Technical note

Effects of re-infection with *Neospora caninum* in bulls on parasite detection in semen and blood and immunological responsesI. Ferre^{a,*}, E. Serrano-Martínez^a, A. Martínez^b, K. Osoro^b, A. Mateos-Sanz^a,
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Abstract

Three bulls with experimentally induced primary infection with *Neospora caninum* were re-infected intravenously with 10^8 live *N. caninum* tachyzoites of the NC-1 isolate at 300 days post-infection to investigate the presence of *N. caninum* in semen and blood, and the associated immune responses. In parallel, three bulls with experimentally induced primary infection with *N. caninum* and three non-infected bulls were also monitored. Re-infected and infected bulls showed an intermittent presence of *N. caninum* DNA in semen with a parasite load ranging from 0.1 to 15.6 (mean 4.4) and 0.1 to 11.1 (mean 4.1) parasites/ml, respectively. Re-infected bulls showed significant and persistent serum-specific IgM and IgG antibody responses. Specific IgG levels were detected in seminal plasma of all infected bulls, but the magnitude of the response was significantly higher in re-infected rather than in chronically infected animals. The mean specific IFN- γ levels in re-infected bulls were significantly increased as early as 3 and 7 days after experimental infection when compared to bulls in other groups. This study showed that the intermittent presence and parasite load of *N. caninum* in the semen of re-infected bulls is very similar to that reported in chronically infected animals. The protozoa could not be isolated from BALB/c *nu/nu* mice inoculated with PCR-positive semen samples and inseminated heifers with pooled semen samples did not show seroconversion. Plasma IFN- γ level seems to be a good indicator of a recent *N. caninum* infection.

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1. Introduction

Since, *Neospora caninum* DNA was reported in fresh and frozen semen from naturally infected bulls [1],

several studies have been carried out to investigate the possibility of venereal transmission in bovine neosporosis. The presence of *N. caninum* DNA in fresh semen of naturally [2] and experimentally [3] infected bulls was intermittent and the parasite load was low (0.1 and 14.5 parasites/ml of semen). A recent experimental study showed that the intrauterine infection via contaminated semen using 5×10^4 tachyzoites caused persistent serum-specific antibody responses in some

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heifers [4]. In persistently infected cattle, the reactivation (recrudescence) of the infection (caused by stress or immunosuppressive diseases) or ingestion of sporulated *N. caninum* oocysts could result in parasitaemia, leading to the dissemination of *N. caninum* throughout the body, including the genital tract [5]. In bulls, this fact could increase the number of parasites in semen and thus, lead to venereal infection risk. The present work is a follow-up study in bulls with experimentally induced primary infection with *N. caninum* which were re-infected with *N. caninum* tachyzoites to evaluate the effect of re-infection on parasite load shedding in semen.

2. Materials and methods

2.1. Bulls and experimental design

Nine bulls of the Asturiana de los Valles breed, ranging in age from 3.9 to 12.6 (mean 5.8) years old, weighing from 806 to 1158 (mean 954) kg, and trained for semen collection were selected for this study. Six of the bulls were infected 10 months before with 10^8 tachyzoites of *N. caninum* [3] and the other three were non-infected. Bulls were allocated into three groups (A–C) composed of three animals each. Groups A and B included bulls with experimentally induced primary infection with *N. caninum* and only bulls in group A were experimentally re-infected intravenously with 10^8 live *N. caninum* tachyzoites of NC-1 isolate. Group C included non-infected control bulls. Tachyzoites used for experimental infections and infection procedure were described previously [3]. The presence of *N. caninum* DNA in semen and blood, serological (IgG, IgG1, IgG2, and IgM) and IFN- γ responses, and specific IgG and IgA levels in seminal plasma of bulls was assessed. Bulls were monitored for 13 weeks. Individual and pooled semen samples of bulls from groups A and B were bioassayed in a BALB/c *nu/nu* mouse model and in synchronized seronegative heifers by means of AI, respectively.

2.2. Sample collection and preparation

Sample collection and preparation was previously explained [3]. For the bioassay in heifers, a weekly pooled semen sample was prepared with excess semen collected from each bull from groups A and B. The pooled semen sample (total volume oscillated between 6 and 15 ml) was stored in a sterile container at 37 °C in a water bath until used. Sample preparation, inoculation of mice, and insemination of synchronized heifers were performed immediately after semen collection from bulls from groups A and B.

2.3. PCR procedures

DNA from whole blood, seminal fluid and the non-sperm cell fraction were analysed separately using nested-PCR as described [1]. *N. caninum* DNA from nested-PCR positive semen samples was quantified by a real-time PCR test [6].

2.4. Bioassay in BALB/c *nu/nu* mice and heifers

The bioassay procedure was previously described [2,3]. In parallel, experimental infections were done with bovine semen artificially contaminated with different quantities of *N. caninum* tachyzoites of the NC-1 strain using BALB/c *nu/nu* mice to assess the capability of the parasite to induce an infection within this animal model in order to assess the sensitivity of this bioassay methodology. Five groups of 6-week-old female athymic mice were used. They were intraperitoneally infected with 10 , 10^2 , 10^3 , 10^4 , and 10^5 tachyzoites resuspended in 400 μ l of semen from a *N. caninum* seronegative bull. Positive (10^3 tachyzoites diluted in PBS) and negative (only semen) control groups were also included. Afterwards, inoculated mice were examined daily and killed with CO₂ gas at 20 weeks post-inoculation. At necropsy, brain was removed aseptically and examined by a nested-PCR procedure [7].

Eleven cyclic heifers of the Asturiana de los Valles breed, ranging in age from 15 to 28 (mean 19) months and weighing from 337 to 420 (mean 380) kg were used for the bioassay. Before starting, blood samples were taken from all 11 heifers and tested for evidence of exposure to *N. caninum*, bovine viral diarrhoea virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), *Leptospira hardjo* and *Brucella abortus* (assays conducted by the Instituto Vasco de Investigación y Desarrollo Agrario, NEIKER, Derio, Spain). The heifers were negative for all five abortifacient agents. The 11 heifers were artificially inseminated using standard techniques with weekly pooled semen samples. One heifer was artificially inseminated each week between 21 and 91 days after the experiment began. Before AI, all cyclic heifers were synchronized hormonally with two doses of a synthetic PGF2 α analogue given 11 days apart (2 ml i.m., Prosolvin, Intervet, Salamanca, Spain). Oestrus detection (day 0; 2.5 days after the second PGF2 α analogue administration on average) was based on monitoring behavioural signs three times daily as serum progesterone levels. The heifers were monitored daily for 100 days post-insemination and pregnancy was diagnosed by transrectal ultrasonography on days 34 and 60 after insemination. Blood samples were collected fortnightly

Table 1

N. caninum DNA detection by nested-PCR in semen and blood samples of three bulls (group A) with experimentally induced primary infection with *N. caninum* which were re-infected with 10^8 *N. caninum* tachyzoites of the NC-1 isolate and three bulls (group B) with experimentally induced primary infection with *N. caninum*

Group	Bull	Days after re-infection (group A)/days after experiment started (group B)														
		0	7	10	14	21	29	36	43	50	57	64	71	78	85	91
A (re-infected)	4852	S (7.8)	P	P	P	–	–	S (1.8)	–	S (0.1)	–	–	–	–	–	–
	90	S (5.8)	P, S (2.0)	P	–	P	P	P	S (4.4)	–	–	S (4.2)	–	S (2.7)	S (4.0)	–
	4375	–	P, S (15.6)	P	P, S (0.1)	–	–	–	–	–	–	–	–	–	–	–
B (not re-infected)	6507	–	S (9.6)	–	–	–	P, S (2.5)	S (1.4)	S (1.6)	–	–	S (3.4)	S (0.1)	S (2.3)	–	–
	1940	S (2.5)	–	–	–	–	S (0.1)	–	–	–	–	S (0.1)	–	S (5.0)	–	–
	6367	–	S (11.1)	–	–	–	S (11.1)	–	–	–	–	–	S (7.2)	–	–	–

Animals were monitored for 91 days.

Parasite load (number of parasites/ml of semen) determined by real-time PCR in nested-PCR positive semen samples is indicated within parentheses. S, detected in semen; P, detected in blood; –, not detected.

by coccygeal venipuncture in plain tubes for serological analysis. Serum samples were assayed for specific IgG antibodies using a commercial kit (CIVTEST^{TM}). Positive and negative controls of the bioassay in heifers were also carried out and published [4].

2.5. Assessment of *N. caninum*-specific immune responses

Serum and undiluted seminal plasma samples were assayed for specific IgM, IgG, IgG1 and IgG2, and IgG and IgA antibodies, respectively using a commercial kit (CIVTEST^{TM}) as previously described [3]. To assess IFN- γ production, duplicate plasma samples were tested using a commercial ELISA kit (Bovigam IFN- γ kit, CSL, Australia) as previously described [3].

2.6. Statistical analysis

Independent two-sample *t*-tests were performed to compare the antibody and IFN- γ responses between bulls from groups A–C. The association between the detection of *N. caninum* DNA in semen and/or blood and serological and IFN- γ specific responses in bulls from groups A and B were analysed by the Wilcoxon Rank-test. Statistical analyses were performed using SAS Software v.8.2. (SAS Institute Inc.). All statistical analyses were considered significant when $P < 0.05$.

3. Results

3.1. Detection of *N. caninum* DNA and bioassays in BALB/c nu/nu mice and heifers

The three bulls from group A showed parasitaemia between 7 and 36 days post-infection (pi), but only one

bull (6507) from group B showed *N. caninum* DNA in blood at 29 days after the start of the experiment (Table 1). The presence of *N. caninum* DNA in semen was intermittent and the number of positive semen samples was similar in bulls from groups A and B (11 versus 14, respectively). In all positive semen samples, specific-DNA was found in the cellular fraction and not in seminal plasma. The parasite load varied from 0.1 to 15.6 (mean 4.41 ± 4.38) and 0.1 to 11.1 (mean 4.14 ± 4.01) parasites per ml of semen in groups A and B, respectively (Table 1). Mean individual parasite load varied from 3.23 (± 4.05) to 7.85 (± 10.96) in group A and 1.93 (± 2.34) to 9.80 (± 2.25) in group B.

A total of 90 athymic mice were inoculated with semen samples. Twenty-five mice were inoculated with PCR-positive semen samples containing an approximate tachyzoite number between 0.04 and 6.24 parasites per dose. No *N. caninum* DNA was detected by nested-PCR in the brain, lung or liver of BALB/c nu/nu mice inoculated. Experimental infections in athymic mice showed that *Neospora* DNA was found in the brains of mice inoculated with semen contaminated with at least 10^3 tachyzoites and clinical signs associated with neosporosis (anorexia, inactivity, rounded back, and pelvic limb weakness) were detected in some mice inoculated with semen contaminated with 10^4 and 10^5 tachyzoites (Table 2). Eight of the 11 heifers were inseminated with a pooled semen sample containing some PCR-positive individual semen samples. The approximate number of tachyzoites included in pooled semen samples oscillated between 0.2 and 27.4. No *N. caninum*-specific antibody responses were detected in any of the heifers inseminated. All but two heifers became pregnant and gave birth to calves that did not show precolostral specific antibodies to *N. caninum*.

Table 2

Presence of *Neospora* DNA in brains of BALB/c *nu/nu* mice inoculated intraperitoneally with bovine semen contaminated with different *N. caninum* (NC-1 isolate) quantities

Number of tachyzoites in semen	<i>Neospora</i> DNA in brain (positives/total tested)
10	0/4
10 ²	0/4
10 ³	3/7
10 ⁴	3/3*
10 ⁵	3/3**
10 ³ (PBS)	2/2***
0 (semen)	0/3

* Two mice showed clinical signs and were sacrificed at 23 days post-inoculation.

** Three mice showed clinical signs and one animal at 23 days and two animals at 28 days post-inoculation were sacrificed.

*** One mouse showed clinical signs and was sacrificed at 22 days post-inoculation.

3.2. *N. caninum*-specific immune responses

Bulls from group A showed significant ($P < 0.05$) increased mean specific IgM antibody levels to *N. caninum* between 7 and 91 days pi (Fig. 1a) when compared with bulls from groups B and C, respectively. In re-infected bulls, mean anti-*N. caninum* IgG levels increased from 7 days pi and were significantly higher ($P < 0.05$) than in groups B and C during the entire experimental period (Fig. 1b). Specific serum IgG antibody levels in bulls from group B were higher than in controls, but in bulls 1940 and 6507, the value fell below the cut-off value between 43 and 92, and 85 and 92 days after the experiment started, respectively. Mean IgG1 levels remained significantly ($P < 0.05$) elevated in all bulls from group A compared to groups B and C, but decreased progressively (Fig. 1c). The mean levels of IgG2 in re-infected bulls increased from 7 days pi and were significantly ($P < 0.05$) higher compared to groups B and C (Fig. 1d). No significant association was found between the presence and quantity of *N. caninum* DNA in semen and the magnitude of specific IgG, IgG1, IgG2 or IgM responses in bulls from groups A and B, respectively. In seminal plasma, mean anti-*N. caninum* IgG levels in re-infected bulls were significantly higher ($P < 0.05$) than in groups B and C from 21 to 91 days pi (Fig. 1e). No differences were detected in seminal plasma IgA antibody levels between groups A and B and control bulls (data not shown). The mean specific IFN- γ levels in re-infected bulls were higher than in groups B and C as soon as 3 (bulls 90 and 4375) and 7 (bull 4852) days pi (Fig. 1f), but decreased soon thereafter. No significant associations were observed

between the presence and magnitude of *N. caninum* DNA in semen and IFN- γ production levels, nor among IgG2 antibody levels, IgG1/IgG2 ratio, and IFN- γ levels in bulls from groups A and B, respectively.

4. Discussion

This study demonstrated that the intermittent presence and the low number of parasites in semen observed in re-infected bulls were very similar to that reported in animals after primary infection [3]. When these results were compared with those reported in naturally infected bulls (probably infected prenatally by the endogenous transplacental route) [2], two significant differences were observed. First, all experimentally infected bulls showed *N. caninum* DNA in semen at some time during the course of the study (100% versus 62.5%, respectively). Second, the mean parasite load was approximately twice the quantity reported in naturally infected bulls (6 tachyzoites/ml versus 3 tachyzoites/ml of semen, respectively). However, the maximum parasite load found in semen samples of re-infected bulls (15.6 tachyzoites/ml) suggests an ejaculate containing barely 200 tachyzoites. Two recent studies support our findings. Staubli et al. [8] found no positive semen samples when investigating five separate semen specimens from 20 seropositive bulls used for AI in Switzerland. In Brasil, only one of 55 seropositive bulls from AI centers showed *N. caninum* DNA in semen samples [9].

Unfortunately, we cannot conclude that live infectious *N. caninum* stages were present in semen because of the inability to isolate the protozoa from positive PCR semen samples. Lack of success in isolating *N. caninum* from semen could be explained by two causes. First, the toxic effects of bovine semen in athymic BALB/c *nu/nu* mouse did not allow us to inoculate more than 400 μ l of fresh semen in each animal and second, there was a low parasite load found in semen in relation to the detection threshold in the murine and bovine models. Parasite viability may not be compromised because positive semen samples were inoculated immediately after semen extraction. The total number of parasites in 400 μ l of fresh bovine semen was less than 6 tachyzoites and the experimental infections in athymic mice with artificially contaminated semen indicate that at least 10³ tachyzoites are necessary in bovine semen to induce an infection in an athymic BALB/c *nu/nu* mouse. An interesting issue of the present work was the insemination of seronegative cyclic heifers with pooled semen from infected bulls in order to evaluate the viability of parasites shed in

Fig. 1. Specific antibody and IFN- γ responses to *N. caninum* in monitored bulls: (a) serum IgM; (b) serum IgG; (c) serum IgG1; (d) serum IgG2; (e) seminal plasma IgG; (f) IFN- γ . For IgM, IgG subclasses, seminal plasma IgG, and IFN- γ , the graphs show the individual OD of three bulls with experimentally induced primary infection with *N. caninum* which were re-infected with 10^8 *N. caninum* tachyzoites of the NC-1 isolate (solid dotted black line), three bulls with experimentally induced primary infection with *N. caninum* (broken dotted white line) and the mean OD $\pm$ S.D. of three control non-infected (vertical white bars) bulls, respectively. The serum IgG results are expressed as relative index $\times$ 100 values and the cut-off value is indicated as a broken line.

semen. However, the low number of parasites shed in semen was probably inadequate to produce an infection in heifers. The maximum number of parasites included in a pooled semen sample only reached 27 tachyzoites. In a recent study, we have demonstrated a threshold level of between 5000 and 50,000 tachyzoites for horizontal transmission via contaminated semen [4], which seems to be very different from the parasite load described in semen of experimentally and naturally-infected bulls.

Inoculation with *N. caninum* tachyzoites in chronically infected bulls resulted in an early increase in specific serum IgM and IgG antibodies, with a higher peak and later decline than those observed after primary infection in the same animals [3]. The specific antibody IgG profile in re-infected bulls was similar to that observed in other re-infected cattle with live [10,11] and inactivated [12] tachyzoites. In bulls with experimentally induced primary infection with *N. caninum*, serum antibody IgG levels were predominantly close to threshold levels, and sometimes fell to seronegative values. During the chronic stage of neosporosis, low-specific antibody titres in serum were near the cut-off level of the techniques employed [13,14] and antibody fluctuations [2] that have been reported in naturally infected bulls. The detection of specific antibodies to *N. caninum* in seminal plasma could be an alternative to serology in the diagnosis of bovine neosporosis. However, experiments in naturally infected bulls, especially from AI centres, should be performed to confirm the data from experimentally infected animals. Although the possible toxic effect of specific antibodies on parasite shedding in semen was not investigated, the presence of *N. caninum* DNA associated with the cellular fraction of semen strongly suggests an association between *N. caninum* and phagocytic cells which could act as carriers protecting the protozoa [2,15]. It is worth mentioning that the early IFN- γ increase highlights the potential use of IFN- γ determination as a tool for the diagnosis of a recent *N. caninum* infection in cattle. The lack of correlation between IgG and IFN- γ levels found in our study has also been reported in naturally [2] and experimentally [16] infected cattle, suggesting that factors other than IFN- γ regulate IgG antibody production in cattle.

The most important question derived from the current work and previous studies [1,15] is the possibility of venereal transmission of bovine neosporosis. Despite the low parasite burden found in semen of naturally [2] and experimentally [3] infected bulls and the elevated infectious dose needed to infect heifers by the intrauterine route [4], at present we are involved in field trials that

include experimentally infected bulls and seronegative heifers under natural services. The future results will show if venereal transmission plays a role in the spread of bovine neosporosis under natural conditions.

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