Research Article

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Evaluation of Different Staining Techniques for Detection of Cryptosporidium Oocysts in Backyard Chickens (*Gallus gallus domesticus*)

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Abstract

The oocysts of *Cryptosporidium* spp. were investigated in backyard chickens, and study the effect of age and sex on the infection rate. All samples taken from feces and intestinal contents were examined microscopically by staining methods, including modified acid-fast stain, Malachite Green Stain at a concentration of 5%, 1%, Nigrosin Stain (1%), and Safranin methylene blue staining. The overall infection rate was 48.7%. The oocyst appeared in an oval to spherical shape with a bright red color in the modified acid-fast stain and without stain with a green background in malachite green stain 5% and light malachite green 1%. The oocyst appeared brighter while in an orange color when using a safranin methylene blue stain, which found the highest infection rate (48.7%). Its efficiency reached 100%, Nigrosin stain (47%), malachite green stain (45.2%), and safranin blue stain (43.5%), while the lowest infection rate was in the light malachite green stain (30.4%). The oocysts were divided into two types of cysts depending on the size of the cysts (large and small cysts) and the wall thickness (thick and thin-walled cysts). The highest infection rate was in young ages less than three months (60.8%), while in adults, the infection rate was 29.2%. No significant difference was recorded between the infection rate in females and males,

Keywords: *Cryptosporidium* spp, backyard chicken, modified acid-fast stain, Malachite Green Stain.

Introduction

Cryptosporidiosis is a parasitic disease infecting humans and animals worldwide (1). Cryptosporidium is a complex protozoon that infects mammals, birds, fish, and reptiles (2). Many researchers have diagnosed the parasite in various bird species, including chickens, Cone of the most important bird species that can be infected with Cryptosporidiosis, and it is the source of infection for other birds and humans (3,4). Chicken droppings are also an important source of contamination of soil, grasses, pastures, and drinking water (5,6).

Cryptosporidium in chicken is characterized by infection of the digestive and respiratory systems (7). The main features of intestinal infection are green or yellow diarrhea with an unpleasant smell and containing mucus. At the same time, respiratory Cryptosporidiosis shows several signs, such as sneezing, mucus secretions, and difficulty breathing (8,9). There are two routes for ofransmitting Cryptosporidium spp transmission, including indirect transmission. тhis type of transmission results from cross-contamination of food, and water, or environmental contamination like soil and water. Direct transmission *Cryptosporidium* Oocysts released with feces are transmitted by accidental infection through the fecal-oral route. The incidence of transmission increases following heavy rain and floods (10,11). *Cryptosporidium* spp has been diagnosed in more than 30 bird species worldwide (12,13) and is an important pathogen with a high prevalence rate in livestock. And wildlife Species of the parasite that infect birds, including poultry: *Cryptosporidium galli*, which affects the real stomach (proventriculus) of chickens and birds; *Cryptosporidium meleagridis, Cryptosporidium baileyi,* and *C. aviam,* (14) Three species of *Cryptosporidium* have been identified in birds, ranging in size from large, medium, small size, such as *Cryptosporidium galli Cryptosporidium baileyi* and *Cryptosporidium meleagridis* (15). In Mosul, Iraq, the cryptosporidiosis parasite was recorded in pigeons with the species *C. baileyi* 30% (16),*C. Baileyi was recorded in the backyard, and broiler chickens, ducks, pigeons, and quail, C. galli, were* also diagnosed in domestic chickens (17). Finding Cryptosporidium depends on finding oocysts in feces. This can be done in several ways, including flotation with Sheather's sugar, sedimentation, Ziehl-Neelsen, malachite green, and harmful staining methods with nigrosin and carbo-fuchsin. You can also use immunological tests and molecular methods like polymerase chain reaction (PCR) to determine the genotype of Cryptosporidium (18). Due to the low number of diagnostic studies on this parasite in poultry and the high death rate in poultry farms, this study aimed to find the Cryptosporidium parasite in backyard chickens and examine how age and sex affect the parasite. This was done by using different staining techniques to find Cryptosporidium oocysts in the feces of backyard chickens and then judging how well these methods work for diagnosing the parasite.

Material and methods

Collection of samples

Eighty fresh feces were taken directly from the cloaca, and 35 samples were taken from

the intestines of chickens killed in different parts of Nineveh Governorate. The chickens were of different ages and genders, and they were kept in clean containers with the date, age, and gender of the animal written on them. The samples were then sent to the College of Veterinary Medicine parasitology lab. The samples were preserved using a 2.5% potassium dichromate solution when handling them (19).

Microscopic examination

Several staining techniques have been used to detect parasite oocyst:

a-Modified Ziehl-Neelsin: The stain was prepared according to the method [20)Where it was placed A drop sediment of fecal sample added to a glass slide, spread out, and allowed to dry. Fixed with alcohol methanol. Then, it was stained with basic carbol fuchsin for 3 minutes. Wash with water. Added acidic alcohol and washed with water. by adding methylene blue, washed in water, and air-dried. Finally, the slide was examined under a light microscope with a 100× lens) (21) .

b-Light Malachite green stain (1%): This stain consists of mixing (1 gram) of the Stain in (100 ml) of water. A drop of the stool sample sediment is added and mixed on a glass slide with a drop of L.M.G.S. 1%, evenly spread over all the slides, and air-dried. The slide was examined under an X100 oil immersion lens (22).

c-Malachite Green Stain (5%) This stain consists of mixing (5g) of the Stain in (100ml) of water, done by adding a drop sediment of a fecal sample (mixed with a drop of M.G.S. 5% and spreading on the slide the smear examined under 100X oil immersion lens (22).

d-Nigrosin Stain (1%): Prepared by dissolving (1g) of Nigrosin stain in (100ml) distilled water, then added (0.05ml) of formalin and mixed well and done by adding one drop sediment of the fecal sample mixed with a drop of Nigrosin Stain 1%, then spread and air-dried. Followed and examined under a 100 X lens (22).

e-Safranin methylene blue staining: the staining method was prepared by (23)a drop from the sediment of stool sample spread on the slide, dried by a flame. And fixed in acid alcohol. Then, the smears were stain with 1% safranin solution for 60 s. Then, rinse with water and add 1% methylene blue for 30 S., rinse by adding 1% methylene blue for 30 S., rinse with water, and dry dry, followed by examination under 100 X lens.

Measurement of *Cryptosporidium* oocysts: An ocular micrometer was used to measure the dimensions 25 of *Cryptosporidium* oocysts (24).

Statistical analysis

The results were compared using the chi-square test (25).

Ethical approval

the work followed the official guidelines of the University of Mosul, College of Veterinary Medicine, Iraq, with UM.VET, 2024, 032.

Results

A total of 80 backyard chickens' fecal samples and 35 intestinal contents were diagnosed by modified zeal-Nielsen Stain, Safranin methylene blue stain, Nigrosin

stain, Malachite green stain 5% and light Malachite green stain (1%) To know the infection rate of *Cryptosporidium* spp and compare the diagnostic efficiency of this stain. The total infection rate with *Cryptosporidium* spp of backyard chicken in Nineveh governorate reached 56 (48.7%) Table. The *Cryptosporidium* oocysts appeared different in shape, size, and color by using different stains such as (Modified Ziehl-Neelsen Stain, Malachite Green Stain 5%, Nigrosin Stain%1, Safranin methylene blue staining, and light Malachite green stain 1%. The oocysts were more oval, round or spherical shapes with pink color on a blue ground in Modified Ziehl-Neelsen Stain as in (fig.1) The oocysts were shown unstained with green background in Malachite Green Stain (fig.2) and L.M.G.S. while in Nigrosin Stain appeared more glowed and brighter; on black back as in(fig.3), while in Safranin methylene blue staining the oocysts were shown stained with orange-pink color(fig.4,5). Results have shown different rates of *Cryptosporidium* oocysts with different staining methods. The highest infection rate was recorded in stains modified Ziehl-Neelsen stain 56(48.7%). The efficiency of this method was 100%. And the lowest infection rate in light Malachite green stain is 1% (30.4). The efficiency of this method has reached 62.4%, as in Table 2.

During the microscopic examination, large and small oocyst species of *Cryptosporidium* parasite were detected using ocular lens measurement (length x width) for 25 oocysts. These species showed variable-sized oocyst (µm). The parasitic oocysts are spherical or oval, surrounded by a single wall. The oocysts also contain a notch at one of its poles, representing where the spores exit. A transparent haloA transparent halo surrounds the oocyst surrounds the oocyst and contains different-sized grains of unclear features; two types of oocysts were distinguished according to the wall thickness: thick-wall oocyst, which were the most common, and thin-wall oocyst, which were the least common (fig.6) table 3. The results of the total sample showed that female and male backyard chickens recorded (49.3%-47.5%,) There is no difference at the level (P<0.05) Table 4.

Young backyard chicken (< 3 Months) recorded a high infection rate of 31(60.8%)and lowest infection rate was 7(29.2%) recorded in the adult with significant differences (P<0.05) table 5.

Table 1: total infection rates with *Cryptosporidium* oocyst in backyard chicken by Ziehl-Neelsen stain.

Samples	Examined No.	Infected No.	infection rate%
fecal sample	80	36	45[a]
intestinal contents	35	20	57.1[b]
Total	115	56	48.7%

various letters indicate difference at(P<0.05).

Table 2: Comparison of staining methods and estimation of the diagnostic efficiency of each stain

Types of methods STAIN	Infected No.	infection rate%	Efficiency rate%
Modified Ziehl-Neelsen Stain,	56	48.7	100
Nigrosin Stain	54	47	96.5
Malachite green stain 5%	52	45.2	92.8
Safranin methylene blue staining.	50	43.5	89.3
Light Malachite Green Stain (1%)	35	30.4	62.4

Table 3: The Size and morphology of *cryptosporidium* oocysts in backyard chicken (25 oocysts measured)

Size of oocyst	Average of length± S E.	Average of width± S E	Morphology	Wall
Larger oocysts	7.6± 0.620	6.1± 0.780	spherical or oval	Single wall (thick or thin wall)
Smaller oocyst	4.8± 0.512	4.05± 0.06		

Table 4: Percentage *of Cryptosporidium* Infection of backyard chicken according to the sex by Modified Ziehl-Neelsen stain

Sex	Examined No.	Infected No.	Infection rate%
Female	75	37	49.3[a]
Male	40	19	47.5[a]
Total	115	56	48.7

Letters indicate no significant (P<0.05).

Table 5: Percentage *of Cryptosporidium* Infection of backyard chicken according to the age by Modified Ziehl-Neelsen stain

age	Examined No	Infected No.	Infection rate%
< 3 Months	51	31	60.8[a]
3-6	40	18	45[b]
> 6 Months	24	7	29.2[c]
total	115	56	48.7

Different letters indicate different (P<0.05)

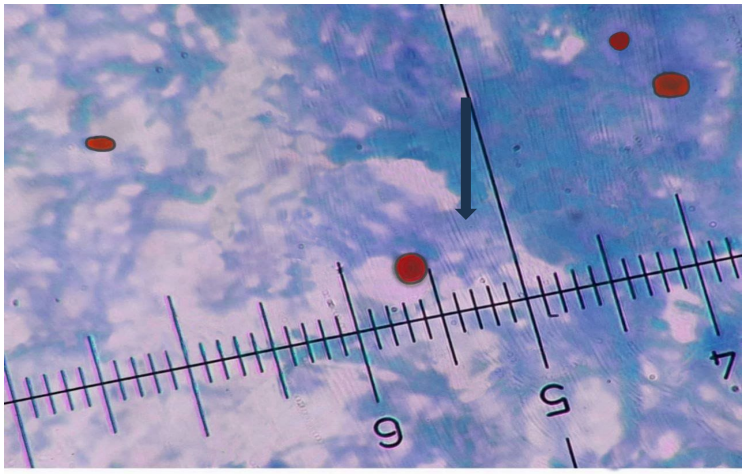

Figure 1: *Cryptosporidium* oocysts stained by M.Z.N.S (X1000).

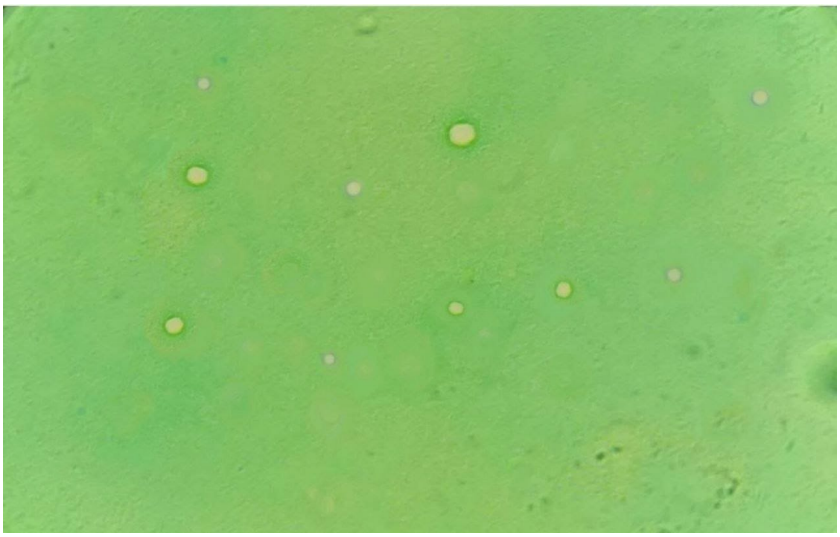

Figure 2: *Cryptosporidium* oocysts in Malachite Green stain 5%,1000x

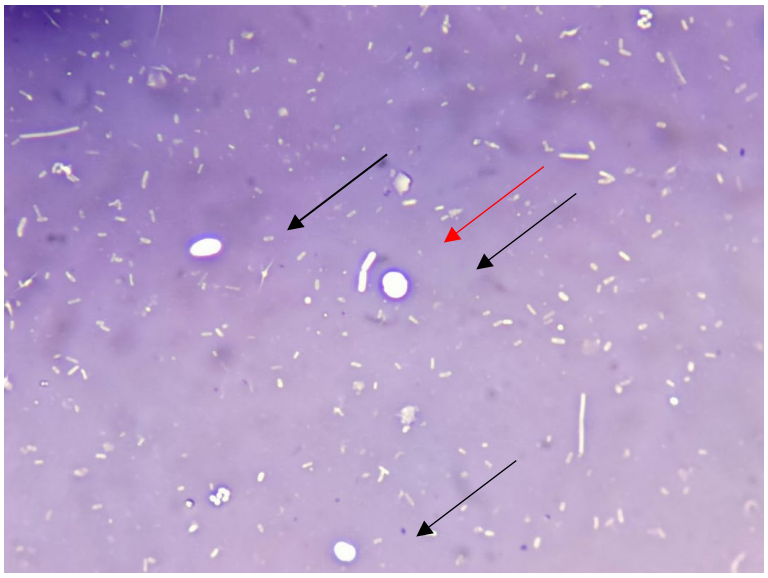

Figure3: spherical and oval *Cryptosporidium* oocysts by Nigrosin stain) Black Arrow) , bacteria (red arrow) 1000x

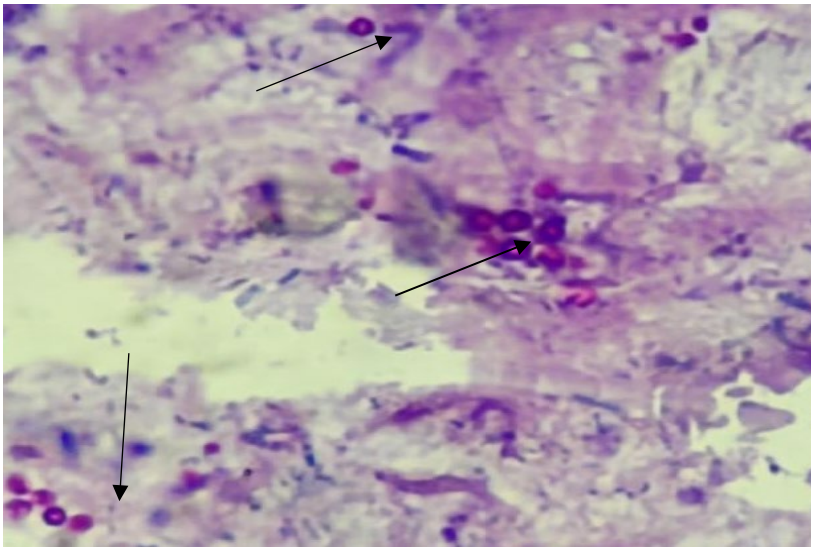

Figure 4: *Cryptosporidium* oocysts in Safranin methylene blue staining 1000x

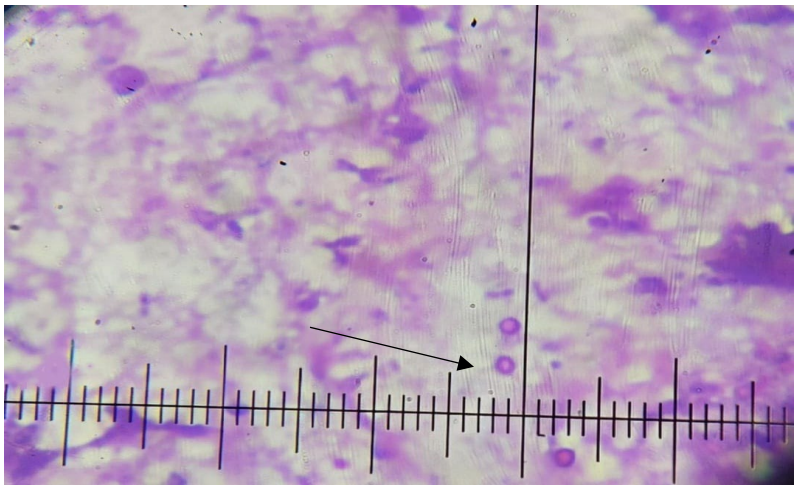

Figure 5: *Cryptosporidium* oocysts by Safranin methylene blue staining under the ocular micrometer 1000x

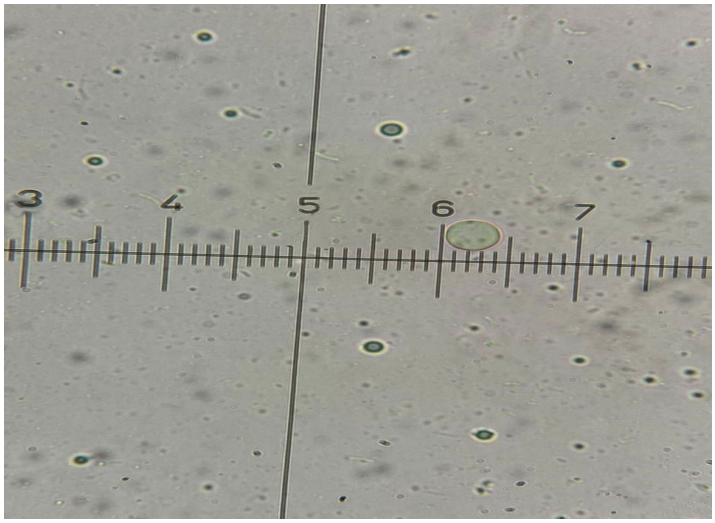

Figure 6: The Size and morphology of *cryptosporidium* oocysts by Malachite Green stain 1%,1000x

Discussion

Cryptosporidium spp is a prevalent parasite in most hosts, including chickens, causing economic losses of weight and egg production and the death of infected chickens (26). chickens are a source of infection for humans and animals, especially after recording infections without clinical signs (27). results of current work by microscopic examination explained infection rate in backyard chickens 56 (48.7%) the results approached with the results of (28) in chickens when they recorded a rate of infection reached 51.4%,46,6%, and 40% respectively. result higher than the study recorded by researchers (29,30,31) with an infection rate in chickens of 11.9%,20.7%, and 38%, respectively, the variance of these infection rates is happening to depend on the effect of different factors such as temperature, humidity, age, months of study, health conditions of the host, methods of animal husbandry and management, and immune status of the animal, and the difference in the number of samples (32). The study showed a significant variation in detecting oocysts using different staining methods. More Cryptosporidium oocystwere detected that were stained by Modified Ziehl-Neelsen stain 56(48.7%). These results revealed that this stain detected all positive samples. This result agreed with (33), who recorded that MZN stain is the best technique because it easily detects the oocysts by counter color, so it is considered a more sensitive and specific stain for the *Cryptosporidium* parasite. All these opinions confirm that the best common technique for detecting *Cryptosporidium* oocyst is the Modified Ziehl-Neelsen stain. The results of the microscopic examination of this study agreed with (34) the modified Ziehl-Nelseen stain, which is considered a more sensitive and specific stain for *Cryptosporidium* parasite., from Saffranin methylene blue stain. The Nigrosin Stain used in this study was second in Iraq and second in the world for the detection and identification of *Cryptosporidium* spp. oocysts. The study revealed that Nigrosin Stain is the second important used for detecting Cryptosporidium oocysts (47 %), due to being very quick and ,and the oocysts appear clear and brighter against the dark black background. However, it is considered less sensitive due to some of the difficulties in diagnosis. The oocysts and yeast remain unstained, but the yeast has shown abnormal size and irregular shape. This finding agreed with(22) that showed similarly; the negative staining with Nigrosin also results in yeasts remaining unstained while the lowest rate was recorded in L.M.G.S (30.4%) while Rekha et al. (22), revealed the important stains of detection of Cryptosporidium are N.S, M.G.S., and L.M.G.S. respectively.

Parasite oocysts in positive samples were circular or oval in shape, and the measurement of oocysts in similar studies with the results recorded by (35). When conducting statistical analysis, no significant difference was observed in the infection rate between males and females .Results of this study were consistent with (36) recorded no significant difference between females and males as well as disagreement with experiences (37). The study indicated that chicken age groups showed a significant difference, with the highest rate recorded in

>3 months 31(60.8%) and the lowest rate at age > 6 Months 7(29.2%). This result is not in agreement with (37), which recorded the lowest infection at the age of two weeks, while This result agrees with (38), which recorded the highest infection rate at the age of (1-2) weeks. However, agreement was reached with (39), who found that young quail had a greater infection rate of 44.2% (19/43) while adult quails had a lower infection rate of 17.2% (24/137). Also revealed that young animals were more susceptible to infection than adult animals, and the infection rate decreased significantly with the increase of host age.

Conclusions

The present study highlights the infection with the parasite *Cryptosporidium* and its importance in backyard chickens. The study included some risk factors, which makes it possible to establish a close relationship between the presence of cryptosporidiosis and other parameters, namely age and sex. A comparison between the staining methods used to diagnose the parasite and the diagnostic efficiency of each stain was estimated. The Modified Ziehl-Neelsen Stain was found to be more efficient. Two types of cysts were identified based on size and wall thickness. Given the economic losses caused by the parasite, routine checks, especially routine checks, especially on chicken farms. Chicken workers: technicians, breeders, and veterinarians must handle the parasite cautiously.

Acknowledgment

This work was done at the University of Mosul, College of Veterinary Medicine.

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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تقييم تقنيات التصبيغ المختلفة للكشف عن أكياس بيض الابواغ الخبيئه في الدجاج المحلي

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الخلاصة

تم دراسة اكياس بيض الطفيلي .*Cryptosporidium* spp في الدجاج المنزلي ودراسة تأثير العمر والجنس على معدل الإصابة. تم فحص جميع العينات المأخوذة من البراز ومحتويات الأمعاء مجهريًا بطرق التلوين بما في ذلك صبغة الصامده للحامض المحورة وصبغة الملكايت الأخضر بتركيز 5٪ و1٪ وصبغة نيكروسين (1٪) وصبغة السفرانين المثيلين الزرقاء. كان معدل الإصابة الإجمالي 48.7٪. ظهرت أكياس البيض بشكل بيضاوي إلى كروي بلون أحمر ساطع في الصبغة الصامدة للحامض المحورة وبدون صبغة مع خلفية خضراء في صبغة الملكايت الأخضر 5٪ والملكايت الأخضر الفاتح 1٪. ظهرت أكياس البيض أكثر إشراقًا، بينما كانت بلون برتقالي عند استخدام صبغة السفرانين المثيلين الزرقاء. وقد وجد أعلى معدل إصابة في الصبغة الصامدة للحامض المحورة (48.7%)، وبلغت كفاءتها 100%، وصبغة النيكروسين (47%)، وصبغة الملكايت الأخضر (45.2%)، وصبغة السفرانين المثليين الزرقاء (43.5%)، بينما كانت أقل نسبة إصابة في صبغة الملكايت الأخضر الفاتح (30.4%). وقد قسمت الأكياس إلى نوعين من الأكياس تبعاً لحجم الأكياس (أكياس كبيرة وأكياس صغيرة) وعلى سمك الجدار (أكياس سميكة الجدران وأكياس رقيقة الجدران). وكانت أعلى نسبة إصابة في الأعمار الصغيرة أقل من ثلاثة أشهر (60.8%)، أما في البالغين فكانت نسبة الإصابة 29.2%، ولم يسجل فرق معنوي بين نسبة الإصابة في الإناث والذكور،

الكلمات المفتاحية :داء الابواغ الخبيئة، الدجاج المحلي، الصبغه الصامدة للحامض المحورة، صبغه الملكايت الخضراء .