Korean Journal of Clinical Medicine

Research Article Open Access

Effect of Dexamethasone in Neospora Caninum Seropositive Calves

Idarraga-Bedoya S[1], Armendano JI[1], Gual I[1], Hecker YP[2], Leunda MR[3], Pereyra S[3], Verna AE[3], Campero CM[3], Odeon AC and Moore DP[1,2*]

[1]Department of Clinical Medicine, National University of Mar del Plata (UNMDP), CP7600 Mar del Plata, Argentina

[2]Department of Clinical Medicine, National Research Council, CP7600 Mar del Plata, Argentina.

[3]Department of Clinical Medicine, National Institute of Agrarian Technology Research, Balcarce, CP7620, Argentina

***Corresponding author:** Dadin Prando Moore , Department of Clinical Medicine, Veterinary Pathology, INTA EEA Balcarce. CP 7620, Argentina. Phone: 54-2266-439100-20. Fax: 54-2266-43-9101. E-mail: moore.dadin@inta.gob.ar

Citation: Moore DP (2020) Effect of Dexamethasone in Neospora Caninum Seropositive Calves. Korean Journal of Clinical Medicine. V1(1): 1-6.

Received Date: Feb 05, 2020 **Accepted Date**: Mar 02, 2020 **Published Date**: Mar 09, 2020

1. Abstract

The aim of this study was to evaluate the effect of Dexamethasone (DXM) on clinical parameters, absolute and differential leukocyte count, specific IgG titers, IFN-γ production and parasitemia in beef calves seropositive to *Neospora caninum*. Fifteen calves were assigned to four experimental groups as follow: group A: 4 seropositive calves treated with DXM; group B: 4 seropositive calves without DXM; group C: 3 seronegative calves treated with DXM, and group D: 4 seronegative calves without DXM treatment. The absolute leukocyte count was higher in both groups A and C in the 3rd day after the first dose of DXM mainly due to a significant neutrophilia ($p<0.05$). No changes in the total specific IgG titers or ratios between their subisotypes or antibodies against NcGRA7 and NcSAG4 antigens were observed in calves from group A compared with those in group B ($p>0.05$). Similarly, IFN-γ levels did not change among experimental groups ($p>0.05$). DNA was detected in calves from groups A and B at 7 Days Post Administration (dpa) and from group A at 14 dpa. Even when DXM administration induced hemotological parameter changes, no reactivation of *Neospora*-infection was observed according to specific IgG titers and presence of the parasite on leukocytes in naturally seropositive beef male calves.

2. Keywords: Calves; Dexamethasone; *Neospora caninum*; Immunology

3. Introduction

Neospora caninum is an apicomplexan parasite closely related to *Toxoplasma gondii*, which causes a disease of major concern for the cattle industry [1]. Specific antibody titers fluctuate through gestation in chronically naturally infected cattle [2, 3]. The increase in antibody titers is a good indicator of parasitemia during the pregnancy in cattle and the down regulation of immune markers like proliferation of citotoxic lymphocytes T, production of IL-12, IFN-γ and IgG_2 seems to favor the reactivation of latent *Neospora*-infections [4]. By applying recombinant protein-based ELISAs utilizing NcGRA7 and NcSAG4 proteins associated to the tachyzoite and bradyzoite stages, respectively, it was possible to identify primo-infection, recrudescence, and chronic *Neospora*-infections [5]. Moreover, gene expressions of the SAG1 protein from tachyzoites and the BAG1 protein from bradyzoites are increased during early and late immune suppression in mice infected with *T. gondii*, respectively [6]. Nevertheless, the parasitic strategies and associated molecular immune mechanisms responsible for the passage from bradyzoites to tachyzoites and vice versa remain unknown in bovine neosporosis.

Animal welfare and stress-related diseases, including bovine neosporosis, are enhanced by husbandry procedures and intensive production systems. In addition, concomitant viral infections and mycotoxicosis or any other immune suppressive event have been mentioned as risk factors for bovine neosporosis [1]. Elevated activity of the hypothalamic-pituitary-adrenocortical axis that accompanies stress has been successfully induced by administration of Dexamethasone (DXM) [7, 8]. The administration of DXM affected cytokine and antibody responses in a toxoplasmosis murine model [9]. Moreover, reactivation of the infection was attributed to a specific Th1 immune response down regulation [9]. In order

to understand the role of the immunosuppression on the physiopathology of the bovine neosporosis, this study was designed to evaluate the effect of DXM on clinical parameters, hematology white blood cell count, specific IgG titers, and IFN-γ production in naturally seropositive beef male calves.

4. Materials and Methods

4.1. Experimental Design

Fifteen Angus ten-month-old male calves weighing 190±21 kg (mean ±SD) belonging to a beef herd located at INTA-Balcarce, Argentina were involved in the experiment. All animal usage was according to protocols from the Animal Ethics Committee at INTA, Argentina. Eight naturally seropositive calves born from seropositive dams and seven seronegative calves born from seronegative dams were randomly assigned to 4 experimental groups as follow: group A: 4 seropositive calves treated with DXM; group B: 4 seropositive calves without DXM; group C: 3 seronegative calves treated with DXM, and group D: 4 seronegative calves without DXM treatment. The serostatus to *N. caninum* was determined by Indirect Fluorescent Antibody Test (IFAT) at a serum dilution of ≥1:25 (Venturini et al., 1999).

The calves were allocated in dog-proof pens and provided with water *ad libitum*, standard hay and commercial cattle concentrate. Calves remained in level 6 (scale of 1 to 9) of body condition and preventive anthelmintic treatment was applied.

All calves from groups A and C were given DXM (Dexametasona* Vet, Schering Plough, Sanidad Animal, Argentina) at a dose of 0.1 mg/kg/day, intravenously for 5 consecutive days [10]. Rectal temperature and clinical signs were monitored daily throughout the experimental period, which were 24 days after post administration of the 1st dose of DXM. Animals with temperatures above 39.5^{0}C were considered to be febrile.

Sampling

Blood samples (with and without anticoagulant) were collected by external jugular venipuncture. Whole blood samples were assessed for absolute and differential leukocyte count at 0, 3, 7, 9, 13, 17 and 24 Days Post Administration (dpa) of the 1st dose of DXM. Similarly, leukocytes were obtained from whole blood samples to perform nested PCR on 0, 3, 7, 9, 13 and 17 dpa. Serum samples were obtained to perform IFAT, several ELISAs and IFN-γ responses at 0, 7, 13 and 24 dpa.

4.2. Leukogram

Leukocyte counts were performed in a modified Neubauer chamber and expressed in thousands of cells per microliter of blood, with Thomas fluid as the diluent. The differential leukocyte count was performed on stained blood smears, with analysis of the differentiation of 100 white blood cells allowing their classification [11]. Humoral immune responses

4.3. Indirect Fluorescent Antibody Test

Indirect Fluorescent Antibody Test (IFAT) was performed as previously described by [12]. *Neospora caninum* specific antibodies were measured using dilutions of serum from 1:25 to endpoint titer [13]. A polyclonal rabbit anti-bovine IgG labeled with fluorescein isothiocyanate (Sigma, St. Louis, MO) diluted 1:200 in PBS was used. Positive and negative control sera were used (VMRD, Inc., USA). Slides were examined with an epifluorescence microscope (Nikon Fluophot, 40 × 1.3). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed distinct whole parasite fluorescence.

4.4. Indirect ELISA for IgG Subisotypes

Specific IgG_1 and IgG_2 levels were assessed in serum of IFAT seropositive calves by indirect ELISAs as previously mentioned [14]. Briefly, one microgram of solubilized *N. caninum* tachyzoite antigens was distributed and adsorbed to each flat bottom well of 96-well plates (Polysorp, Nunc). After blocking, negative and strong positive control sera, and serum samples were added. Anti-bovine IgG_1 or IgG_2 mAbs (Serotec TM, Oxford, UK), anti-mouse IgG mAb conjugated to peroxidase (Jackson®) and 3% H_2O_2/0.04M ABTS were used to assess the immune-assay. A kinetic reading was determined at an OD 405 when *N. caninum* C++ with anti-IgG_1 reached 1.0 ±25%. Data were expressed as a ratio of OD values for IgG_1/OD value for IgG_2.

4.5. Indirect ELISA For The Recombinant Proteins NcGRA7 and NcSAG4

This ELISA was performed as mentioned by [5]. Briefly, coating buffer containing 0.15µg and 0.1µg of rNGRA7 and rNSAG4, respectively, was added to each well of a polystyrene microtiter plate (Immuno Plate Maxisorp, Nunc, Roskilde, Denmark) and incubated overnight at 4^{0}C. After blocking for 2h at room temperature with PBST containing 1% BSA, the wells were washed three times with PBST and incubated 1h at 37^{0}C with sera diluted 1:100. After three washes, a monoclonal mouse anti-bovine IgG conjugated with peroxidase was added and left for 1 h at 37^{0}C. Wells were rinsed three times with PBST and antibody reaction was detected

by using 2, 2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid substrate (ABTS) (Sigma). After 20 min, the reaction was stopped by adding oxalic acid. Absorbance was measured as O.D. values at 405 nm using a microplate reader (Multiskan RC 6.0, Labsystems). Serum samples were analyzed in duplicate. Data were expressed as a ratio of OD values for NcGRA7/OD values for NcSAG4.

4.6. Assessment of *N. caninum*-Specific IFN-γ Responses

Immune stimulation was performed as mentioned [15]. Briefly, 0.9 ml of heparinised whole blood was dispensed into each of two wells of 24-well tissue culture plates (Cellstar Greiner, USA) and cultured with 0.1 ml of PBS (unstimulated control), concanavalin A (Con-A, Sigma, St. Louis, USA) at 10µg/ml to ensure cellular ability to respond to stimulation and secrete IFN-γ, and with native antigen from the *N. caninum* NC-1 strain (1µg/ml) (Moore et al., 2011). Heparinised whole blood samples were incubated in a 5% CO_2 atmosphere for 16h at 37^{0}C. Plasma was harvested from each well and frozen at - 20^{0}C until testing. To assess IFN-γ production, plasma samples were tested using a commercial ELISA kit (Bovigam IFN-γ kit, CSL, Australia), according to the manufacturer's recommendations. Briefly, 100 ml of each sample (1:2 dilution) was dispensed in anti-bovine IFN-γ antibody-coated plates and incubated for 60 min at RT. Plates were washed six times, incubated for 60 min at RT with 100 ml of horseradish peroxidase-anti-bovine IFN-γ antibody conjugate, washed again and incubated for 30 min at RT with 100 ml of TMB substrate. Fifty µl/well of stopping solution (0.5M sulphuric acid) was added, and plates were read at A450 nm in an ELISA plate reader (Labsystems Multiskan®, Plus; Finland).

N. caninum nested PCR

DNA was isolated from leukocyte samples using Qiagen DNA easy kit (Qiagen, USA) according to the manufacturer's recommendations. The concentration of DNA was determined by spectrophotometric analysis at A 260/280 and all the samples were diluted to a final concentration of 60 ng/ml. DNA samples were stored at -20ºC until PCR analysis.

Neospora DNA was detected by a nested PCR on the Internal Transcribed Spacer (ITS1) region with four oligonucleotides as described by [16]. Positive (purified *N. caninum* tachyzoite DNA) and negative controls (DNA from normal bovine brain, paraffin control, and double-distilled water) were included in each PCR. Secondary amplification products were visualized by 1.8% agarose gel electrophoresis and ethidium bromide staining under UV light.

4.7. Statistical Analysis

Rectal temperature, absolute and differential leukocyte counts antibody titers and IFN-γ levels were expressed as least square means ± standard error (LS means ± SE) and analyzed by using linear mixed models (Proc. GLIMMIX, SAS Studio v3.6, SAS Institute Inc., Cary, NC, USA). The group, time and their interaction were included as fixed effects, the animal as a random effect and a continuous first order autoregressive covariance structure was assumed. Parametric assumptions were assessed using graphical methods, modelling the heteroscedasticity when it was needed.

PCR positivity were expressed as percentage ± SE and analyzed by using generalized linear mixed models (Proc. GLIMMIX, SAS Studio v3.6), assuming a binomial distribution and a logit link function. In all analysis, multiple pairwise comparisons were adjusted using the Tukey-Kramer's method and significance level was set to α=0.05.

5. Results

5.1. Clinical signs and immune parameters

There were no significant differences in the rectal temperature neither among groups nor through the time ($p>0.05$). However, there were statistical differences in the absolute leukocyte count being higher in both groups A and C in the 3rd day after the first dose of DXM ($p>0.05$) (Figure 1a). This increased value was due to a significant neutrophilia in the same experimental groups ($p<0.05$) (Figure 1b). Although there were no statistical differences for the other leukocyte population neither among groups nor through the time ($p>0.05$), the highest and lowest counts for eosinophils and lymphocytes were recorded for groups A and C, respectively (data no shown).

There were differences in the IFAT titers among groups being higher in groups A and B compared with C and D from the beginning of the trial and there after ($p<0.05$) (Figure 2a). Note worthy, the administration of DXM did not induce any change in the specific IgG titers in calves from group A compared with those in group B ($p>0.05$). Moreover, the levels of the IgG_2 remained stable through the time and IgG_1/IgG_2 ratio were >1.5 in all calves from groups A and B. Similarly, the ratios for antibodies against NcGRA7 and NcSAG4 did not have statistical differences between calves from groups A and B ($p>0.05$) (Figure 2b). The IFN-γ levels did not change among experimental groups ($p>0.05$). *Neospora*-DNA was detected in calves from groups A and B at 7dpa and from group A at 14dpa (Table 1) being no statistically different ($p>0.05$).

6. Discussion

Changes in the absolute and relative leukocyte count are common findings in cattle treated with DXM (Anderson et al., 1999). Both experimental groups A and C developed an increased absolute leukocyte count due to neutrophilia in the 3rd day after the first dose of DXM. Although blood levels of DXM were not tested in the present study, the dose and administration route were good enough to change these in clinical immune parameters.

Specific antibody titers are good indirect parameters to test parasitemia increasing after parasite reactivation in naturally infected pregnant cows [2, 3]; however, the administration of DXM did not induce any change in the specific IgG titers in calves receiving DXM. Although the reactivation of the infection has been attributed to a specific Th1 immune response down regulation associated with decreased IgG_2 titers [4, 9], the levels of the IgG_2 remained stable through the time and IgG_1/IgG_2 ratio were >1.5 in all calves from groups A and B. Moreover, the recrudescence and chronic *Neospora*-infections have been successfully characterized by applying recombinant protein-based ELISAs [5]. Higher ratios for antibodies against NcGRA7 and NcSAG4 suggest the conversion of bradyzoite to tachyzoite but no statistical differences between calves from groups A and B were observed in this trail.

IFN-γ has a critical role in *Neospora*-infections [4, 9]. Although DXM administration may induce reactivation of a latent infection with BHV-1 in calves [10] and induced immunosuppression has been largely used for studing neosporosis in mice [1], the IFN-γ levels did not change among the *Neospora* seropositive calves involved in this trail. This fact could partially explain why DXM administration seems to be safe in *Neospora* seropositive calves. Moreover, [7], reported that IFN-γ levels were not affected by DXM adminis-

A)

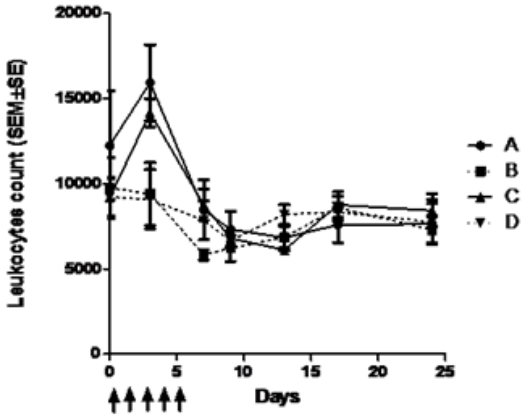

Figure 1A: absolute leukocyte

B)

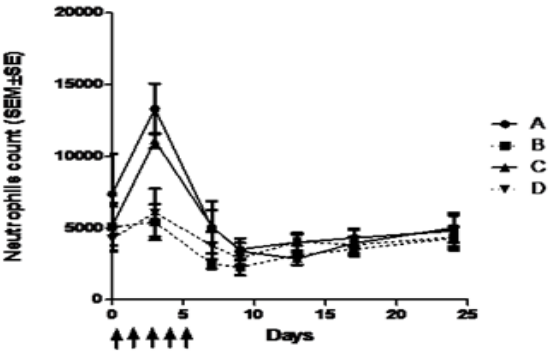

Figure 1B: neutrophil counts for all experimental groups

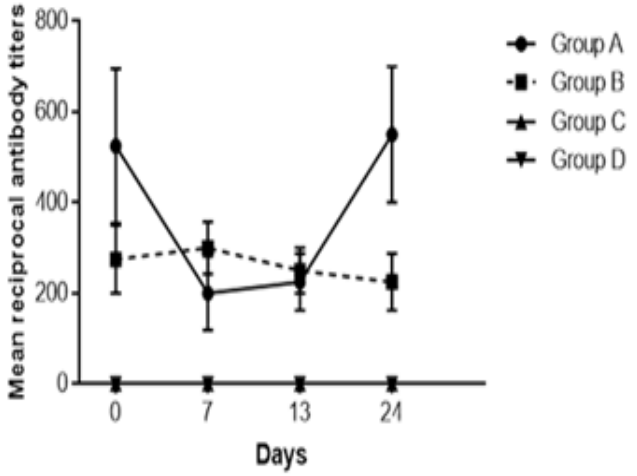

Figure 2A: Means (±SEM) of the reciprocal specific antibody titers for all experimental groups;

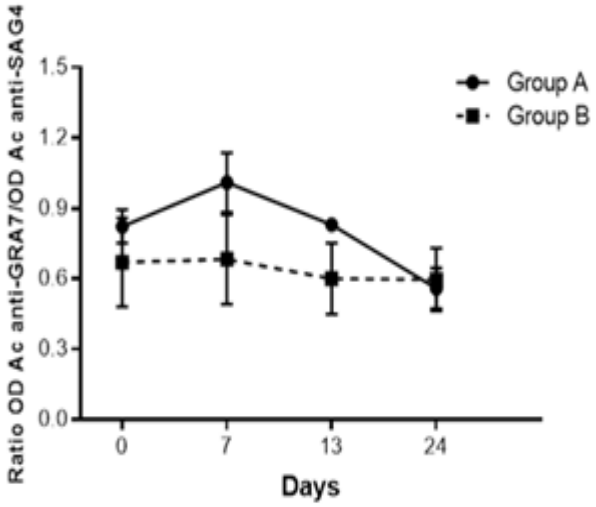

Figure 2B: ratios for antibodies against NcGRA7 and NcSAG4 between calves from groups A and B (p>0.05).

Table 1: *Neospora caninum* DNA detected by nested PCR in animals from groups A, B, C and D. Dexamethasone was administrated on day 0 for 5 consecutive days.

Days							
Group	**Animal**	**0**	**3**	**7**	**9**	**13**	**17**
A	**1**	-	-	+	-	+	-
	2	-	-	+	-	+	-
	3	-	-	+	-	-	-
	4	-	-	-	-	-	-
B	**5**	-	-	-	-	-	-
	6	-	-	+	-	-	-
	7	-	-	+	-	-	-
	8	-	-	+	-	-	-
C	**9**	-	-	-	-	-	-
	10	-	-	-	-	-	-
	11	-	-	-	-	-	-
D	**12**	-	-	-	-	-	-
	13	-	-	-	-	-	-
	14	-	-	-	-	-	-
	15	-	-	-	-	-	-

tration. On the other hand, several hormonal or endocrinal factors associated to pregnancy rather than IFN-γ production alone seem to be crucial in the reactivation of latent *N. caninum* stages [3, 4].

In agreement with [17] the detection of *Neospora*-DNA is a feasibly procedure seropositive cattle; however, the frequency of *Neospora*-DNA detection did not change by DXM administration. Even when elevated activity of the hypothalamic-pituitary-adrenocortical axis that accompanies stress has been successfully induced by administration of DXM [7,8], such treatment did not induce the reactivation of *Neospora*-infection according to clinical parameters, specific IgG titers, and presence of the parasite on leukocytes in naturally seropositive beef male calves.

Finally, immunosuppressive mechanisms associated to DXM administration were not enough to induce *Neospora* reactivation in seropostive beef calves. Even when any immunosuppressive conditions have been postulated as risk factors in bovine neosporosis, DXM may be used safely on *Neospora* seropositive cattle.

7. Acknowledgements

We gratefully acknowledge Drs G. Alvarez-Garcia, J. Regidor-Cerrillo and L. M. Ortega-Mora from SALUVET, Universidad Complutense of Madrid, Spain, for performing the iELISA for the recombinant proteins NcGRA7 and NcSAG4 and critical review of this article. We also acknowledge the students, technicians and colleagues from the Animal Health Group at INTA Balcarce. This study was funded by the grant BID-PICT 2011-1179, Argentinean Government.

References:

1. Dubey JP, Schares G, Ortega-Mora LM. Epidemiology and control of neosporosis and Neospora caninum. Clin. Microbiol. Reviews. 2007; 20: 323-67.
2. Stenlund S, Kindahl H, Magnusson U, Uggla A, Bjorkman C. Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with Neospora caninum. Vet. Parasitol. 1999; 85: 227-34.
3. Guy CS, Williams DJL, Kelly DF, McGarry JW, Guy F, Bjorkman C et al. Neospora caninum in persistently infected, pregnant cows: spontaneous transplacental infection is associated with an acute increase in maternal antibody. Vet. Rec. 2001; 149: 443-9.
4. Innes EA, Andrianarivo AG, Bjorkman C, Williams DJ, Conrad PA. Immune responses to Neospora caninum prospects for vaccination. Trends in Parasitol. 2002; 18: 497-504.
5. Aguado-Martinez A, Alvarez-Garcia G, Fernandez-Garcia A, Risco-Castillo V, Arnaiz-Seco I, Rebordosa-Trigueros X eta l. Usefulness of rNcGRA7- and rNcSAG4-based ELISA tests for distinguishing primo-infection, recrudescence, and chronic bovine neosporosis. Vet. Parasitol. 2008; 157: 182-95.
6. Selseleh M, Modarressi MH, Mohebali M, Shojaee S, Eshragian MR, Selseleh M et al. Real-time RT-PCR on SAG1 and BAG1 gene expression during stage conversion in immunosuppressed mice infected with Toxoplasma gondii Tehran strain. Korean J. Parasitol. 2012; 50: 199-205.
7. Anderson BH, Watson DL, Colditz IG. The effect of dexamethasone on some immunological parameters in cattle. Vet. Res. Comm. 1999; 23: 399-413.
8. Maeda Y, Tanaka R, Ohtsuka H, Matsuda K, Tanabe T, Oikawa M. Comparison of the immunosuppressive effects of dexamethasone, flunixin meglumine and meloxicam on the in vitro response of calf

peripheral blood mononuclear cells. J. Vet. Med. Sc. 2011; 73: 957-60.

9. Kang Ki-Man, Choi In-Uk, Shin Dae-Whan and Lee Young-Ha. Cytokine and antibody responses of reactivated murine toxoplasmosis upon administration of dexamethasone. Korean J. Parasitol. 2006; 44: 209-19.
10. Perez SE, Bretschneider G, Leunda MR, Osorio FA, Flores EF, Odeon AC. Primary Infection, Latency, and Reactivation of Bovine Herpesvirus Type 5 in the Bovine Nervous System. Vet. Pathol. 2002; 39: 437-44.
11. Jain NC. Normal hematology of cattle, sheep and goats, p.1075-1084. In: Ibid. (Ed.), Schalm's Veterinary Hematology. 5th ed. Lea and Febiger, Philadelphia. 2000; 1344p.
12. Dubey JP, Hattel AL, Lindsay DS, Topper MJ. Neonatal Neospora caninum infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 1988; 193: 1259-63.
13. Venturini MC, Venturini L, Bacigalupe D, Machuca M, Echaide I, Basso W et al. Neospora caninum infections in bovine foetuses and dairy cows with abortions in Argentina. Int. J. Parasitol. 1999; 29: 1705-8.
14. Moore DP, Echaide I, Verna AE, Leunda MR, Cano A, Pereyra S et al. Immune response to Neospora caninum native antigens formulated with immune stimulating complexes in calves. Vet. Parasitol. 2011; 175: 245-51.
15. Serrano-Martinez E, Ferre I, Martinez A, Osoro K, Mateos-Sanz A, del-Pozo I etal. Experimental neosporosis in bulls: parasite detection in semen and blood and specific antibody and interferon-gamma responses. Theriogenology. 2007; 67: 1175-84.
16. Buxton D, Maley SW, Wright S, Thomson KM, Rae AG, Innes EA. The pathogenesis of experimental neosporosis in pregnant sheep. J. Comp. Pathol. 1998; 118: 267-79.
17. Okeoma CM, Williamson NB, Pomroy WE, Stowell KM, Gillespie L. The use of PCR to detect Neospora caninum DNA in the blood of naturally infected cows. Vet. Parasitol. 2004; 122: 307-15.