Plant Archives Vol. 20, Supplement 2, 2020 pp. 7-10 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

MOLECULAR DIAGNOSIS OF *NEOSPORA CANINUM* IN BRAIN TISSUES OF LOCAL BREED DOMESTICATED CHICKENS (*GALLUS GALLUS DOMESTICUS*) AT AL-FALLUJAH DISTRICT, IRAQ

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Abstract

The aim of this study was conducted to estimate the prevalence of *Neospora caninum* infection in domesticated chickens by using the conventional molecular technique (PCR). A total 100 brain samples were examined by the target Nc-5 gene with an infection rate 6% (6/100).

This study is the first molecular diagnosis of *N. caninum* in domesticated chickens in Iraq, and the results are highlight on the role of these animals in the maintain and spread the infection to canids in the environment.

***Keywords*:** *Neospora*, *caninum*, Molecular Diagnosis, Domesticated Chickens, PCR.

Introduction

Neospora caninum is intra-cellular apicomplexan protozoan parasite of worldwide distribution and it have been implicated in abortion and reproductive disorders in livestock mainly in ruminants (Dubey *et al*., 2007). The parasite is a common cause of abortion in cattle with a significant economic impact in the dairy and beef industries (Trees *et al*., 1999). It was first reported as a parasite of the domesticated dogs (*Canis familiaris*) associated with encephalomyelitis and myositis (Bjerkas and Presthus, 1984), which are definitive hosts of the parasite, since a sexual phase occurs in the intestine of them , and oocysts are shed in their feces (McAllister *et al*., 1998; Gondim *et al*., 2004), and also other canids, such as Australian dingo (*Canis lupus dingo*) (King *et al*., 2010), the coyote (*Canis latrans*) (Gondim *et al*., 2004) and the gray wolf (*Canis lupus*) (Dubey *et al*., 2011).

It is not completely understand the role of birds in the life cycle of *N. caninum*, but some previous studies have been shown that the presence of them in dairy farms increases the prevalence and causes reproductive problems in cattle, that suggest they may be an important intermediate host contribute to the transmission of the parasite to definitive hosts (Otranto *et al*., 2003).

The diagnosis of the parasite can be done by many classical and conventional methods, but PCR is a highly sensitive and specific technique for DNA detection of the parasite, which applied for tissues, blood, CSF and other body fluids by using specific repetitive Nc5 gene or the internal transcribed spacer 1 (ITS1) of the rRNA gene as the most common markers used for routine detection (Dubey and Schares, 2006). In the last years, many studies using molecular techniques have been shown that small mammals and birds are an intermediate hosts of the parasite (Truppel *et al*., 2010).

The aim of this study was conducted for the first time to estimate the prevalence of *N. caninum* in local breed domesticated chickens in Al-Fallujah District, Iraq.

Materials and Methods

One hundred brain samples were collected randomly from the different ages and of both sexes of local breed domesticated chickens during the period 1/12/2018- 1/9/2019 at different areas of Al-Fallujah District (Al-Fallujah Center, Al-Shehabi, Al-Saglawia and Al-Karma). For each animal, half of the brain tissue sampled was homogenized and DNA was obtained from about 20 mg of tissue by using WizPrep™ gDNA Mini Ki (Cell/Tissue) Kit, Wizbiosolutions, Korea and was done according to company instruction.

DNA Estimation

The extracted genomic DNA from brain samples was examined by using Nanodrop spectrophotometer, which checked and measured the purity of DNA through reading the absorbance at a wavelength 260 / 280 nm as following steps:-

1- After opening up the Nanodrop software selection the suitable application (Nucleic acid, DNA).

2- Dried wipe was taken and cleaned the measurement pedestals several times, and for blank the Nanodrop system was carefully added 2µl of free nuclease water onto the surface of the lower measurement pedestals.

3- The sampling armed was lowered and clicking (ok) to start the Nanodrop, then cleaning off the pedestals and 1µl of DNA was added to measurement.

Conventional Polymerase Chain Reaction

The conventional PCR technique was performed for detection *N. caninum* based on *Nc5* gene for all genomic DNA samples extracted from chicken brain samples according to the fallowing steps:-

1- Primers

The PCR primers *Neospora caninum Nc5* for detection the parasite was novel designed in this study synthesized by Alpha DNA Ltd (Canada) based on *NC5* gene.

Table 1 : Primers designed and used in this study.

Primers	Sequence 5' – 3'		Product size
Nc5	F	5' CCCAGTGCTCCAATCCTGTA 3'	155 bp
	R	5' ACAAACCACGTATCCCACCT 3'	

Primers Preparation

The primers working solution was prepared from the lyophilized primers after dissolved in nuclease free water according to the manufacture to make a stock solution with a concentration of 100 µl for each primers and stored at -20°C. A working solution with a concentration of 10 µl was prepared by diluting 10µl of primers stock solution in 90 µl of nuclease free water and stored at -20°C until used.

2- PCR Product

The PCR master mix was prepared by using 2×EasyTaq® PCR Super Mix which done according to company prescript (Table, 2).

3- PCR Thermocycler Conditions

All PCR tubes products were homogenized by vortex and transferred into Micro Spin Centrifuge and centrifuge at 3000rpm for 5 sec, then placed in PCR Thermocycler. Conventional PCR and thermocycler conditions were done by using the PCR thermocycler system. (Table 3)

Table 2 : Components of PCR Master Mix used in the study.

Reagents of Master Mix	1 Reaction
2×EasyTaq® PCR Super Mix	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Template	3 µl
Nuclease free water	7.5 µl
Total volume reagent master mix	25 µl

Table 3: The PCR Thermocycler Conditions.

PCR Steps	Temp.	Time	Cycles
Initial Denaturation	95 C^{o}	5 min.	1
Denaturation	95 C^{o}	20 sec.	35
Annealing	59 C^{o}	30 sec.	
Extension	72 C^{o}	20 sec.	
Final extension	72 C^{o}	7 min.	1
Hold	4 C^{o}	-	-

4) Agarose Gel Electrophoresis

After PCR amplification, the presence of amplification were confirmed by using Agarose Gel Electrophoresis 2% and then PCR products 155 bp were visualized by using the UV Transilluminator.

Results

Genomic DNA Estimation

The DNA extraction from brain samples, which were checked by using Nanodrop Spectrophotometer with concentration between 5-50 ng/ ìl, with purity 1.6-1.8 at the wave length 260 /280 nm absorbance.

PCR Technique Analyses

After PCR was analyzed by an Agarose Gel Electrophoresis (2%), that stained by gel stain by using voltage at 100 volts and 80 AM for 1 hour. The positive DNA bands were 155 bp. (Fig. 1)

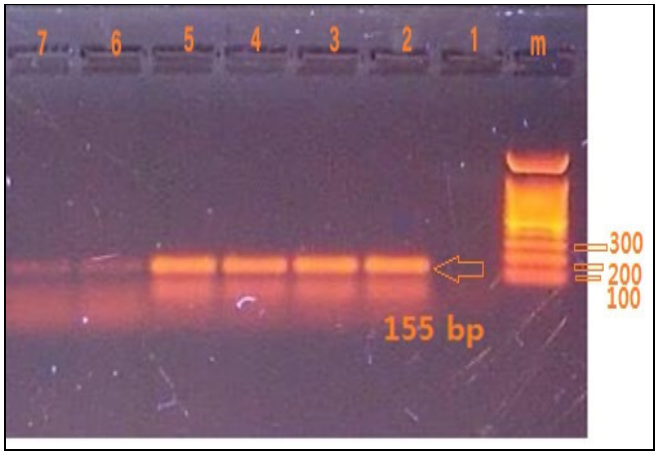

Fig. 1 : Agarose Gel Electrophoresis showed the PCR product analysis of Nc5 gene in *Neospora caninum*. lines 2,3, 4, 5, 6 and 7 are 155 bp positive, and 1 is a negative samples in 2% agarose gel, 100 volts, 80 AM and 1 hr, and M is a molecular marker (100 bp).

Total infection rate of *N. caninum* in Domesticated local breed chickens

According to conventional PCR examination, the total infection rate of Domesticated local breed chickens was 6% (6/100) of brain samples. (Tables,4).

Table 4 : Total infection rate of *Neospora caninum* in the brain samples of domesticated local breed chickens by using conventional PCR.

Tissue	No. of Brain Samples Examined	Positive	Percentage (%)
Brain	100	6	6

Discussion

This study was done for the first time in Iraq , that used the molecular technique for identification the *Neospora caninum* from the brain samples of domesticated local breed chickens in Al-Fallujah District by using the conventional PCR technique with designed a specific forward and reverse primers at a positive band of 155 bp . The total infection rate was 6% (6/100) .In the previous reports in the world, different molecular techniques have been used to study *N. caninum* such as RAPD-PCR (Schock *et al* ., 2001) and amplification of targets genes such as ITS1, Nc5 and the á and â-tubulin (McInnes *et al*., 2006). Also the presence of parasite DNA has been reported in tissues of different species of wild birds (Gondim *et al*., 2010; Darwich *et al*., 2012; Rocchigiani *et al*., 2017) and free-range chickens (Costa *et al*., 2008; Goncalves *et al*., 2012; Romero *et al*., 2016). Recently, studies have been conducted to detect *N. caninum* DNA in different avian species by PCR targeting the Nc5 gene, did not observe a DNA amplification in any of the tested eared doves (*Z. auriculata*) from Southern Brazil, it shows that probably this species of doves do not bear a chronic infection of parasite without development the cysts in the brain tissues (Barros *et al.,* 2017). On the other hand, Lukasova *et al*. (2018) targeting the same gene (Nc5 gene) in brain samples from 110 wild and domestic birds in South Africa, but didn′t found any animal positive for the parasite. Also Feng *et al*. (2017) studied 77 brain samples from ostriches (*Struthio camelus*) in China and didn′t observed a positive results by PCR targeting the same gene, but the results of the present study agreed with Goncalves *et al*. (2012) that recorded 6% an infection rate of *N. caninum* in the free ranging chickens in Bahia State, Brazil and disagreed with Rocchigiani *et al*. (2017) that register a high infection rate (28.6 %) in the waterfowl. Also disagreed with Somayeh *et al*. (2016) who recorded a peak of the parasite was 9.8% in pigeons in the Southwest Iran, but it was applicable with the result (7.5%) that recorded by Gondim *et al*. (2010) in sparrows from Northeast of Brazil. On the other hand, Darwich *et al*. (2012) showed an infection rate (1.5%) less than our study in the wild birds and Abdoli *et al*. (2015) recorded a less an infection rate of *N. caninum* (3.6%) in sparrows from Iran.

The finding of DNA in seronegative birds is not unexpected. It could be explained by a decreased in the antibodies to an undetectable level and/or by a sampling occurring after infection, but before seroconversion, as hypothesized by (Mineo *et al*., 2009), but it has been found that the DNA of the parasite can be detected in brain tissue of seronegative beef cattle (Santos *et al*., 2010). The difference between our study and that of the previously reports may be attributed to the topographic and climatic features of the studied region. The regions that characterized by a high rainfall and the presence of many hills and mountains lead to increasing persistence and dispersion of oocysts of the parasite (Amdouni *et al*., 2018). Other interpretation may be due to a higher genetic resistance of local breeds to *N. caninum* in comparison with other breeds and this may be a novel area of research or due to the higher body temperature of birds that may be prevents the establishment of a viable infection and a recent *in vitro* experiments support this hypothesis (Rezende-Gondim *et al*., 2017 and Balkes *et al.,* 2015) and differences in virulent amongst isolates were reported (Dellarupe *et al*., 2014) It is clear that these isolates behaved differently in animal models and cell culture, that if they were derived from asymptomatic calves or sheep appear less virulent compared to those isolates obtained from symptomatic calves (sick calves or aborted foetus) (Rojo-Montejo *et al.*, 2009). In a previous study by Darwich *et al*. (2012) attributed that the presence of parasite DNA in the magpies brain for commensal of this bird with humans and easily adapt to urban and rural areas and their diet includes insects, young birds and eggs, vegetable substances and carrion.

In conclusion, the results of this study provide evidence that domesticated local breed chickens retain *N. caninum* in their tissues (Brain), and could be serve as a potential reservoir for canids infection in the environment, for that a further advance studies of different bird species will be needed to elucidate the role these animals in the Epidemiology of Neosporosis.

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