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MOLECULAR DETECTION OF CRYPTOSPORIDIUM PARASITE IN CHICKENS (BROILER AND LAYER) IN THI-QAR PROVINCE, IRAQ

Moatamed R.K. AL-Ghezey* and Mohammed T.S. Al-Zubaidi

Department of Parasitology, College of Veterinary Medicine, University of Baghdad, Al-Ameria, 10070, Baghdad, Iraq.

Abstract

Cryptosporidium is a widespread parasite in most hosts, including birds, in addition to that it causes economic losses through the death of infected birds or the loss of weight and egg production. Birds are also considered one of the reservoirs and important vectors for infection in humans and animals for this parasite, especially after recording infections without clinical signs. By using the molecular method (nested PCR), the incidence of Cryptosporidium parasite was investigated in Dhi Qar Governorate, (south of Iraq region, located 360 Km south of the capital Baghdad, Iraq) by tested A total of fifty fecal sample from broiler chickens and the same number of laying chickens. The infection rate was in Broiler and laying hens 64%, 36%, respectively. Fourteen samples seven of broiler chickens and the same number of laying hens were selected from pre-diagnosed samples by the PCR nested, where DNA analyzes were identified for them. Four types were shown in laying hens, namely *C. baileyi*. (3/7). *C. parvum* (2/7). *C.galli* (1/7). *C.meliagredis* (1/7).In broiler chickens, DNA analyzes have four types, *C.baileyi.* (2/7). *C.parvum* (1/7). *C.galli* (2/7). *C.meliagredis* (2/7).

Key words : broiler chickens; cryptosporidium; diagnosis; nested PCR; Thi-Qar.

Introduction

Cryptosporidium is enteric protozoan parasite caused cryptosporidiosis in mammals, birds and fish the disease transmitted by contaminated food, water or dust (Smith *et al.,* 2007). Jackson Clark was the first individual who observed the parasite in 1895 in the mucous layer of intestine of rat and was known as swarm spores (xiao *et al.,* 2004*)*. In 1910, Tyzzer called cryptosporidium, which it is a Greek term capability hidden spores, because the difficulty of diagnosing the four crescent sporozoite in the oocyst, in contrast to different sorts of coccidian. Also he described the cryptosporidium in caeca of poultry (Fayer and Xiao., 2008). The most important clinical signs of intestinal cryptosporidiosis is Green or yellow diarrhea with an unpleasant smell and containing mucus, while respiratory cryptosporidiosis show several signs, the most important of which are difficulty breathing, sneezing and mucous respiratory secretions from the nostrils. The diagnosis of cryptosporidiosis in birds is depend generally on microscopy, histopathological, immunological, and molecular methods (Lindsay *et al.,* 1989; OIE, 2008). Because of the widespread prevalence of this parasite and its veterinary and economic importance to it, the study designed to explore the spread of the Cryptosporidium parasite in chickens (broiler and layer) and identified of the species by sequences analysis of some samples.

***Author for correspondence :** E-mail: mohabood24@gmail.com

Materials and Methods

Collection of samples

Fifty dropping samples were randomly selected from layer chicken and 50 from broiler chickens dropping for nPCR screening. The *18S rRNA* gene-based nPCR was aimed at the detection of *Cryptosporidium* species from the fecal samples of chickens and as per the method used by (Yu *et al.,* 2009 and Ruecker *et al.,* 2013).

Extraction of Genomic DNA

Dropping-sample based extraction was conducted by AccuPrep® stool DNA Extraction Kit and per the company protocol (Bioneer from Korea).

PCR screening

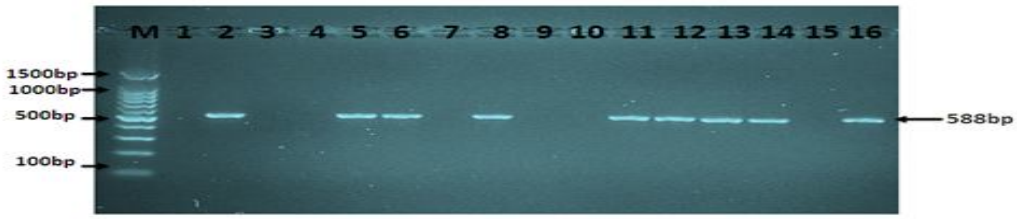
The *18SrRNA* gene-based primers of the nested PCR for the detection of *Cryptosporidium* spp. were designed via the NCBI-Genbank and primer 3 plus. Macrogen company, Korea, from where those primers were purchased (Table 1). The components(Primer (10pmol, Product (PCR) and H_2O (Molecular Biology Use)) inserted into standard Maxime PCR PreMix tubes containing other ingredients such as DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, and $MgCl_2$. Then, the final mixture was vortexed, 3000rpm-centrifuged for 3mins, and placed in a thermocycler (MyGene-Bioneer, Korea).

Results and Discussion

A total of 100 fecal samples were randomly taken from chickens (fifty samples from laying hens and fifty from broiler chickens), are submitted to molecular methods. The molecular diagnosis showed a higher infection rate (64% in broilers and 36% in layers. Genomic DNA extracted from layer and broiler chicken fecal samples submitted to molecular analysis by nested PCR using small subunit ribosomal RNA gene-specific primers in order to identify the *Cryptosporidium* spp. The nested PCR results of all 100 samples employed in the study exhibited a distinct band of (588) bp) Nested PCR product size on agarose gel confirming the presence of *Cryptosporidium* spp. in both broiler and layer chickens (Fig. 1-1).

Through the current study and previous studies, a clear difference was observed of the infection rates that recorded in different regions of the world, Perhaps the reason for this difference is due to the use of different methods of diagnosis (molecular or microscopic methods) Add to the sample origin (fecal or tissue sample) And other influencing factors such as temperature, humidity, age, months of study, management, the hygiene, and geographical location (Helmy, 2017; Al.Zubaidi *et al.,* 2018).

Ghiidaa and Ikhlas., (2015) They found the infection rate in broiler chickens (42.9%), (Itakaura *etal*., 1985) he revealed that the prevalence rate was in broiler more than layer chickens (36.8- 33.3) respectively, (Kichaw *et al.,* 1996) and (Alzubidi *et al.,* 2018) in broiler chickens they recorded a rate of infection reached 36%, 35% respectively while Al-Attar and Abdul Aziz (1985), in Baghdad, Al- Taei (2015) in Babylon and Kucukerden *et al.,* (1999) in Turkey in broiler chickens, who recorded an infection rate of 8.8%,14%,4.4% , respectively. The results of the study in layer chickens differed with what Itakura (1984) recorded in the United States of America and with what was recorded by Rongjun *et al*., (2010) in china. The infection rates were (5.9%, 10.6%), respectively. The reason for the high rate of infection in broiler chickens compared to laying hens is that the laying hens are raised in cages, while broiler chickens are raised on the ground in addition to the inverse relationship between immunity and weight, as laying hens have high immunity while broiler chickens have a high a conversion efficiency with Less immunity, as well as different results according to the type of breeding from one region to another, (Bakr, 2005); AL-Khayat, (2015).



Figure(1-1): Agarose gel electrophoresis image that showed the Nested PCR product analysis of small subunit ribosomal RNA gene in Cryptosporidium spp. From layer chickens feces samples. Where M: marker (1500-100bp) Lanes (1-16) some Positive amplification Cryptosporidium spp. at (588bp) PCR product.

Table 1: *Cryptosporidium* primers of the nested PCR.

Primer		Sequence 5'-3'	Amplicon
18SrRNA geneCryptosporidium sp PCR	F	CGGGTAACGGGGAATTAGGG	665bp
	R	ACCTCCAATCTCTAGTCGGCA	
18SrRNA geneCryptosporidium Nested PCR	F	CGCGCAAATTACCCAATCCT	588bp
	R	ACCTCCAATCTCTAGTCGGCA	

Fourteen samples randomly selected from both broiler chickens 7 isolates and layer chickens 7 isolates were positive by nested PCR, of *Cryptosporidium* DNA samples were successfully sequenced. Four types of species were efficiently discriminated using a nested-PCR based tool, namely *C. baileyi*, *C.meliagredis*, *C. parvum* and *C. galli* (Table 1-1). The most predominant detected parasite was represented by *C. baileyi* (5/14). Followed by *C. galli*, *C. parvum*, *and C. meleagridis* all spp (3/14). According to the analysis, the C bailey was shown in three samples of laying hens (MT645522, MT645523 and MT645534), but In broiler chickens, this spp was shown in two samples (MT645532 and MT645533) while The *C. galli* was recorded in two samples in broiler chickens (MT645525 and MT645531) and in one sample in laying hens (MT645529). However *C. meliagredis* appeared in two samples in broiler (MT645524 and MT645526) and one sample in layer

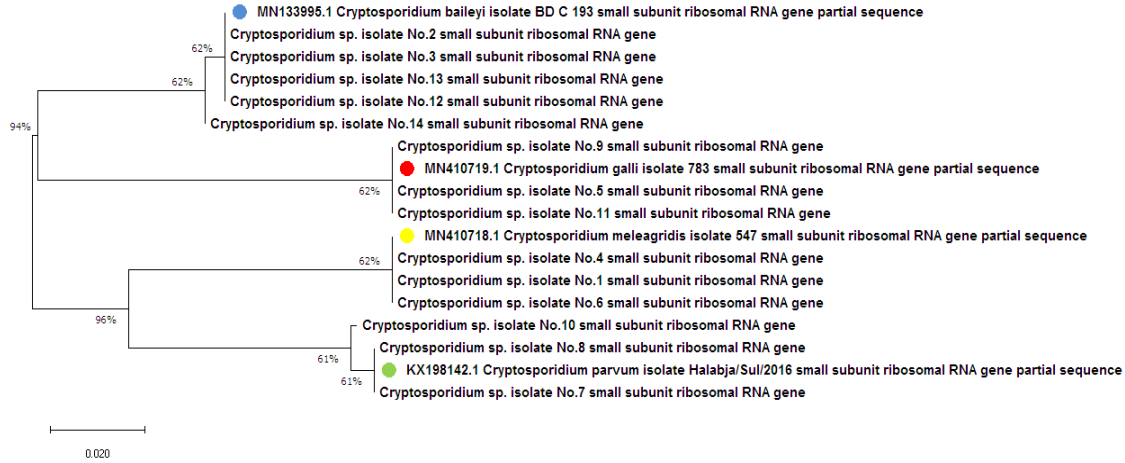


Figure (1-2): Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local Cryptosporidium sp. Chicken isolates that used for genetic species identification . The phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model tree method) in (MEGA X version). The local Cryptosporidium sp. chicken isolate (No.1, No.4, and No.6) were showed genetic closed related to NCBI-BLAST Cryptosporidium meleagridis (MN410718.1). The local Cryptosporidium sp. chicken isolate (No.7, No.8, and No.10) were showed genetic closed related to NCBI-BLAST Cryptosporidium parvum (KX198142.1). The local Cryptosporidium sp. chicken isolate (No.5, No.9, and No.11) were showed genetic closed related to NCBI-BLAST Cryptosporidium galli (MN410719.1). The local Cryptosporidium sp. chicken isolate (No.2, No.3, No.4, No.6, No.12, No.13 and No.14) were showed genetic closed related to NCBI-BLAST Cryptosporidium baileyi (MN133995.1) at total genetic changes (0.020%).

Table 2: The NCBI-BLAST Homology Sequence identity (%) between local Cryptosporidium sp. chicken isolates and NCBI-BLAST submitted Cryptosporidium species isolates.

Type of chicken	Cryptosporidium spp. isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)			Identity (%)
			Identical NCBI BLAST Cryptosporidium species	Genbank Accession number	Country	
Layer	Cryptosporidium sp. isolate No.1	MT645521	Cryptosporidium meleagridis	MN410718.1	China	100%
Layer	Cryptosporidium sp. isolate No.2	MT645522	Cryptosporidium baileyi	MN133995.1	Bangladesh	100%
Layer	Cryptosporidium sp. isolate No.3	MT645523	Cryptosporidium baileyi	MN133995.1	Bangladesh	100%
Broiler	Cryptosporidium sp. isolate No.4	MT645524	Cryptosporidium meleagridis	MN410718.1	China	99.67%
Broiler	Cryptosporidium sp. isolate No.5	MT645525	Cryptosporidium galli	MN410719.1	China	100%
Broiler	Cryptosporidium sp. isolate No.6	MT645526	Cryptosporidium meleagridis	MN410718.1	China	99.55%
Broiler	Cryptosporidium sp. isolate No.7	MT645527	Cryptosporidium parvum	KX198142.1	Iraq	100%
Layer	Cryptosporidium sp. isolate No.8	MT645528	Cryptosporidium parvum	KX198142.1	Iraq	100%
Layer	Cryptosporidium sp. isolate No.9	MT645529	Cryptosporidium galli	MN410719.1	China	99.34%
Layer	Cryptosporidium sp. isolate No.10	MT645530	Cryptosporidium parvum	KX198142.1	Iraq	99.12%
Broiler	Cryptosporidium sp. isolate No.11	MT645531	Cryptosporidium galli	MN410719.1	China	100%
Broiler	Cryptosporidium sp. isolate No.12	MT645532	Cryptosporidium baileyi	MN133995.1	Bangladesh	100%
Broiler	Cryptosporidium sp. isolate No.13	MT645533	Cryptosporidium baileyi	MN133995.1	Bangladesh	100%
Layer	Cryptosporidium sp. isolate No.14	MT645534	Cryptosporidium baileyi	MN133995.1	Bangladesh	99%

chickens (MT645521) Finally, the *C. parvum* was recorded in one sample in broiler chickens (MT645527) and two samples in laying hens (MT645528 and MT645530) (Table 4-11). The analysis confirmed the documentation of *C. bailey C. galli, C. mliagredis* and *C. parvum* since (99 - 100% homology was detected with their respective species sequences reported on Gen-Bank accession numbers (MN133995.1) in Bangladesh, (MN410719.1, MN410718.1) in China and (KX198142.1) in Iraq respectively (Table 1-1). The Phylogenetic tree analysis firstly used to resolve the disagreement of the classification of the genus *Cryptosporidium*. Phylogenetic tree made for all 14 sequenced isolates of *Cryptosporidium* species with respective reference sequences retrieved from GenBank. Phylogenetic tree made for sequences of *C. bailey, C. galii*, *C. meliagredis* and *C. parvum* isolates separately to highpoint the variances between these 4 species by DNA STAR in chickens (Fig. 1-2). Phylogenetic tree established the grouping of *C. bailey, C. meliagredis*, *C. galli* and *C. parvum* in chickens. The Datasets for these species provide strong support for the genetic distinctiveness among these species.

Cryptosporidium species in chickens: *C. bail*ey, *C. galli, C. meliagredis* and *C. parvum*. Our results were in agreement with results recorded by Nakamura *et al* (2009) from Brazil, qi *et al.,* (2011) from China and (Ghiidaa and Ikhlas., 2015) in Iraq.

Furthermore, *C. baileyi*, *C. meleagridis* and *C. parvum* are highly prevalent species and were identified in a wide range of birds belong to several orders around the world. High occurrence of *C. meleagridis* 21% and less *C. baileyi* 0.6% was identified in the red-legged partridge from an aviary in the Czech Republic (Máca and Pavlásek 2015). *Cryptosporidium meleagridis* and *C. baileyi* were detected also in chickens and turkey in Algeria (Laatamna *et al.,* 2017), Ewald *et al*. (2017) from Brazil recorded *C. meleagridis* in 57 (62.6%), *C. baileyi* in 15 (16.4%) and *C. parvum* in 3 (3.2%) in free-range chicken. *Cryptosporidium baileyi* 7.0% (33/471) and *C. meleagridis* 3.2% (15/471) were identified in farmed chickens in China (Liao *et al.,* 2018). While (Helmy., 2017) detected the *Cryptosporidium parvum* in turkeys (7/86) and in broiler (5/158) while in layers (1/12). While *Cryptosporidium baileyi* was detected in broiler only (2/256). *C. baileyi* infection very important in chickens because the infection occur on the peaks of intestinal villi, the infiltration of inflammatory cells in the layers of the intestine is only a response caused by the extensive damage and destruction of the epithelial cells also caused respiratory problems. (AL-Khayat, 2015 and al-Zubaidi *et al.,* 2018) While *Cryptosporidium galli* recorded in the big ages of broiler and laying hens, and this is contrary to what was reported by (Pavlasek, 2001) which indicated that this species occurrence of infection at the age of 9 day and does not infect the chickens with a lifespan of more than 40 days.

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