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High Prevalence of *Cryptosporidium meleagridis* in Domestic Pigeons (*Columba livia domestica*) Raises a Prospect of Zoonotic Transmission in Babylon Province, Iraq

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ABSTRACT

Cryptosporidium is one of the most common protozoan's parasites with remarkable infectivity of a wide range of animals, including mammals and birds. Domestic pigeons (*Columba livia domestica*) act as a potential reservoir for several species of *Cryptosporidium* because they live in close proximity to humans. This study was conducted to assess the genetic diversity of *Cryptosporidium* in domestic pigeons in Iraq. A total of one hundred samples obtained from feces of domestic pigeons in Babylon province were included. After being exposed to microbial examination, all fecal samples were subsequently screened by nested polymerase chain reaction (PCR) for the possible recognition of *Cryptosporidium* species. Microscopy tests detected only 14/100 (14%) of infection with *Cryptosporidium*, while molecular tests detected 21/100 (21%) of the same targeted parasite. Sequencing experiments showed a high prevalence of *C. meleagridis* with 13/21 (61.90%), followed by *C. baileyi* with 7/21 (33.33%), while only one infection was detected with *C. hominis* (1/21) (4.76%). No co-infection with mixed *Cryptosporidium* spp. was observed, and sex factor was not found to affect the infection rate. In conclusion, this study informed a high prevalence of *C. meleagridis* in domestic pigeons than both *C. baileyi* and *C. hominis*, respectively, signifying a higher zoonotic potential of *C. meleagridis* between domestic pigeons and their handlers. This finding may raise more questions with regard to the increasing infectivity of *C. meleagridis* in human. This is the first important screening study in Iraq that uses molecular methods for the detection of *Cryptosporidium* in domesticated pigeons.

Keywords: pigeons, *Cryptosporidium*, zoonosis, detection, nested PCR

INTRODUCTION

Cryptosporidiosis is a pathological condition induced by infection with the protozoan *Cryptosporidium*, which occurs in many classes of birds, whether being domestic or wild worldwide (1). *Cryptosporidium* is considered an emerging pathogen and is currently one of the most prevalent parasites infecting a wide range of birds (2). Avian cryptosporidiosis is mainly caused as a result of infection with four species of *Cryptosporidium*, including *C. meleagridis* (3), *C. baileyi* (4), *C. galli* (5), and *C. avium* (6). In addition to these species, thirteen genotypes of *Cryptosporidium* have been identified in many birds (7). However, the infection with *Cryptosporidium* spp. may

affect the digestive or respiratory tract of birds; and has increasingly been reported in many species of birds such as Java sparrows (8), canaries (9), psittacines (10), companion birds (11), and several other birds (12).

Among these birds, domestic pigeons may deserve more attention due to their wide distribution in cities. In addition to that, the domestic pigeons usually found in urban environments living side by side with man and other animals. These pigeons can transmit microorganisms to human, signifying a medical concern in spreading some zoonosis to human as well as being a reservoir for several parasitic diseases (13). People who own domestic pigeons whether as a hobby, food source, or for experimental purposes are at a higher risk to be infected with cryptosporidiosis (14). Furthermore, various studies have recognized a noticeable occurrence of *C. parvum* and *C. hominis* in pigeons, which are reported to be involved in human pathogenesis (15). This evidence may signify the possibility of zoonotic transmission induced by several *Cryptosporidium* species via pigeons (16). However, the infections with several species of *Cryptosporidium* have increasingly been reported in pigeons in several portions around the world, such as Iran, Thailand, China, and Brazil (17- 20).

The growing evidence of *Cryptosporidium* spp. in pigeons that share the same ecology with the human population underscores the importance of a need for continual world-wide surveillance of these birds to improve knowledge of their potential implication in the epidemiology of human cryptosporidiosis (21). To the best of our knowledge, no molecular-based studies have been conducted in Iraq to detect the occurrence of *Cryptosporidium* spp., in domestic pigeons and only a preliminary description of microscopy-based diagnosis has been reported in pigeons (22) and other study reported in chicken (23). Therefore, this study aimed to use the molecular-based techniques for assessing the potential of domestic pigeons to harbor *Cryptosporidium* spp. in Iraq.

MATERIALS AND METHODS

The study was conducted in six breeding areas from Babylon province in Iraq. Fresh stool specimens were collected from 100 caged pigeons, including 43 males and 57 females of different ages raised by six pigeon breeders within Babylon province. The collection period was lasted to up10 months, starting from January to October 2019. Samples were collected after isolation of each pigeon in a single clean cage, immediately after dropping. Each sample was preserved in a disposable, sterilized, and labeled container stored in a cool box until being processed. Part of each sample was screened microscopically for the presence of *Cryptosporidium* oocysts using a modified-Ziehl-Neelson staining method (24). Stained slides were examined by microscope under oil immersion lens ×100 for the detection of the protozoan oocysts.

Genomic DNA Extraction

The genomic DNA of all fecal samples was extracted from the stool of domestic pigeons according to the instructions recommended by manufacturers (PrestoTMgDNA extraction kit, STLD100, Geneaid, Taiwan). The validity of the extracted genomic DNA was evaluated using a Nanodrop (BioDrop µL ITE, BioDrop Co., UK). The isolated DNA samples were used as templates for PCR.

Nested Polymerase Chain Reaction (nested PCR)

Two sets of PCR primers pairs were utilized in this study. The outer primer set, CPr I; 5′-AAACCCCTTTACAAGTATCAATTGGA-3′ and CPr II; 5′-TTCCTATGTCTGGACCTGGTGAGTT-3′, was used in the first round of PCR, which was covered 676 bp of the small ribosomal subunit of *Cryptosporidium*. The inner set of primers, CPr III; 5′- TGCTTAAAGCAGGCATATGCCTTGAA-3′ and CPr IV; 5′-AACCTCCAATCTCTAGTTGGCATAGT-3′, was utilized in the second round of PCR to cover only 285 bp within the same amplified ribosomal locus (25). The lyophilized primers were purchased from Bioneer Company.

The PCR reaction of primers was performed using AccuPower PCR premix (Cat # K -2012, Bioneer, Daejeon, South Korea). Each 20 µL PCR premix was contained 1 U of top DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in PCR thermocycler (Mastercycler-nexus, Eppendrof, Hamburg). The amplification was begun by initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min, and the final step was extension at 72 °C for 10 min. Amplification was confirmed by electrophoresis on an ethidium bromide (0.1 mg/mL) pre stained 1.5% agarose gel in 1× TBE (2 mM of EDTA, 90 mM of Tris Borate, pH 8.3) buffer.

DNA Sequencing

The observed PCR products from both ends were sequenced (Macrogen Inc. Geumchen, Seol, South Korea). The reference databases of the referring rRNA sequences of *Cryptosporidium* were retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov). The sequencing results of PCR products were edited, aligned, and analyzed as long as with respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNA STAR, Madison, WI, USA). Sequence variations chromatograms were visualized using SnapGene Viewer Ver. 4.0.4, (http://www.snapgene.com). The witnessed *Cryptosporidium* variants were deposited in NCBI under accession numbers **MN729293 - MN729299, MN729300**

- MN729312, and **MN729313** to represent *C. baileyi, C. meleagridis*, and *C. hominis*, respectively.

Phylogenetic Analysis

A specific comprehensive *Cryptosporidium* tree was constructed in this study according to the protocol described by Al-Shuhaib *et al.* (26). The observed protozoal variants were compared with their neighbor homologous reference sequences using the NCBI-BLAST server (27). Next, the blast results of the observed variants were combined and aligned together using a Clustal Omega based tools (28). A full inclusive tree, including the observed variants, was visualized as a polar cladogram using iTOL (Interactive Tree of Life) tool (29). The nucleic acid sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

Statistical Analysis

The computer software, SPSS Version 23.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to verify the association between the presence and absence of this parasite with each of the studied variables (diagnostic methods and the sex) depending on Chi-square test. The variable database was created with Microsoft Office Excel 2016. Differences were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

All fecal samples of domestic pigeons were being submitted to two types of tests to assess the pattern of *Cryptosporidium* spp. prevalence within Babylon province: a classical microscope-based modified-Ziehl-Neelson staining method (Figure 1) and a molecular nested PCR-based method. Out of screened 100 fecal samples, only 14 samples showed indications for *Cryptosporidium*, 6 males and 8 females, which accounted for only 14% of the total examined feces.

Figure 1. *Cryptosporidium* oocysts in feces of pigeons diagnosed using modified-Ziehl-Neelson (MZN) staining method, magnification 100x

No significant differences were observed between the microscopy test and its downstream nested PCR test or between males and females (P>0.05). Nested PCR test showed a noticeably higher number of the identified *Cryptosporidium* which was being witnessed by the detection of 21 *Cryptosporidium* spp. (all sent for sequencing), 11 males and 10 females within the same examined fecal samples (Table 1).

The screening of our detected *Cryptosporidium* sequences was performed with the most homologous sequences using the NCBI-BLAST tool (30).

Table 1. Prevalence of *Cryptosporidium* spp. (%) using microscope and nested PCR in domestic pigeons

Host	Faecal samples	Conventional microscope		Molecular-nested PCR	
Domestic pigeons	Total No.	No. positive	%	No. positive	%
Male	43	6	13.95	11	25.58
Female	57	8	14.03	10	17.54
Total	100	14	14	21	21

The pattern of genetic diversity of fecal samples infected with *Cryptosporidium* spp. has been described in this study. Three types of species were efficiently discriminated using a nested-PCR based tool, namely *C. meleagridis*, *C. baileyi*, and *C. hominis*. Nucleic acid sequences similarity results indicated 99%–100% homology with the referring sequences of three different species within *Cryptosporidium*, namely *C. meleagridis* (GenBank acc. No. MN410718.1), *C. baileyi* (GenBank acc. no. MN461549.1), and *C. hominis* (GenBank acc. No. KJ019854.1), respectively. With regard to *C. meleagridis*, only three nucleic acid substitutions were detected in 5 strains out of 13, namely T>G 70 and G>A 71 in IQB18 and T>A 150 in IQQB10, IQB11, OQB13, and IQB15 strains, while the other strains had not shown any detectable variations compared with the deposited referring sequences. Considering *C. baileyi*, two nucleic acid substitutions were observed to be distributed in five strains, namely T>C 117 in the IQB2 and

C>A 167 in IQB3, IQB4, IQB5, and IIQB7. Meanwhile, IQB21, the only one strain of *C. hominis* detected in this study, did not exhibit any nucleic acid substitution and showed 100%

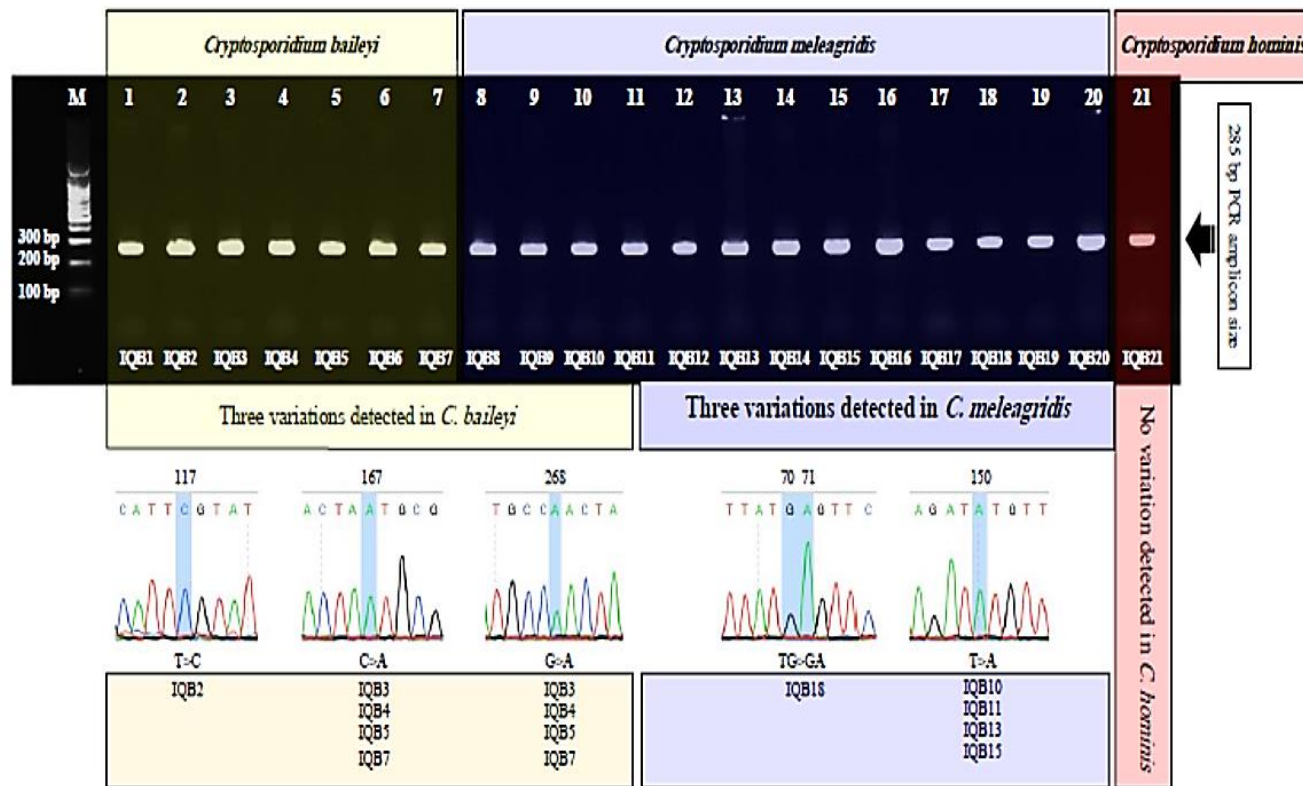homology with the deposited referring sequences (Figure 2).

Figure 2. Agarose gel electrophoresis profile for the observed 285 bp of PCR amplicons of *Cryptosporidium* spp.). Sequence reaction interpretation of the corresponding identity of each identified *Cryptosporidium* spp. with all observed nucleic acid variations

The construction of neighbor joining- based comprehensive tree was provided further details with regard to our identified *Cryptosporidium* spp. These details were being analyzed using the-state-of-the-art online annotations to evaluate the phylogenetic relationships among the investigated sequences. Despite close connections observed for the detected IQB1 – IQB21 strains within the *Cryptosporidium* spp., the constructed comprehensive tree had provided clear discrimination amongst these sequences without any phylogenetic confusion. Thus, considerable inclusive data were observed from the currently constructed tree, in which our detected sequences were obviously categorized into three main clades, namely *C. baileyi*, *C. meleagridis*, and *C. hominis*. All the detected *C. baileyi* IQB1–IQB7 strains showed clear positioning toward Chinese strains isolated from several birds' fecal samples. Meanwhile, in the present study, it was detected *C. meleagridis* IQB9–IQB20 exhibited several patterns of distributions within its species. considering *C. hominis*, a clear positioning of *C. hominis* IQB21 strain was

being observed in the inclusive tree within the *C. hominis* group since no mutations were observed in this identified nucleic acid sequences (Figure 3).

The most predominant existence of these detected parasites was represented by *C. meleagridis* 61.90% (13/21), which was highly exceeded the other detected two species in domestic pigeons' fecal samples. In accordance with the present findings, *C. meleagridis* has been found in the feces of pigeons in Thailand with high prevalence (18). The multi-phylogenetic positioning of *C. meleagridis* observed in this study indicated a broader phylogenetic distribution of this species than both *C. baileyi* and *C. hominis*, respectively. This may be due to the high host adaptation of *C. meleagridis*, which is combined with a considerable ability to infect animals and birds in distinctly different species localized in a variety of geographical areas (31). Furthermore, a study of 442 stool samples from South African children found a high prevalence of *C. meleagridis*, implying a tendency of this species towards expanding hosts (32). However, the ability of *C. meleagridis* to be
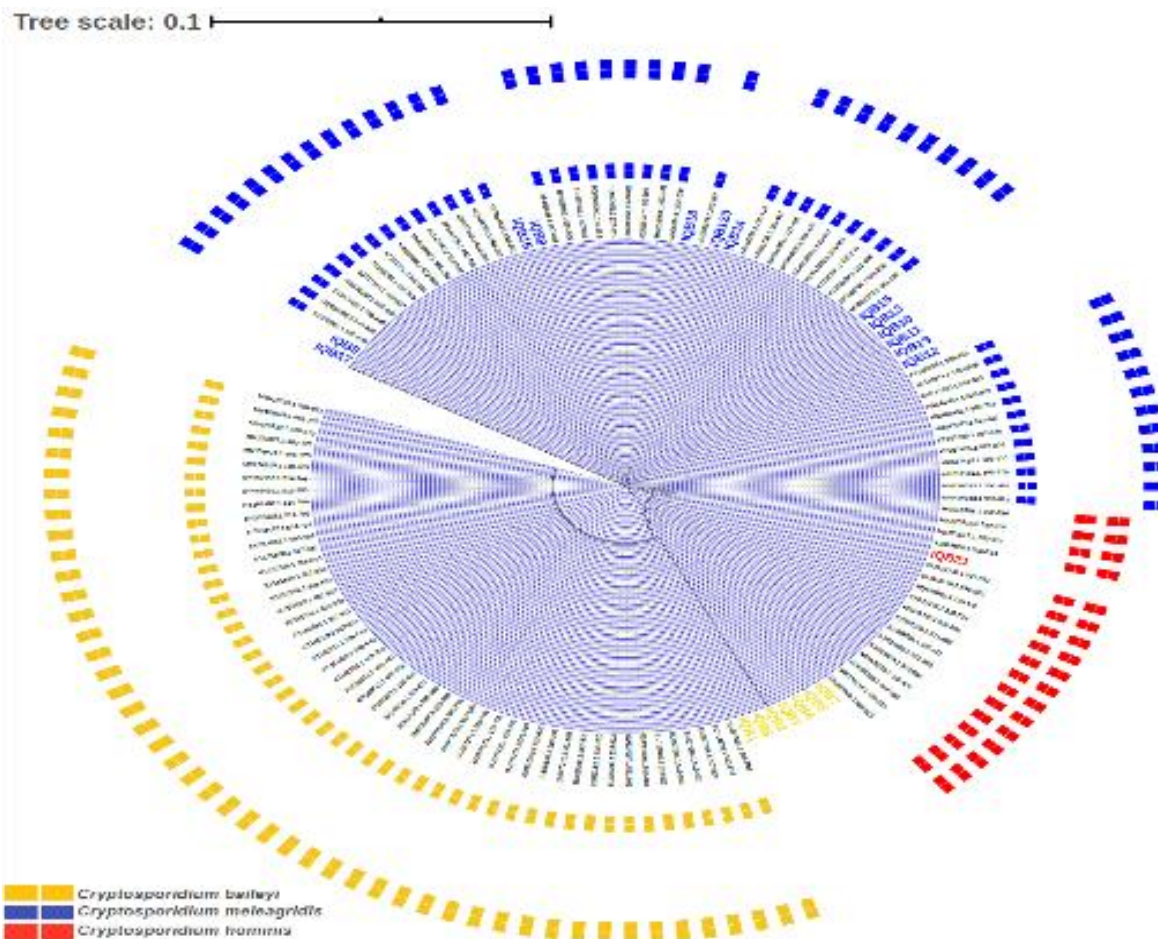
Figure 3. Comprehensive neighbor-joining phylogenetic tree of *Cryptosporidium* spp. detected in this study. The analyzed 18S ribosomal RNA sequences and their phylogenetic neighboring accession numbers were obtained from the NCBI Genbank databas(https://www.ncbi.nlm.nih.gov). Ribosomal sequences were aligned using multiple sequence alignment and phylogenetic tree tools of the online Clustal omega server. Aligned sequences were used for the phylogenetic analysis conducted with the iTOL server (https://itol.embl.de/). The number 0.1 at the bottom of the tree refers to the phylogenetic distance measure by a bootstrap scale. Yellow, cyan, and red colors refer to *Cryptosporidium baileyi*, *Cryptosporidium meleagridis*, and *Cryptosporidium hominis*, respectively.

transmitted from birds to humans has been confirmed via direct contact (33). Furthermore, *C. meleagridis* has been reported to be responsible for about 10% of human cryptosporidiosis (34). Therefore, its ability to be involved in cross-transmission between birds and humans is being evident (35, 36). Thus, the potential of *C. meleagridis* to be involved in zoonotic transmission and inducing considerable public health concerns has been confirmed (37-39).As long as *C. meleagridis* can infect both human and birds (40); its high infectivity may occupy more potential risk factors than the other species. In agreement with the present findings, a serious concern for *C. meleagridis* dissemination has recently been confirmed between birds and human (1). Moreover, the occurrence of *C. meleagridis* in colons of immunocompetent patients may induce active pathological development within those patients (41), this current observation may raise more public concerns for *C. meleagridis* in terms of its zoonotic potential from birds to

human. Following *C. meleagridis*, this study has also detected the infection of fecal samples of domestic pigeons with *C. baileyi*. Though such infection was less than *C. meleagridis*; and it is not unusual to detect the presence of this species in birds (42). In addition to *C. meleagridis*, *C. baileyi* showed infection percentage of 33.33% (7/21) which has also been described in the feces of pigeons (19). However, *C. baileyi* has not been associated with any zoonotic effects since no report of cross-contamination between birds and other mammals have suggested any role for this species in this zoonotic transfer (43, 44). In addition to the prevalence of both *C. meleagridis* and *C. baileyi*, this study has demonstrated the presence of only one sample of *C. hominis* 4.76% (1/21), being an anthroponotic species in domestic pigeons. However, this detection has also been detected in Spanish pigeons with few infectivity ratios (15). Noteworthy, it has widely been accepted that *C. hominis* may act as a causative agent in infecting human (45-47).

However, this infection may be an accidental occurrence of *C. hominis* in fecal samples that may suggest a minor source for pigeons to be involved in the mechanical dissemination of this protozoan to human. For this reason, it can be stated that both *C. meleagridis* and to a less extent *C. hominis* may signify a possible role for domestic pigeons in zoonotic distribution via direct contact with human. This finding emphasizes an increasing role for infection with cryptosporidiosis in the environment under study. So that, methods of epidemiological surveillance should be adopted to reduce the development of clinical cryptosporidiosis transmitted via domestic pigeons.

In conclusion, although several *Cryptosporidium* species have been found in this study, *C. meleagridis* was the most detected in the feces of domestic pigeons from Babylon, Iraq. Therefore, one must consider more potential risks posed by this zoonotic species to public health.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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الانتشار العالي لطفيلي *Cryptosporidium meleagridis* في الحمام الداجن (*Columba livia domestica*) يؤشراحتمال انتقال الاصابة الى الانسان في محافظة بابل، العراق

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فرع الطفيليات، كلية الطب البيطري، جامعة بغداد

الخلاصة

يعتبر طفيلي الابواغ الخبيئة احد أكثر الاوالي شيوعًا حيث ينتشر بشكل ملحوظ وعلى نطاق واسع بين الحيوانات، بما في ذلك اللبائن والطيور. ان الحمام الداجن(*Columba livia domestica*) يعمل كخازن محتمل للعديد من أنواع الابواغ الخبيئة لأنه يعيش بالقرب من الانسان. أجريت هذه الدراسة لتقييم التنوع الجيني لطفيلي الابواغ الخبيئة في الحمام الداجن في العراق. جمعت مائة عينة تم الحصول عليها من براز الحمام الداجن في محافظة بابل. وبعداجراء الفحص المجهري لهذه العينات، تم فحصها مجددا باستخدام طريقة تفاعل سلسلةالبلمرةالمتداخل (nested PCR) للتعرف على أنواع الطفيليات المحتملة. اظهرالفحص المجهري نسبة اصابة14٪ فقط (100/14)،بينما كشفت الاختبارات الجزيئية نسبة اصابة 21٪ (100/21) لنفس الطفيلي المستهدف. أظهرتحليل تسلسل القواعد النيتروجينية نسب اصابة مرتفعة بطفيلي *C. meleagridis*13/21 بنسبة (61,90٪)، يليها طفيلي *C. baileyi* 7/21 بنسبة (33,33٪)، بينم تم اكتشاف إصابة واحدة فقط بطفيلي *C. hominis*1/21 وبنسبة (4,76٪).لم يلاحظ وجود اصابة مشتركة بأكثر من نوع واحد من الطفيلي. كما لم يكن لعامل الجنس تأثير على معدل الاصابة. أخيراً، سجلت هذه الدراسة نسبة اصابة بطفيلي *C. Meleagridis* في الحمام المنزلي أعلى من كل من طفيلي *C. baileyi* و طفيلي *C. hominis* على التوالي، وفي هذا دلالة على احتمال وجود اصابة مشتركة ذات نسبة عالية بين الحمام الداجن و الاشخاص الملامسين له. وقد تثير هذه النتيجة المزيد من التساؤلات فيما يتعلق بالإصابة المتزايدة بطفيلي *C. meleagridis* في الانسان. وتعتبر هذه أول دراسة مهمة في العراق تستخدم فيها الطرق الجزيئية للتحري عن الاصابة بطفيلي الابواغ الخبيئة في الحمام الداجن.

الكلمات المفتاحية: الحمام، طفيلي الابواغ الخبيئة،الأمراض المشتركة، التحري، تفاعل سلسلة البلمرة المتداخل

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