Research Article

Molecular Detection of *Leucocytozoon* spp. in Broiler Chickens in Baghdad City

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Abstract | The purpose of this study was to detect the infection by *Leucocytozoon* spp. parasite in broiler chickens by using thin blood smears Nested and quantitative real time (qRT)- Polymerase Chain Reaction methods in Baghdad city between 1/11/ 2021 and 31/3/2022. Fifty jugular venous blood samples (about 5ml) were collected. The total infection rate of *Leucocytozoon* spp. in the blood smears was 10% (5/50), which divided into males 6% and in females 4%, while by qRT-PCR was 4% (2/50) with an equal ratio between males and females (2%). In conclusion, it is the first diagnosed study in Baghdad city to detect *Leucocytozoon* spp. in broiler chickens by using qRT– PCR.

Keywords | *Leucocytozoon*, Broiler, Chickens, q RT–PCR, Haemosporidian, Avian, Birds

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INTRODUCTION

The industry of domestic poultry plays a very important foundation of protein includes eggs and meat for humans, and plays a crucial role as a revenue provider in the national economy (Mirzaei *et al.*, 2020). There are more than 200 morphologically identified avian haemosporidian species in avian (Bell *et al.*, 2015). Their infections are the most prevalent infections of the different parasitic diseases that caused by haemosporidian parasites as a vector-borne parasites that infect 30 mammals, birds, amphibians, reptiles, over the world (Valkiünas, 2005). According to Takang *et al.* (2017) avian blood parasites are diseases that negatively affects 33 poultry productivity and results in economic losses; because they are known to causes pathogenic effects in their host that resulting in Emaciation, anemia, reproductive failure, growth retardation, decreased productivity, and high mortalities, as well as potential harmful effects on their community structure and behavior (Dunn *et al.*, 2011; Gimba *et al.*, 2014; Ogbaje *et al.*, 2019). *Plasmodium*, *Haemoproteus* and *Leucocytozoon* well known as avian haematozoan infections of domestic and wild birds that can be decreased their productivity with high mortality and maybe affects their population's dynamics (Mirzaei *et al.*, 2020). Avian blood parasites that called leucocytozoids are infect a wide variety of avian hosts (Valkiünas *et al.*, 2010; Zhao *et al.*, 2014). All *Leucocytozoids* have a host-specific pathogenicity in the levels of the order, family and occasionally species (Forrester and Greiner, 2009). According to Levine *et al.* (1980) *Leucocytozoon* belongs to the Phylum Apicomplexa's, suborder Haemsporonia and Its infection has been documented in a variety of birds and regions including Africa (Huchzermeyer, 1993; Permin *et al.*, 2002; Sehgal *et al.*, 2006), New Zealand (Hill *et al.*, 2010), Spain (Merino *et al.*, 1997), Turkey (Ozmen *et al.*, 2005, 2009), and the United States (Stuht *et al.*, 1999). It

has been commonly arisen in domestic chickens (*Gallus gallus domesticus*) in Thailand, (Worasing *et al*., 2001; Tongkamsai and Napoon, 2015; Takang *et al*., 2017; Prasopsom *et al*., 2020), and in the other countries of Southeast Asia (Paperna *et al*., 2008).

Three distinct species of *Leucocytozoons*, including *L. macleani* (Sambon, 1908), *L. caulleryi* (Mathis and Léeger, 1909) and *L. schoutedeni* (Vandenbranden and Bequaert, 1913) were found in domestic chickens (Valkiünas, 2005; Forrester and Greiner, 2009). The pathogenicity of *Leucocytozoon* was determined by the severity of the infection (Smith *et al*., 2015). *L. schoutedeni* and *L. macleani* are much less pathogenic than *L. caulleryi* (Valkiünas, 2005). It affects circulating leucocytes (Suprihati and Yuniarti, 2017), as well as tissue erythrocytes, macrophages and endothelial cells. In addition, it leads to the formation of enormous tissue schizonts that can grow to 700 µ in length (Atkinson and Van Riper III, 1991). In Southeast Asia, the most two species affecting chickens are *L. caulleryi* and *L. sabrazesi* (Suprihati and Yuniarti, 2017). Acute clinical symptoms in the farmed poultry showed tachypnea, lethargy, anemia, green feces, reduced appetite, loss diarrhea, disturbances in the CNS (Central Nervous System) and leukocytosis (Legowo *et al*., 2017).

The molecular diagnosis techniques by using Polymerase Chain Reaction (PCR) mentioned high diversity of these parasites in bird's communities (Ricklefs *et al*., 2007; Kim and Tsuda, 2010). Real Time- PCR is a highly effective diagnostic method in avian to determine the haemosporidian parasites in blood and liver samples (Bell *et al*., 2015; Lutz *et al*., 2015). Prasopsom *et al*. (2020) was found that the microscopically and molecular studies discovered the similarity in morphology and phylogenetic tree of some specific parasite (*Leucocytozoon*) in both fighting cocks and chickens.

Due to the very lack information about the use of qRT-PCR for detection *Leucocytozoon* in broiler chickens in Baghdad city, this study was conducted.

MATERIALS AND METHODS

Samples collection

From 1/11/2021, until 31/3/ 2022, fifty (33 males and 17 females) jugular venous blood samples (about 5 ml) were collected in EDTA tubes from broiler chickens, which had been obtained from several local markets in Baghdad city. Blood samples were divided into an equal part, the first one (about 2 ml) was utilized for thin blood smears and stained by Giemsa stain according to Soulsby (1982) and the second part (about 3 ml) was kept at -20 °C for molecular analysis by using nested and qRT –PCR. The second primers of Nested PCR were also used for qRT-PCR illustrate as follows:

The nested PCR program was done according to Suprihati and Yuniarti (2017) and The reaction components of second primers of nested PCR as follows:

Components	(Final volume) 25µL
Master mix or GoTaq® green master mix	12.5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
Reaction from PCR 1	1.5µl
Distill water	9µl

The optimal conditions for detection PCR as follows:

No.	Phases	Tm (°C)	Time	No. of cycles
1-	Initial denaturation	94°C	3 min	1 cycle
2-	Denaturation -2	94°C	45 Sec	35 cycle
3-	Annealing	56°C	30 Sec	
4-	Extension-1	72°C	1min	
5-	Extension -2	72°C	7 min.	1 cycle

DNA extraction

DNA extraction from blood samples was performed by using Quick-gDNA™ Blood MiniPrep (Catalog No. D3025 - USA) according to the manufacture procedure and the eluted DNA was stored at - 20°C and it was used in qRT-PCR.

Agarose gel electrophoresis

Electrophoresis was carried out according to Sambrook *et al*. (1989), after DNA was extracted in order to evaluate the interaction of PCR. The size of the band was 503bp of the bundle produce by the interaction of the PCR and the agarose gel should be distinguished when the standard DNA is present, that was previously prepared by add 1.5 g agarose to TBE buffer 100 ml and Red Safe stain melting with it. The agarose gel was created through condensation.

2-Primers:

Gene	Primers	Primers sequence	Tm (°C)	GC (%)	Size of product (bp)	Reference
Cyt b	Forward	5′- ATGTGCTTTAGATATATGCATGCT -3′	50.55	33	503	Suprihati and Yuniarti (2017)
	Reverse	5′ GCATTATCTGGATGTGATAATGGT-3′	52.26	38		

Conducting q real-time- PCR

Master Mix (2X) (KAPA SYBR FAST PCR) which contains a unique DNA polymerase designed for PCR and using SYBR Green fluorescent dye, MgCl2, and dNTPs. Based on the following table of each component that is needed:

Components	Volume (µl)	Final concentrations
qPCR Master Mix KAPA, SYBR FAST (2X) global	10	2X
Forward primer	1	0.2µM
Reverse primer	1	0.2µM
Nuclease-free water	3	-
Template DNA sample volume	5	1pg-100ng
Final volume	20	-

Cycling program of qRT- PCR

KAPA SYBR FAST PCR Master Mix (2X) is designed for high-performance Real-Time PCR. The kit contains a novel DNA polymerase engineered via a process of molecular evolution resulting in a unique enzyme specifically designed for quantitative real-time PCR (PCR) using SYBR green I dye chemistry. KAPA SYBR FAST DNA Polymerase has been engineered to perform optimally in stringent qPCR reaction conditions, exhibiting dramatic improvements in signal-to-noise ratio (fluorescence), quantification cycle (Cq), linearity, and sensitivity and proprietary buffer system improves the amplification efficiency of difficult targets, including both GC- and AT rich templates. KAPA SYBR FAST PCR Master Mix (2X) Kits are a ready to-use cocktail containing all components (except primers and template) for the amplification and detection of DNA in PCR. The KAPA SYBR FAST PCR Kit is supplied as a 2X master mix with integrated antibody-mediated hot start, SYBR Green Fluorescent Dye, $MgCl_2$, dNTPs, and stabilizers.

The qReal-Time PCR conditions were illustrated as follows:

Steps	Temp.(°C)	Time	Cycles
Enzyme activation	95	5:00 min.	Hold
Denaturation	95	30 sec.	40
Annealing	51	30 sec.	
Extension	72	30 sec.	
	90	15 sec.	100

Statistical analysis

Chi square was use to evaluate the effects of *Leucocytozoon* spp. infection in the broiler chickens under the significant level P ≤ 0.05% (Al-Mohammed *et al*., 1986).

RESULTS AND DISCUSSION

The total infection rate of *Leucocytozoon* spp. by thin blood smears in broiler chickens was 10% (5/50) ,which divided in the females 11.76 % (2/17) and in the males 9.09 % (3/33) with significant differences (P≤ 0.05) (Table 1; Figure 1) ,while by qRT-PCR the infection rate was 4% (2/50) ,in males 1/33 (3.03%) and in females 1/17 (5.88%) with significant differences (P≤ 0.05) (Table 2; Figures 2, 3).

Table 1: Shows the infection rate of *Leucocytozoon* spp. in broiler chickens by thin Giemsa stained blood smears.

No. of samples examined	No. of positive (%)	Males positive (%) (No. 33)	Females positive (%) (No. 17)
50	5(10)	3(9.09)	2(11.76)
χ^2	45		

P≤0.05

Table 2: The infection rate of *Leucocytozoon* spp. in broiler chickens by using qRT-PCR.

No. of samples examined	No. positive (%)	Males positive (%) (No. 33)	Females positive (%) (No.17)
50	2(4)	1(0.03)	1(5.88)
χ^2	48		

P≤0.05

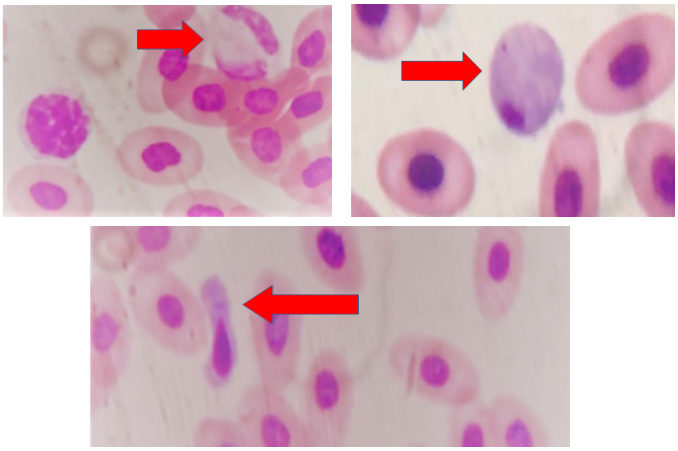

Figure 1: Thin blood smears Giemsa stained, shows different forms of *Leucocytozoon* spp. in boiler chickens (X100).

A highest diversity of haemosporidian parasites in the birds has been possess, including the different genera: *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* (Valkiūnas, 2005). *Haemoproteus* and *Leucocytozoon* are the best-known species, and both are rather host-specific and closely related to the other species (Bennett *et al*., 1993; La Pointe *et al*., 2012), while the most common blood parasites were *Leucocytozoon* species and *Plasmodium* species (Aiyedu *et al*., 2022). The infections with *Leucocytozoon* were found in chickens and in different birds Southeast Asia (Prasopsom *et al*., 2020).

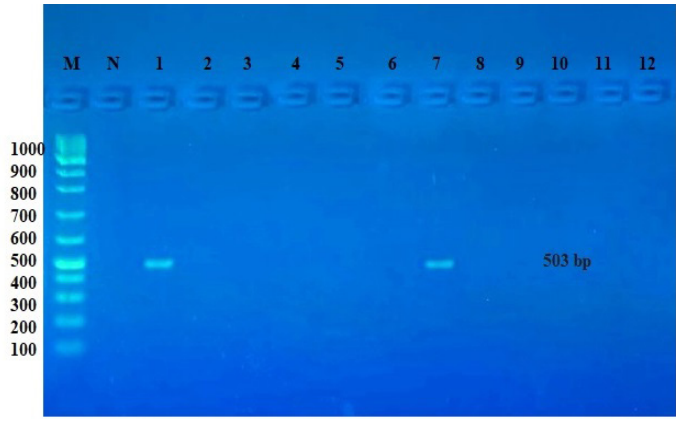

Figure 2: PCR products (bp503) of electrophoresis in agarose (1.5%) at 5 Volt /cm^2 for1:30 hours. Lanes 1 and 7 positive, M: DNA ladder (100).

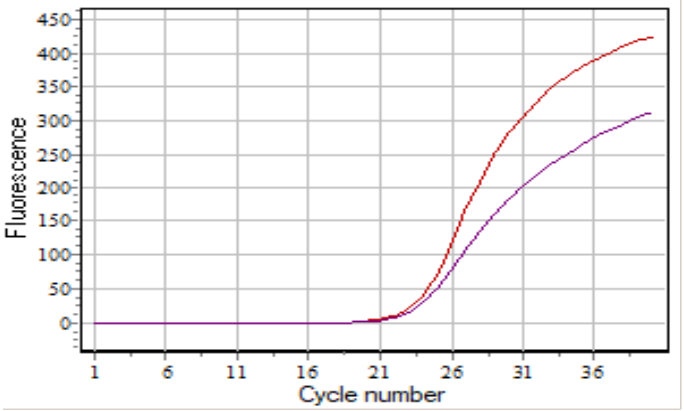

Figure 3: The *Leucocytozoon* spp. positive results in qRT-PCR (red and violate color) with Cp. Fam. 23.4 and 23.1.

It had been identified morphologically with molecular tests (Martinsen *et al.*, 2006). All these diagnostic examinations have differences in their advantages and disadvantages, but the combination of them was the best choice for diagnosis of this parasite and other avian haemosporidian (Cosgrove *et al.*, 2006; Valkiūnas *et al.*, 2008; Garamszegi, 2010). The stained thin blood smears were used for finding the characteristic gametocytes (Valkiūnas, 2005; Forrester and Greiner, 2009). The total infection of the *Leucocytozoon* in the present study by Giemsa stained blood smears was 10% that was to be relatively agree with Legowo *et al.* (2017) who recorded from Giemsa blood smears of chicken breeder's, 11 samples were positive (6.88%), while disagree with Takag *et al.* (2017) who found infection rates of backyard chickens that involved of *L. caulleryi* (0.47%) and *L. sabrazesi* (72.66%). Also, Prasopsom *et al.* (2020) found an infection rate in chickens and fighting cocks was 46% by Giemsa blood-stained smears.

The total infection rate of *Leucocytozoon* spp. by using qRT-PCR was 4% that disagree with Win *et al.* (2020) who firstly microscopic detection and molecular identification of *Leucocytozoon* parasites from seven different areas of Myanmar and in the blood smears were detected the gametocytes from village chickens 17.6% (81/461), while by nested PCR targeting mitochondrial cytochrome b (*cyt b*) genes of the parasite (17.6%). Nevertheless, based on microscopic inspection, Piratae *et al.* (2021) discovered that 22 out of 250 (8.8%) samples had confirmed Leucocytozoon infections, but using nested-PCR, 50 samples tested positive, 5 of which were *L. schoutedeni* (2%), and 45 of which were *Leucocytozoon* sp. (18%). On than same hand, in the indigenous Thai chickens the prevalence of *L. sabrazesi* was 68.61% of 446 Giemsa stain thin blood smears from 108 farms in Nan province, Thailand (Jaijan *et al.*, 2012). On the other hand, it was lower than the prevalence of *L. schoutedeni* infected village chickens (18.3%) in Uganda and Cameroon (Sehgal *et al.*, 2006). The blood samples composed from 345 birds (19 guinea fowls and 326 chickens) examined by thin blood smear for detected blood parasites, the most prevalent blood parasite was *Leucocytozoon* sp. (42.90%) then followed by *Plasmodium* spp. (33.62%) was recorded by Aiyedun *et al.* (2022). *Leucocytozoon* was present in 2.9% of the 335 birds samples (Mirzaei *et al.*, 2020). Approximately seventy-two blood samples (from 26 chickens, 22 pigeons and 24 ducks) were used in a different investigation. *Leucocytozoon* 146 species were found in the blood of chickens (34.6%), ducks (58.3%), and in pigeons (22.7%), as *Haemoproteus* species and *Leucocytozoon* sp. (Momin *et al.*, 2014). Ahmadov *et al.* (2019) had been recorded an infection rate of *Leucocytozoon* of 125 chickens (*Gallus gallus domesticus*) 12.8 % (16/125). Additionally, of 17 wild birds from 9 species (n=1332) were discovered to have the *leucocytozoon* and the overall frequency was 1.3% (Shurulinkov and Golemansky, 2003), and Mirzaei *et al.* (2020) found the prevalence of the parasite was 2.9% in the avian blood parasite. In general, PCR technologies is acknowledged as a technical innovation which is important to the identification of microorganisms, enhancing the sensitivity, accuracy, and precision of the diagnosis of many infectious illnesses of farm animals, including avian species. The PCR technology, RT has arisen as technical innovations that are playing an ever-increasing role in clinical diagnostics and research laboratories. It is the ideal approach for the quick identification of infections, particularly those difficult to grow. The screening of illnesses that are challenging to cultivate in the lab is another use for it. Due to its ability to identify microorganisms early, PCR technology has found use in Parasitology as well, assisting in the accurate diagnosis and treatment of parasitic infections (Das *et al.*, 2017). A highly successful method for detecting about the haemosporidian parasites in avian blood and liver samples was qRT-PCR. This methodology is an important instrument in the rapidly developing field of avian haemosporidian research since the real-time PCR test demonstrated superior efficacy to two commonly used molecular transmission approaches, traditional PCR and

nested PCR (Bell *et al*., 2015). In the past, it was discovered that parasites were present in a different samples (Teal *et al*., 2012; Xu *et al*., 2015). A number of *Leucocytozoon* spp. genes were created in the similar manner, followed by the cytochrome b gene (*cyt b*). According to Hellgren *et al*. (2004) and Bernotienè *et al*. (2016), it was frequently the gene that was utilized the most. For these parasites, slower molecular clocks have been proposed than for the host cells (Bensch *et al*., 2013). Generally, the differences between the results of present study and the previous studies may have been due to geographical and seasonal differences (Prasopsom *et al*., 2020) or to the ecological conditions on farms that adds a lot to the rate of the Leucocytozoon infections (Legowo *et al*., 2017). Additionally, it is anticipated that changes in the environment will have an impact on how common and widespread vector-borne haemosporidian parasites are (Van Hemert *et al*., 2019). Conversely, the prevalence of haemosporidian infections of chickens in was substantially correlated with chicken species, breeds of poultry, age, and sex (Aiyedun *et al*., 2022). The protocol for real-time screening of samples has dramatically increased, but the throughput of sample screening or exact species fertility is unknown due to high genetic diversity, deficient sampling in highly diverse regions, and the limitations of any screening method for haemosporidian, whether using a straight forward microscope or molecular techniques (Bell *et al*., 2015). The infections has low intensity can also be overlooked by such molecular approaches, such as nested PCR (Valkiūnas *et al*., 2008). The diversity of *Leucocytozoon* may be a great in areas through a diverse bird population (Lutz *et al*., 2015), at some host populations besides that (Reeves *et al*., 2015). According to studies (Lutz *et al*., 2015). The difficulty in detecting haemosporidian DNA is further exacerbated by the fact that host DNA are significantly more concentrated in samples than parasite DNA (Freed and Cann, 2006). Restriction digestion and the microscopy protocol (Beadell and Fleischer, 2005), income significantly more time than the nested PCR was quiet necessary to amplify DNA for sequencing, even though it took a lot longer than real-time PCR (Bell *et al*., 2015), for its aptitude to generate both quantitative and qualitative results, RT- PCR is regarded as a quick and perfect platform for diagnosing a variety of infectious diseases (Avlami *et al*., 2010), as a result, its cost was safely increased significantly without compromising sensitivity, and screening time was reduced (Bell *et al*., 2015). On the other hand, the slightly elevated occurrence of infections with *Plasmodium* spp. and *Leucocytozoon* spp. revealed the area's mosquitoes and *Simulium* spp. insect vectors were convenient (*Simulium* spp. and Mosquitoes) in the area (Aiyedun *et al*., 2022; Prasopsom *et al*., 2020) referred to the high blood parasite infection rate in both backyard chickens and FCs that may have been due to the free-range habitats that could increase intermediate insect host contacts. According to Valkiūnas *et al*. (2008) the primary way to these parasites identifying was the microscopic inspection of blood films. Although, analysis of blood films is an efficient means to recognize and quantify parasites, the prevalence estimates may rely on the method employed in their detection and necessitates experience in creating, staining, and studying such films (Fallo *et al*., 2005).

CONCLUSIONS AND RECOMMENDATIONS

Based on the data of the results, this is the first study in Baghdad city concluded to detection of *Leucocytozoon* spp. infection in broiler chickens diagnosed by using (qRT-PCR), a highly successful method for detection the presence of haemosporidian parasites in avian blood. This methodology is an important tool in the rapidly developing field of avian haemosporidian research since the real-time PCR test demonstrated superior efficacy and commonly used as molecular screening approaches, single PCR and nested PCR.

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NOVELTY STATEMENTS

It is the first study in Baghdad city, Iraq to detect *Leucocytozoon* spp. infection in broiler chickens that diagnosed by using nested and qRT-PCR. The early diagnosis of parasite have an economic benefit through reduce the infection in the hosts (avian and birds) and using the suitable treatment to limit its spread in the area.

AUTHOR'S CONTRIBUTION

RMI: Research article, experiment design preparing materials, work the real time qPCR, get the results and data creation. HMAA-R: Proposal and writing article.

Conflict of interest

The authors have declared on conflict of interest.

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