Molecular study to detect the *Eimeria* species in sheep in Al-Diwaniyah province, Iraq

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Abstract

Sheep eimriosis is one of the most important and common disease which infects sheep in all ages but it is more effective in lambs. The diarrhea with or without blood is the main signs of infection. *Eimeria* protozoan required single host to complete its life cycle which pass in different stages including schizogony, gametogony and sporogony. The study was designed for detection of sheep *Eimeria* species through the molecular method. This study was conducted in Al-Diwanyah province during the winter months of 2019. In which 200 sheep fecal samples were collected and examined traditionally to investigate the *Eimeria* oocytsts. Ninety-seven samples of highly intensity infection with *Eimeria* oocysts were selected to subject for DNA extraction process. The extracted DNAs were tested through amplification of internal transcribed spacer 1 (*ITS-1*) gene by conventional PCR, and then phylogenetic analysis was made to diagnose the sheep *Eimeria* species. All samples that examined microscopically were showed positive results of infections with *Eimeria* protozoan. Out of 97 molecularly examined samples, forty-five (46.39%) were given positive result in conventional PCR technique, where *Eimeria* spp. detected through succeeded amplification of internal transcribed spacer 1 (ITS-1) gene. Then phylogenetic analysis referred to that there are five species of *Eimeria* confirmed in sheep in Al-Diwanyah province including 6 (33.33%) samples diagnosed as *E. ahsata*, 4 (22.22%) samples *E. weybridgensis*, 3 (16.66%) samples *E. ovinoidalis*, 3 (16.66%) samples *E. bovis* and 2 (11.11%) samples *E. auburnensis*. So, the *Eimeria* protozoan appears as an endemic parasite and can infect sheep with different species in study area. The sheep can infect with both specific and nonspecific species.

دراسة جزيئية لتحديد أنواع طفيلي الايميريا في الأغنام في محافظة الديوانية، العراق

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الخلاصة

تُعد كوكسيديا الأغنام من أكثر الأمراض شيوعًا حيث تصيب الأغنام في جميع الأعمار ولكنها أكثر تأثيراً في الحملان. يعتبر الإسهال مع أو بدون دم هو أحد أهم العلامات الرئيسية للعدوى. يحتاج طفيلي الايميريا مضيفا واحدًا لإكمال دورة حياته التي تمر بمراحل مختلفة تتضمن التكاثر الانشطاري، تكوين الأمشاج، والتبوغ. تم تصميم الدراسة لتحديد أنواع الايميريا في الأغنام من خلال الطرق الجزيئية. أجريت هذه الدراسة في محافظة الديوانية خلال أشهر الشتاء ٢٠١٩. حيث تم جمع ٢٠٠ عينة برازية من الأغنام وفحصها تقليديًا للكشف عن بويضات الإيميريا. تم إخضاع سبعة وتسعين عينة ظهرت فيها أكياس البيضة للايميريا لعملية استخلاص الحمض النووي وقد تم اختبار الحمض النووي المستخلص من خلال تضخيم الجين الفاصل الداخلي ١ بواسطة تفاعل سلسلة إنزيم البولمر التقليدي، ثم تم إجراء

التحليل الوراثي لتشخيص أنواع إيميريا الأغنام. جميع العينات المفحوصة أظهرت نتيجة إيجابية لوجود أكياس البيضة للايميريا مع تباين في شدة الإصابة. ظهر من بين ٩٧ عينة تم اختيارها للفحص الجزيئي على أساس مستوى الإصابة العالية فيها أن ٤٥ (٤٦,٣٩٪) عينة منها أعطت نتيجة إيجابية في تقنية تفاعل إنزيم البولمر التقليدي، حيث تم تشخيص جنس الايميريا من خلال التضخيم الناجح للجين الداخلي الفاصل ١. لقد أشار التحليل الوراثي إلى وجود خمسة أنواع من الإيميريا في الأغنام في محافظة الديوانية، منها ٦ (٣٣,٣٣%) عينة تم تشخيصها على أنها *E. ahsata* و ٤ (٢٢,٢٢%) عينات *E. weybridgensis*، و ٣ (١٦,٦٦%) عينات *E. ovinoidalis*، و ٣ (١٦,٦٦%) عينات *E. bovis* و ٢ (١١,١١%) عينات *E. auburnensis*. لذلك يظهر طفيلي الايميريا كطفيلي مستوطن ويمكن أن يصيب الأغنام بأنواع مختلفة في منطقة الدراسة ويمكن أن تخمج الأغنام بأنواع خاصة وغير خاصة.

Introduction

Coccidiosis is an importance economic disease in all animals which can be significant problem in the young's members (1). Coccidiosis in sheep can be a serious disease that causes severe diarrhea, emaciation and sometimes death (2). The disease is more serious when sheep are reared in a closed breeding system, in particularly; lambs kept in overcrowded barns or on irrigated pastures during winter months (3).

The parasite has two phases of life cycle, endogenous phase in which the parasite undergoes numerous divisions in the intestinal cells, Where the ingested sporulated oocysts release sporozoites in intestinal lumen (excystation); and the exogenous phase which takes place outside of body in the environment under certain conditions (4).

Ingestion of contaminated food and water are the main source of infection and the symptoms of the disease begin with diarrhea, sometimes containing mucus or/ and blood, loss appetite, weight loss, anemia, fatigue, wool breaking and finally death of the animal (5).

The morbidity of the disease may be reach 10-40% and the mortality about 10% (5,6) *Eimeria* highly hosts specific and the disease is usually caused by sporulated oocysts (7,8).

Different species of *Eimeria* parasitize the sheep intestine and mixed infections with a number of *Eimeria* spp. are common in natural infections (9).

Different sheep *Eimeria* species were recorded worldwide including *E. ahsata, E. bakuensis, E. parva, E. pallida, E. crandallis, E. weybridgensis, E. ovina, E. ovinoidalis, E. granulosa, E. intricata, E. faurei* and *E. marsica* (2). Among these species only *E. ovinoidalis* and *E. crandallis* are appeared to be pathogenic and lead to the most severe infections (10).

Differentiation among these different species is depending mainly upon shape and measurements of oocysts, infection site and sporulation time, but may be unreliable methods due to the large overlapping in size and shape of these different species (11, 12).

Many methods were used in the diagnosis of *Eimeria* depending upon fecal examination, serological and molecular (13).

Sensitive and specific fecal examination results can get by use of molecular techniques (14). PCR based on amplification of DNA that has been used for the diagnosis of *Eimeria* in different hosts and have proved to give accurate results in different used samples (15).

As sheep are regarded as the good source of protein for the study area population, therefore the needing to increase the protein sources requires understanding any disease aliment such as coccidian infection which can limit the production of small stocks. So, given that most traditional diagnostic examinations have a significant error rate, we consider studying the diagnosis of *Eimeria* species based on molecular methods.

Materials and methods

Two hundred sheep fecal samples 5-10 gram were collected directly from animals' rectum and put in clean plastic labeled containers, then transported with ice bags to a Parasitology laboratory in the College of Veterinary Medicine, University of Al-Qadisiya. These samples were getting from different regions of Al-Diwaniyah province during the winter months of 2019.

All samples were examined by flotation method (16), to investigate the *Eimeria* oocysts. DNA extraction was done for certain positive samples selected based on the density of oocysts existing using fecal DNA extraction kit (Favorgen Biotech Corp®, Bioneer, Korea) according to manufacturer directives. The purity of extracted DNA was measured by using a Nanodrop spectrophotometer (THERMO. USA), at 260 /280 nm absorbance, then stored at -20 ℃ till used.

Conventional PCR technique was performed to amplification the extracted DNA at 18SrRNA (*ITS-1*) gene according to a method described by (15), where *Eimeria* common primers were used (IDT, Canada).

The up-and downstream sequences in internal transcribed spacer 1 (*ITS-1*) region were: F: 5´- GCA AAA GTC GTA ACA CGG TTT CCG -3´, R: 5´- CTG CAA TTC ACA ATG CGT ATC GC-3´ with expected product sizes of 348-546 bp. PCR construction was prepared by using (AccuPower PCR PreMix Kit), whereby for 50 µl volume of PreMix Kit tube (Taq DNA polymerase, dNTPs, Tris. HCl pH: 9.0, KCl, MgCl₂, stabilizer, and tracking dye) 5 µl of extracted DNA and 1.5 µl (10 pmol) of both forward

and reverse primers were added, then the volume completed with nuclease free distilled water.

Reaction conditions set as an initial denaturation at 94ºC for 30 sec followed by 35 cycles at 94ºC for 10 sec, 55ºC for 45 sec, and 72ºC for 20sec with final extension at 72ºC for 2min using PCR thermocycler (Thechne/ USA). The PCR products were analyzed by 1.5 % agarose gel electrophoresis.

To detect the *Eimeria* species, PCR positive samples were subjected to DNA sequencing (Bioneer / Korea) by AB DNA sequencing system. The Phylogenetic analysis was performed based on NCBI-Blast Alignment identification and Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

Results

All samples 100% that examined microscopically showed positive results for *Eimeria* oocysts in different intensity of infections (Figure 1). Ninety-seven samples were selected according to their heavy infection for DNA extraction. Where 45 46.39% extracted DNA showed positive results when succeeded amplification of internal transcribed spacer 1 (*ITS-1*) gene through conventional PCR technique to produce 348-546 bp product (Figure 2).

Figure 1: Sheep *Eimeria* oocysts.

For getting perfect sequencing results, 18 positive samples of more specific PCR products were subjected to DNA sequencing to detect sheep *Eimeria* species. Where the results revealed that 6 (33.33%) samples diagnosed as *E. ahsata* (accession no. MN269978, MN269980, MN306558, MN269981, MN269982, MN269984), 4 (22.22%) *E. weybridgensis* (MN306559, MN306560, MN306561, MN306563), 3 (16.66%) *E. ovinoidalis* (MN306556, MN306565, MN306566) and 3 (16.66%) *E. bovis* (MN306557, MN306562, MN306564) and 2 (11.11%) *E. auburnensis* (MN269979, MN269983) (Table 1; Figure 3).
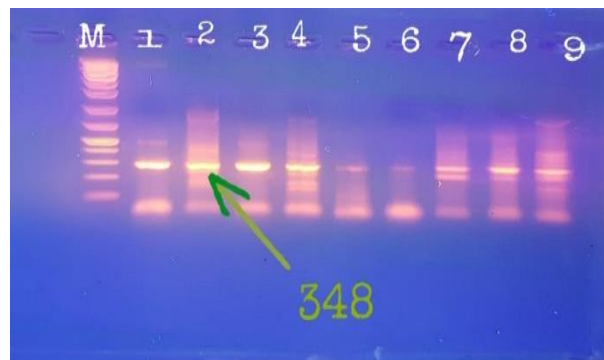

Figure 2: PCR product of agarose gel electrophoresis of *Eimeria ITS-1* gene (348- 546 bp), where; M: 1000bp DNA ladder; Lanes 1-9 Positive samples.
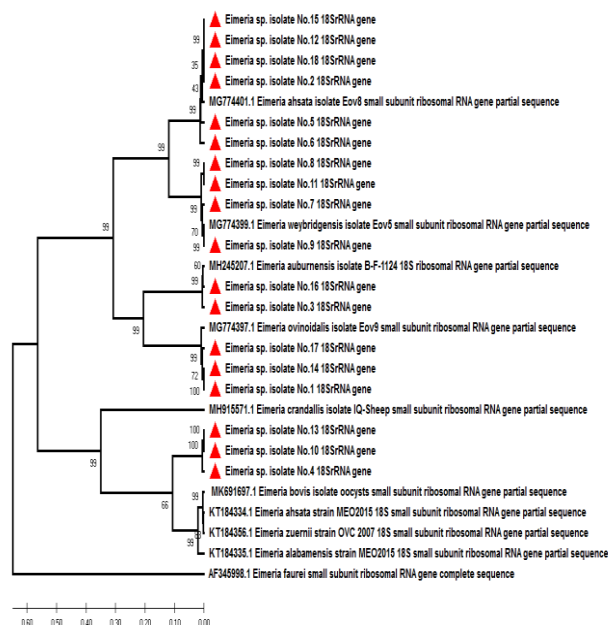

Figure 2: Phylogenetic tree analysis based on 18S rRNA gene partial sequence in local *Eimera* spp. isolates that used for genetic identification. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version) at total genetic changes (0.10-0.60%).

Discussion

Eimeria protozoan is a principal etiologic agent for coccidiosis in livestock, where the sickness causes economic loss in sheep as a result of the drop in milk and meat production, paleness, and the increment of the prospect to induce mortality (17).

Table1: NCBI-Blast homology sequence identity (%) among local *Eimeria* spp. and NCBI-BLAST submitted *Eimeria* spp. isolates

Eimeria spp isolates No.	Homology sequence identify			
	Genbank Accession number	NCBI - Blast isolates	Gene bank AN.	Identify %
1	MN306556	*E. ovinoidalis*	MG774397.1	98.19%
2	MN269978	*E. ahsata*	MG774401.1	98.84%
3	MN269979	*E. auburnensis*	MH245207.1	99.28%
4	MN306557	*E. bovis*	MK691697.1	99.75%
5	MN269980	*E. ahsata*	MG774401.1	98.84%
6	MN306558	*E. ahsata*	MG774401.1	98.27%
7	MN306559	*E. weybrigensis*	MG774399.1	98.84%
8	MN306560	*E. weybrigensis.*	MG774399.1	98.52%
9	MN306561	*E. weybrigensis*	MG774399.1	100.00%
10	MN306562	*E. bovis*	MK691697.1	99.25%
11	MN306563	*E. weybrigensis*	MG774399.1	98.52%
12	MN269981	*E. ahsata*	MG774401.1	98.84%
13	MN306564	*E. bovis*	MK691697.1	99.24%
14	MN306565	*E. ovinoidalis*	MG774397.1	98.17%
15	MN269982	*E. ahsata*	MG774401.1	98.84%
16	MN269983	*E. auburnensis*	MH245207.1	99.28%
17	MN306566	*E. ovinoidalis*	MG774397.1	97.79%
18	MN269984	*E. ahsata*	MG774401.1	98.84%

Molecular and phylogenetic studies for *Eimeria* species identification in ruminants are few compared with studies conducted on avian. The *ITS1*- rRNA genes has been showed to an effective target for some *Eimeria* species phylogenetic analysis (15,18).

The results of the current study showed that all 100% microscopically examined samples have infection with different *Eimeria* spp. This result higher as compared with the results of studies were performed in India 70.44% and Iraq 72% by Om *et al.* (19) and Kareem and Yücel (20) respectively. These differences in outcomes may be due to differences in environmental, breeding and health care conditions.

Molecularly the results recorded five different species of *Eimeria* in sheep that which *E. ahsata* 33.33% *E. weybridgensis* 22.22%, *E. ovinoidalis* 16.66%, *E. bovis* 16.66% and *E. auburnensis* 11.11%, where the first three species are a specific for sheep, while the two later a specific for cattle. These results are agreed with the result of Platzer *et al.* (21) who recorded three species included *E. ovinoidalis*, *E. weybridgensis* and *E. ahsata* in Austria, Hashemni *et al.* (22) who recorded *E. ahsata* in Western Iran and Kara (23) who confirmed *E. ovinoidalis* in Turkey.

Most Iraqi previous studies depend upon classical method for detection of sheep *Eimeria* species (20,24), except the study carried by Al-Saadoon (25) in Wasit who fixed 10 *Eimeria* species (*E. ahsata, E. parva, E. pallida, E. bakuensis, E. ovinoidalis, E. crandallis, E. faurei, E. Intricate, E. weybridgensis, E. granulosa*) by both classical and molecular methods recorded 11 species of *Eimeria* (*E.*

bakuensis, E. weybridgensis, E. marsica, E. ovinoidalis, E. ahsata, E. parva, E. pallida, E. crandallis, E. faurei, E. intricata and *E. granulosa*) in Sulaimaniya, also in other study.

In this study two cattle species (*E. bovis* and *E. auburnensis)* were detected in sheep. These species were observed in previous studies in their specific host, where Ibrajim *et al.* (26) recorded *E. bovis* 25.84% and *E. auburnensis* 7.78%, in Saudi Arabia in Subclinical bovine coccidiosis, also Bangoura *et al.* (27) showed *E. bovis* was more effective and highly pathogenic.

The appearing of these two cattle *Eimeria* species may be attribute to the common grazing in most fields between sheep and cattle that may lead to some hosts being infected with non-pathogenic or pathogenic *Eimeria* spp. of other host. In other hand the *E. auburnensis* is recorded for the first time in Iraq, at both cows or sheep, where previous studies have not indicated its recordation (20,24,25).

The typical diagnosis of pathogens is important in understanding the biology and life cycle of them. Therefore, traditional methods of diagnosing coccidiosis do not achieve accurate diagnosis because of the significant overlap in the properties of different species (12).

Conclusions

The sheep eimeriosis is an endemic disease in Al-Diwaniyah province and there are different *Eimeria* species can infect sheep in the study area. The molecular techniques are an accurate method in detection of *Eimeria*

species, in addition to that sheep can infect with nonspecific species (*E. bovis, E. auburnensis*).

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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