Research Article

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Comprehensive Analysis of Morphologic and Molecular Studies on *Eimeria tenella* Infection in Broiler Chicks in Sulaimani Province, Iraq.

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Abstract

Coccidiosis is a major parasitic disease that affects domestic fowl, turkeys, ducks, and geese around the world. It is caused by protozoan parasites of the phylum Apicomplexa originating from the genus *Eimeria.* This study was recommended due to the lack of data on chicken *Eimeria* species in the Kurdistan region. There are nine recorded species of *Eimeria* in chickens, *and E. tenella* is the most common coccidia in poultry. *It* is distinguished by its easily identifiable injuries and significant losses in early broilers and pullet layers. The research examined naturally infected broiler chicks aged between 3 and 6 weeks with *Eimeria tenella.* The samples were taken from caecal content and examined by standard necropsy and microscopic examinations. Microscopic examination revealed a large number of coccidian oocysts in the caecal content. Histopathological analysis showed severe sloughing of the lining mucosa with hemorrhage and necrosis, along with the presence of different stages of *Eimeria*, including oocytes, macrogametes, microgametes, and schizonts in the submucosal layer. Hematoxylin and eosin staining facilitated a histopathological study on the affected caecum tissue. The study highlights an important stage that streamlines parasite isolation for molecular diagnosis by directly using cecum samples for DNA extraction. This method allows molecular diagnostic procedures to be completed more efficiently, regardless of the parasite's stage.

Keywords: Broiler chick; Coccidiosis; Diagnosis; *Eimeria tenella*; Infection.

Introduction

Coccidiosis is considered economically significant due to its high mortality rate, expensive treatment and control costs, and a decrease in productivity rate (1). It is estimated that coccidiosis costs the chicken business $3 billion annually on a global scale (2). enteric diseases, such as coccidiosis, which affect poultry and gamebirds, cause diarrhea, dehydration, weight loss, and high morbidity and mortality rates (3).

Coccidia are parasites lives inside the cell in the phylum of Apicomplexa that belong to the genus *Eimeria.* There are nine recorded species of *Eimeria* in chickens, only seven are important, they are: *E. tenella; E. acervuline*; *E. maxima; E. necatrix; E. miti; E. praecox; E. brunett* (4).

The locations of these species in the intestinal tract and their capacity to cause mortality and disease vary (5). The two species that are most pathogenic are *E. tenella* and *E. necatrix*. Common and mild to somewhat pathogenic species are: *E. acervuline*; *E. maxima*; *E. mivati*. *E. brunetti* is rare, but dangerous when it does occur. *E. hagani*; *E. praecox; E. mitis* are species that are not particularly pathogenic (6). The most common coccidia in poultry records is *E. tenella*, which is distinguished by its easily identifiable injuries and significant losses in early broilers and pullet layers (7). It lives in the caecum and produces a serious illness identified by bleeding in the intestinal tract, high morbidity and mortality, emaciation and decreased weight gain, paleness of skin pigmentation, and a bloody mass in the caecum (8). Birds between the ages of three and eighteen weeks are affected by most Eimeria species (9). The disease is common in areas that are tropical or subtropical, where deep litter is commonly are used in farm management practices to create an environment that facilitates the growth of Eimeria species (10). Different methods are used for the diagnosis of coccidiosis and characterization of eimeria, such as the morphology and size of eimeria oocysts, characteristic gross lesions at necropsy, time taken for sporulation, and cross-immunization tests (11).

Recently, DNA amplification has been used to distinguish between coccidian parasites, and the ITS-1 gene of ribosomal DNA (rDNA) is a prime candidate for the polymerase chain reaction (PCR) targeting of genomic DNA (12). In the current study, broiler farms in Sulaimani province will examine the presence of *Eimeria tenella*.

Materials and Methods

Sample examination

The samples were taken from 40 broiler chicks aged between the ages of 3 and 6 weeks on six broiler farms of various discrete locations in Sulaimani province/Iraq. 5 chicks per 10,000 is sufficient for the diagnosis of coccidiosis (13). After a clinical examination was carried out on each chick, the chicks were sacrificed for postmortem examination. Clinical signs are diarrhea, weight loss, lethargy, dehydration, pale comb and wattles, huddling and dropped wings. A wet smear was prepared from the caecum where

the lesion was located for examination of *E. tenella* under microscopic.

Positive samples for sporulation were stored in petri dishes with 2.5% potassium dichromate and cultured for 48 hours at 30°C (11). After incubation, centrifuge the plate content using the sedimentation procedure. Identification of *E. tenella* was done on the basis of criteria such as location of lesion in intestine, size, shape of oocyst and its time needed for sporulation (14).

Histopathological examination and scoring evaluation

Caecal specimens were fixed in 10% neutral buffered formalin for 48 hrs, and then subjected to a series chemical process for histopathological analysis. Thin 4µm sections were prepared and stained with hematoxylin and eosin. Microscopic lesions were evaluated by light microscopy (Leica, Japan) and photographed with a digital camera (AmscopeTM, Japan) in the Research Centre of the College of Veterinary Medicine, University of Sulaimani/Iraq. The microscopic lesions were evaluated by a blind or independent pathologist to record any pathological characteristics and confirm the life cycle of the Eimeria stages of life, then each of the lesions was scored according to their severity as follows: A score of 0 indicates a normal lesion, a score of 1 indicates a swollen lesion without bleeding, a score of 2 indicates a swollen lesion with minimal bleeding, a score of 3 indicates a marked lesion with bleeding, and a score of 4 indicates a severe dilatation with extensive bleeding and sloughing of the mucosal lining(15).

DNA extraction

In this study, molecular diagnosis was performed on pooled caecal mucus derived from three chicks for each of the six broiler farms. No specific kit was used for the DNA extraction of parasites. Instead, total nucleic acid extraction was performed using the AddPrep viral nucleic acid extraction Kit (Addbio, Korea).

PCR Amplification and Sequencing

The PCR amplification procedure adhered to the constructor's guidelines and was conducted using the Add Star Taq master mix PCR kit (Addbio, Korea). 20 µL of reaction was prepared by mixing 10 µL of master mix, 3 µL of DEPC water, 5 µL of DNA sample, and 1 µL (10 pmol) of each forward primer (ET-F: AATTTAGTCCATCGCAACCCTTG) and reverse primer (ET-R: CGAGCGCTCTGCATACGACA), with the aim of amplifying a segment of 278 bp of the internal transcribed spacer 1 (ITS 1) gene (16). The PCR amplification program for the conventional thermocycler, Techne TC-4000 thermal cycler (Techne Ltd., UK), involved an initial denaturation phase of 5 minutes at 95 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension phase of 3 minutes at 72 °C.

The PCR product was monitored by putting 7 µL of it on a 1% agarose gel in 1× TBE buffer. The gel was stained with safe stain (7 µL) (Addbio, Korea), and the electrophoresis was set at 100 volts for 1 hour using electrophoresis system. 100- bp

DNA ladder was used to analyze the migration pattern of the PCR amplicons.

Thereafter, the amplification product, which had distinct DNA bands, was selected and sent to Macrogen Company in South Korea for sequencing. The amplified DNA sequences were sequenced using Sanger DNA. Both the forward and reverse primers were used to identity each nucleotide sequence.

Results

Clinical and post mortem findings

The majority of the birds displayed signs of depression, reduced feed intake, frequent eye closures, and huddling, along with the presence of blood in their faeces. Upon postmortem examination of the sick birds, it was found that the small intestine was severely distended, accompanied by gas and petechial hemorrhages that appeared without the need for gut opening. The most prominent lesion was observed in the ceca. The caecum was greatly enlarged and distended. The cecal content became watery, mixed with mucus and blood (Fig. 1).

Microscopic Findings

After post-mortem investigation, the caecal content was studied under a microscope, which revealed a large number of oocysts (Fig. 2) and supported the parasite coccidia is present. The collected oocysts were oval-shaped. The unsporulated oocysts contain zygotes filling the entire space of the oocyst (Fig. 2a). In contrast, the sporulated oocysts have four sporocysts, each including two sporozoites (Fig. 2b). The oocysts measured (19–24) µm long and (16–20) µm wide (size determined by image J software). The oocysts sporulated after 48 hours.

Histopathological Finding

The gross lesions of the chicken's caecum showed severe swelling with excessive hemorrhage on the surface of the serosa, a large amount of blood exudate in the lumen leading to caecal dilation (Fig. 3a, b), and severe falling of mucosal lining also seen (Fig. 3c).

The microscopic features demonstrated that extensive sloughing of the mucosal lining epithelium and the shedding of necrotic material in the lumen, marked atrophy of the mucosal fold that led to blunting of the mucosa with atrophy of the caecal crypts and replaced by chronic inflammatory cells that diffused throughout the caecal layers (Fig. 3a-c). Different stages of development of *Eimeria* in the submucosal layer include; immature and mature oocyst that are characterized by the wall of the oocyst and central nucleus, gametocytes such as macrogametes with a central nucleus and eosinophilic bodies lining their border, and microgametes, have many crescent-shaped merozoites in addition to growing schizonts arranged in the cluster (Fig. 3d. e).

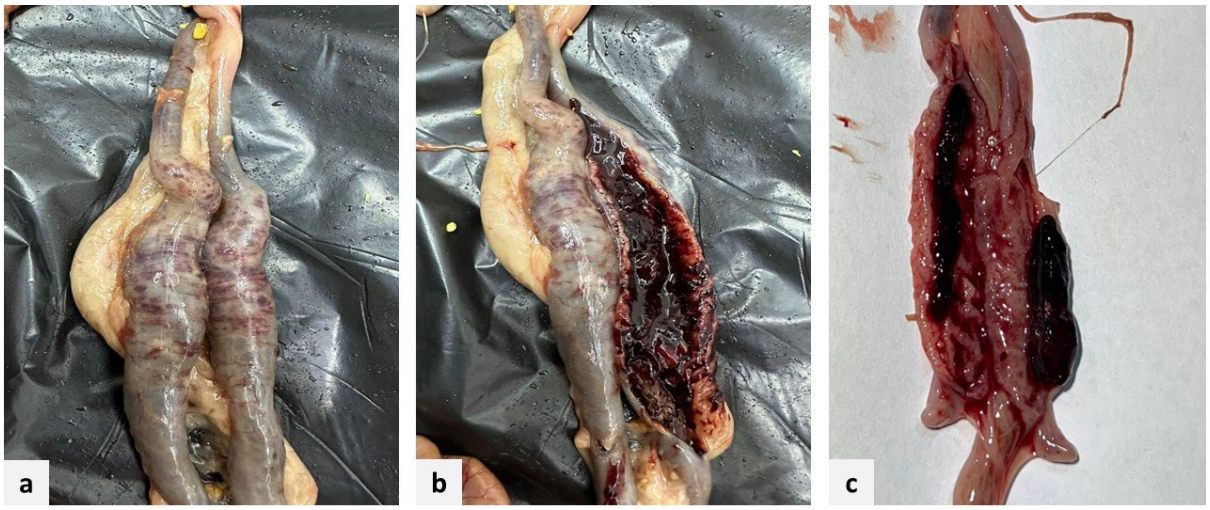

Figure 1: Marked congestion and dilatation of the caecum filled with a lot of blood exudate (A). A friable caecal wall with a lack of lining mucosa, especially in figures B and C.

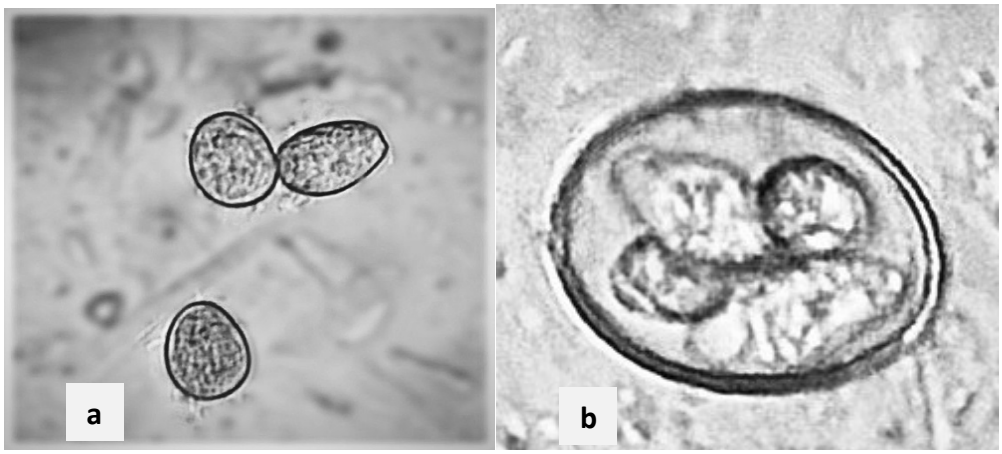

Figure 2: Oocysts of *E. tenella* from broiler chick. a; Unsporulated oocysts. b; Sporulated oocyst.

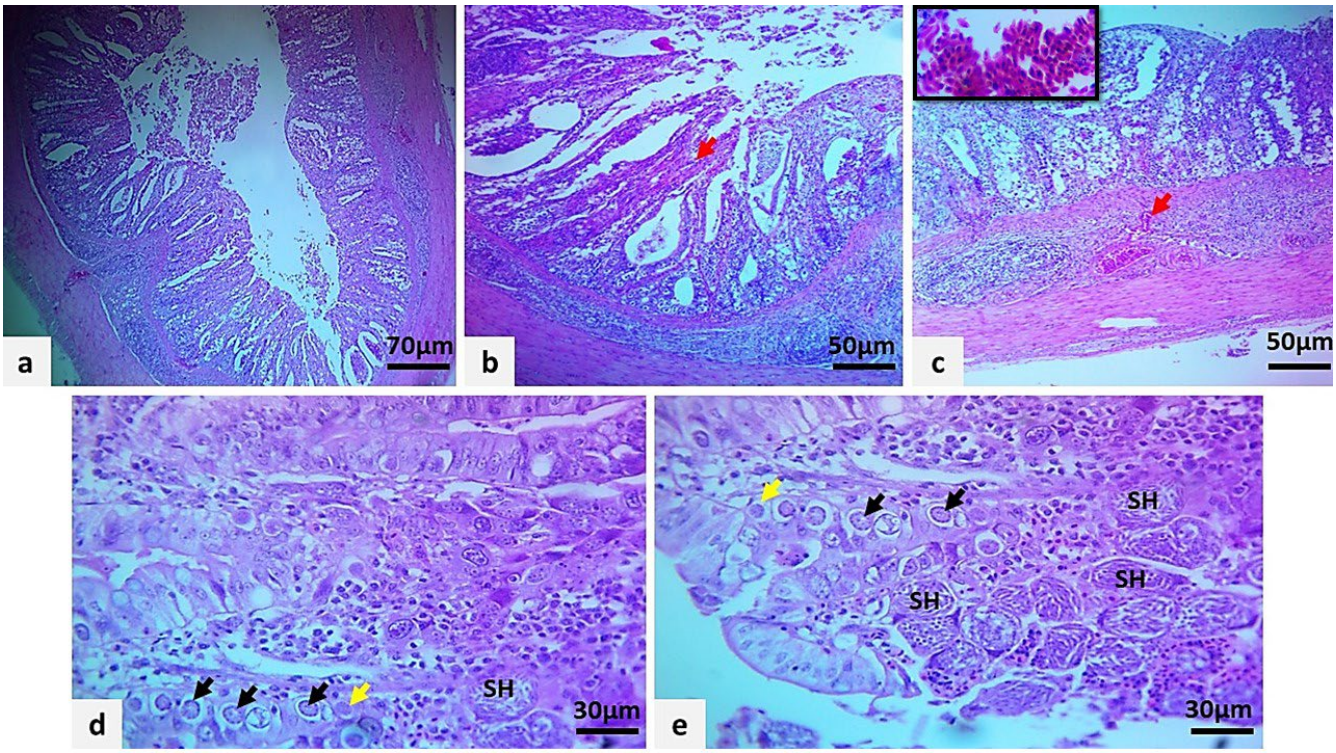

Figure 3: Microscopic sections of the caecum in naturally infected chickens with *Eimeria* species revealed; a-c: Severe sloughing of the lining mucosa with necrotic debris in the lumen, atrophy of the mucosal folds, and the crypts with transmural inflammatory cell infiltration with marked hemorrhage (red arrows and inset). d and e: Presence of different stages of *Eimeria,* including oocytes, macrogametes (black arrows), microgametes (yellow arrows), and schizonts (SH) that contain many eosinophilic cerement merozoites in the submucosal layer (H&E stain).

Molecular Finding

This research employed molecular identification techniques to identify *E. tenella*. The findings revealed a clear amplification of the *E. tenella* DNA, specifically targeting a 278- bp region of the ITS-1 marker (Figure 4). The identity was verified through sequencing, and the resulting sequence was deposited in the NCBI Gene Bank with the accession number PP229210.

Discussion

Poultry rearing is currently quite popular and profitable. Most individuals, being non-vegetarians, have a preference for poultry meat and eggs. Coccidiosis significantly impacts commercial chicken production since it can result in global losses of about $3 billion a year (6). There are nine distinct species of Eimeria found in the boiler chicks, *E. acervuline, E. brunetti, E. tenella E. maxima, E. necatrix, E. mivati, E. mitis, E. praecox and E. hagani* (4). Until now, there has been no data on these species among chicks in Sulaimani province. The post-mortem examination revealed that the ceca were greatly enlarged and filled with blood. Hyperaemia and enteritis were present in the intestine.

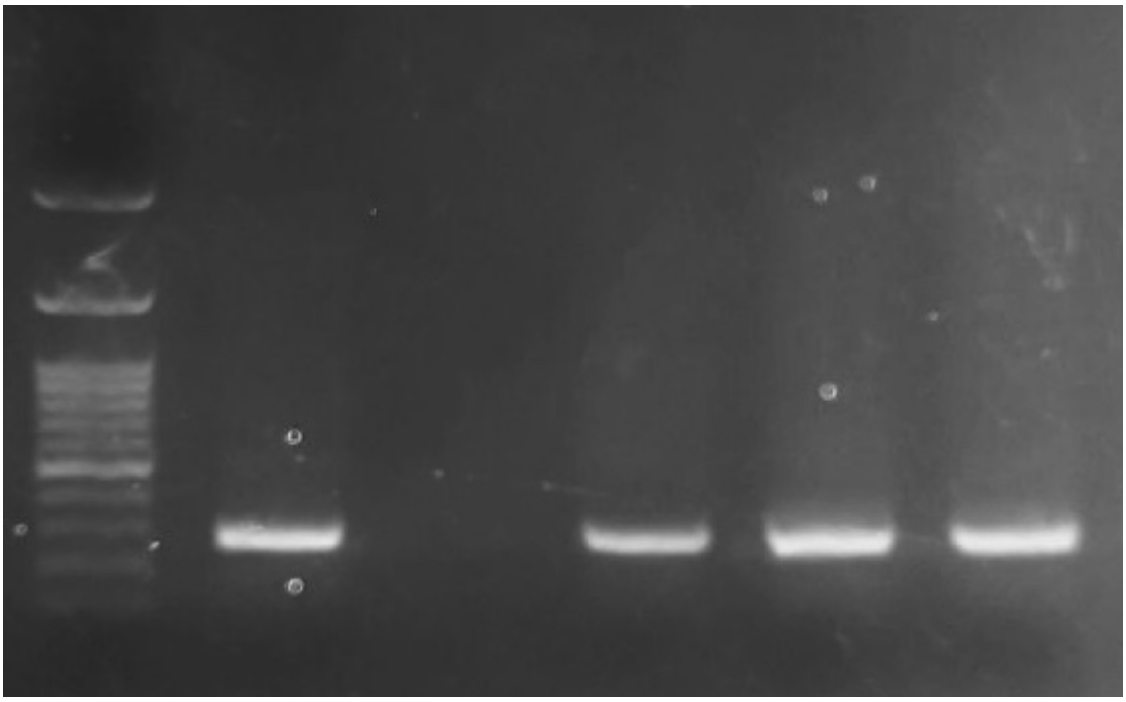

Figure 4: The agarose gel electrophoresis pattern shows PCR amplification of the *E. tenella* ITS-1 gene. Lane L: hyper DNA ladder 100 bp, Lane P: positive control, Lane N: negative control, Lanes 1-3 represent 278 bp of *E. tenella* from pool sample of broiler farms.

These findings are consistent with previous publications (17). Histopathological study revealed extensive sloughing of the mucosal lining epithelium and the shedding of necrotic debris in the lumen and numerous stages of development of *E. tenella*. These outcomes were consistent with the findings of these workers (18). A study of the morphological properties of the oocyst showed that its wall was double, ovoid in shape, and the sizes were (19–24) µm long and (16–20) µm wide. Other workers also described *Eimeria tenella* as having similar characteristics (19).

The molecular analysis outcomes were carried out using conventional PCR, using the amplified segment of the marker (ITS-1) about 278 base pairs of *E. tenella*. In this study, we used a straightforward approach using direct PCR from cecal mucus samples, targeting all stages of parasite development, whether it is dead or alive, bypassing additional procedures typically performed by other researchers for the isolation and sporulation of *E. tenella* (16,20, 21). We used special extraction kits for viruses already present in the molecular laboratory (22). Interestingly, it demonstrated a robust amplification of the ITS-1 marker of the *E. tenella* genome. This finding indicates that a molecular method can directly identify the Eimeria species from a sample, eliminating the need for a special procedure for parasite sporulation.

Conclusion

This is the first report on *E. tenella* infection in broiler farms in Sulaimani province. Our findings showed that *E. tenella* are our region's most abundant pathogenic species. This study suggests that E. tenella could significantly reduce the productivity of broiler chickens.Coccidian infection may be a major factor in the financial losses of chicken farms in this region.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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الدراسات المورفولوجية والجزيئية التحليلية الشاملة حول عدوى الأيميريا تينيلا في افراخ اللحم في محافظة السليمانية، العراق

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الخلاصة

الكوكسيديا مرض طفيلي رئيسي يصيب الطيور الاليفة والديك الرومي والبط والأوز في جميع أنحاء العالم. وهو يسببه طفيليات أولية من شعبة Apicomplexa من جنس *Eimeria* . وبسبب عدم وجود بيانات عن أنواع *Eimeria* في الدجاج في منطقة كردستان فقد ارتئينا لهذه الدراسة. هناك تسعة أنواع مسجلة من *Eimeria* في الدجاج. *E. tenella* هي الكوكسيديا الأكثر شيوعًا في الدواجن والتي تتميز بإصاباتها التي يمكن التعرف عليها بسهولة والخسائر الكبيرة في افراخ اللحم والدجاج البياض.تم فحص افراخ اللحم المصابة بشكل طبيعي بـ *Eimeria tenella* في البحث التي تراوحت أعمارها ما بين 3 و 6 أسابيع. تم أخذ العينات من محتويات الأعور وفحصها بالطرق القياسية مثل التشريح والفحوصات المجهرية. كشف الفحص المجهري عن وجود عدد كبير من أكياس الكوكسيديا في محتوى الأعور. أظهر التحليل النسجي المرضي انسلاخا شديدًا للغشاء المخاطي المبطن مع نزيف ونخر فضلا إلى جانب وجود مراحل مختلفة من الأيميريا بما في ذلك البويضات والأمشاج الكبيرة والأمشاج الصغيرة والانقسامات في الطبقة تحت المخاطية. اظهر صبغ الهيماتوكسيلين والإيوسين إجراء دراسة نسجية مرضية على أنسجة الأعور المصابة. سلطت الدراسة الضوء على مرحلة مهمة تبسط عزل الطفيلي للتشخيص الجزيئي من خلال استخدام عينات الأعور مباشرة لاستخراج الحمض النووي. سمحت هذه الطريقة بإكمال إجراءات التشخيص الجزيئي بكفاءة أكبر بغض النظر عن مرحلة الطفيلي.

الكلمات المفتاحية: افراخ اللحم ؛ الكوكسيديا؛ التشخيص؛ إيميريا تينيلا؛ العدوى.