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Prevalence and molecular characterization of *Cryptosporidium parvum* in pet lovebirds from Babylon province, Iraq

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Abstract

Objective

Cryptosporidium parvum is a globally important enteric protozoan with recognized veterinary and zoonotic significance; however, data on its occurrence in pet birds in Iraq remain limited. The aim of this study was to investigate the prevalence of *Cryptosporidium parvum* in pet lovebirds (*Agapornis spp*.) in Babylon Province, Iraq, using both conventional microscopic methods and molecular techniques. It also aimed to characterize the detected isolates through sequence and phylogenetic analyses.

Materials and methods

A total of 100 fecal samples were collected from pet lovebirds in various farm locations in Babylon, Iraq between October 2025 and January 2026. The Ziehl Neelsen stain was applied for the detection of *Cryptosporidium parvum.* Molecular detection of *Cryptosporidium parvum* was performed using a nested polymerase chain reaction (nested-PCR) targeting the partial region of the 18S rRNA gene. DNA was extracted from fecal samples. Polymerase chain reactions (PCR) with 50 µL final volume were performed. PCR products were electrophoresed on 1% agarose gel. Ten representative PCR-positive samples were selected for sequencing analysis. The Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software made the phylogram, and Clustal W did the alignments. The NCBI blast n server was used to compare the sequences. SPSS software was used to analyze the data.

Results

Molecular analysis revealed a significantly higher prevalence (34%, 34/100) compared to microscopic detection (21%, 21/100) ($\chi^2 = 6.70, p < 0.05$), highlighting the superior sensitivity of PCR-based methods. Sequence analysis of the partial 18S rRNA gene showed the highest similarity to *Cryptosporidium parvum* isolates deposited in GenBank (99.15-99.79%). Phylogenetic tree analysis (PX907836, PX907837, PX907838, PX907839, PX907840, PX907841, PX907842, PX907843, PX907844, PX907845) targeting the partial 18S rRNA gene

demonstrated that all isolates clustered closely with international reference strains, indicating low genetic divergence and possible epidemiological linkage. Spatial distribution showed the highest prevalence in Al-Kifl district (44%, 11/25), followed by Al-Hilla (36%, 9/25), while lower rates were observed in Al-Hashimia and Al-Musayyib (28%, 7/25 each), with no significant variation ($p > 0.05$). Minimal differences were observed between males (35.3%, 23/65) and females (31.4%, 11/35).

Conclusion

Collectively, these findings confirm the endemic presence of *Cryptosporidium parvum* in pet birds in Babylon Province and emphasize the importance of integrating molecular and phylogenetic approaches to enhance diagnostic accuracy and better understand transmission dynamics.

Keywords: 18S rRNA, *Cryptosporidium parvum*, Lovebirds, Nested-PCR, phylogenetic analysis

Paper Type: Research Paper.

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Introduction

Intestinal protozoan infections represent an important public health concern, particularly in low- and middle-income countries where environmental conditions and inadequate sanitation facilitate parasite transmission among humans and animals (Badri et al., 2025; Mosa et al., 2023). Pet birds are increasingly maintained as companion animals worldwide, primarily for ornamental and recreational purposes (Shivambu et al., 2024). This growing trend has heightened attention toward their health status, particularly infectious diseases that may adversely affect bird welfare and pose potential public health risks (Nakhaie et al., 2026). Among these diseases, intestinal protozoan infections represent a major parasitological concern due to their high prevalence, ease of transmission, and impact on both avian health and productivity (Sarkar, 2026). Intestinal protozoa such as *Giardia spp*., *Eimeria spp*., and *Cryptosporidium spp*. are commonly reported in pet birds and are associated with a range of gastrointestinal disturbances, including diarrhea, malabsorption, weight loss, poor growth, and reduced reproductive performance (Odumosu et al., 2025). Although infections are often subclinical (Das et al., 2025), chronically infected birds may act as asymptomatic carriers, facilitating continuous parasite circulation within aviaries, pet shops, and household environments (Salman Al-Adilee et al., 2025). In Iraq, and particularly in

Babylon Province, studies addressing the prevalence and of intestinal protozoa in pet birds remain scarce (Alabdali and Alewi, 2025). This lack of data highlights the need for comprehensive investigations integrating traditional parasitological techniques. Moreover, biochemical tests are incapable of distinguishing different types of microorganisms, such as bacteria, viruses, and parasites (Ahsani et al. 2010). Genomic techniques such as PCR and sequencing are the most modern practical technology in diagnosing infectious diseases and compared with classical techniques, it has been shown to be more rapid, with results obtained in a few hours, and also more reliable (Mohammadabadi et al., 2004). Genomic techniques allow a faster identification directly from clinical samples (Shahdadnejad et al. 2016). Therefore, the aimed of the current investigation was to study the prevalence of *Cryptosporidium parvum* in pet lovebirds (*Agapornis spp*.) in Babylon Province, Iraq, using both conventional microscopic methods and molecular techniques. Moreover, this study aimed to characterize the detected isolates through sequence and phylogenetic analyses.

Materials and methods

Collection of samples: A total of 100 fecal samples were obtained from pet lovebirds in various farm locations in Babylon between October 2025 and January 2026, using the techniques detailed below. The fecal samples, each weighing about five grams, were deposited in sterile acrylic containers labeled with the region's name and date. The samples were subsequently taken to the Department of Parasitology at the College of Veterinary Medicine at Al-Qasim Green University for microscopic examination.

Microscopic examination: Approximately 1 g of fecal material was thoroughly mixed with distilled water and filtered through a fine mesh sieve prior to microscopic examination. Before being inspected under a light microscope (at 10x and 40x magnification), one or two drops of the liquid were put on a glass slide with a coverslip. Iodine (Lugol) solution was dropped onto a glass slide to produce colored streaks in order to see cysts and eggs (Yokota et al., 2020). The Sheather's solution using to investigate cysts and oocyst according to Abbassi et al. (2000). The Ziehl Neelsen stain has been widely applied for the detection of coccidian protozoa, particularly *Cryptosporidium parvum*, whose oocysts exhibit acid-fast properties. Due to their small size and transparent nature, Cryptosporidium oocysts are often difficult to detect using routine wet mount preparations. Ziehl-Neelsen staining enhances the visibility of these oocysts, which typically appear as bright red, spherical or ovoid bodies against a blue background, facilitating accurate identification.

Molecular detection: Molecular detection of *Cryptosporidium parvum* was performed using a nested polymerase chain reaction (nested-PCR) targeting the partial region of the 18S rRNA gene. DNA was extracted from fecal samples based on method described by Russell et al. (2024), ensuring high purity and concentration suitable for PCR amplification. The primers were provided lyophilized (Macrogen, Korea) and were dissolved in a high pure water to give a final concentration 10 µM (Table 1). These were kept at - 20 °C until further use.

Table 1. Primer sequences used for nested PCR detection of *Cryptosporidium parvum*

PCR Round	Primer	Sequence (5′ → 3′)	Target Gene	Amplicon Size
First Round PCR	Forward	CTTTAAGCACTCTAATTTTCTC	18S rRNA	450 bp
	Reverse	GGGTTGTATTTATTAGATAAAGAAC	18S rRNA	
Second Round PCR (Nested PCR)	Forward	GACTTTTTGGTTTTGTAATTGGAATG	18S rRNA	165 bp
	Reverse	TAAATTATTAACAGAAATCCAACTAC GAGC	18S rRNA	

Polymerase chain reactions (PCR) with 50 µL final volume were performed in standard microtubes. Each tube contained 4 µL (50-100 ng/µL) of DNA (template), 2.5 mM $MgCl_2$, 25 pmol of each primer, 250 µM each of dNTPs, and 2 U Taq polymerase. The thermal profile included step 1 (initial denaturation for 5 min at 94 °C), step 2 (included 35 cycles with denaturation for 30 s at 94 °C, annealing for 45 s at 50 °C, and synthesis for 35 s at 72 °C), and step 3 (final extension for 10 min at 72 °C). PCR products were electrophoresed on 1% (w/v) agarose gel. Ten representative PCR-positive samples were selected for sequencing analysis to Macrogen company (Korea) by DHL service. Sanger sequencing technique was used for DNA sequencing. After receiving the sequences, the generated sequences were trimmed from noisy signals and submitted to NCBI for accession numbers. The phylogenetic tree elucidates the genetic proximity or disparity among species, enhances comprehension of evolutionary relationships, and facilitates a more refined classification of organisms grounded in evolutionary history rather than mere physical resemblances (Al-Khafaji and Al-Musawi, 2025). The Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software made the phylogram, and Clustal W did the alignments. The NCBI blast n server was used to compare the sequences that were analyzed.

Statistical analysis: SPSS software was used to analyze the data. Percentages were used to indicate the prevalence of *Cryptosporidium parvum*. The Chi-square (χ^2) test was used to evaluate differences between groups (diagnostic techniques, sex, and geographic locations), with Yates' adjustment added as necessary. The threshold for statistical significance was fixed at $p < 0.05$. The detection percentage of microscopic inspection was compared to PCR as the reference technique. The findings were shown as percentages and frequencies (Rahmati et al., 2022).

Results

The present study aimed to compare molecular and conventional diagnostic methods in detecting *Cryptosporidium parvum* in fecal samples. A total of 100 fecal samples were collected and each sample was examined using both conventional microscopic methods and nested-PCR for the detection of *Cryptosporidium parvum* (Figure 1). The results revealed a higher prevalence rate using molecular diagnosis (34%, 34/100) compared to conventional microscopic diagnosis (21%, 21/100) (Table 2). This difference was statistically significant ($\chi^2 = 6.70$, $p < 0.05$),

indicating the superior sensitivity and diagnostic accuracy of molecular techniques over conventional methods.

Table 2. Comparison between molecular and conventional microscopic diagnosis for detection of *Cryptosporidium parvum*

Diagnostic Method	No. of Examined Samples	No. of Positive Samples	Percentage (%)
Molecular	100	34	34%
Microscopic	100	21	21%
X^2		6.70	
p-value		≈0.01	

The molecular diagnosis of *Cryptosporidium parvum* according to geographic area showed variations in infection rates among the studied regions, as presented in Table 3. The highest infection rate was recorded in Al-Kifl District (44%, 11/25), followed by Al-Hilla Center (36%, 9/25), while both Al-Hashimia District and Al-Musayyib recorded lower infection rates (28%, 7/25 each). The overall infection rate was 34% (34/100). However, statistical analysis revealed no significant differences among the studied geographic areas ($\chi^2 = 1.29$, $p \approx 0.73$), indicating that the distribution of *Cryptosporidium parvum* infections was relatively similar across the different regions.

Table 3. Molecular diagnosis and infected rate of *Cryptosporidium parvum* according to geographic area

Area	No. of sample	No. of positive samples	Percentage %
Al-Kifl district	25	11	44%
Al-Hashimia district	25	7	28%
AL-Hilla center	25	9	36%
AL-Musayyib	25	7	28%
Total	100	34	34%
X^2		1.29	
P-Value		≈ 0.73	

The molecular detection of *Cryptosporidium parvum* according to sex is presented in Table 4. The infection rate was slightly higher in males (35.3%, 23/65) than in females (31.4%, 11/35). The overall infection rate was 34% (34/100). However, statistical analysis showed no significant difference between males and females regarding the prevalence of *Cryptosporidium parvum* infection ($\chi^2 = 0.158$, $p = 0.69$). This finding indicates that sex had no significant effect on the distribution of infection among the examined samples. The result of NCBI-BLAST Homology Sequence identity (%) between local *Cryptosporidium parvum* (PX907836, PX907837, PX907838, PX907839, PX907840, PX907841, PX907842, PX907843, PX907844, and PX907845) and NCBI-BLAST deposited isolates were shown in Table 5. Phylogenetic analysis based on the partial 18S rRNA gene (Figure 2) demonstrated that all Iraqi *Cryptosporidium parvum* isolates were grouped within the same major clade as reference isolates obtained from GenBank. Several nodes were supported by a bootstrap value of 100%, indicating strong phylogenetic reliability. The Iraqi isolates showed very short branch lengths and clustered closely with isolates originating from

China, Iran, Turkey, Japan, Sweden, Brazil, and Latvia. The low branch divergence observed among the sequences suggests a high degree of genetic similarity and conservation within the analyzed 18S rRNA region. The short branch lengths observed in the phylo genetic tree indicate minimal genetic divergence among the analyzed isolates.

Table 4. Molecular detection of *Cryptosporidium parvum* according to sex

Sex	No. of sample	No. of positive samples	Percentage %
Male	65	23	35.3%
Female	35	11	31.4%
Total	100	34	34%
X^2		0.158	
P-Value		0.69	

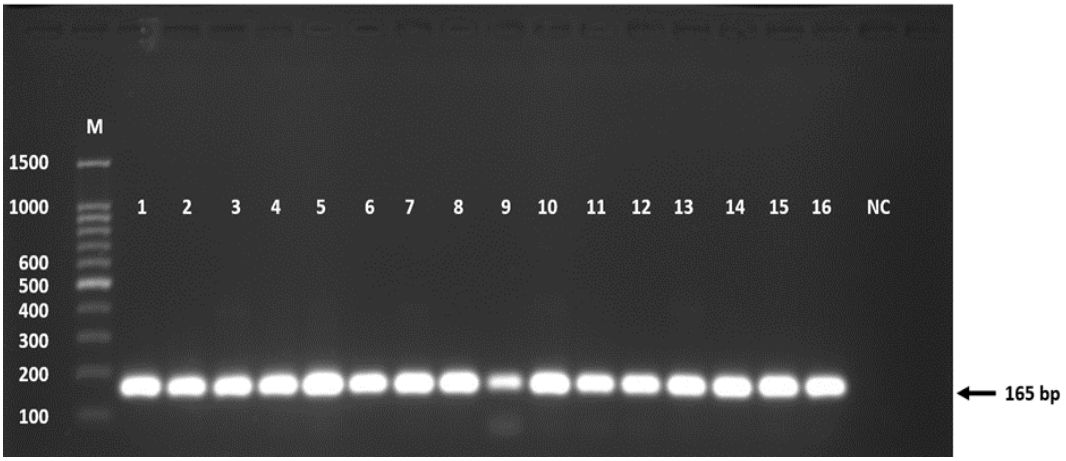

Figure 1. Agarose gel electrophoresis (1.5%) of nested-PCR products targeting the partial 18S rRNA gene of *Cryptosporidium parvum*. Lanes 1-16 are positive samples; NC is negative control; and M is 100-1500 bp DNA ladder

Discussion

The present study demonstrated a significantly higher detection rate of *Cryptosporidium parvum* using molecular methods (34%) compared to conventional microscopy (21 %), confirming the superior sensitivity of PCR-based techniques. The lower prevalence observed with microscopic examination is likely due to its limited ability to detect low parasite burdens and intermittent oocyst shedding, leading to underestimation of infection rates (Mahmudunnabi et al., 2024). The statistically significant difference ($\chi^2 = 6.70$, $p < 0.05$) supports the reliability of molecular diagnostics as a more accurate tool for epidemiological investigations. These findings are consistent with previous reports highlighting PCR as a gold standard for protozoan detection due to its high sensitivity and specificity (Mosa and Zenad, 2020; Fitri et al., 2022).

Table 5. The NCBI-BLAST Homology Sequence identity (%) between local *Cryptosporidium parvum* isolates (PX907836, PX907837, PX907838, PX907839, PX907840, PX907841, PX907842, PX907843, PX907844, and PX907845) and NCBI-BLAST deposited isolates

Samples	Obtained Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Genbank Accession number	Country	Identity (%)
1	PX907836	*Cryptosporidium parvum*	MF074634	China	99.79
2	PX907837	*Cryptosporidium parvum*	MF074678	China	99.15
3	PX907838	*Cryptosporidium parvum*	MK301397	Brazil	99.15
4	PX907839	*Cryptosporidium parvum*	OK429260	Latvia	99.57
5	PX907840	*Cryptosporidium parvum*	KU892560	Sweden	99.36
6	PX907841	*Cryptosporidium parvum*	MN540759	Japan	99.57
7	PX907842	*Cryptosporidium parvum*	OR485185	China	99.36
8	PX907843	*Cryptosporidium parvum*	OK425871	China	99.79
9	PX907844	*Cryptosporidium parvum*	MN918203	Turkey	99.79
10	PX907845	*Cryptosporidium parvum*	KF830255	Iran	99.57

Although variations in prevalence were recorded across different geographic locations, no significant differences were observed, suggesting a relatively uniform distribution of infection within the study area. Similarly, the slight difference between males and females indicates that sex is not a major risk factor for infection (GBD, 2022; Jańczak et al., 2026). The identification of *Cryptosporidium parvum* with high genetic similarity to global isolates highlights its epidemiological importance and potential zoonotic risk. Overall, these results emphasize the necessity of incorporating molecular techniques into routine diagnostic and surveillance programs to improve detection accuracy and support effective control strategies (Liu, et al., 2023; Mosa et al., 2025). The prevalence recorded in the present study (34%) was comparable to several reports from neighboring countries. Similar prevalence rates have been reported in pet and ornamental birds from Iran and Turkey, whereas lower prevalence rates have been documented in some European countries. Such differences may be attributed to variations in husbandry practices, environmental conditions, diagnostic methods, and sample size. Several environmental and management factors are likely involved in the study areas that facilitated the transmission of oocysts among domestic birds and led to the relatively high prevalence of Cryptosporidium species observed in the present study.

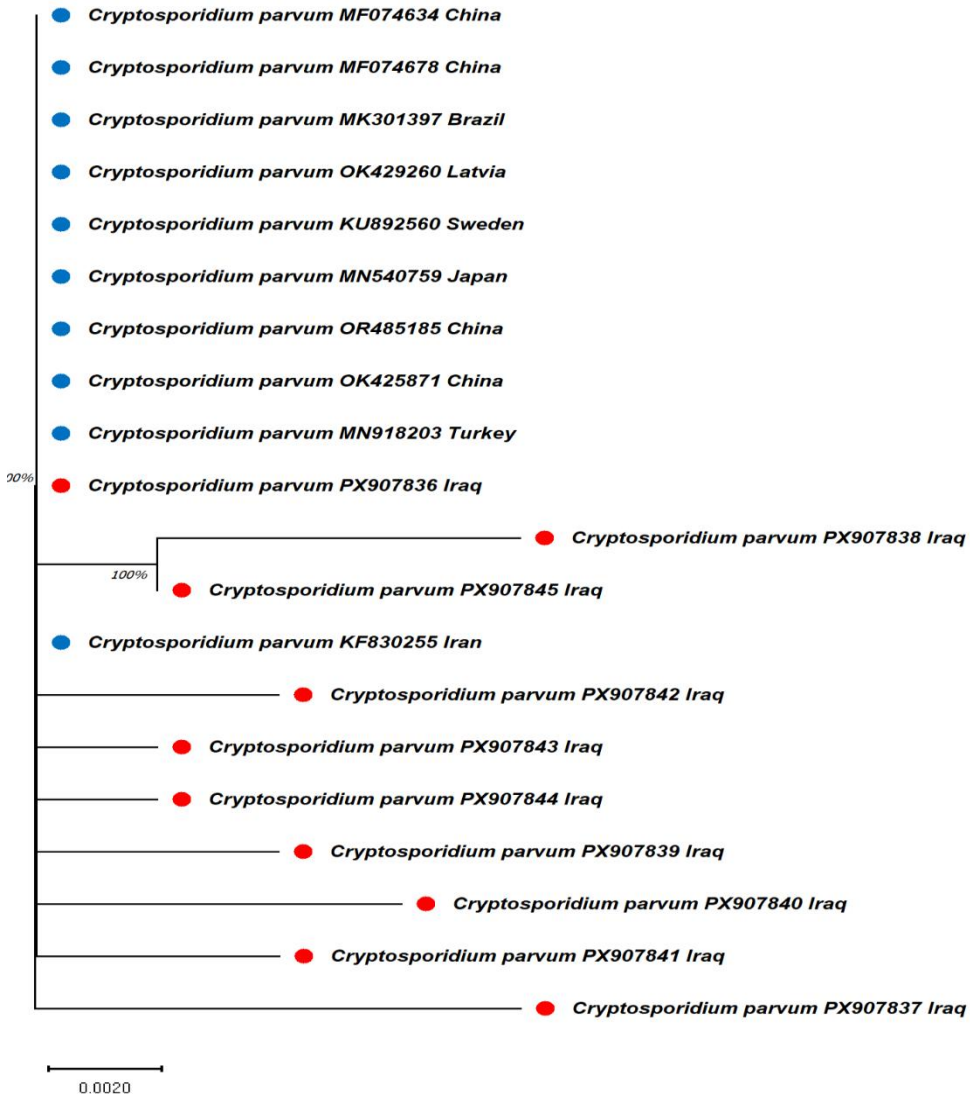

Figure 2. Evolutionary phylogenetic analysis by Maximum Likelihood method of *Cryptosporidium parvum* targeting partial region within 18S rRNA gene using the Maximum Likelihood method, Tamura-Nei model, and The Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software

Cryptosporidium oocysts are highly resistant in the environment and can survive for long periods in contaminated water, feed, and cages (Jańczak et al., 2026). Therefore, contaminated drinking water and feed may be important sources of infection for caged lovebirds. In this regard, various studies have reported that poor sanitary conditions and inadequate hygiene significantly increase the risk of Cryptosporidium infection in bird populations (Fitri et al., 2022; Alabdali and Alewi, 2025; Jańczak et al., 2026). In addition, the common practice of keeping several birds in common cages may contribute to the rapid spread of infection through the fecal-oral route. Because lovebirds are highly social birds and are often kept in close contact with other birds in

poultry farms, pet stores, and breeding centers, they provide favorable conditions for parasite transmission. Exposure to wild birds is another possible source of infection. Since this may also contaminate feed, water, or cage environment with infectious oocysts. No significant differences were observed between geographical areas in the present study, it can be concluded that these risk factors are likely widespread throughout Babylon province, leading to a relatively uniform distribution of infection. The persistence of Cryptosporidium infection among domestic birds may also be attributed to the biological characteristics of this parasite (Mahmudunnabi et al., 2024). Cryptosporidium species have a direct life cycle and require only a low infectious dose to cause infection. Furthermore, infected birds may remain asymptomatic while continuously shedding oocysts into the environment, thereby acting as a reservoir of infection. This silent shedding could facilitate undetected transmission within bird populations and complicate control measures. Consequently, routine screening and strict hygiene measures are essential to reduce the prevalence of Cryptosporidium in captive bird populations.

Conclusion: The results of the present study showed that *Cryptosporidium parvum* is present among domestic lovebirds in Babil province, Iraq. It also showed that nested-PCR has superior diagnostic performance compared to conventional microscopy. Molecular characterization and phylogenetic analysis showed a high degree of genetic similarity between local isolates and international reference strains. This could indicate a possible genetic relatedness. The high genetic similarity observed between local and international isolates may indicate the widespread distribution of closely related *Cryptosporidium parvum* genotypes. No significant differences were observed between geographical locations and between the two sexes. This could indicate that the infection is widely distributed in the study area. These findings emphasize that molecular diagnostic tools should be included and considered in routine surveillance programs to improve the detection, monitoring and control of Cryptosporidium in domestic birds and reduce potential public health risks.

Author contributions

S.F.S. designed the study, conducted laboratory work, analyzed the data, and drafted the manuscript. S.M.K. supervised the research, contributed to data interpretation, and reviewed and edited the manuscript.

Data availability statement

Data generated in this study will be available from the corresponding author upon reasonable request.

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Ethical considerations

All procedures involving animal samples were conducted in accordance with institutional and national ethical guidelines for animal research. Sample collection was performed with minimal harm to animals and under appropriate veterinary supervision.

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Conflict of Interest

The authors declare no conflict of interest.

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شیوع و شناسایی مولکولی *Cryptosporidium parvum* در مرغ عشق‌های زینتی استان بابل، عراق

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چکیده

هدف: *Cryptosporidium parvum* یک پروتوزوآی روده‌ای مهم در سطح جهانی است که از نظر دامپزشکی و بیماری‌زایی مشترک بین انسان و حیوان اهمیت دارد، اما اطلاعات مربوط به حضور آن در پرندگان خانگی در عراق محدود است. هدف این مطالعه بررسی شیوع *Cryptosporidium parvum* در مرغ عشق‌های زینتی (*Agapornis spp.*) در استان بابل عراق با استفاده از روش‌های میکروسکوپی متداول و تکنیک‌های مولکولی بود. همچنین، شناسایی ایزوله‌های به‌دست‌آمده از طریق آنالیز توالی و بررسی فیلوژنتیکی انجام شد.

مواد و روش‌ها: تعداد ۱۰۰ نمونه مدفوع از مرغ عشق‌های زینتی در مناطق مختلف پرورش در استان بابل، عراق، بین اکتبر ۲۰۲۵ تا ژانویه ۲۰۲۶ جمع‌آوری شد. برای شناسایی *Cryptosporidium parvum* از رنگ‌آمیزی زیل-نیلسن استفاده شد. تشخیص مولکولی با استفاده از نستد PCR (Nested-PCR) و با هدف تکثیر بخشی از ژن 18S rRNA انجام گرفت. DNA از نمونه‌های مدفوع استخراج شد و واکنش PCR با حجم نهایی ۵۰ میکرولیتر انجام شد. محصولات PCR بر روی ژل آگارز ۱٪ الکتروفورز شدند. ده نمونه مثبت به عنوان نماینده نمونه‌ها برای تعیین توالی انتخاب گردید. نرم‌افزار MEGA11 برای ترسیم درخت فیلوژنتیکی و Clustal W برای هم‌ترازی توالی‌ها استفاده شد. مقایسه توالی‌ها با استفاده از سرور BLAST در NCBI انجام گرفت و داده‌ها با نرم‌افزار SPSS تحلیل شدند.

نتایج: آنالیز مولکولی، شیوع به‌طور معنی‌داری بالاتری (۳۴٪) نسبت به روش میکروسکوپی (۲۱٪) نشان داد ($\chi^2 = 6.70, p < 0.05$) که بیانگر حساسیت بیشتر روش‌های مبتنی بر PCR است. تحلیل توالی بخشی از ژن 18S rRNA بیشترین شباهت را با ایزوله‌های *Cryptosporidium parvum* ثبت‌شده در GenBank (بین ۹۹/۱۵٪ تا ۹۹/۷۹٪) نشان داد. بررسی درخت فیلوژنتیکی برای ایزوله‌های PX907836 تا PX907845 نشان داد که همه آن‌ها به‌طور نزدیک با سویه‌های مرجع بین‌المللی خوشه‌بندی می‌شوند و اختلاف ژنتیکی کمی دارند که می‌تواند نشان‌دهنده ارتباط اپیدمیولوژیک باشد. از نظر توزیع جغرافیایی، بیشترین شیوع در منطقه الکفل (۴۴٪) مشاهده شد، پس از آن الحله (۳۶٪)، در حالی که مناطق الهاشمیه و المسیب کمترین میزان (هر کدام ۵٪۲۸) را نشان دادند و این تفاوت‌ها از نظر آماری معنی‌دار نبودند ($p > 0.05$). همچنین تفاوت اندکی بین نرها (۳۵/۳٪) و ماده‌ها (۳۱/۴٪) مشاهده شد.

نتیجه‌گیری: مجموعه یافته‌های این مطالعه حضور بومی *Cryptosporidium parvum* را در پرندگان خانگی استان بابل تأیید می‌کند و بر اهمیت استفاده هم‌زمان از روش‌های مولکولی و فیلوژنتیکی برای افزایش دقت تشخیص و درک بهتر الگوهای انتقال تأکید دارد.

کلمات کلیدی: تحلیل فیلوژنتیکی، مرغ عشق، نستد PCR، 18S rRNA، *Cryptosporidium parvum*

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