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MOLECULAR IDENTIFICATION OF *CRYPTOSPORIDIUM* SPECIES FROM OSTRICHES IN CENTRAL AND SOUTH PARTS OF IRAQ

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Abstract

Farming Ostriches is a new birds livestock production in Iraq which infected with varying parasites, including Genus *Cryptosporidium* from apicomplexan protozoal parasites which infected many species of birds. Molecular study by Nested PCR was done to differentiate between *Cryptosporidium* spp.in Ostriches. The results showed total infection rate 26.5% (53/200) Young's Ostriches < 9 Months recorded 30.19% (32/106), while the lowest 22.34% (21/94) recorded in the adult > 9 Months Depending on the nested PCR results, 5 Ostriches male were found infected with *Cryptosporidium* out of 24 with rate 20.83 % (5/24) while female Ostriches recorded 22.86% (16/70). Seven provinces in central and south parts of Iraq including : Wasit, Baghdad, Babylon, Diyala, Karbala, Al-Najaf and Al-Qadisyiah recorded 30% (6/20), 16.67%(8/48), 31.58%(6/19), 11.11%(3/27), 30.77%(8/26) 38.24% (13/34) and 34.62% (9/26) respectively. In relation to months of study. April recorded highest rate of infection72.73% (8/11) while the lowest rate 7.14% (2/28) recorded in July. The sequences analysis of nested PCR products revealed the presence of four *Cryptosporidium* species in Ostriches at central and south provinces of Iraq, namely *C. parvum* (4/20), *C. baileyi* (9/20), *C. meleagridis* (5/20) and *C. galli* (2/20). Confirmation and identification of C. *baileyi* was 100% homology was observed with their respective species sequences reported on Gen Bank on accession numbers (MN410723.1) in China while, *C. parvum* identity was 99.76 % on accession number (KM870602.1) in Thailand. *C. meleagridis* 99.53 % homology sequence identity on accession number (MN410718.1) in China and homology sequence of *C. galli* identity was 99.28 % on accession number (GU816045.1) in Brazil.

***Keywords*:** *Cryptosporidium* species, Ostriches, Nested PCR, Iraq

Introduction

Ostriches and other ratite infected with many intestinal protozoa *Balantidium struthionis, Giardia spp., Trichomonas spp., Cryptosporidium spp., Histomonas meleagridis* and *Hexamita spp.*. Which causes gastrointestinal infection and lead to wasting, anorexia, diarrhoea and death for many ratite spp. (Tully and Shane, 1996) and causing most serious economic losses in ratites through the world. (Nemejc and Lukesova, 2012). *Cryptosporidium* is one of the most widespread protozoan parasites that infects domestic and wild animals (Khan *et al*., 2018). Avian *Cryptosporidium* has been recorded in more than 30 species of domestic and wild birds are *C. baileyi, C. galli* and *C. meleagridis* (Ng *et al.*, 2006). Molecular techniques is used for confirmation *Cryptosporidium* spp detected, is also important since they cannot be identified by morphological and biological traits, in addition to inadequate culture methods, the identification of Cryptosporidium species isolates is solely based on molecular assays, which includes PCR-based genotyping, DNA sequencing of PCR products (Robinson *et al.,* 2006; Feng *et al*., 2007).

In china many studies showed that the Cryptosporidium infection are presented in Zheng Zhou Ostriches 2.48% (10/404) the pattern of oocysts shedding was different in Ostriches ,which has multiple peaks (Sun *et al*., 2007). Zhu *et al.* (2008) found that the total infection rate was 1.7% (14/829). The most positive *Cryptosporidium* isolates come from (20-40 days) old Ostriches, which enhanced that young ostriches chicks more susceptible to *Cryptosporidium* infection than adult. The percentage of *Cryptosporidium* avian genotype in ostriches in the central of Vietnam was 23.7%, prevalence of *Cryptosporidium* varies widely among age groups of Ostriches 45 days, 45-60 days, 61-90 days, 12 months and more than 12 months was 23.1%, 33.3%, 35.2%, 0, and 5.8% respectively (Nguyen *et al*., 2013).

Material and Methods

Molecular Diagnosis

Two hundred fecal samples were collected selectively from farming Ostriches and used for nPCR screening. The nPCR technique was performed for detection *Cryptosporidium* spp. based small subunit ribosomal RNA gene from Ostriches fecal samples. This method was carried out according to Yu *et al.* (2009) and Ruecker *et al.* (2013) A nested PCR was performed to detect *Cryptosporidium* spp. based on 18S ribosomal rRNA gene were design in this study based on NCBI-Genbank *Cryptosporidium* sp. Small subunit ribosomal gene sequence (DQ002931.1) and primer 3 plus design. These primers was provided from Macrogen company, Korea included the first primer pair:

forward (5–CGGGTAACGGGGAATTAGGG–3) and reverse (5-TCGTCTTCGATCCCCTAACT-3), then placed in PCR Thermocycler. Secondary PCR master mix was prepared by using (Maxime^{TM} PCR PreMix Kit (*i*-Taq)) included second primer pair: forward (5-CCTGAGAAACGGCTACCACA-3) and reverse (5-GCCCCCAACTGTCCCTATTAA-3), then placed in PCR Thermocycler.

The two PCR rounds were done under the same conditions: Nested PCR mixtures contained 1x PCR buffer, 5 mM $MgCl_2$, 200 lμ each deoxynucleoside triphosphate, 100 nM each primer and 1.25 U Hot Start Taq polymerase. Cycling conditions consisting of a hot start at 94˚C for 5 min followed by 30 cycles with denaturation at 94˚C for 30 seconds, annealing at 58˚C for 30 seconds, and at 72˚C for 30 seconds, and a final extension at 72˚C for 5 minutes then holding at 4 ˚C forever (Ruecker *et al.,* 2013).

DNA Sequencing Method

DNA sequencing method was performed for species typing of some positive local *Cryptosporidium* isolates and

constructed a phylogenetic tree for our *Cryptosporidium* versus NCBI-Blast-GenBank. Positive PCR 18S rRNA gene were analyzed for DNA sequencing (Molecular Evolutionary Genetics Analysis version 6.0) and Multiple sequence alignment analysis (ClustalW). Evolutionary distances were computed by Maximum Composite Likelihood as described by Tamura *et al.* (2013). Data was statistically analyzed using Chi-square test as described by Petrie and Watson, (2006).

Results

Nested PCR revealed total infection rate with *Cryptosporidium* spp.in Ostriches 26.5% (53/200) (Table -1). All stool samples exhibited a distinct band of 459 bp on agarose gel for *Cryptosporidium* spp. as shown in Figure 1, below.

Table 1 : Total rate of infection with *Cryptosporidium* in ostriches by Nested PCR

Host	No. of samples examined	No. of Positive	%
Ostriches	200	53	26.5

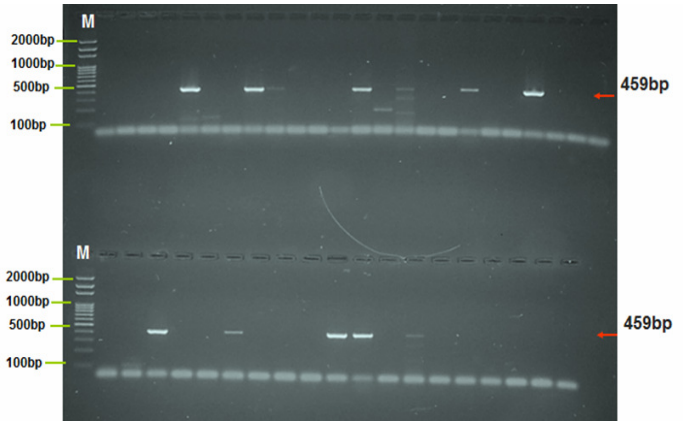

Fig. 1 : Agarose gel electrophoresis image showed the PCR product analysis of small subunit ribosomal RNA gene in *Cryptosporidium* sp. from Ostriches fecal samples. Where M: marker (2000-100bp), lanes showed some positive *Cryptosporidium* sp. at (459bp) PCR product .

The results showed a significant difference (P<0.05) in infection rate among age groups. The higher rate recorded in Young's Ostriches < 9 Months 30.19% (32/106), while the lower 22.34% (21/94) recorded in the adult > 9 Months (Table 2). There was age-related distribution of *Cryptosporidium spp.* among the Ostriches , *C. baileyi* and *C. parvum* was dominant species in young Ostriches 40% (8/20),15% (3/20) respectively, while *C. meleagridis* was dominant species in adult Ostriches > 9 Months age 15% (3/20) although *C. galli* was occurred in adult less than 2 years 10%(2/20)

Table 2 : Rates of infection with in ostriches by nested PCR in relation to age groups

Age groups		No. of samples examined	No. of Positive	%
Young's	< 9 Months	106	32	30.19 *
Adult	> 9 Months	94	21	22.34
Total		200	53	26.5

Significance * (P<0.05)

Depending on the nested PCR results, 5 Ostriches male were found infected with *Cryptosporidium* out of 24 with rate 20.83 % (5/24) while Ostriches female recorded rate 22.86% (16/70). without significant differences.(P<0.05) (Table - 3).

Table 3 : Rates of infection with *Cryptosporidium* in ostriches by nested PCR in relation to sex.

Sex of Ostriches	No. of samples examined	No. of Positive	%
Male	24	5	20.83
Female	70	16	22.86
Total	94	21	22.34

Molecular study by Nested PCR in seven provinces in central and south parts of Iraq including : Wasit, Baghdad, Babylon, Diyala, Karbala, Al-Najaf and Al-Qadisiyah rates of infection 30%(6/20), 16.67%(8/48), 31.58%(6/19), 11.11%(3/27), 30.77%(8/26), 38.24%(13/34) and 34.62% (9/26) respectively. Al-Najaf province showed the highest prevalence rate (38.24%) while Diyala province showed the lowest rate (11.11%).with significant differences among provinces (P<0.05) (Table- 4).

Table 4 : Rates of infection with *Cryptosporidium* in ostriches by nested PCR in relation to Iraqi provinces

Province	No. of samples examined	No. of Positive	%
Wasit	20	6	30
Baghdad	48	8	16.67
Babylon	19	6	31.58
Diyala	27	3	11.11
Karbala	26	8	30.77
Al-Najaf	34	13	38.24 **
Al-Qadisiyah	26	9	34.62 *
Total	200	53	26.5

Significance * (P<0.05)

Nested PCR revealed significant difference (P<0.05) between prevalence of *Cryptosporidium* infection in relation to months of study. April recorded highest rate of infection72.73% (8/11) while the lowest rate 7.14% (2/28) recorded in July. (Table -5)

Table 5 : Rates of infection with *Cryptosporidium* by nested PCR in relation to months

Year	Months	No. of samples examined	No. of Positive	%
2018	December	15	3	20
2019	January	12	4	33.33
	February	12	6	50
	March	18	13	72.22 *
	April	11	8	72.73 **
	May	23	7	30.43
	June	26	4	15.38
	July	28	2	7.14
	August	29	3	10.34
	September	26	3	11.54
Total		**200**	**53**	**26.5%**

Significance * (P<0.05)

Twenty positive PCR Ostriches fecal samples with *Cryptosporidium* species strains were compared with Gen-bank revealing *C. parvum*, *C. baileyi*, *C. meleagridis* and *C. galli* (Table-6).

Table 6 : The NCBI-BLAST Homology Sequence identity (%) between local *Cryptosporidium* spp. Ostriches isolates and NCBI-BLAST submitted *Cryptosporidium* spp. isolates

Local Ostriches *Cryptosporidium* sp. No.	Gen –Bank accession No.	NCBI BLAST Homology sequence identity		
		NCBI BLAST *Cryptosporidium* sp.	Gen –Bank accession No.	Identity (%)
1	MN515110	*C. meleagridis*	MN410718.1	98.82
2	MN515111	*C. baileyi*	MN410723.1	99.53
3	MN515112	*C. meleagridis*	MN410718.1	99.29
4	MN515113	*C. parvum*	KM870602.1	99.07
5	MN515114	*C. baileyi*	MN410723.1	99.52
6	MN515115	*C.meleagridis*	MN410718.1	99.53
7	MN515116	*C. baileyi*	MN410723.1	98.80
8	MN515117	*C. galli*	GU816045.1	99.27
9	MN515118	*C. baileyi*	MN410723.1	99.28
10	MN515119	*C. baileyi*	MN410723.1	99.28
11	MN515120	*C. baileyi*	MN410723.1	100
12	MN515121	*C.baileyi*	MN410723.1	99.52
13	MN515122	*C. baileyi*	MN410723.1	99.26
14	MN515123	*C. baileyi*	MN410723.1	99.29
15	MN515124	*C. parvum*	KM870602.1	99.29
16	MN515125	*C.meleagridis*	MN410718.1	98.80
17	MN515126	*C.galli*	GU816045.1	99.28
18	MN515127	*C.meleagridis*	MN410718.1	99.53
19	MN515128	*C. parvum*	KM870602.1	99.76
20	MN515129	*C.baileyi*	MN410723.1	99.52

Genomic DNA of 20 sequenced isolates of *Cryptosporidium* species from Ostriches were described in phylogenetic tree with respective reference sequenced retrieved from GenBank. Phylogenetic tree was constructed for sequences of *C. meleagridis*, *C. parvum*, *C. baileyi and C. galli* isolated separately to highlight the differences between these four species DNA STAR in Ostriches (Fig. 2).

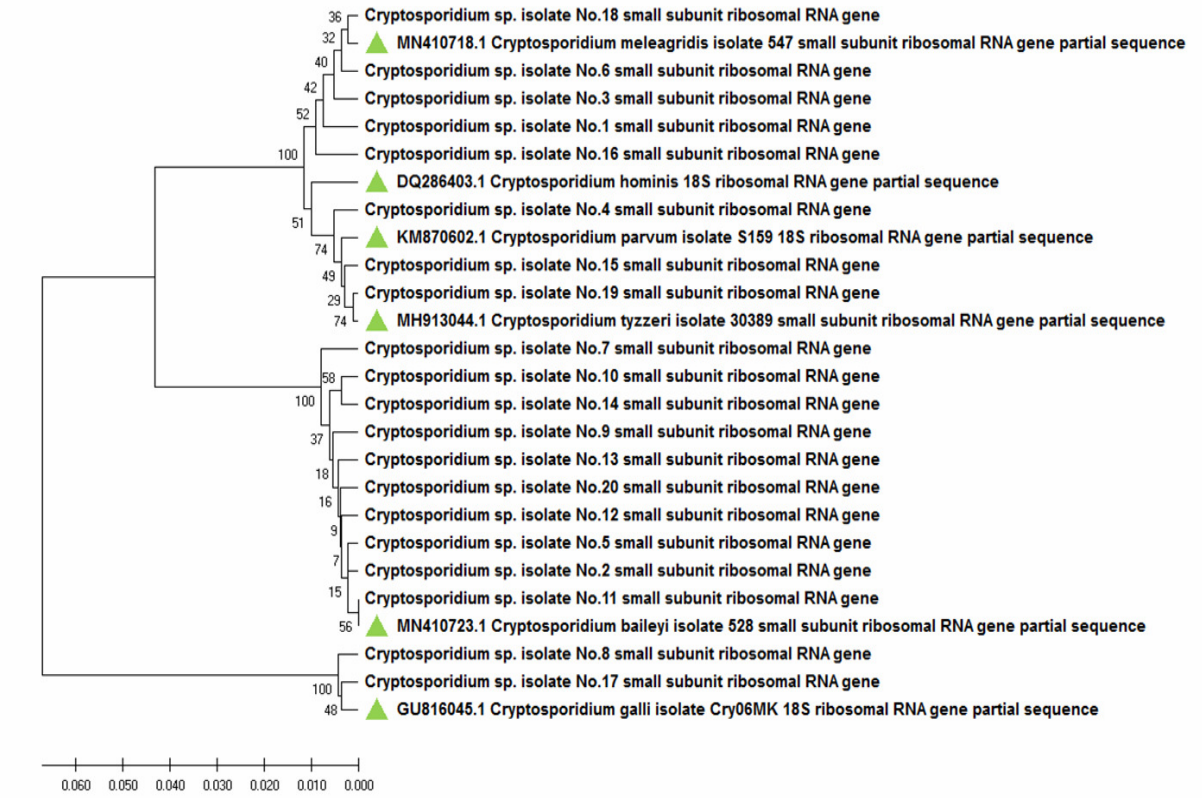

Fig. 2 : Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local *Cryptosporidium spp*. Ostriches isolates that used for genetic *Cryptosporidium* species identification . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA .X version). The local *Cryptosporidium* isolate IQ. Ostrich (No.1 No.3 No.6 No.16 and No.18) were showed closed related to NCBI-BLAST *Cryptosporidium meleagridis* (MN410718.1). The local *Cryptosporidium* isolate IQ. Ostriches (No.2 No.5 No.7 No.9-No.14 and No.20) were showed closed related to NCBI-BLAST *Cryptosporidium baileyi* (MN410723.1). The local *Cryptosporidium* isolate IQ. Ostrich (No.4, No.15 and No.19) were showed closed related to NCBI-BLAST *Cryptosporidium parvum* (KM870602.1). The local *Cryptosporidium* isolate IQ. Ostrich (No.8 and No.17) were showed closed related to NCBI-BLAST *Cryptosporidium galli* (GU816045.1) at total genetic changes (0.010-0.060%).

Discussion

The current molecular study by Nested PCR of *Cryptosporidium* spp. infected Ostriches is the first in Iraq. There are many species of Cryptosporidium were detected by nested PCR protocol targeting the 18S rRNA gene which has been shown highly sensitive and having successfully amplified the DNA from just one oocyst (Xiao *et al*., 1999; Santin *et al.,* 2008; Karanis *et al*., 2010; Thigeel, 2016). The nPCR more sensitive as a compared to modified Ziehl-Neelsen (mZN) staining technique (Uppal *et al* 2014). Therefore the 18S rRNA gene fragment was targeted in the current study to detected *Cryptosporidium* spp. Nested PCR revealed total infection rate with *Cryptosporidium* spp. in Ostriches was 26.5%.This finding was comparable with the results of Nguyen *et al*. (2013) whom recorded infection rate 23.7 % , Silva *et al.* (2010) reported 24.5% from adult birds in Brazil . On the other hand, the infection rate was lower in study of Wang *et al.* (2011) in China which recorded overall prevalence 11.7%, Ng *et al.* (2006) in Australia recorded infection rate in (6.3%). Nakamura *et al*. (2009) reported infection rate 4.86% in captive birds in Brazil.

These differences were attributed to husbandry practices, stressing condition, poor type of feed, water or hygiene management, area of sampling (farming / captive) Ostriches, environmental conditions, the sampling method and samples size (Caccio and Putignani, 2014).

The prevalence showed a significant differences among age groups .the higher rate recorded in Young's Ostriches < 9 Months 30.19% while the lower infection rate 22.34% recorded in the adult > 9 Months age group. These results were agreement with previous studies showed that young Ostriches chicks more susceptible to *Cryptosporidium* infection than adult from the first day of life (Zhu *et al*., 2008). There was age-related distribution of *Cryptosporidium* spp. among the Ostriches, *C. baileyi* and *C. parvum* was dominant species in young Ostriches 40% (8/20), 15% (3/20) respectively, while *C. meleagridis* was dominant species in adult ostriches > 9 Months age 15% (3/20) although *C. galli* was occurred in adult less than 2 years 10% (2/20). These results were accordance with (Meireles and Figueiredo 1992; Pavlasek, 1993; Sreter and Varga, 2000; Morgan *et al*., 2001) whom recorded that *Cryptosporidium baileyi* is a dominant parasite of various species of young birds, including chickens, turkeys, ducks, cockatiels, a brown quail, gulls and Ostrich.

In relation to sex nested PCR results showed that Ostriches male were found infected with *Cryptosporidium* at rate 20.83 %while Ostriches female recorded rate 22.86% without significant differences. Which was accordance with many previous studies (Mohammed, 2010; Bamaiyi *et al*., 2013)

The rates of infection in seven provinces at central and south parts of Iraq including : Wasit, Baghdad, Babylon, Diyala, Karbala, Al-Najaf and Al-Qadisyiah revealed prevalence 30% 16.67%, 31.58% ,11.11% , 30.77% , 38.24% and 34.62% respectively. Al-Najaf province showed the highest prevalence rate (38.24%) while Diyala province showed the lowest rate (11.11%). with significant differences among provinces. The highest results in Al-Najaf was due to many reasons including poor management systems, density of breeding farms, contaminated of water sources as compared to Diyala province.

Nested PCR revealed significant difference between infection rate with *Cryptosporidium* infection in relation to months of study. April recorded highest rate of infection72.73% while the lowest rate 7.14% recorded in July. the result agreement with the Wang *et al*.(2011) which clarify that the extremely shedding of oocysts are the most responsible for high prevalence of *Cryptosporidium* spp. particularly during spring season, and they recorded highest prevalence rate in spring season was 15.6% Goodwin and Brown (1989) recorded high prevalence rate 9% in spring with seasonal period peak of detection at the rainy season. and lowest rate in winter 3.5%, Muchiri *et al.* (2009) showed the peak of prevalence occurred in March and April (19 % and 19 %) respectively of each tow year of study. The differences of low infection in summer it may be the all examined ostriches were adult and have high resistant to infection as compare with young chicks.

Results of DNA sequencing revealed the presence of four *Cryptosporidium* species in Ostriches at central and south provinces of Iraq, *C. parvum*, *C. baileyi*, *C. meleagridis* and *C. galli*. Our results were in agreement with results Xiao and Ryan, (2004) in Korea, Behzadi *et al*., 2009) in Iran whom recorded the species infecting birds are *C. baileyi, C. galli* and *C. meleagridis*. Current *et al.*(1986) recorded the validity of *Cryptosporidium meleagridis* and *C. baileyi* as distinct species in Ostriches, Santos *et al*., 2005 in Brazil reported the species infected bird are *C. baileyi*, *C. meleagridis* and *C. galli*. (Ng *et al*., 2006; Xiao and Fayer, 2008; Qi *et al*., 2014) in China whom showed that *C. baileyi*, *C. meleagridis* and *C. galli* are the commonest *Cryptosporidium* species and have been identified in many avian hosts. in brazilain Ostrich, Meireles *et al*. (2006) detected *C. baileyi*, *C. parvum*, *C. meleagridis*. In China Wang *et al*. (2011) reported *C. baileyi* only in five farms, zoo, and an animal rescue center in Zhengzhou, Henan Province.

The result of phylogenic tree analysis indicated a high genetic variation among 20 local *Cryptosporidium* spp. isolates and other NCBI BLAST *Cryptosporidium spp*. sequences of Ostriches obtain from GenBank with (0.010-0.060%) as total genetic changes and recorded that dominant Ostriches species, *C. baileyi* 45% (9/20) was detected in different age groups of Ostriches, following *C. meleagridis* 25% (5/20), *C. parvum* 20% (4/20) and finally *C. galli* 10% (2/20). The results were accordance with(Meireles and Figueiredo 1992; Pavlasek, 1993; Sreter and Varga, 2000; Morgan *et al*., 2001) whom recorded that *Cryptosporidium baileyi* is a dominant parasite of various species of young birds, Wang *et al*. (2011) recorded that the most common *Cryptosporidium* of Ostriches in China is *C. baileyi*. Zhu *et al*. (2008) recorded *C. baileyi* in Ostrich from many areas in Henan Province, China.

Conclusion

Molecular prevalence by Nested PCR recorded 26.5% infection rate with *Cryptosporidium* in Ostriches, the highest rate recorded in Young's Ostriches < 9 Months, while sex recorded percentage without significant differences among male and female Ostriches. Al-Najaf province showed the highest *Cryptosporidium* prevalence rate than other provinces in central and south parts of Iraq, phylogenic analysis revealed the presence of four *Cryptosporidium* species in

Ostriches : *C. parvum*, *C. baileyi*, *C. meleagridis* and *C. galli*. At First time in Iraq.

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