samples appears 160(80%) were positive ,depending on this technique three species of Eimeria were diagnosed including *E. tenella* which form the highest percentage (66.8%) in comparison to other species ,While the *E. necatrix* form (30%)of the total positive samples and the last species that diagnosed was *E. maxima* which form (3.13%) as in the table(4).

Table 4: The percentage of the three species of Eimeria diagnosed by Molecular assay in Karbala and Babylon provinces:

Species	Positive samples	Percentage
E.tenella	107	66.8%
E.necatrix	48	30%
E.maxima	5	3.13%
Total	160	

The used of qRT-PCR techniques in the specific detection of Eimeria species showed a fluorescence of SYBER Green dye which appeared very clear through formed of

amplification plot to the positive samples during the cycle 30 as shows in the fig.(2,3 &4).

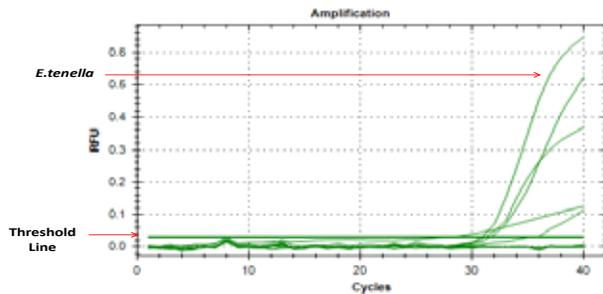


Figure 2: Amplification Plot of region ITS1 of *E.tenella* species, in which the fluorescence of the dye SYBER Green represent samples positive located above of Threshold while the control samples located below the threshold.

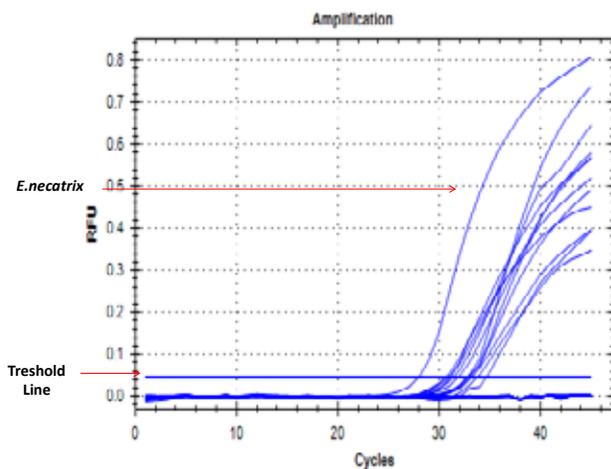


Figure 3: Amplification Plot of region ITS1of *E.necatrix* species, in which the fluorescence of the dye SYBER Green represent samples positive located above of Threshold while the control samples located below the threshold.

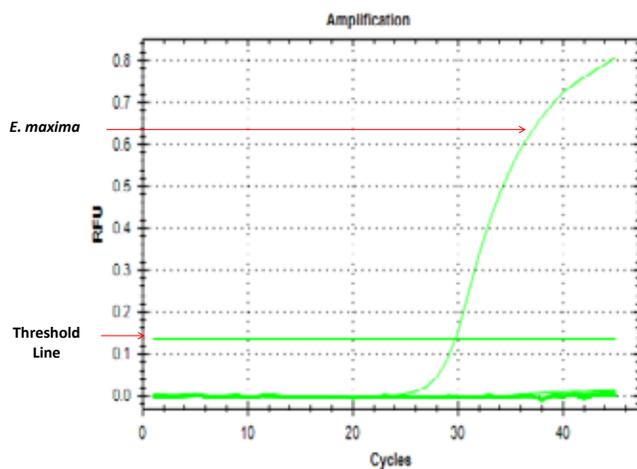


Figure 4: Amplification Plot of region ITS1of *E.tenella* species ,in which the fluorescence of the dye SYBER Green represent samples positive located above of Threshold while the control samples located below the threshold.

DISCUSSION

In chickens is clearly central to a better understanding of epidemiology and dynamics of the disease in intensive and extensive chicken establishments. This is particularly important for planning an effective prevention and control program of coccidiosis. Traditionally, diagnosis has been achieved by detecting *Eimeria* oocysts excreted in the feces of chickens by measuring oocyst and sporocyst size or notes of the site and pathological lesions in the intestine of chickens. Although the microscopic examinations can absolutely show the positive fecal samples, but the traditional methods very limited in the specific diagnosis or identification of *Eimeria* species also These methodes are unreliable in mix infection when there are a multiple species of *Eimeria* infect a single host and there is Similarity in the size , shape of oocysts and the sites of infection (25).

During recent years, there have been significant advances in the development of molecular-diagnostic tools like the Polymerase chain Reactions(PCR) and the Real-time PCR, to comparison between both of them : the qRT- PCR has several advantages: there is no need to post-amplification analysis, which reduces risks of contamination; the ability to better differentiate between different species of *Eimeria* infections; and numerical results which are much easier to distinguish than the visualization of a stained gel in the conventional PCR(14) .

Several studies that used of PCR technique targeting different regions of the *Eimeria* genome,such as the 5S rRNA (the small subunit rRNA (12, 13), the sporozoite antigen gene EASZ240/160 (14) and ITS-1 (8, 15,17) and ITS-2 (18-20) genomic regions. Since the ITS regions are less conserved than the rRNA genes, big variations in this region of DNA sequence between the Eimerian species , makes the design of primers straight forward and reduces the risk of cross reactions among different species (6).

The REAL-TIME assay have been shown to be directly comparable in terms of sensitivity and robustness, capable of detecting 10 parasite genomes but not one, unaffected by the presence of DNA derived from the host or the other test species (26).

Every sporulated oocyst contains eight eimerian genomes, suggesting that the DNA equivalent of a single oocyst will be consistently detectable given normal experimental replication (between one and 10 genomes detected per reaction).Mature intracellular stages represent in the order of 10 to 100 eimerian genomes (depending on the species and stage (27). Suggesting that even a fraction of one may be counted (25).

Apart from an accurate identification of *Eimeria* species, molecular methods can also be helpful in epidemiological study of the parasite, an aspect that has been less

investigated to date at yet, there has not been any documentary report related to the specific identification and epidemiological pattern of the pathogenic *Eimeria* species in the chickens, in Karbala and Babylon provinces. Therefore, the results of the present study are the first study depend on molecular methods to detection the *Eimeria* species the causal agent of poultry coccidiosis in Kerbala and Babylon provinces.

To compared the results of the present study with the resultes of researchers in various Arab and foreign countries ,in which the study which was conducted in Arab Republic of Egypt has been diagnosed prevalence of five species of *Eimeria* mainly include of *E. tenella* then *E. necatrix*, *E. acervulina*, *E. maxima* and finally type *E. mitis* using a technique REAL TIME- PCR (28).

Researcher Muhammad, L. et al held a study on the diagnosis of infection in poultry coccidiosis t in Jordan using technique Quantitative REAL TIME- PCR and identified five species of *Eimeria* where the species *E. tenella* comes in front and reached a rate of about 80.5%, while the *E. maxima* (66%), *E. necatrix* (45%), *E. acervulina* (12%) and finally the species *E. mitis* (3.5%) (29).

Also this technique were used in the Kingdom of Saudi Arabia to diagnosed the infection with coccidiosis in chickens, in which the stool samples collected from six fields scattered across the Kingdom and identified of four species of *Eimeria* in which the species *E. necatrix* appeared high precentage then the species *E. tenella*, *E. maxima* and *E. acervulina* (10).

In comparison of the present study with the results of studies in foreign countries, including Australia, where the researcher use the REAL TIME PCR for the detection and diagnosis of seven species of *Eimeria* which infect chickens and targeted one genetic area which is called the ITS2 located in the ribosomal DNA (rDNA) using Taqman probs found that among the seven species the most prevalent species are the species *E. tenella* compared with the other six species (30).

As in Japan used the technique SYBR Green-based Real-Time PCR for the diagnosis of five species of *Eimeria* using Primers sets were designed based on the sequences area ITS1 in Ribosomal DNA specific to each species of the five and the researcher concluded that Melting Curves for each species has one Peak and the values of Melting temperature are specific for each species. The researcher concluded that the species *E. brunett* is the most prevalent species after *E. maxima* then *E. necatrix* *E. tenella* and finally the species *E. acervulina* ,so the REAL- TIME PCR technique is not only easy but also fast and efficient in qualitative diagnosis of poultry coccidiosis (31).

It also conducted some studies on the diagnosis of poultry coccidiosis in a manner REAL- TIME PCR in some de-

veloping countries such as Ethiopia ,held a study on the prevalence of species of *Eimeria* in Ethiopian villages, by using this technique were reached to diagnosis of seven species of *Eimeria* where the percentage of species *E. praecox* was 95% and then species *E. maxima* (60%) and then species *E. mitis* (28%) and then come successively species *E. tenella*, *E. necatrix* and *E. brunette* (32).

Carried out a diagnostic study is the first of its kind in Bangladesh for poultry coccidiosis following the molecular methods such as REAL TIME PCR technique .Where diagnosed seven species of *Eimeria* the most prevalent are *E. tenella* then *E. necatrix* , *E. acervulina* , *E. maxima*, *E. brunetti*, *E. mitis* and finally the species *E. praecox* (16).

Efforts towards educating the chicken farmers to control coccidiosis in through good management practices, and the proper use of anticoccidial drugs should be considered. However, to control this economically important parasitic disease of poultry, further studies need to be undertaken to devise sustainable and cost-effective prevention and control methods

CONCLUSION

The following conclusions were conducted from the present study:

- 1-Although the routine tests such as clinical examination, macroscopic and microscopic diagnosis important, but it is unable to qualitative diagnosis of *Eimeria* that cause infection.
- 2- Using of the molecular methods such as REAL-TIME PCR which characterized by highly accuracy, but these methods expensive economically compared to routine methods.
- 3-The use of specific primers for diagnosis of ITS1 region are important in molecular detection of *Eimeria* species that isolated from intestines and feces of suspected chickens infection with Coccidiosis.
- 4- The *E. tenella* consider the most prevalent species (66.8%), Then *E. necatrix* (30%) and finally *E. maxima* which represented (3.13%).
- 5- The coccidiosis among chicken farms of Kerbala and Babylon is highly prevalent. *E.tenella* was the most prevalent species.

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