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Phynotypic and genotypic identification of *Eimeria* species in backyard chicken in Nineveh governorate, Iraq

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Abstract

Coccidiosis is an intestinal disease caused by a parasite of the genus *Eimeria*. This parasite mainly affects poultry species and causes great economic losses in the poultry industry. This study was designed to estimate the prevalence of coccidiosis in the local breed of domestic chicken in Nineveh Governorate, Iraq. 450 faecal swabs and intestinal samples (intestinal scraping) were collected from different local breeds of home-bred chickens from October 2020 to the end of March 2021. All fecal samples were examined using the flotation method by using sugar solution, and *Eimeria* was confirmed by the polymerase chain reaction method. Fecal examination results showed that 32.6% of the total samples were positive for *Eimeria* oocysts, classified into six species including *E. brunetti, E. mitis.E. maxima E. acervulina E. necatrix, E. tenella* with infection rates are 57.5, 44.2, 42.1, 26.5, 20.4, 16.3%, respectively. The phenotypic results were genetically confirmed by the result of the reaction of 455 base pairs. The prevalence of coccidiosis was highest in chickens less than three months of age 49.2% and lowest in chickens older than 6 months 23.9%**.**

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Introduction

There are many parasitic diseases, which affect in backyard poultry which are due to poor management (1). Avian coccidiosis is enteric disease caused by protozoan parasite of the genus *Eimeria* phylum Apicomplex caused significant economic losses in poultry industry worldwide which annually estimated about three billion dollars due to of high incidence of morbidity and mortality lead to low productivity, lack of growth, animal death, costs of treatment and disease control (2).

The disease occurs associated with poor management by ingestion the sporulated oocysts of contaminated food and water (3). The disease clinically characterized by diarrhea develop to bloody diarrhea due to acute intestinal inflammation and, low feed conversion rate, poor growth, low production, and high morbidity and mortality.

In poultry there are seven important species belonges to genus *Eimeria* (*E. brunetti*, *E. maxima*, *E. necatrix*, *E. tenella*, *E. mitis*, *E. acervulina* and *E. praecox*) characterized by high degrees of host and site specificity (4). For example, *E. tenella* consider to be most pathogenic and wide spread in chicken compared with other species in Poultry (5). Phenotypically there are different criteria used previously for identification and characterization of *Eimeria* species such as parasitized intestine zone, gross appearance of the lesion, oocyst morphology, minimum sporulation period, minimum prepatent time, parasite location in the intestinal epithelium, and cross-immunization tests (6,7).

The present study was designed to estimate the prevalence of coccidiosis in local breed of backyard chicken in Nineveh governorate, Iraq using phenotypic (morphological) and genotypic (PCR) methods to identify the species and genus of *Eimeria*.

Materials and methods

Sample collection

A total of 450 samples of fresh fecal swabs (n=390) and intestine contain (intestinal scraping smear) (n=60) were collected randomly from backyard chicken from different locations in Nineveh governorate, Iraq. The study was conducted from October 2020 to March 2021. Fresh fecal samples were collected using spatula and preserved in a clean polypropylene tube. The samples were labelled with the name of farm (owner), location of the farm, date of collection, and health status. Samples were transported to the laboratory of Department of Microbiology, University of Mosul, Mosul, Iraq and kept in 4ºC until further analysis.

Phenotypic (morphological) examination

Microscopic identification of *Eimeria spp* in the present study was based on following criteria: size (length and width µm/µm) and shape of oocyst, presence or absence of micropyle, sporulation time and location of lesion in the gut. The intestine (duodenum, jejunum, ileum, large intestine: caeca) was opened and gross examination of pathological lesions were recorded and sample was scraped. All fecal swabs and intestinal content samples were examined microscopically by simple floatation technique using saturated sugar solution as described by Urquhart *et al.* (8). Of each sample 25 oocytes at least were examined morphometrically and oocyst was measured (length and width µm/µm) using calibrated ocular microscope at X40 magnification (9).

The statistical analysis of oocytes measurements of size (length and width µm/µm) were analyzed using the Ocular Micrometer under the magnification power of 40 x and the true dimensions of the egg sack were found according to the following equation: true egg sac dimensions = measurement of egg sac dimensions in the planned eyepiece × microscope factor (6).

Sporulation of parasite

Oocyst were diluted into 2.5% aqueous potassium dichromate and kept in Petri dishes for sporulation using shaker water bath at 29ºC. The dichromate solution 2.5% was used to provide sufficient moisture and destroyed other bacteria. After sporulation, oocysts were recovered by centrifugation with saturated sugar solution as described by Duszynski and Wilber (10) and used in subsequent analysis. Microscopic examination was performed at various times to determine the sporulation of oocysts (4). The shape (length/width) of the sporulated oocysts were determined by using the method for species identification as described by Hadipour *et al.* (11). The calculated oocysts shape index values were then compared with the standard diagnostic guide provided by Teixeira and Lopes (12) to determine the species encountered in the study.

Nucleic acid extraction

DNA was extracted from oocysts of *Eimeria* spp using QIAamp fast DNA Stool Mini Kit in accordance with the recommended procedure of manufacturer (Qiagen, Hilden, Germany as following. A total of 220 µg of fresh feces was initially suspended in 1 mL inhibit buffer (Qiagen) and incubated for 5 min at 70ºC with vortexed intervals for 15s. The suspension was centrifuged at 900×g for 1 min and 200 µL of supernatant was transferred in to 1.5 mL Eppendorf tubes. Afterwards, proteinase K (15 µL) and 200 AL lysis buffer (Qiagen, Hilden, Germany) were added and the suspension was incubated at 70ºC for 10 min. This was then heated at 70ºC for 10 min, then 200 µL of absolute ethanol was added and vortexed. Final suspension was treated with spin column (Qiagen) following the manufacturer's instructions. The purified DNA was quantified using NanoDrop (Thermo Fisher Scientific, Darmstadt, Germany).

Eimeria-based PCR assay

The DNA was further confirmed by protocol targeting of the 18S rRNA gene using universal PCR primers (Forward primer: CGCGCAAATTACCCAATGAA and revers primer: ATGCCCCCAACTGTCCCTAT) (13) resulting in an amplicon of ~455 base pairs. The gDNA was used as template for PCR amplification. Each 20 µL PCR reaction mixture comprised of 2 µL gDNA, 1 µL of each forward and reverse primer 10 pM, 10 µL Master Mix and 6µL aqua dest. PCR amplification cycles were: initial denaturation at 95ºC for 10 min, followed by 35 cycles of 95ºC for 45s, 65ºC for 10s and 72ºC for 12s, and a final extension at 72ºC for 40s. The amplified PCR products were electrophoresed on 1.5 agarose gel matrix, stained in ethidium bromide 0.5 µg mL^{-1} and visualized at 302 nm on a UV transilluminator using the Geldoc 2000 gel documentation system (BioRad, Munich, Germany).

Results

A total of 147 (32.6%) from 450 fecal and intestinal contain samples were phenotypically positive of *Eimeria spp* oocysts. The highest prevalence rate was recorded March and lowest was in October with significant difference (Table 1). Six different *Eimeria* species were recorded including: *E. tenella* 57.5 %, *E. necatrix* 44.2%, *E. acervulina* 42.1%, *E. maxima* 26.5%, *E. mitis* 20.4% and *E. brunetti* 16.3%, respectively (Table 2). Mixed infection with more than two species of *Eimeria* was recorded. Morphological Characterization of oocysts of sporulated and nonsporulated oocyst was illustrated in (Fiqure1-6). The prevalence rate was higher in chicken of age < 3 month and lower in chicken of age > 6 months (Table 3).

According to the clinical signs the prevalence rate of *Eimeria* oocytes were observed in Backyard chicken was

clinically healthy and no signs of disease were recorded (Table 4). The sporulation time was recorded as following: *E. maxima* after 30 h, *E. mitis* and *E. acervulina* after 18 h and *E. necatrix*, *E. tenella*, *E. brunetti* after 20 h. and the morphometric form of *Eimeria* oocyst (Table 5). The of molecular results revealed that all the samples investigated in the present study showed amplification size 455 bp of genus *Eimeria* which was considered positive PCR reaction (Figures 1-7).

Table 1: Distribution of positive *Eimeria* oocyst in backyard chicken in the present study

Periods	Number	Number (%) of positive
10.2020	85	20 (23.5)
11.2020	75	30 (40)
12.2020	65	17 (26.1)
01.2021	70	16 (22.8)
02.2021	80	29 (36.25)
03.2021	75	35 (46.6)
Total	450	147 (32.6)

Table 2: Percentage of six *Eimeria* species identified backyard chicken in the present study

Species	Number (%) of positive samples
E. tenella	84 (57.5)
E. necatrix	65 (44.2)
E. acervulina	62 (42.2)
E. maxima	39 (26.5)
E. mitis	30 (20.4)
E. brunetti	24 (16.3)

Table 3: Distribution of coccidiosis according to the age of backyard chicken investigated in the present study

Age of chicken	Number (%)	
	examined samples	+ve samples
> 3 months	126	62 (49.2)
3-6 months	119	36 (30.2)
< 6 months	205	49 (23.9)
Total	450	147 (32.6)

Table 4: Prevalence of coccidiosis in backyard chicken according to clinical signs

Eimeria	Oocyst size (µm)	Mean of Width and length (µm)	Sporulation time (h.)	Morphology	Site of infection	Gross lesion
E. tenella	19.57×22.5	17-22× 25.5-20	20	Ovoid, thin smooth wall	Caecum	Hemorrhagic and filled with clotted and unclotted blood
E. necatrix	16.7×19.1	12.5-18 × 22.5-14	20	oblong ovoid, thin wall	Jejunum	Petechial hemorrhage and mucoid
E. acervulina	15.23×19.2	17.9-21.5 × 14.2-16	18	Ovoid, thin smooth wall narrow anterior end	Duodenum	Congestion and thickening of mucosa with whitish transverse lesion
E. brunetti	19.74×23.4	21-28.2 × 18.5-22	20	Ovoid, thick, brown wall	Ileum	Mucoid content and thickening of mucosa
E. maxima	22.3 × 28.5	23-30.5 × 18-24	30	Large ovoid, thick wall, yellow- brown	Jejunum and Ileum	Petechial hemorrhage and thickening of intestinal wall
E. mitis	14.7×15	11.5-17.5× 12.5-18	18	Sub spherical, thin wall	Ileum	Enteritis

Table 5: Morphological and morphometric characterization of *Eimeria* species in backyard chicken

Diarrhea	No. examined	No. (%) positive
Without diarrhea	334	121 (36.22)
With diarrhea	116	26 (22.41)
Total	450	147 (32.6)

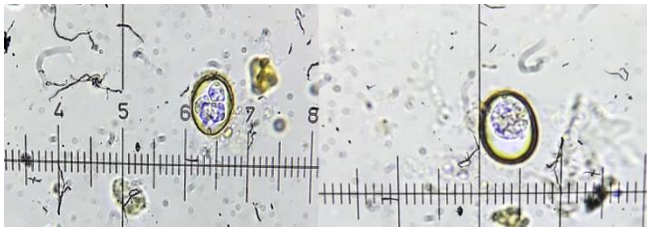

Figure 1: Sporulated and nonsporulated oocyst of *E. tenella* (40X).

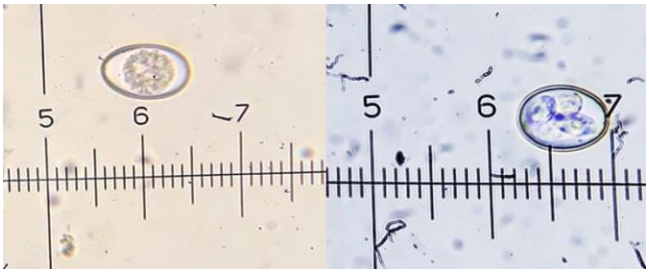

Figure 2: Oocyst of *E. acervulina* (40X).

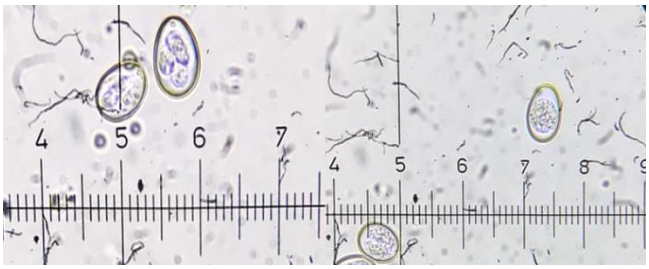

Figure 3: Oocyst of *E. necatrix* (40X).

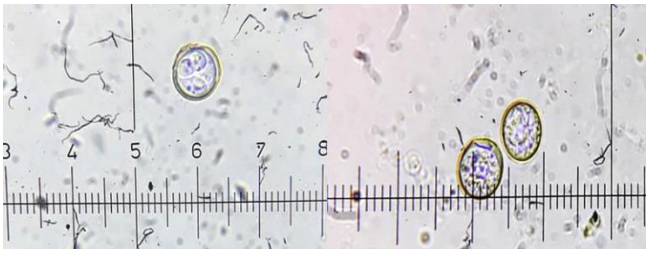

Figure 4: Oocyst of *E. mitis* (40X).

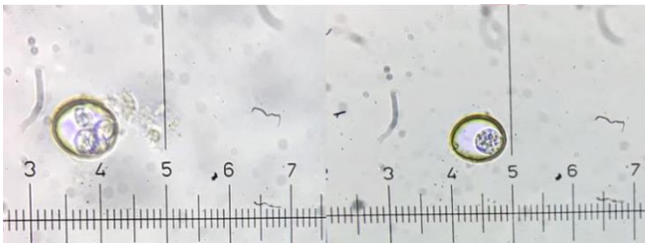

Figure 5: Oocyst of *E. brunetti* (40X).

Figure 6: Oocyst of *E. maxima* (40X).

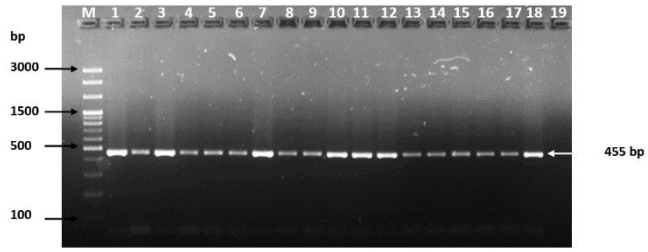

Figure 7: Agarose gel electrophoresis of 18S rRNA gene of genus *Eimeria* showing amplification product size ca. 455 bp. M: Marker 100 bp. Wells 1-18 are positive samples, well 19 is negative control.

Discussion

In present study the prevalence of coccidiosis in local breed of backyard chicken was 32.6 %, the result was agreement with Kabouudi *et al.* (14) and Nnadi and George (15) were recorded 31.8% and 35.5% of coccidiosis in local breed chicken in Tunisia and Nigeria (11), respectively. In Turkey (16), in Ethiopia and in Egypt (17) were recorded the coccidiosis in 54.3%, 64%, 53.6% and 21.24% in chicken. These differences in prevalence rates could be attributed to the conditions of breeding, the study area, the efforts made to control the disease, the different climatic and environmental conditions from one region to another, the season of sample collection during the year, in addition to the difference in the number of samples examined and the ages of the chickens examined (18). Diarrhea is a major clinical sign of coccidiosis in the chicken (19) and it is worth mentioning the prevalence of subclinical coccidiosis in backyard chicken in the present study was significantly higher than clinical coccidiosis which considered as carrier of oocyst without showing any signs of disease. Previous studies also mentioned that subclinical coccidiosis in local breed chicken are most prevalent and acting a source of infection and also effect on production performance of chicken (20). This attributed to repeated exposure to different species of *Eimeria* and development of immunity against the parasite. These birds are become a source of oocyst fecal shedding and lead to contaminate of surrounding farm environment (e.g. housing surfaces, pasture, water, feed, soil, and more others). In the present study six *Eimeria* species were identified and this species were recorded also previously (21). The most prevalent species was *E. tenella* 57.5% which agreement with other previous studies (5,15,22). But other previously study reported that *E. acervulina*, and *E. tenella* are most prevalent species (23). In the present study mixed infection with more than two *Eimeria* species was observed in local breed chicken as reported previously (24). In the present study, the age of local breed of chicken was studied as one

of the main factors in the incidence and spread of coccidiosis in poultry, but *Eimeria* species can be infected all live of bird in different ages (25). The higher prevalence of coccidiosis in young chicken 0-3 months compare with chicken >6 months this result was agreement with Wondimu *et al.* (26) who reported high incidence of coccidiosis in young bird. Highest prevalence rate of infection recorded in March while lowest infection rate was in October. This may be attributed to the relative moderation of temperatures in this month, with the presence of humidity and rainfall that provides adequate conditions for the sporulation of *Eimeria* oocyst and occurrence of infection. The morphology of oocyst and sporulation time and gross lesion may help in identification of *Eimeria* species in present study oocyst length, width and morphology were an indicative for *Eimeria* species identification. Most of *Eimeria* oocysts were identified in ovoid shape which agreement with Soulsby (9) which reported the length and width of *E. tenella* was 19.5-26 and 16.5-22.8 µm (20,26) were reported that length and width *E. acervulina* 15.7-20.3 and 14.5- 18.6 µm and *E. maxima* 27.9-34.5 and 18.6-26.4 µm and *E. brunetti* 20.6-26.3 and 16.9-21.6 µm.

Conclusion

This study demonstration the *Eimeria* species parasite in digestive system of backyard chicken by using microscopy and nested PCR.

Acknowledgments

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Conflicts of Interest

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

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تحديد النمط المظهري والوراثي لأنواع الايميريا في دجاج التربية المنزلية في محافظة نينوى، العراق

عدنان محمد أحمد و هيثم صديق البكري

فرع الأحياء المجهرية، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

يعد داء الكوكسيديا مرضا معويا يسببه طفيلي يعود لجنس الايميريا ويصيب هذا الطفيلي بشكل رئيسي أنواع الدواجن ويتسبب في خسائر اقتصادية كبيرة في صناعة الدواجن. صممت هذه الدراسة لتقدير نسبة انتشار الكوكسيديا في السلالة المحلية من الدجاج المنزلي في محافظة نينوى، العراق. إذ تم جمع ٤٥٠ مسحة برازيه وعينة معوية (كشط معوي) من سلالات محلية مختلفة من دجاج التربية المنزلية من شهر تشرين الأول ٢٠٢٠ إلى نهاية شهر اذار ٢٠٢١. تم فحص جميع عينات البراز باستخدام طريقة التطويف بالمحلول السكري وتم تأكيد الإصابة بجنس الايميريا باستخدام طريقة تفاعل السلسلة المتبلمر. أظهرت نتائج فحص البراز أن ٣٢,٦٪ من مجموع العينات كانت موجبة بأكياس بيض الايميريا، مصنفة إلى ستة أنواع تشمل *E. brunetti E. mitis.E. maxima E. acervulina E. necatrix, E. tenella* وبنسب إصابة ٥٧,٥، ٤٤,٢، ٤٢,١، ٢٦,٥، ٢٠,٤، ١٦,٣٪ على التوالي. تم تأكيد النتائج المظهرية وراثيا وبواقع ناتج تفاعل٤٥٥ زوجا قاعدياً. كانت نسبة انتشار الكوكسيديا الأعلى في الدجاج الذي بعمر أقل من ٣ أشهر ٤٩,٢٪ وأقل نسبة في الدجاج الأكبر من ٦ أشهر ٢٣,٩٪.