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STUDY OF CELLULAR IMMUNITY RESPONSE (IL4) IN CATTLE INFECTED WITH NEOSPORA CANINUM IN AL-MUTHANNA PROVINCE –IRAQ

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ABSTRACT

This study was conducted to determine the cellular immunity response (IL4) in dairy & Cross breed of beef cattle infected with Neospora caninum 800 serum samples of cows were examined by using Indirect Enzyme Linked Immuno Sorbent Assay (ELISA) kit, The age of cattle were ranged between 1-8 years, during the period from March -2010 to June -2011.The overall seroprevalence percent of N.caninum in cows was 17.5 %. The results showed also increased in interleukin 4 (IL4) concentration at the age group 6-8y that was (88.52±16.64) pg/ml as compared with other age groups. The results showed increase in interleukin concentration in infected aborted cows (93.03±21.06) pg/ml as compared with non aborted cows (78.56±18.07) pg/ml. with statistically significant difference ($P \leq 0.05$). Moreover, the results showed increase in interleukin4 (IL4) concentration in infected aborted cows in 6 month and 7 month of gestation (second to third trimester of gestation),(71.83±13.25) pg/ml and (86.22±14.42) pg/ml respectively, with statistically significant difference at ($P \leq 0.05$). in conclusion , we notice that IL4 increase in infection with Neospora caninum especially in aborted cattle and this is the first report of determine of cellular immunity response of infected cattle with N.caninum in AL-Muthana Province, Iraq.

KEYWORDS: Cellular Immunity (IL4); N. Caninum; Cattle, AL-Muthanna Province-Iraq

INTRODUCTION

Neosporosis is a major cause of abortion in cattle (Dubey and Lindsay, 1996; Dubey et al., 2007). Neospora caninum was classified in the family Sarcocystidae, subclass Coccidiasina of the phylum Apicomplexa (Ellis et al., 1994). McAllister et al., (1998) discovered that the dogs are the definitive host of N. caninum. Initially, it was suggested that the parasite had a life cycle like T. gondii, with a carnivore as definitive host (Dubey, 1992). In general, it is very similar in structure and life cycle to T. gondii with two important differences: (1) Neosporosis is primarily a disease of cattle, and dogs and related canids are

definitive hosts of N. caninum whereas (2)Toxoplasmosis is primarily a disease of humans, sheep, and goats, and felids are the only definitive hosts of T.gondii. (Dubey et al.,2007). Neosporosis has been related with epizootic and sporadic abortion in dairy herds worldwide. Since the discovery of neosporosis, some studies have been conducted to assess the prevalence and to identify factors related to the disease. Prevalence's have been estimated in ranges between 16.8% and 70% (Pare et al., 1996; Pare et al., 1997; Thurmond et al., 1997; Waldner et al., 1998).

Cattle generally show few clinical symptoms following infection with N. caninum. Specific antibody and cell mediated immune responses involving proliferation of cells and production of gamma interferon (IFN)-γ have been observed in both naturally and experimentally infected cattle with either tachyzoites or oocysts (Lunden et al., 1998; DeMarez et al., 1999).

Barr et al.,(1991) found that the timing of placental/foetal infection in gestation is crucial to the outcome of the pregnancy.

Foetal trophoblast cells produce IL-10, which floods the maternal immune system, locally creating a Th2 cytokine environment at the maternal-foetal interface (Wegmann et al., 1993).

In non-pregnant cattle infected with N. caninum, cell-mediated immunity (CMI) involving IFN-γ and TNF-α seem to play an important role in protection, this was demonstrated by the ability of exogenously administered gamma interferon (IFN-γ) to inhibit replication of the parasite in bovine brain cells (Innes et al ,1995). Khan et al., (1997) found that N. caninum was able to induce significant amounts of IL-12 and IFN gamma, they suggested that N. caninum induces a T-cell immune response that is at least partially mediated by IL-12 and IFN gamma. Nishikawa et al.,(2001) found that IL-4 seems to have a modulating effect on the toxic effects of IFN-γ, while IFN-γ is indispensable for protection.

During pregnancy in cattle and mice, it has been found that expression of pro inflammatory cytokines like IFN-γ and TNF-α levels are down regulated while regulatory cytokines like IL-10, TGF-β and IL-4 are up regulated. (Piccinni, 2002). Dealtry et al.,(2000) confirmed that the fetal trophoblasts and placental tissue secrete cytokines like IL-4 and IL-10 , TGF-β and a decrease in IFN-γ levels occurs during mid-gestation and found that the IL-4 levels do appear to influence vertical transmission rates in mice.

Protection against N. caninum is mediated by a T-helper 1 (Th1) response, therefore it is possible that the depression in the Th1 response is responsible for recrudescence of the disease in pregnant animals and leads to transmission of the parasite (Pare et al,.1997)

2. Materials and Methods:

The study was done in Al-Muthana province -Iraq that made use sera of 800 dairy & beef cows (Cross breeds of Holstein-Friesian , Iraqi local breeds) that where found in farms and villages of Al- Muthana province, Their age ranged between 1 -8 year according to (Yadave, 2010), during the period from March -2010 to June -2011 . Blood samples were collected from cows to detect N. caninum antibody and measurement of interleukin 4 (IL4) by Elisa test.

2.1. Blood Samples Collection (Cows) :

The blood samples were collected from aborted and non aborted cows after cleaning the area by using 70% medical spirit, 5 ml of venous blood was taken in a 10 ml vacutinar disposable tube. The blood samples were centrifuged at 3000 rpm for 5 minutes and serum samples then transferred to 3 ml sized micro test tube with screw cap and stored at 4 – 8°C for 24–48 hours. If longer periods of storage required sera kept in deep freeze at -20 °C. After that, the samples were transported to the laboratory in Al-Samawa hospital by cooling box. At laboratory, sera samples were examined by Elisa test according to manufacturer's instructions

2.1.1 Assay Procedure: (ELISA Test For Detect Neospora caninum antibody)

1.The reagents were allowed to come to room temperature (18-25°C) at least 30 minutes before use. Individual serum: Individual serum and controls have to be diluted 1/100 in sample diluent solution. The positive and negative controls must always be run in duplicate. Twenty µL of prediluted 1/20 positive control was added to wells A1 and B1. Twenty µLof prediluted 1/20 negative control was added to wells A2 and B2. Twenty µLof prediluted 1/20 samples were added for testing to the remaining wells. Eighty µL of sample diluent solution was added to each well occupied by controls and samples which was then mixed gently and the plate was covered with an adhesive plate cover (included in the kit). Then Incubated for 1 hour at 37±2°C**.**

2-The adhesive cover was removed and the plate was washed 4 times with diluted washin solution. Then all the wells were filled to the top for each was (volume per well:300µL). All liquids from the wells were empted and the plate was taped hard to remove the last traces of liquid. Alternatively, the plate was washed 4 times on a automatic plate washer using a well volume of 300 µL.

3. One hundred µL of Conjugate solution was added to each well.

4. The plate was mixed gently and covered with a new adhesive cover and incubated for 1 hour at 37±2°C.

5.The adhesive cover was removed and the plate was washed 4 times with diluted washing solution, all the wells were filled to the top for each was (volume per well: 300 µL). all liquid from the wells were empted and the plate was taped hard to remove the last traces of liquid. Alternatively, the plate was washed 4 times on a automatic plate washer using a well volume of 300 µl.

6. One hundred µlof substrate solution was added to each well, then mixed gently for 2 seconds.

7. The chromogenic reaction was developed for 10 minutes at room temperature (18-25 ºC) in the dark. The plate didn't cover.

8. One hundred µL of stop solution was added to each well, the stop solution was added in the same order as the substrate solution was added, the plate was mixed by gently for 2 seconds.

9. The under-surface of the plate free was wiped of dust with a soft tissue. Finally, the plate was rea using a microtiter plate reader at 450 nm, or at dual wave length 450-620 nm on a microplate reader.

2.1.1.1Calculations:

For the interpretation of results, an IRPC value was required (Relative Index x100). The following formula was applied to obtain the IRPC value (using mean DO_{405} values obtained for controls).

$$IRPC = \frac{(OD_{405}\ \text{Sample Mean} - \text{Mean}OD_{405}\ \text{NegativeControl})}{(\text{Mean}\ OD_{405}\ \text{PositiveControl} - \text{Mean}OD_{405}\ \text{NegativeControl})} \times 100$$

Calculation of results:

Individual Serum :

SAMPLE	*IRPC VALUE
Negative	≤ 5.0
Positive +	5 < IRPC < 25
Positive + +	25 < IRPC < 50
Positive + + +	50 < IRPC < 100
Positive + + + +	> 100

*IRPC: Interpretation of Calculation.

After completed ELISA test of 800 cows, We also examined 44 infected cows to measure Interleukin 4 (IL4) Conc. ratio in these cows by ELISA using bovine IL4 kit according to manufacturer's instruction:

2-1-2.Assay procedure: (Elisa test for measurement of IL4)

All reagents and samples were brought to room temperature before use.

1- A blank well was seated without any solution, 50 µL of standard or sample was per well. Added standard need test in duplicate.

2- Fifty µL of HRP-conjugate was added to each well (not to blank well), then 50µl of antibody was added to each well. After that mixed well and incubated for 2 hours at 37°C.

3-Each well was filled with wash buffer (about 250 µL) ,stayed for 10seconds and spinning. The process was repeated for a total of three washes , removal of liquid completely at each step was essential to good performance ,after the last wash the remaining wash buffer was removed by aspirating or decanting the plate was inverted and blotted it against clean paper towels.

4-Fifty µL of substrate A and substrate B was added to each well, then mixed well and incubated for 15 minutes at 37°C. the plate was kept away from drafts and other temperature fluctuation in the dark.

5-Fifty µL of stop solution was added to each well when the first four wells containing the highest concentration of standards that obvious color was developed. If the color does not appear uniform, gently tap the plate to ensure thorough mixing.

6-The optical density of each well was determined within 10 minutes using a microplate reader set to 450 nm.

3-Results:

3-1-Serological Results:

3-1-1.Indirect Enzyme linked immunosorbent assay: (iELISA)

The results of serological examination by indirect ELISA of Neosopora caninum in cattle (Dairy and cross breed Beef), showing the total percentage of infection was(140 out of 800) 17.5%. (Table 3-1)

Interleukin 4 (IL4) Assay:

The measurement of cytokines (IL4) in the present study, that examined (44) infected cows randomly to measurement of Interleukin 4 (IL4) concentration in these cows. The results showed increased mean of interleukin 4(IL4) concentration in age group (6-8y) that was (88.52±16.64)pg/ml as compared with other age groups (1-3y) and (4-6y) (72.04±12.83) pg/ml,(65.93±11.93) pg/ml respectively with statistically significant difference (P≤ 0.05)between groups. (Table 3-2, Figure 3-1).

In table (3-3), Figure (3-2) the results showed increase in mean interleukin 4 concentration in infected aborted cows (93.03±21.06) pg/ml compared with non aborted cows (78.56±18.07)pg/ml. with statistically significant difference(P≤0.05).

In table (3-4) and Figure (3-3) the results showed increase in mean of interleukin concentration in infected aborted cows in (6 month and 7 month) of gestation age that was (71.83±13.25)pg/ml and (86.22±14.42)pg/ml respectively ,with statistically significant difference (P≤ 0.05).

3-2.Statistical Analysis:

Statistical analysis was conducted to determine the statistical differences among different groups using differences among different groups using ready – made statistical design statistical package for social science. Probabilities of (P≤ 0.05) were considered statistically significant.(SPSS version 13).

3-2-1.Calculation of Results (IL4):

Statistical analysis were conducted by using readymade (Standard curve Expert 1.3) .((Sorlie, 1995).

4-Discussion:

Neosporosis is a parasitic disease caused by Neospora caninum. It is recognised as intracellular protozoan parasite of dog and livestock distributed worldwide. In cattle, it is considered as one of the main causes of abortion (Dubey et al., 2006).

N .caninum is an obligate intracellular parasite, cell-mediated immunity (CMI) is expected to have a major role in protection. Initial evidence for this came from in vitro studies showing that with IFN-γ significantly inhibited intracellular multiplication of N. caninum (Innes et al., 1995).

It has been reported in many countries with different prevalence rates since the disease was recognized in 1988 (Campero et al.,1998;Cabaj et al,2000, Waldner et al.,2001).

4-1. Bovine Interleukin 4(IL4) Assay:

Interleukin 4 (IL-4) is secreted by a small number of cells and, more specifically, by type-2 CD4+TL, Basophils, mastocytes and certain CD8+ TL can also be a source of IL-4. A factor for the activation and differentiation of TL and B lymphocytes (BL), it increases the expression of class-II MHC antigens and triggers IgE isotype switching (Filisetti and Candolfi., 2004).

The result showed increased of interleukin 4 (IL4) concentration in age group(6-8y) that was (88.52±16.64)pg/ml as compared with other age groups, this result may be due to that more the age groups which infected of N. caninum was in (6-8 y) ,also regulatory cytokine 4(IL4) will increase during infection of N. caninum that mean it was able to stimulate Th type-2cells and this cell produce interleukin 4(IL-4).(Almeria et al., 2003).

Also the results showed increase in interleukin concentration in infected aborted cows (93.03±21.06) pg/ml compared with non aborted cows (78.56±18.07) pg/ml. with statistically significant difference ($P \leq 0.05$). These differences in infection rate may be due to foetal immune responses, that Dealtry et al.,(2000) confirmed that the fetal trophoblasts and placental tissue secrete cytokines like IL-4 and IL-10 , TGF-β and a decrease in IFN-γ levels occur during mid-gestation.

While Entrican (2002) found that the microenvironment in the placenta favours the more regulatory Th2-type cytokines, such as IL-10, IL-4 and transforming growth factor beta (TGF-B) whose role is to counteract the inflammatory responses induced by the Th1-type cytokines.

Also, the results showed increase interleukin concentration in infected aborted cows in (6 - 7 month) of gestation age(second to third trimester of gestation) that was

71.83±13.25pg/ml and 86.22±14.42 pg/ml respectively ,that this result is in agreement with Wetson et al., (2005).

Innes et al., (2005) and Quintanila-Gonzallo et al (2000), Dubey and Lindsay (1996) They found that the abortion occurred in mid and third trimester of gestation was due to low immune responses of cows during this period and the ability of cattle infected with N. caninum to mount antigen-specific cell proliferation were found to decrease significantly around mid-gestation compared with pre-pregnancy or early gestation, while increased in interleukin 4 in this period may be due to that the fetal trophoblasts and placental tissue secrete cytokines like IL-4 and IL-10 TGF-β and a decrease in IFN-γ levels occurs during mid-gestation (Dealtry et al.,2000).

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Captions

Table (3-1): Positive number and total percentage of infected cows

Total No. of samples	No. of (+ve)sample	%
800	140	17.5

Table (3-2): Interleukin-4(IL4) concentration in infected cows in different age.

Age (year)	IL4 Concentration(pg/ml) (mean±SD) in Positive samples	IL4 Concentration(pg/ml) (mean±SD) in Negative samples(control)
1-3 y	AB,a 72.04±12.83	A,b 29.38±3.54
4-6 y	A,a 65.93±11.93	A,b 32.20±6.52
6-8 y	B,a 88.52±16.64	B,b37.08±4.31

Differences A, B are significant (P≤0.05) to comparison columns
Differences a, b are significant (P≤0.05) to comparison rows

Table(3-3):Interleukin-4(IL4)concentration in aborted and non aborted Cows

Aborted/Non aborted Cows	IL4 Concentration(pg/ml) (mean±SD) in Positive samples	IL4 Concentration(pg/ml) (mean±SD) in Negative samples(control)
Aborted	A,a93.03±21.06	A,b 41.01±15.52
Non aborted	B,a78.56±18.07	A,b 43.42±14.96

Differences A, B are significant (P≤0.05) to comparison columns
Differences a, b are significant (P≤0.05) to comparison rows

Table (3-4): Interleukin-4(IL4) concentration in aborted cows according abortion time

Date of abortion (month)	IL4 Concentration(pg/ml) (mean±SD) in positive samples	IL4 Concentration(pg/ml) (mean± SD) in Negative samples(control)
6 months	A,a 71.83±13.25	A,b 31.20±12.27
7 months	B,a 86.22±14.42	B,b 47.73±14.28
8-9 months	A,a 69.93±13.66	A,b 30.05±11.02

Differences A, B are significant (P≤0.05) to comparison columns
Differences a, b are significant (P≤0.05) to comparison rows

Captions:

Fig.(3-1): Relation of Interleukin 4 concentration and age groups.

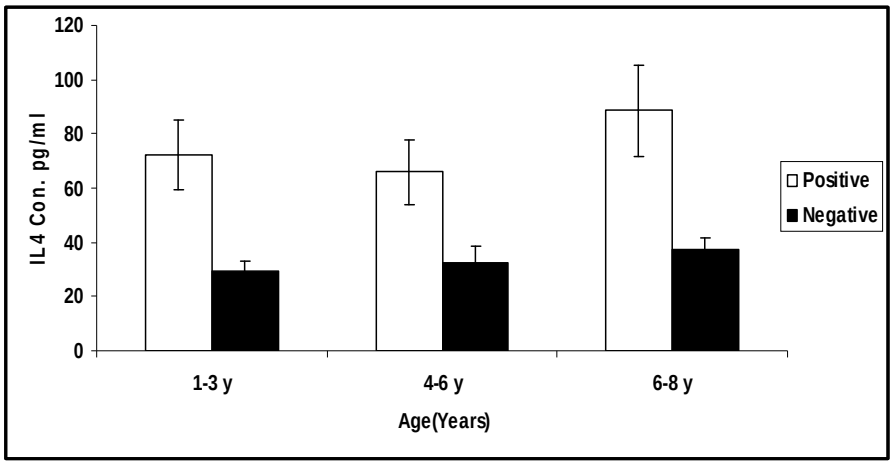

Fig.(3-2): Relation of Interleukin 4 Concentration and Aborted and non Aborted cows.

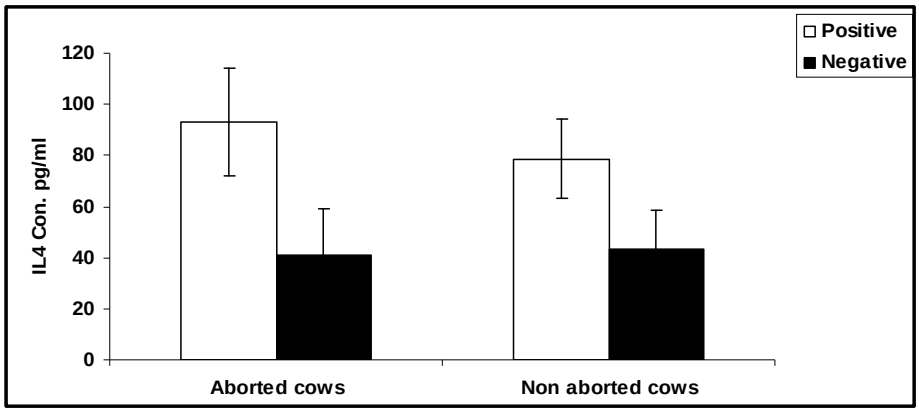

Figure (3-3): Relation of Interleukin- 4 concentration and abortion time.