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

†Ayoub Ibrahim Ali and ‡Haider Mohammed Ali Al-Rubaie
1College of Veterinary Medicine, University of Fallujah, Al-Anbar, Iraq,
2College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.
*Corresponding Author : ayoub119688@gmail.com

Abstract

The aim of this study was conducted for the first time 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 from 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 following 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 NCS gene.
Table 1: Primers designed and used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’ – 3’</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5’ CCCAGTGCTCCAATCCTGTA 3’</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’ ACAAACCACGTATCCCTACCT 3’</td>
<td>155 bp</td>
</tr>
</tbody>
</table>

Primer Preparation

The primers working solution was prepared from the lyophilized primers after dissolved in nuclease free water according to the manufacturer to make a stock solution with a concentration of 100 µl for each primer 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 2xEasyTaq® 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 centrifuged 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.

<table>
<thead>
<tr>
<th>Reagents of Master Mix</th>
<th>1 Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2xEasyTaq® PCR Super Mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Template</td>
<td>3 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Total volume reagent master mix</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Table 3: The PCR Thermocycler Conditions.

<table>
<thead>
<tr>
<th>PCR Steps</th>
<th>Temp.</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 min.</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>20 sec.</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>59 °C</td>
<td>30 sec.</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>20 sec.</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>7 min.</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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/ µl, 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)
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.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Brain Samples Examined</th>
<th>Positive</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>100</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

**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 â and â-tubulin (McInnes et al., 2009), but it has been found 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 foetuses) (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.

**References**


Darwich, L.; Cabezon, O.; Echeverria, I.; Pabon, M. and Molina-Lopez, R.; Alarcia-Alejos, O.; Lopez-Gatius,


