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IDENTIFICATION AND PREVALENCE OF CRYPTOSPORIDIUM SPECIES FROM SELECTED CAPTIVE BIRDS IN DISTRICT KASUR

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ABSTRACT

The most prevalent protozoan that can infect a variety of animals, including birds and mammals, is *Cryptosporidium*. Certain species of Avian *Cryptosporidium spp*. are zoonotic and can cause fatal respiratory and digestive disorders in birds. Because they are in close proximity to humans, captive birds have the potential to serve as reservoirs. The current study reveals the molecular characteristics and prevalence of *Cryptosporidium* spp. from selected captive birds kept in district kasur. Fifty fresh fecal samples from captive birds belonging to three bird orders and five species were collected. To identify *Cryptosporidium* species, a polymerase chain reaction (PCR) technique that targets the small subunit 18S rRNA gene was employed. The prevalence rate of *Cryptosporidium* in captive birds was 6%. Of the three samples, *Cryptosporidium baileyi* 10% (1/10), *Cryptosporidium meleagridis* 5.5% (1/18) and *Cryptosporidium parvum* 10% (1/10). The rate of occurrence *Cryptosporidium spp*. infecting captive birds in Kasur having zoonotic potential is probably low. These findings provide baseline information to support future research on the genetic epidemiology and management of *Cryptosporidium* infection in avian species in Kasur.

Keywords: Captive birds, *Cryptosporidium*, molecular identification, PCR, phylogenetic analysis, zoonosis.

INTRODUCTION

The morbidity and death of pet birds from gastrointestinal (GI) parasite disorders are significant, and they are also considered as an important source of pathogens for humans (Sivajothi and Sudhakara, 2015). The parasitic unicellular protozoan *Cryptosporidium* is a member of the phylum Apicomplexa (Robertson *et al.,* 2020). Coccidia from the suborder *Eimeriorina* and the family *Cryptosporidiidae* form the genus *Cryptosporidium* (Doneley, 2009; Ryan, 2010). *Cryptosporidium* was initially identified in birds of the order Galliformes and has since been documented in over 30 avian species throughout the world (Ryan, 2010). In 1929, *Cryptosporidium* was first identified in chickens (Tyzzer, 1929). The identification of *Cryptosporidium meleagridis* was reported in the ileum of Turkeys, but no specific species were named until 1955 (Slavin, 1955).

Cryptosporidium species have a wide range of hosts and a worldwide distribution (Wang *et al*., 2021). *Cryptosporidium* was first discovered in humans in 1976, and it was assumed to be closely related to immunological suppressive people (Zaheer *et al*., 2021). *Cryptosporidium* species exhibit direct life cycles, where oocysts shed in feces from infected host's respiratory tract. Once shed, the oocysts become infectious and spread through inhalation or ingestion. Sporozoites are released inside

the new host and enter epithelial cells, mostly in respiratory and GI tract. Other possible targets include conjunctiva, bursa and urinary system (Doneley, 2009; Ryan, 2010). The primary causes of infections in humans and animals are food and water contaminated with *Cryptosporidium* oocysts (Xiao, 2010). In addition, the parasite can be transferred directly from infected animals to individuals. Therefore, it is regarded as a zoonotic disease (Ryan, 2010). The oocysts of *Cryptosporidium* are extremely resistant to chlorination and typical household disinfectants, and survive in the environment for a long time (Xiao and Ryan, 2004).

Cryptosporidium hominis, Cryptosporidium parvum, Cryptosporidium andersoni, Cryptosporiuum muris, and *Cryptosporidium canis* are mammal specific *Cryptosporidium* species and are infrequently observed in avian species (Ferrari *et al.,* 2018). 90% of human cryptosporidiosis infections are caused by *C. hominis* and *C. parvum* (Xiao, 2010). According to a study *C. meleagridis* oocysts can infect healthy humans and cause GI symptoms including diarrhea (Chappell *et al*., 2011). Research on the incidence of *Cryptosporidium* species infections in kids in Kenya indicated that the predominant species was the *C. hominis*, whereas *C. meleagridis* and *C. muris* (Gatei *et al*., 2003). Other species capable of infecting individuals are *C. andersoni, C. canis, C. meleagridis*, *C. felis,* and *C. suis* (Xiao, 2010).

Numerous domestic, pets, commercial and wild birds such as turkey, chicken, quail, pheasant, sea gull, ostrich, finches, cranes, sparrow, pigeon, vulture, flamingo, waterfowl, crow, dove, geese, falcon, and duck, have been found to harbor *Cryptosporidium* species (Li *et al.,* 2015; Kabir *et al*., 2020). Five species have been found in a variety of birds such as *C. meleagridis, C. proventriculi, C. galli, C. avium,* and *C. baileyi* (Wang *et al.*, 2010; Liu *et al.,* 2019). Three distinct species *(C. galli, C. baileyi* and *C. meleagridis)* were regarded as predominant species in birds (Slavin, 1955; Ryan *et al*., 2003).

Bird's family including *Fringillidae*, *spermicide*, and domestic hens are hosts for the parasite *C. galli* (Huber *et al*., 2007). *C. baileyi* was found in the large intestine, ileum, bursa of Fabricius and cloaca of hens, but later research revealed that the species also caused renal and respiratory problems (Lindsay *et al.*, 1990). It causes thicker stroma, lamina propria infiltration and hyperplasic hypertrophied epithelium in the Fabricius bursa. In the proventriculus of chickens, *Cryptosporidium galli* was discovered (Pavlasek, 2001).

Microscopy, histological, immunological, and molecular techniques are mostly used to diagnose cryptosporidiosis in birds (Xiao, 2010). Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium*, leading to GI illness characterized by diarrhea. It is a significant public health concern due to its high transmissibility through contaminated water, food, and surfaces, especially affecting immune GI-deficient individuals, young children, and in settings with inadequate sanitation. This study aims to isolate, identify and determine the prevalence of *Cryptosporidium* from selected captive bird.

MATERIALS AND METHODS

Ethical Statement

All the fecal samples were collected from the floor of cage. As the sampling process was take place from the feces, no permission regarding laws on animal protection was required.

Study site

The proposed study was carried out at Avian Research and Conservation center, Department of wildlife and Ecology, University of Veterinary and Animal Sciences, Ravi Campus (C-Block) Pattoki.

District Kasur located in Lahore Division of Punjab, Pakistan. Kasur town is situated 55 kilometers south of Lahore, between the latitudes of 30° 40’ and 31° 20’ north, and the longitudes of 73° 38’ and 74° 41’ east.

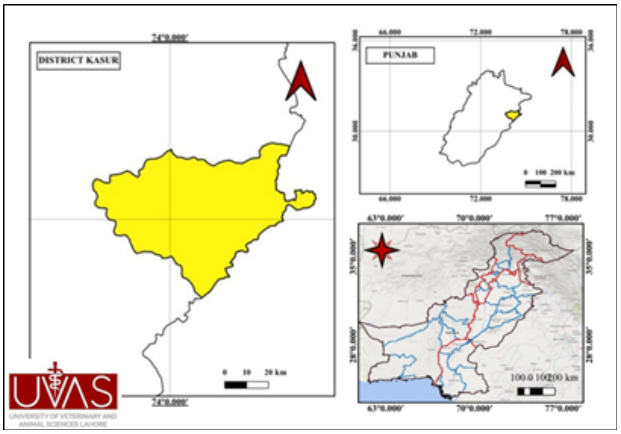

Figure 1: location map of study area

Sample collection

Total 50 fecal samples of bird were collected from pigeon (18), turkey (10), peafowls (7), ring-necked pheasant (10) and ring-necked parakeet (5). Using a disposable wooden spatula and medical cotton swab, the fecal droppings from each captive bird that had not come in to contact with ground were collected, put in a clean and labeled sampling bags or 2ml microtubes (containing 0.9% sodium chloride solution) and stored at 4℃ until purification and molecular analysis (Jian *et al.*, 2021).

Processing of fecal samples

Initially, the fecal samples were first mixed in a Sheather’s sugar solution, prepared by using 1% Tween 20 and phosphate buffered saline (PBS). Afterward, they were subjected to centrifugation at 800Xg for 10 minutes. The resulting supernatant was split into 2 equal portions, and both were subsequently transmitted to new tubes. Both samples underwent two successive washes: the first wash employed a 0.1% Tween 20 with PBS solution, followed by a second wash using PBS with 0.01% Tween 20. To one of these pellets, 10% formaldehyde was added and it was utilized for microscopic detection, while the other pellet was frozen at a temperature of -20°C for later DNA extraction (Oliveira et al., 2017).

Microscopic analysis

Cryptosporidium oocysts detection through microscopic analysis was carried out by employing the malachite green negative staining technique (Oliveira *et al*., 2017) and with modified acid-fast technique (Altamimi and Zubaidi, 2020).

Prevalence assessment

Prevalence was checked using by this formula:
Prevalence= (No. Of positive samples/ Total No. Of samples) × 100 (Tefera and Mebrie, 2014).

DNA Extraction

DNA was extracted from birds feces using the QIAamp DNA extraction stool Mini Kit (Qiagen), following the manufacturer’s instructions (Oliveira *et al*., 2017). Before being subjected to PCR analysis for *Cryptosporidium*, the extracted DNA samples were stored at -20℃ (Altamimi and Zubaidi, 2020).

Amplification of 18s rRNA of Cryptosporidium

PCR reaction targeting the 18S rRNA gene was performed to identify *Cryptosporidium* species-specific DNA sequences (Xiao, 2010). The following PCR conditions were utilized: a total reaction volume of 50µl containing DNA 12 µl, 25 µl of 2X master mixture, 2 µl of each primer and 9 µl of water (ABE *et al.,* 2016). The PCR amplification reaction for 18SrRNA gene was performed under

the following conditions: an initial denaturation step at 95 ˚C for 180 seconds, followed by 35 cycles consisting of 45 sec of denaturation at 95 ˚C. Annealing at 54 ˚C for 45 sec and extension at 72 ˚C for 60 seconds. A final extension cycle was at 72 ˚C for 5 minutes, and the reactions were cooled at 15˚C. PCR amplified products were identified using 1.5% agarose gel electrophoresis and visualization facilitated by ethidium bromide (Hasapis *et al*., 2023).

Table 1: Conditions for PCR amplification

Target gene	Primer pair	Sequence(5'-3')	Amplicon size	References
18S rRNA	PMF	5′-TTCTAGAGCTAATACATGCG-3′	1325bp	Pineda *et al*., 2020
	PMR	5′--CCCTAATCCTTCGAAACAGGA -3′		

Sequencing and phylogenetic analysis

By sequencing the PCR results, the gene sequence information was obtained. Using the secondary PCR primers, positive PCR results from captive birds were sequenced in both directions. The positive sequences were subjected to BLAST searches at NCBI GenBank for identification of the *Cryptosporidium* species (Hasapis *et al*., 2023). In MEGA 10 software, a phylogenetic tree was created using the maximum likelihood technique with Bootstrap 1000 replicates. The Kimura two-parameter model was used to calculate evolutionary distances (Hasapis *et al*., 2023).

RESULTS AND DISCUSSION

Identification of Cryptosporidium spp. in captive birds

Cryptosporidium oocysts detection through microscopic analysis was carried out by performing two different staining methods**:**

Table 2: Microscopic identification of *Cryptosporidium*

Staining Techniques	Results	Interpretation
Malachite green	Not Effective	Malachite Green is not suitable for *Cryptosporidium* oocysts.
Acid fast staining	Red/pink oocysts	Presence of *Cryptosporidium* oocysts

Microscopic examination of fecal smears demonstrated the existence of oocysts of *Cryptosporidium* in certain samples. The modified Ziehl-Neelsen method detected *Cryptosporidium* in 16.6% (3/18) of the pigeon samples, 20% (2/10) of the Ring-necked pheasant samples, and 10% (1/10) of the turkey samples. By using PCR, the positive rate for *Cryptosporidium* was 6% (3/50).

Detection of Cryptosporidium spp. in different captive birds

Cryptosporidium parvum, C. meleagaridis and *C. baileyi* were determined by sequencing, and *C. Baileyi* being the most prevalent species in birds. *C. meleagridis* was detected in this study in 5.5% of pigeon (1/18). *C. baileyi* was detected in 10% (1/10) of turkey. *C. parvum* was identified in 10% (1/10) of ring-necked pheasant.

Table 3: Presence of *Cryptosporidium* species in captive birds

Species	No. of samples	No. of microscopy positive	No. of PCR positive
Pigeons	18	3	1
Turkey	10	1	1
Peafowls	7	0	0
Ring-necked parakeet	5	0	0
Ring-necked pheasants	10	1	1

DNA extraction from the fecal samples

By using Qiagen DNA extraction kit, the DNA was extracted from the fecal samples and the confirmation showed in figure No. 2.

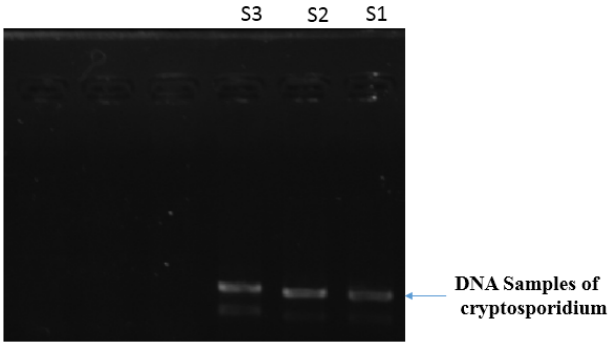

Figure 2: S1 (Pigeon) S2 (Turkey) and S3 (Ring-necked pheasant) samples of DNA extracted from the feces of avian species

Amplification of 18S rRNA gene

By using species specific primer pairs the 18SrRNA region was amplified, yielding a product size of around 940 base pair. The targeted DNA was exclusively amplified by genus-specific primer pair.

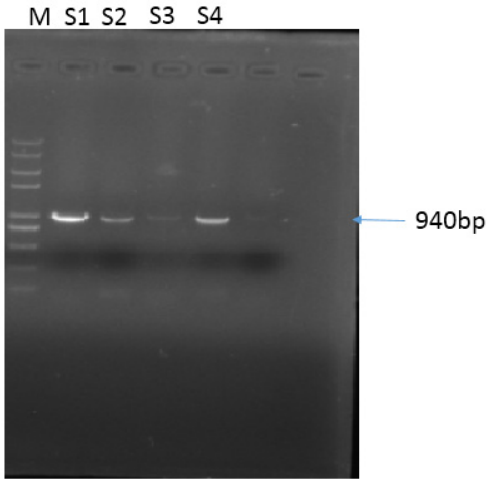

Figure 3: PCR products obtained after agarose gel electrophoresis. Lane 1, 2 and 4 PCR products amplified by using genus specific primers

Prevalence of Cryptosporidium spp. from captive birds

Cryptosporidium species were tested positive for most of the captive birds in different areas of Kasur, Pakistan. The prevalence of *Cryptosporidium spp.* was low in turkey and ring-necked pheasant and high among pigeons. *C. meleagridis, C. parvum* and C. baileyi were identified in captive birds. However, we did not detect *Cryptosporidium spp.* in peafowls and ring-necked parakeet.

Table 4: Prevalence and associated risk factors of *Cryptosporidium* spp. in fecal samples of captive birds

Variable	Category	Total Examined	Infested	Prevalence (%)	Chi-Square	P Value
Gender	Male	29	1	3.44	0.797	0.379
	Female	21	2	9.5		
Age	Adult	31	0	0	5.207	0.04
	Young	19	3	15.7		
Health Status	Healthy	42	2	4.7	0.713	0.41
	Unhealthy	8	1	12.5		
Season	Summer	17	1	5.88	1.595	0.66
	Winter	14	0	0		
	Autumn	9	1	11.1		
	Spring	10	1	10		

In case of risk factor of age, young birds show more prevalence while adults revealed no incidence of disease. The present study revealed that young birds were more infected (15.7%) as compared to the adults (0%) with *Cryptosporidium oocysts.* The P-value shows that the association between age and *Cryptosporidium* presence with respect to age factor is significant results 0.04 ($P \leq 0.05$) (Table 5). In case of health status, the highest prevalence rate was investigated in unhealthy birds. While healthy birds exhibited less presence of *Cryptosporidium spp*. The prevalence rate of *Cryptosporidium spp.* in unhealthy birds was 12.5% and in healthy birds, the prevalence was 4.7%. The association of health status with *Cryptosporidium spp.* existence is although not significant ($P \leq 0.05$) (Table 4).

The prevalence of *Cryptosporidium spp.* regarding gender was studied. It was higher in female 2/21 (9.5%) than male 1/29 (3.4%). The female birds were more likely to be positive for *Cryptosporidium spp*. The statistical analyses showed not significant result ($P \leq 0.05$) (Table 4). Samples collected during the autumn had a higher prevalence of *Cryptosporidium* species. (11.1%) than spring season in which the prevalence rate was 10%, low prevalence was found in winter and summer with rates of 0% and 5.8% respectively. The association of season with the *Cryptosporidium* existence is statistically non – significant ($P \leq 0.05$) (Table 4).

Sequence and Phylogenetic analysis

The Neighbor-Joining approach was used to infer the evolutionary history. With branch lengths presented in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is depicted to scale. The evolutionary distances are expressed in base differences per site and were calculated using the p-distance method. The results included 19 nucleotide sequences in the analysis. Evolutionary analyses were conducted in MEGA 10. Overall genetic difference was 0.393.

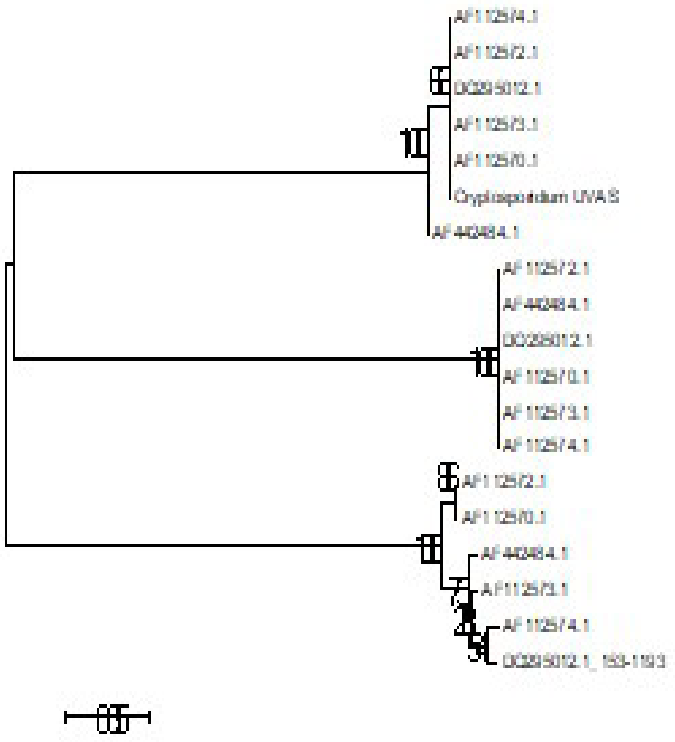

Figure 4: Phylogenetic relationship of *Cryptosporidium* species

A significant pathogen that can cause severe diarrhea in the world in humans and animals is *Cryptosporidium*. We assessed the incidence of *Cryptosporidium* spp. in captive birds from Kasur district, Pakistan in order to investigate the role that captive birds play in the epidemiology of the above mentioned infection. The prevalence of *Cryptosporidium* in captive birds varies widely, often influenced by factors such as species, environmental conditions, and hygiene practices. Studies have reported infection rates ranging from low to moderate, with some captive bird populations showing significant levels of the parasite. *Cryptosporidium* can cause GI issues in birds, potentially impacting their health. The incidence rate of *Cryptosporidium* species in captive birds in district Kasur was determined in this study. Worldwide, *Cryptosporidium* species have been found in a variety of wild and domestic birds (Nakamura and Meireles, 2015). China has reported cases of *Cryptosporidium* spp. in Java sparrows (Yao *et al.,* 2017), quails (Wang *et al.*, 2012), parrots (Zhang *et al.*, 2015), chickens (Liao *et al.*, 2018), ostriches (Qi *et al.*, 2014), domestic pigeons (Li *et al.*, 2015), and ruddy shelduck (Amer *et al.*, 2010). In the current study, PCR-based amplification of the 18S rRNA gene was utilized for the effective identification of *Cryptosporidium* spp. similar conclusions have been reported by Liao *et al.*, (2021). In this study incidence of *Cryptosporidium* was 6%, whereas the infection incidence in Jiangxi Province was 9.52%. The reasons behind these discrepancies could be due to variations in the age range of captive birds, management styles, and climate. In contrast to our findings, investigations conducted on pigeons revealed significantly higher occurrence rates by Lobo *et al.,* (2006), Li *et al.,* (2014) in chicken and Tavalla *et al.,* (2018) in exotic birds.

This study results showed the prevalence of *Cryptosporidium* infection was 6% which was higher than that recorded in Tunisia (4.5%) by (Soltane *et al*., 2007), 5.1% reported in Germany according to Helmy *et al*., (2017), (2013) in Zaria, Nigeria, 7.4% prevalence result of Bamaiyi *et al*., (2013), and 8.1% by Meng *et al.*, (2011) in China, previous researches employed both the diagnostic approach of microscopic examination and molecular characterization. According to researches on captive birds, rate of occurence are as follows: 3.22% in North China's pet parrots (Zhang *et al.*, 2015); 2.3% in

Brazil (da Cunha *et al.*, 2017), 9.1% in Japan's companion birds (Iijima *et al.,* 2018); 10.64% in Brazil's wild captive psittacines (Oliveira *et al.*, 2017b), 19.1% in Santiago, 5% in Brazil's caged exotic psittacines (Ferrari *et al.*, 2018), Chile's introduced monk parakeets (Briceno *et al.*, 2017). In China, reports of *C. baileyi, C. meleagridis,* and *C. galli* in birds are frequent. Worldwide, avian species have also been found to harbor a few rare species of *Cryptosporidium*, including *C. andersoni, C. muris,* and *C. parvum* (Nakamura and Meireles, 2015). Positive results for *Cryptosporidium* spp. in birds show the presence of these zoonotic species in China. In this investigation, a turkey was found to have C. parvum. Humans are susceptible to zoonotic *Cryptosporidium* species, such as *C. meleagridis* and *C. parvum*, from contaminated food and water or through direct or indirect contact with infected birds. The current investigation revealed *Cryptosporidium baileyi* in Turkey. *Cryptosporidium baileyi* has been recorded commonly in poultry and ducks worldwide (Chvala *et al.*, 2006; Huber *et al.*, 2007). Previous research has revealed the existence of *Cryptosporidium* species in birds, but with varying incidence across different regions. This variability may be attributed to variations in climate, breed, sex, and bird farm management practices.

CONCLUSIONS

The current study identified the prevalence of *C. parvum, C. baileyi and C. meleagridis* in captive birds in district Kasur. Further research is needed to assess the zoonotic risks associated with the frequent infection of birds by *C. parvum*. It is imperative to conduct a thorough comparison of prevalence rates between flocks under varying management approaches in order to discover origins and risk factors of cryptosporidiosis infection in captive birds. Specifically, a comparison between birds kept entirely in captivity and birds in the wild should be included.

CONFLICT OF INTEREST

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

AUTHOR'S CONTRIBUTION

Zarnain Rana contributed to various aspects of this research, including concept, study design, methodology and writing review. **Kashaf Nokhaiz, Ifra Saleem**, and **Anisa Tahir** helped with the analysis, data acquisition and interpretation. **Saddam Hussain** and **Muhammad Shakeel Shabbir** contributed to the project's conceptualization, validation and writing evaluation. **Arshad Javid** and **Waqas Ali** assisted in providing resources, writing reviews, and critically revising the article for intellectual content. **Syed Mohsin Bukhari** contributed to the conceptualization, resource acquisition, writing assessment, and project editing.

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