Molecular and bioinformitic analysis of ITS1 region of three Eimeria species in Kerbala and Babylon provinces, Iraq

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الخلاصة

الانتاج العالمي للدواجن قد ازداد بمقدار ثلاثة اضعاف خلال العقدين الماضيين ويعتبر الان من المصادر الرئيسية لانتاج البروتينات الغذائية حيوانية المنشا على المستوى العالمي. تتعرض الدواجن للعديد من الامراض التي تسببها الاحياء المجهرية والتي تقلل من فعاليتاها الحيوية والانتاجية، مرض Coccidiosis الذي يسببة نوع من البدائيات apicomplexan protozoa العائدة الى جنس Eimeria يعتبر واحدا من اهم الامراض التي تصيب الدواجن. ان فهم طفيليات ال Eimeria على المستوى الحياتي يعزز في تطوير عقارات ولقاحات جديدة والذي بدوره يؤدي الى تحسين الامن الغذائي العالمي.

خمسه عشر عينة من الدنا DNA بواقع خمس عينات لكل واحدة من الانواع الثلاث من Eimeria تم تحديد تسلسل القواعد النتروجينيه اعتمادا على صف التسلسل التتابعات المتعددة باستخدام قواعد التحليل عبر الشبكة الدولية للمعلومات للجين المحدد ITS1 (Internal Transcribed Spacer 1) والذي سبق ان تم تضخيمة بعملية تفاعل انزيم البلمرة التسلسلي المقارنة بين تتابعات القواعد النتروجينية للعزلات المحلية لعزلة ال Eimeria مع العزلات العالمية والموثقة في بنك الجينات Gene bank ومقارنة الغربلة الجزيئية في الدراسة الحالية اظهرت صحة ودقة التشخيص لثلاثة انواع من Eimeria تحليل الشجرة التطورية phylogenetic tree باستخدام برنامج الحاسوب المعروف ب (MEGA6) تم اعتمادها لتحليل الشجرة الوراثية Genetic tree لتحليل الانواع لغرض مقارنة الانواع المحلية الثلاثة مع السلالات العالمية لل Eimeria وجد تشابة في تطابق التسلسلات للعزلات المحلية لل Eimeria tenella مقارنة مع بنك الجينات NCBI-Gene bank Eimeria tenella (JX853830) وباستخدام ال NCBI-BLASTE اظهرت النتائج 99% ،98% بينما التشابة في تطابق التسلسلات للعزلة المحلية Eimeria necatrix بالمقارنة مع عزلة بنك الجيناتNCBI-Gene bank Eimeria necatrix (JX83832.1) كانت 100% ،91% والتشابة في تطابق التسلسلات للعزلة المحلية Eimeria maxima الى عزلة بنك الجينات NCBI-Gene bank Eimeria maxima (JX853828.1) كانت 98%.

الكلمات المفتاحية

الإنتاج العالمي للدواجن، الأمراض التي تصيب الدواجن، طفيليات الـ(Eimeria).

Abstract

Global production of chickens has trebled in the past two decades and they are now the most important source of dietary animal protein worldwidE.Chickens are subject to many infectious diseases that reduce their performance and productivity. Coccidiosis, caused by apicomplexan protozoa of the genus Eimeria, is one of the most important poultry diseases. Understanding the biology of Eimeria parasites underpins development of new drugs and vaccines needed to improve global food security.

A Fifteen of DNA samples (five samples for each one of three species) of Eimeria has been sequenced and analyzed in which multiple sequence alignment online based analysis for the ITS1 (Internal Transcribed Spacer 1) region that previously amplified by polymerase chain reaction, A comparison between the sequences of bases of local isolates of Eimeria with global isolates that recorded in Gens Bank and the comparative molecular screening of the present study results revealed the Validity and accuracy of diagnosis of three Eimerian species.

Phylogenetic tree analysis using the program (MEGA 6) were adopted to determine genetic tree of the species analysis to compare the three of local species with global strains of Eimeria and found the Homology sequence identity of Eimeria tenella local isolates in comparison with NCBI-Gen bank Eimeria tenella (JX853830). Using NCBI-BLAST the results showed 98% and 99%, while the Homology sequence identity of Eimeria necatrix of local isolates in comparison with NCBI-Genbank Eimeria necatrix (JX853832.1) were 91% and 100 % and the Homology sequence identity of Eimeria maxima of local isolates to NCBI-Genbank Eimeria maxima (JX853828.1) was 98%.

Key words

Poultry Coccidiosis, Eimeria species, ITS1, PCR, DNA sequencing, Iraq.

Introduction

Chickens are the world's most popular food animal and the development of improved drugs and vaccines to eliminate poultry diseases are vital for worldwide food security. Protozoan parasites of the genus Eimeria cause coccidiosis, a ubiquitous intestinal disease of live stock that has major impacts on animal welfare and agro-economics [1].

It is a particularly acute problem in poultry where infections can cause high mortality and are linked to poor performance and productivity. Eimeria belong to the phylum Apicomplexa, which includes thousands of parasitic protozoa such as Plasmodium species that cause malaria, and the widely zoonotic pathogen Toxoplasma gondii [2]. Eimeria species have a direct oral-faecal life cycle that facilitates their rapid spread through susceptible hosts especially when these are housed at high densities [3]. Unsurprisingly, resistance to anticoccidial drugs can evolve rapidly under these conditions and there is a continuing need to develop novel therapies [4].

More than 1200 species of Eimeria are described [2] and virtually all of these are restricted to a single host species. The chickens can be infected by nine Eimeria species, each of which colonises a preferred region of the intestine causing symptoms of differing severity [5]. Five species induce gross pathological lesions and four of these are the most important in terms of global disease burden and economic impact (E.acervulina, E.maxima, E.necatrix and E.tenella) [6].

Diagnosis of coccidiosis is based on clinical features and pathology of host, parasite characteristics such as morphology at different

stages of parasitism, and the pre-patent period [7,8]. Analysis of these characteristics is labor intensive for diagnosis and does not provide accurate data for identification of the Eimeria species [9].

Identification and genetic characterization of different species of Eimeria genus are central to prevention, surveillance, and control of coccidiosis. This is particularly important with regard to the appearance of a widespread anticoccidial resistance of Eimeria species and the complications associated with drug residues [10].

Due to difficulties in the morphologic identification of some of chicken Eimeria spp., diagnostic laboratories are increasingly utilizing DNA-based technologies for the specific identification of the parasite [8].

So far, there is limited knowledge on the epidemiology of Eimeria infections under different rearing conditions in Iraq. In The present study, together with morphometric diagnosis, PCR assay, based on the amplification of internal transcribed spacer 1 (ITS1) regions of ribosomal DNA (rDNA) [11], and that amplified DNA would used in sequencing and Phylogenetic tree analysis using (MEGA 6) program were adopted to determine genetic tree of the species analysis (Test UPGMA tree) (Unweighted Pair Group Method with Arithmetic) to compare the local species with global strains of Eimeria which recorded in the Genbank in the Website (www.genome.jp).

1. Materials and methods

2. 1. Stool sample collection

From August 2013 to July 2014 about 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 Eppendrof tubes and stored in freezing (-80 C˚) until used in DNA extraction. [12]

DNA extraction from stool

Genomic DNA was extracted from stool samples of chicken by using AccuPrep® Stool DNA Extraction Kit (pioneer, Korea) Table (1) and done according to company instructions.

2. 2. DNA profile

For detection of DNA that extracted from stool samples through the use of a Nanodrop spectrophotometer (THERMO. USA) detects the percentage of purity and measuring the concentration of nuclic acids (DNA and RNA), Where is detected DNA concentration (ng / µl) and measuring the purity of the DNA by reading the absorbance at a wavelength at (280-260 nm).

2. 3. PCR- protocols

The DNA samples which extracted from stool samples would used in thermal cycler machine to amply the ITS1 region of rDNA using the forward and reverse primers which designed by NCBI site Table (1), according to the PCR program shown in the Table (2).

In which 50 µl of PCR master mix used for amplification of ITS1 region.

Also (5µl) of DNA template that extracted from stool samples was added then 1.5 µl of each type of Primers (forward and reverse) added to the master mix and then blend well using Exispin vortex centrifuge, then this tubes would transferred to the thermocycler machine, which has been programmed by the previous program for amplified of ITS1 region. The PCR products were electrophoresed in agarose gel and visualized on UV transllluminator and then photographed using photo documentation system.

Table (1): The sequence of the forward and reverse primers that used in the present study with their PCR product sizE.

Primer	Sequence (¯5-¯3)		Amplicon Bp
E.tenella	F	TGCAAAAGTCGTAACACGGT	525
	R	TCCAAGCAGCATGTAACGGA	
E.necatrix	F	TGCTGCTGGACTTACAGGTT	501
	R	TTCGAGCAAAAGAGTATCGCC	
E.maxima	F	AGAGCCCTCTAAAGGATGCA	503
	R	AATGCAAGACACTTCATACAGC	

Table (2): Thermal cycler program of PCR technique

Step	Temperature and duration	
Initial denaturation	95oC for 4 min	
Denaturation	94oC for 30sec	30 cycles
Annealing	59oC for 30 sec	
Elongation	72oC for 1 min	
Final elongation	72oC for 5 min	

2. 4. Measurement of DNA concentration

The DNA concentration Of the all fifteen samples were measured by Nanodrop machinE. All selected samples gave more than 100 concentration ng/ml, which consider the lowest concentration required in the process of identifying DNA sequences [13].

2. 5. DNA sequencing methods

DNA sequencing method performed for confirmative detection and Phylogenetic analysis of three local species of Eimeria that responsible for coccidiosis based on ITS1 region by Phylogenetic tree analysis using the program (MEGA 6), while the Test type was UPGMA treE.(525 bp) PCR product of the species *E.tenella*, (501 bp) PCR product of species *E.necatrix* and (503 bp) PCR product of the species *E.maxima* were purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). The purified DNA from PCR product samples were sent to Bioneer Company in Korea for performed the DNA sequencing (AB DNA sequencing system).

3. Statistical analysis

The results of present study analyzed statistically by Program The Statistical Analysis System (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. [3]

Results

Out of 200 DNA samples that extracted from stool and intestins collected from chickins that clinically suspected coccidiosis were tested by convential PCR assay, only 160 samples which appeared positive and identified three species of Eimeria in both of Kerbala and Babylon provinces in Iraq. The identified species were *E.tenella* with 525 bp PCR product of ITS 1 region (Fig. 1).

Also *E.necatrix* was identified at 501 bp PCR product of ITS1 region on agarose gel electrophosis (Fig. 2).

While the last species diagnosed was *E.maxima* at 503 bp PCR product of ITS1 region as shown in the (Fig. 3).

Sequence analysis of fifteen positive samples from three species of Eimeria (Five samples for each one) were performed to confirm the PCR results. The Multiple sequence alignment analysis of ITS1 region of *E.tenella* was shown in the (Fig. 4)

while the Phylogenetic relationship tree analysis was constructed based on the five local samples of species *E.tenella* compare with other species of Eimeria through MEGA 6 program used of the test from type (UPGMA tree) as shown in the (Fig. 4).

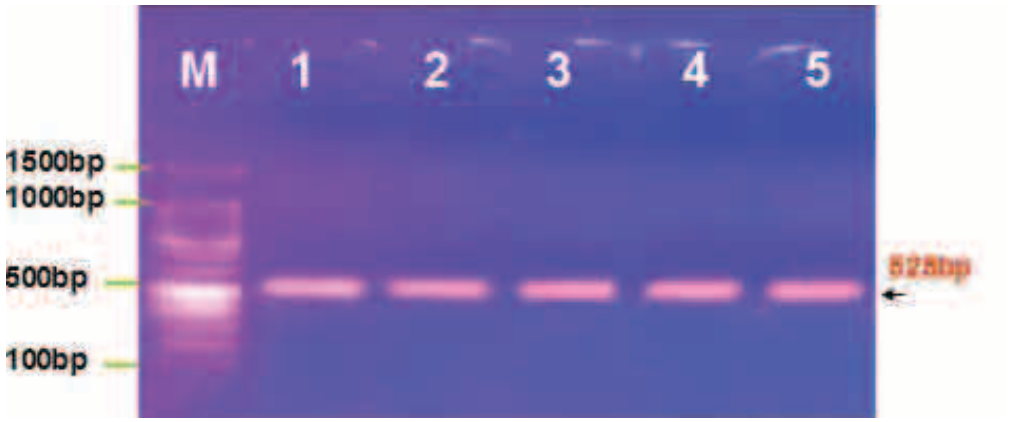

Fig. [1]: Agarose gel electrophosis show the PCR product results for E.tenella of ITS1 region where M: 1500bp ladder, Lane [1-5] are 525pb positive samples.

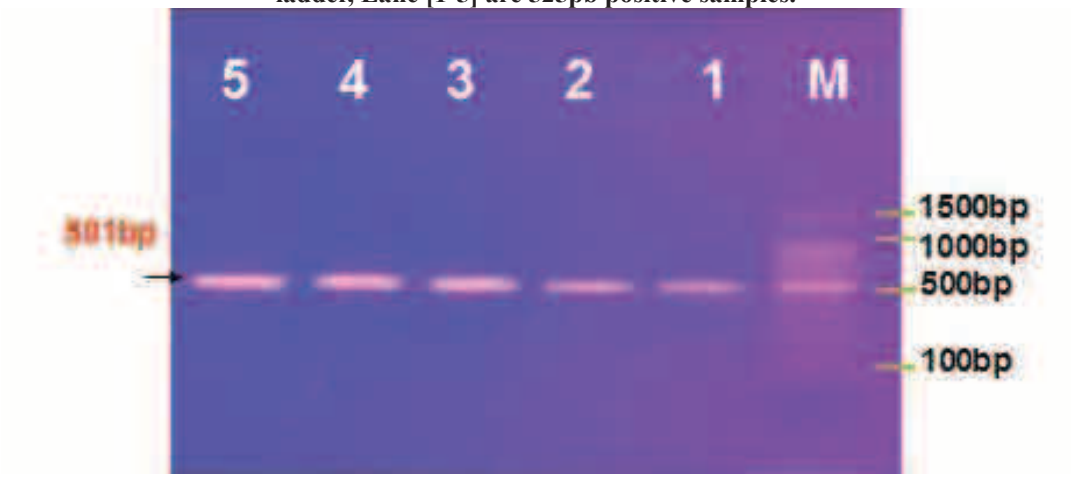

Fig. [2]: Agarose gel electrophosis show the PCRproduct results for E.necatrix of ITS1 region where M: 1500bp Ladder, Lane [1-5] are 501 bp positive samples.

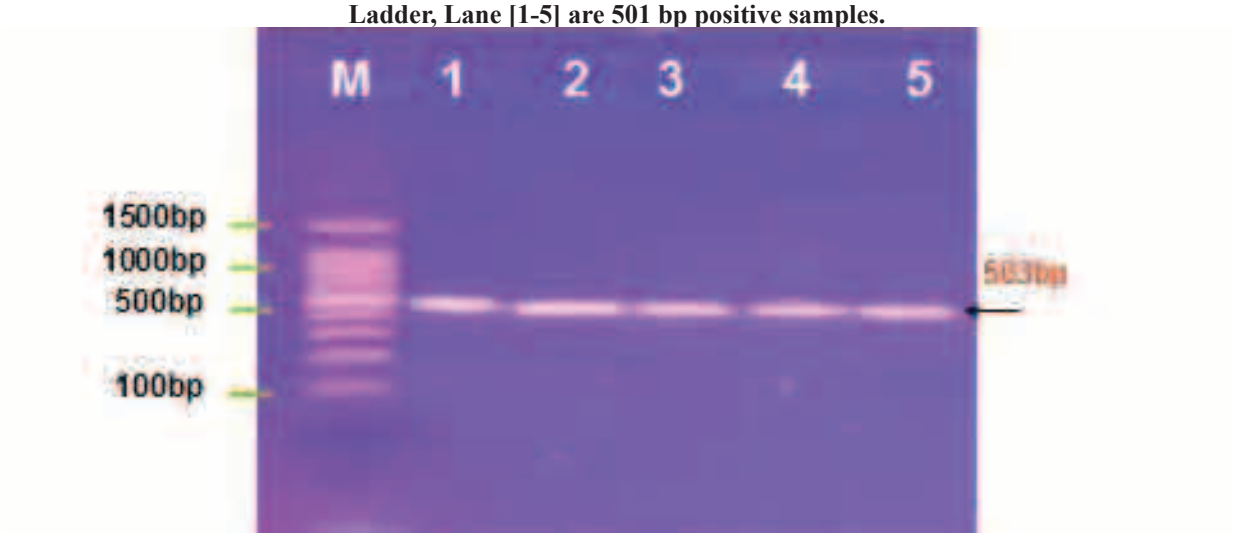

Fig. [3]: Agarose gel show the PCR product results for E.maxima of ITS1 region where M: 1500bp Ladder, Lane [1-5] are 503bp positive samples.

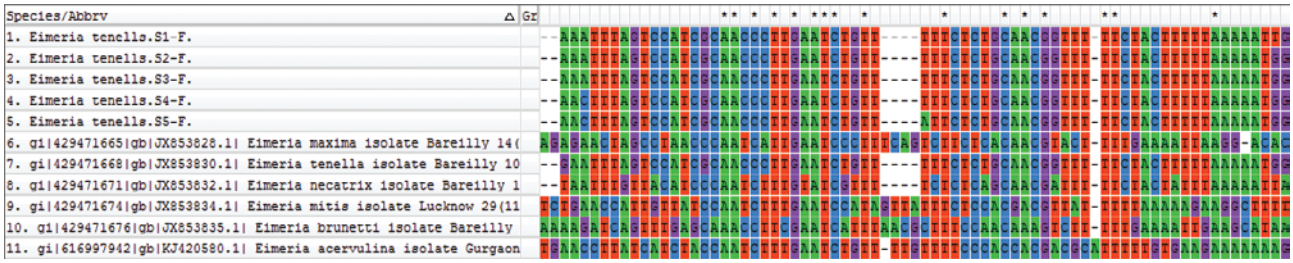

Fig. 4: The multiple alignment analysis of five local positive samples [S1, S2, S3, S4, S5] of E.tenella Comparison with other species of Eimeria.

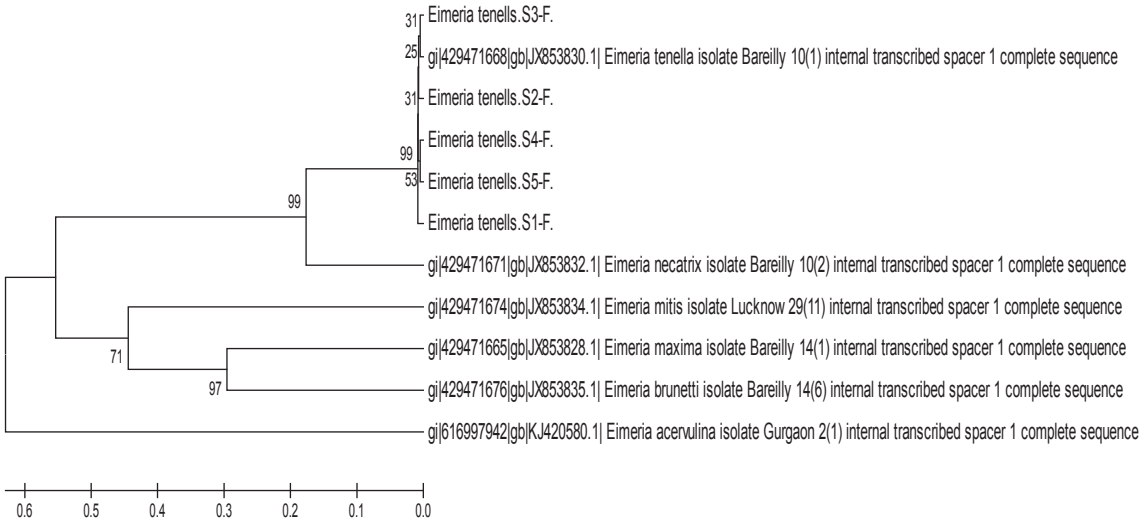

Fig. 5: The comparison between the phylogenetic Tree analysis of five local samples [S1, S2, S3, S4, S5] of E.tenella with other Eimerian species.

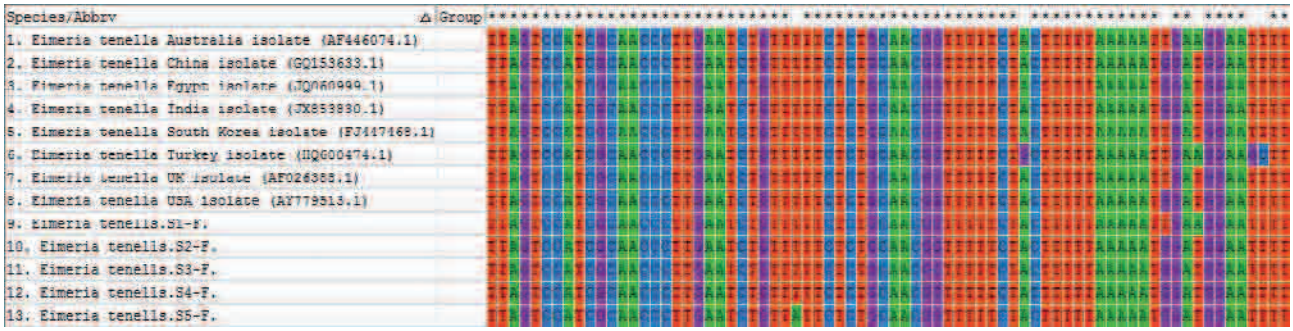

Fig. 6: The comparison in the multiple alignment analysis five local positive samples [S1, S2, S3, S4, S5] of E.tenella with global strains of species E.tenella.

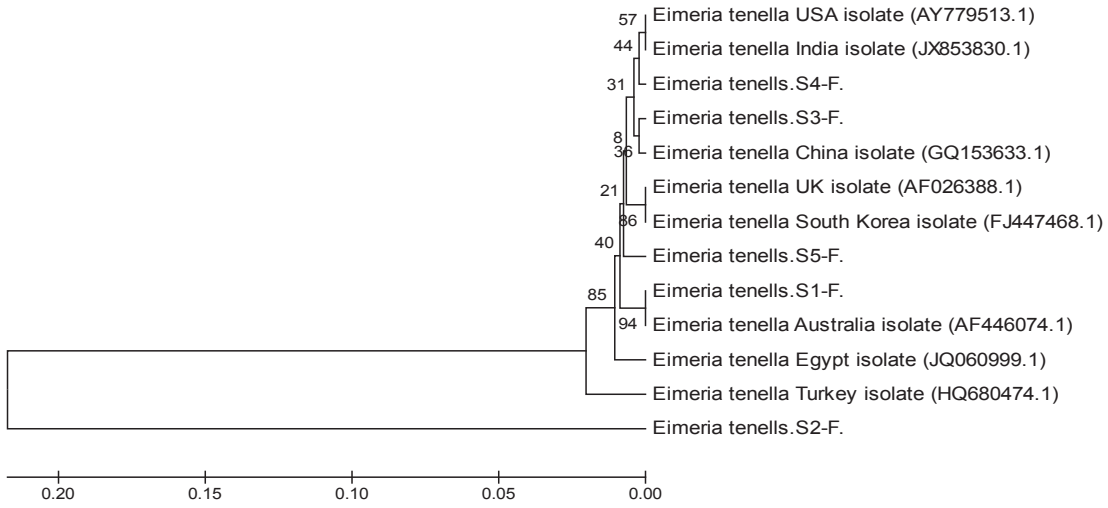

Fig. 7:The comparison between the phylogenetic tree analysis of five local samples of E.tenella with global strains of E.tenella by used of program [MEGA 6].

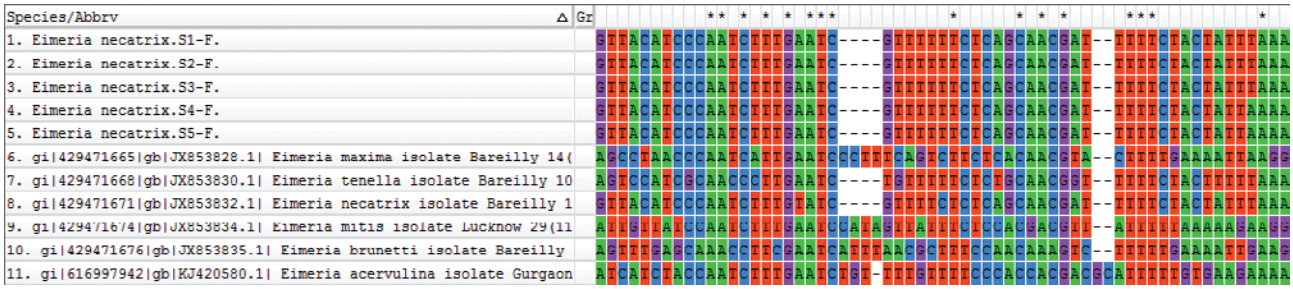

Fig. 8: The multiple alignment analysis of five local positive samples [S1, S2, S3, S4, S5] of E.necatrix comparison with other species of Eimeria.

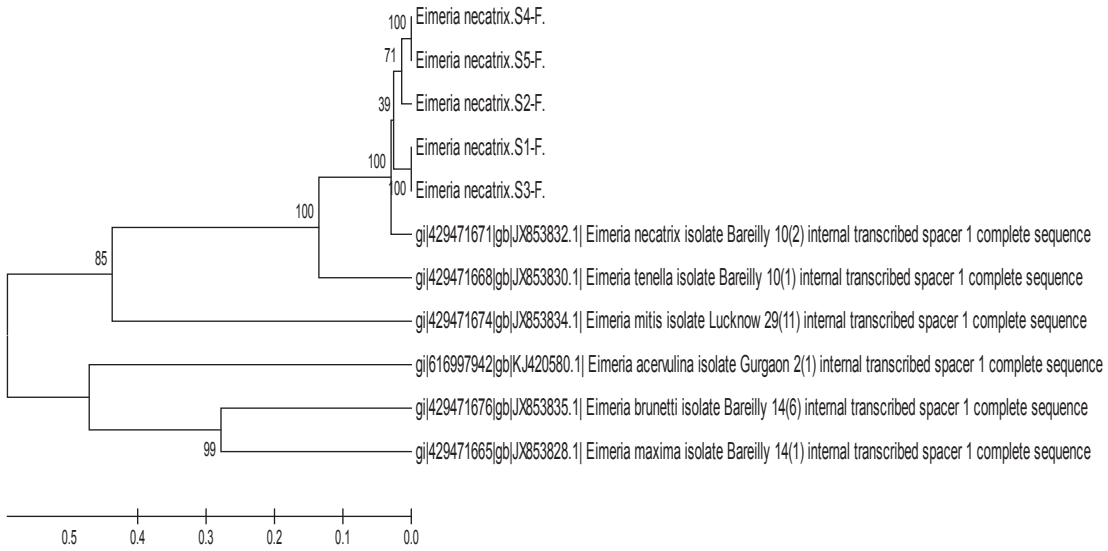

Fig. 9: The comparison between the phylogenetic tree analysis of five local samples [S1, S2, S3, S4, S5] of E.necatrix and the other Eimerian species.

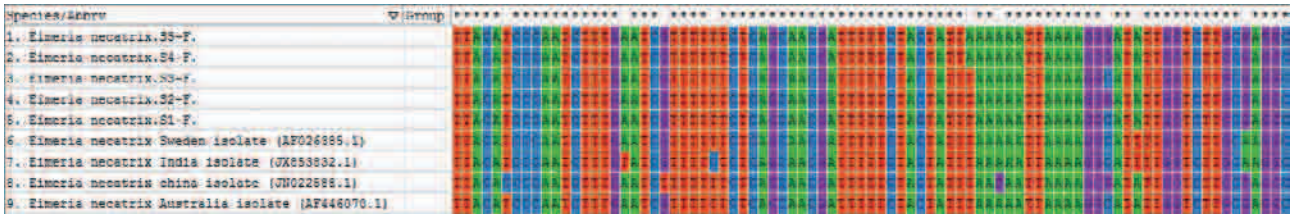

Fig. 10: The comparison of the multiple alignment analysis of five local positive samples [S1, S2, S3, S4, S5] of E.necatrix with global strains of species E.necatrix

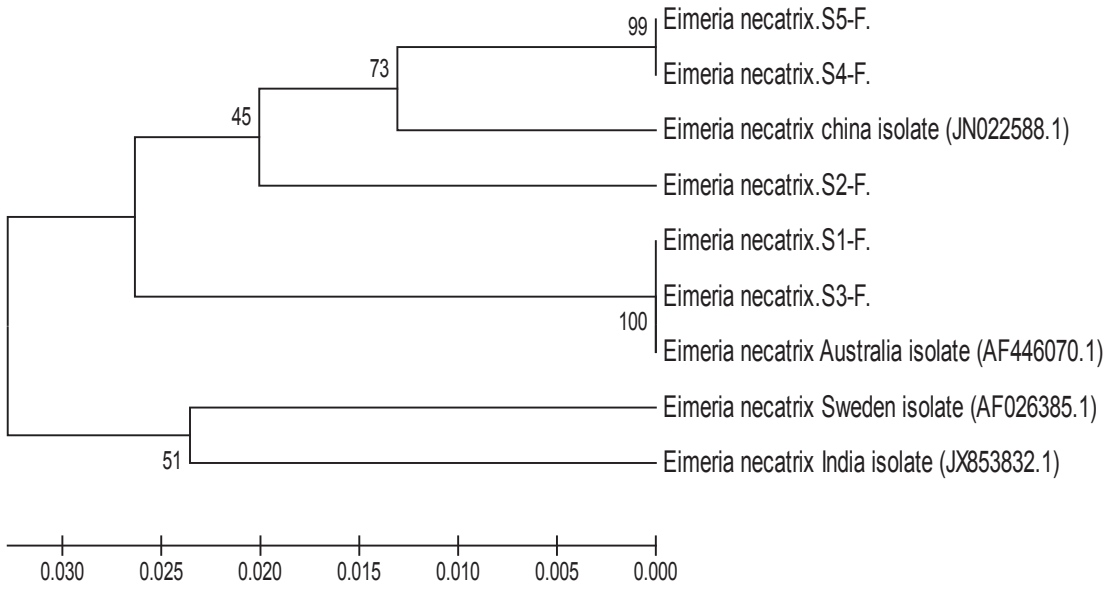

Fig. 11: The comparison between the phylogenetic tree analysis of five local samples of E.necatrix with global strains of *E.necatrix* by used of program [MEGA 6].

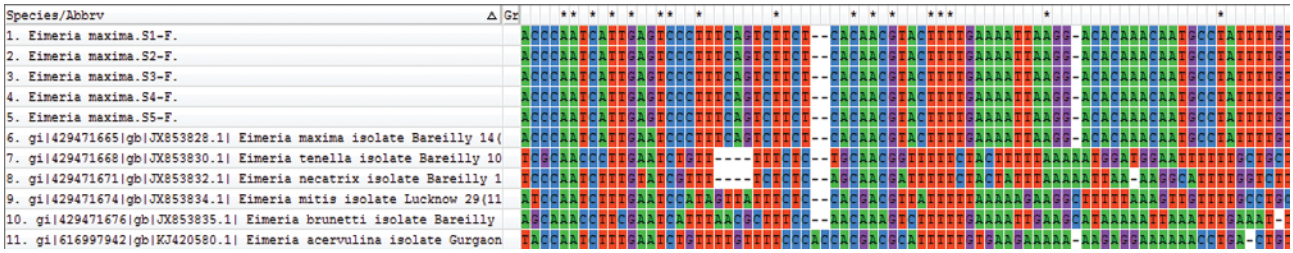

Fig. 12: Shows the multiple alignment analysis of five local positive samples [S1, S2, S3, S4, S5] of E.maxima comparison with other species of Eimeria.

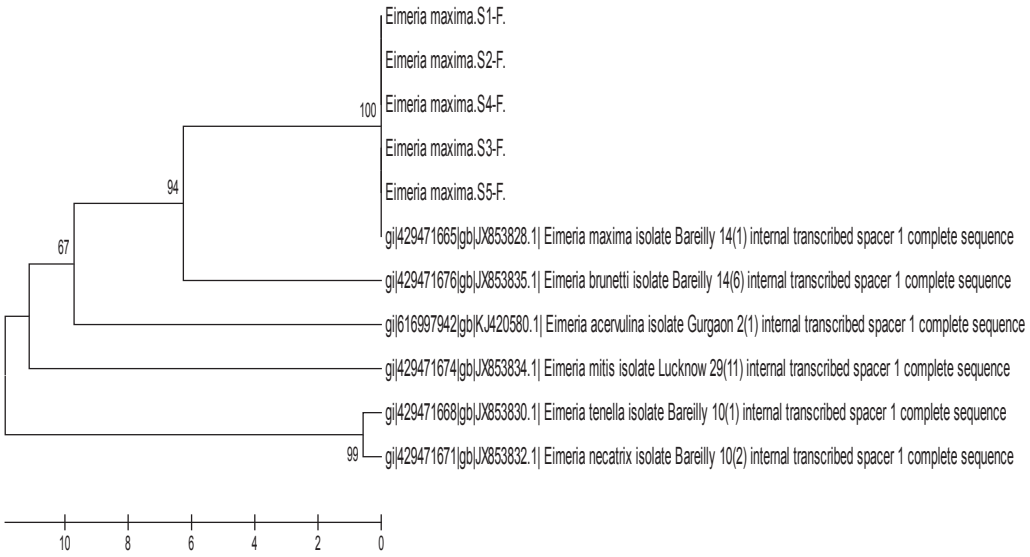

Fig. 13:The comparison between the phylogenetic Tree analysis of five local samples [S1, S2, S3, S4, S5] of E.maxima and the other Eimerian species.

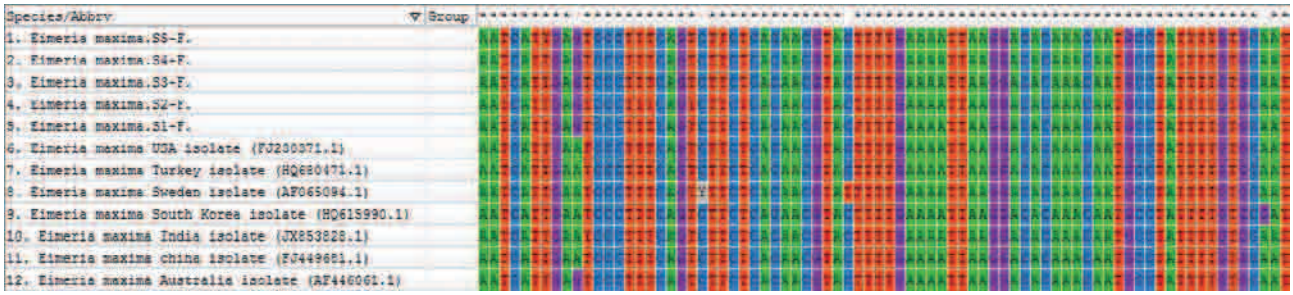

Fig. 14:The comparison of the multiple alignment analysis of five local positive samples [S1, S2, S3, S4, S5] of E.maxima with global strains of species E.necatrix.

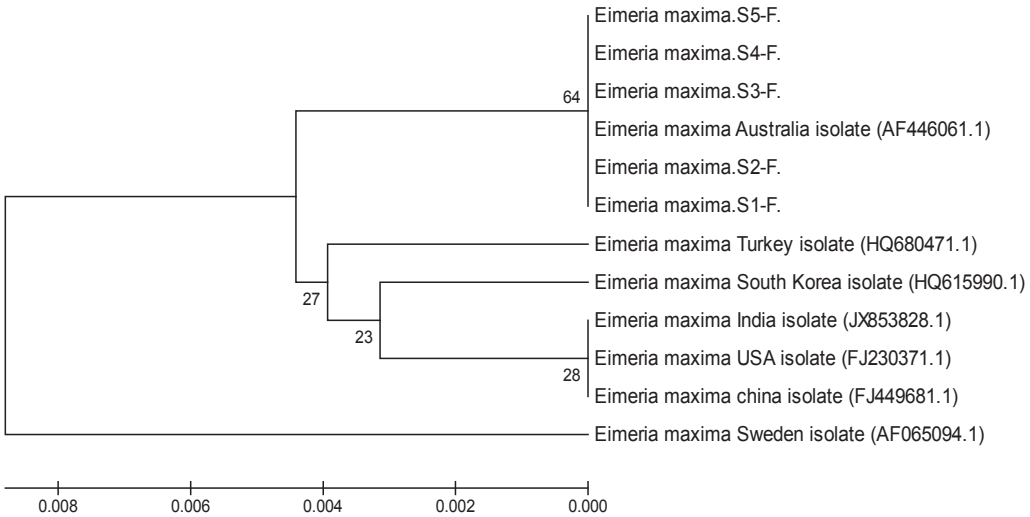

Fig. 15:The comparison between the phylogenetic tree analysis of five local samples of E.maxima with global strains of E.maxima by useing of MEGA 6 program.

4. Discussion

The specific diagnosis of Eimeria infections 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 dimensions or assessing the site and extent of the pathological lesions in the intestine of chickens [14].

Although the microscopic examinations can absolutely show the negative fecal samples, such traditional methods have generally had major limitations in the specific diagnosis of coccidiosis and identification of Eimeria species. These approaches are unreliable, particularly when multiple species of Eimeria simultaneously infect a single host and there is overlap in the size and shape of oocysts and the sites of infection in the intestines [8].

During recent years, there have been significant advances in the development of molecular-diagnostic tools. Several PCR based assays targeting different regions of the Eimeria genome have been described, such as the 5S rRNA, the small subunit rRNA [12, 21], 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, detecting variations in this region of DNA sequence, makes the design of primers straightforward and reduces the risk of cross reactions among different species [15].

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 occurrence and epidemiological pattern of the pathogenic Eimeria species of chickens in Iraq. Therefore, the results of the present study are the first on the prevalence of Eimeria species in the region, based on the molecular methodes.

In the present study, 200 samples of stool and intestines were collected from suspected infected chickens with coccidiosis 160 samples (80%) were positive which identified by used of molecular techniques, including Conventional PCR, by followed this technique three species of poultry Eimeria were diagnosed in the Kerbala and Babylon provinces and that species are E.tenella, E.necatrix and E.maxima the results of present study did not compare with any local studies and that due to the lack of a similar study.

Nowzari et al. in a large study including 5 provinces of Iran showed that E.maxima, E.mitis, E.brunetti, E.tenella and Eacervulina were distributed all over Iran. They identified E.mitis and E.brunetti for the first time by PCR [16]. E.brunettihas been found uncommon in broiler flocks [17]. In our study, E.tenella was the dominant species. This finding suggests that in poor management conditions, poultry houses may encounter acute coccidiosis in Kerbala and Babylon provinces due to highly pathogenic species, E.tenella.

Razmi et al, reported that prevalence of subclinical coccidiosis was 38% in Mashad,

north east of Iran and E.acervulina was the most prevalent species in broiler chicken farms [19]. In north-west of Iran, Tabriz, five Eimeria spp., E.acervulina, E.tenella, E.necatrix, E.maxima and E.mitis, were identified by morphometric study and E.acervulina was the most prevalent species [20].

Three species of Eimeria (E.acervulina, E.maxima and E.praecox (has been identified in Carolina in North America depending on PCR technique by used the amplified ITS1 of DNA that excreted from oocyst, Where the researcher recorded E.acervulina species the largest proportion compared to other species which is usually a medium pathogenesis [11].

In Australia, the researcher used PCR technique for diagnosis and detection the sequences of ITS1 region of rDNA of chickens Eimerian so, seven species were identified (E.tenella, E.necatrix, E.maxima, E acervulina, E.brunetti, E.mitis and E.praecox). The DNA sequences for each species analyzed and compared with European strains [7].

The traditional methods are not sufficiently reliable for specific diagnosis of Eimeria species in chickens. Moreover, occurrence of multiple infections in a single bird and the fact that, Eimeria species with low oocysts frequency in the mixture maybe missed, indicates that PCR based amplification of DNA sequence of parasite, could resolve this problem and overcame the limitation in analysis of small amounts of oocysts in mixed infections. On the other hand, this protocol can even identify strains of Eimeria species, characterized by different drugs resistance phenotypes [16, 18].

In Norway the samples collected from waste and chickens stool from 85 poultry farm and the researcher compared between two methods of diagnosis the first method depend upon the oocysts morphology while the other method is molecular assay (PCR) in which the oocysts isolated and identified depend on ITS1 region for rDNA, five species of Eimeria were identified E.acervulina, E.tenella, E.maxima, E.praecox and E.necatrix there was not a the perfect match between the two methods, with the proportion of compatibility 45% [21].

In Sweden, described the polymerase chain reaction (PCR) had been adopted to detect, identify and distinguish between Eimeria species the causal agent of poultry coccidiosis by used of ITS1 region of the rDNA as a variable and perfect for differentiation between Eimerian species, so a proper primers were designed and led to diagnosed the species (E.acervulina, E.brunetti, E.necatrix and E.tenella) and this study concluded that the ITS1 region of the Eimerian species contain enough variation to design primers can be applied in the PCR technique to detect and distinguish between different species Which constitute excellent indicators of epidemiological studies in the future [5].

The present study used the PCR assay for diagnosis of Eimerian species in which PCR product that represent the amplified ITS1 region of rDNA were analyzed to study the DNA sequencing of three local Eimeria species and compare with global strains of Emiria that recorded in GenBank at the site NCBI data basE.

However, the molecule also possesses phylogenetically informative variable regions that are useful for determining relationships among species and these region represent the

ITS1 region which located in rDNA, so the results from the present study illustrate the percentage of similarity (98% – 99%) between local isolated E.tenella and E.tenella isolate Bareilly (JX853830.1), which refers to a highly match percentage in the DNA sequencing between the local and global strains that recorded in the NCBI data basE.

The Phylogenetic relationship tree analysis according to (MEGA 6) program from type (Test UPGMA tree) to compare between the local E.necatrix and global strains shows identical percentage (91% – 100%) with E.necatrix isolate Bareilly (JX853832.1) in the site NCBI – data base While the Phylogenetic relationship tree analysis of E.maxima comparsion with global strains shows identical percentage (98%) with E.maxima isolate Bareilly (JX853828.1).

The results of Multiple sequence alignment analysis of ITS1 region in the PCR product of five samples of species E.tenella (S1, S2, S3, S4 and S5) with global strains shows great affinity with E.tenella Australia isolate (AF446074.1), E.tenella China isolate (GQ153633), E.tenella U.K isolate (AF026388.1), E.tenella Turkey isolate (HQ680474.1), E.tenella India isolate (JX853830.1), E.tenella South Korea isolate (FJ447468.1) and E.tenella Egypt isolate (JQ060999.1).

While the phylogenetic tree analysis of five samples of the species E.tenella (locally isolation) with global strains shows a high percentage of similarity between the S1 (local strain) and E.tenella Australia isolate (AF446074.1), S2 with. E.tenella Turkey isolate (HQ680474.1) and E.tenella Egypt isolate (JQ060999.1), S3 with E.tenella China isolated (GQ153633.1), S4 with E.tenella USA isolate (AY779513.1) and E.tenella India isolate (JX853830.1) and S5 with E.tenella South Korea isolate (FJ447468.1) and E.tenella UK isolate (AF026388.1).

The comparison between local E.necatrix (S1, S2, S3, S4 and S5) and the global strains by useing of multiple sequence alignment analysis of ITS1 region of PCR product which appear a percentage of similarity between the local strains of E.necatrix and E.necatrix Sweden isolate (AF026385.1), E.necatrix India isolate (JX853832), E.necatrix China isolate (JN022588) and E.necatrix Australian isolate (AF446070.1) according to (MEGA 6) program.

Also the phylogenetic tree analysis of five samples of the species E.necatrix appeared a closely relation between the local samples (S1, S3) and E.necatrix Australia isolate (AF446074.1), while the similarity between (S4, S5) and E.necatrix China isolate (JN 022588.1) were great while the local S2 appear more closely with E.necatrix China Isolate (JN022588.1).

A multiple sequence alignment analysis was conducted for a comparison between the local samples of the species E.maxima and the global strains in which a percentage of similarity appear between the local samples and E.maxima USA isolate (FJ230371.1), E.maxima Turkey isolate (HQ680471.1), E.maxima Sweden isolate (AF065094.1), E.maxima South Korea isolate (HQ615990.1), E.maxima India isolate (JX853828.1), E.maxima China isolate (FJ449681.1) and E.maxima Australia isolate (AF44601.1).

The phylogenetic tree of five samples for the

species E.maxima were analyzed to compare with global strains of the same species and that analysis appeared a similarity between all the five samples with E.maxima Australia isolate (AF446061.1), E.maxima Turkey isolate (HQ 680471.1), E.maxima South Korea isolate (HQ615990.1), E.maxima India isolate (JX853828.1), E.maxima USA isolate (FJ230371.1) and E.maxima China isolate (FJ449681.1).

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