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Characterization of Immune Cell Infiltration in the Placentome of Water Buffaloes (*Bubalus bubalis*) Infected with *Neospora caninum* During Pregnancy

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Summary

Neospora caninum infection in cattle stimulates host immune responses, which may be responsible for placental damage leading to abortion. Susceptibility of water buffaloes (*Bubalus bubalis*) to neosporosis is not well understood, although vertical transmission and fetal death have been documented. The aim of this study was to characterize the immune response in the placentome of water buffalo following experimental infection in early gestation with the Nc-1 strain of *N. caninum*. Placentomes were examined by immunohistochemistry using antibodies specific for T-cell subsets, natural killer cells and $CD79_{\alpha cy}$ cells. Placental inflammation was characterized by the infiltration of $CD3^+$ and $CD4^+$ T cells and T cells expressing the γδ T-cell receptor. The distribution of these cellular subsets in buffalo placentomes was similar to that previously described in cattle infected with *N. caninum* in early gestation, but the lesions were milder, which may explain the lower number of abortions observed in this species after infection.

Keywords: immunity; *Neospora caninum*; placentome; water buffalo

Neospora caninum is a pathogenic protozoan parasite, for which a wide range of warm-blooded animals act as intermediate hosts (Dubey *et al.*, 2007), but the organism causes disease only in cattle and dogs (Buxton *et al.*, 2002). Evidence of *N. caninum* infection in water buffalo (*Bubalus bubalis*) has been reported (Huong *et al.*, 1998; Rodrigues *et al.*, 2004).

After experimental infection of pregnant water buffaloes in early gestation with *N. caninum*, vertical transmission was confirmed and lesions were observed in placentomes and fetuses. This demonstrated the potential of *N. caninum* as an abortifacient in water buffaloes (Chryssafidis *et al.*, 2011; Konrad *et al.*, 2012).

N. caninum produces fetal and placental lesions severe enough to cause mortality (Barr *et al.*, 1990; Maley *et al.*, 2003). Additionally, *N. caninum* stimulates a T helper (Th) 1 immune response, which limits multiplication of the organism (Innes *et al.*, 1995); however, this response may also cause placental damage leading to abortion (Innes *et al.*, 2002; Maley *et al.*, 2006).

The aim of the present study was to characterize the inflammatory cell infiltrate in placentomes collected from pregnant water buffalo infected experimentally with *N. caninum* in early gestation (Konrad *et al.*, 2012). Twelve Mediterranean adult pregnant *Neospora*-seronegative water buffaloes were divided into four groups. Three animals in group A were infected at 70 days of gestation (dg) and culled at 28 days post infection (dpi). Three animals in group B were infected at 90 dg and culled at 28 dpi. Four animals in group C were infected at 90 dg and culled at 42 dpi. Two control animals in group D received uninfected Vero cells at 70 or 90 dg and both were killed at 28 dpi. Challenged animals each received 1×10^8 tachyzoites of the Nc-1 strain of *N. caninum* (Dubey *et al.*, 1998) intravenously.

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After infection, no clinical signs were observed; however, one fetus from one dam infected at 70 dg was found to have died before the dam was killed. Non-suppurative inflammation was frequent in placentomes and fetal tissues of infected animals. No lesions were observed in control fetuses. *N. caninum* was identified by immunohistochemistry (IHC) or polymerase chain reaction (PCR) in placentomes and fetuses from the infected animals. Placentome and fetal tissues from the two control animals were negative by PCR and IHC (Konrad *et al.*, 2012).

During necropsy examination randomly-selected placentomes were collected and fixed in zinc salts fixative (ZSF; pH 7.0–7.4; González *et al.*, 2001) for IHC and in 10% neutral buffered formalin for in-situ hybridization (ISH). Phenotypic characterization of the cellular infiltrates was performed using the IHC technique described by Maley *et al.* (2006) and the IHC was scored as described by Cantón *et al.* (2013b). Sections were incubated overnight with monoclonal antibodies (mAbs) specific for the T-cell marker CD3 (MM1A; VMRD, Pullman, Washington USA), the Th-cell marker CD4 (IL-A11; VMRD), the cytotoxic T-cell marker CD8 (CC58; AbD Serotec, Kidlington, Oxfordshire, UK), T cells expressing the γδ form of the T-cell receptor (γδ T cells; IL-A29; VMRD), natural killer cells (NKp46; CD335; AbD Serotec) and B cells expressing $CD79_{\alpha cy}$ (HM57; Dako, Glostrup, Denmark). Sections of ZSF-fixed water buffalo lymph nodes were used as positive control tissues.

The scores from individual placentomes were averaged into a single score for each animal. Given the limited sample sizes, the potential effects of time of infection or culling were not considered. Non-parametric two-tailed Mann–Whitney tests allowing for ties were conducted on the pooled data in order to investigate differences in the distribution of scores between infected and control animals for each cell type. Statistical significance was assessed at the 5% level.

A mild to severe $CD3^+$ T-cell infiltrate surrounded necrotic foci in the caruncle or within necrotic fetal villi (FV) in placentomes from dams of group A (Fig. 1). Higher scores were obtained in the placentome from the animal carrying a non-viable fetus compared with those from dams carrying viable fetuses. Group B and C placentomes were also infiltrated with T cells, but to a lesser extent than for those of group A. In caruncles from group D animals, there was sparse to mild infiltration of T cells.

A sparse to mild infiltration of $CD4^+$ T cells surrounded necrotic areas in the caruncle in all of the placentomes from group A dams and these scores were higher in the dam carrying the non-viable fetus. In group B animals there was sparse to mild infiltration in most of the samples, with a few placentomes that had marked infiltration. Infiltrates in group C animals were sparse and restricted to the caruncles, as were infiltrates in animals of group D.

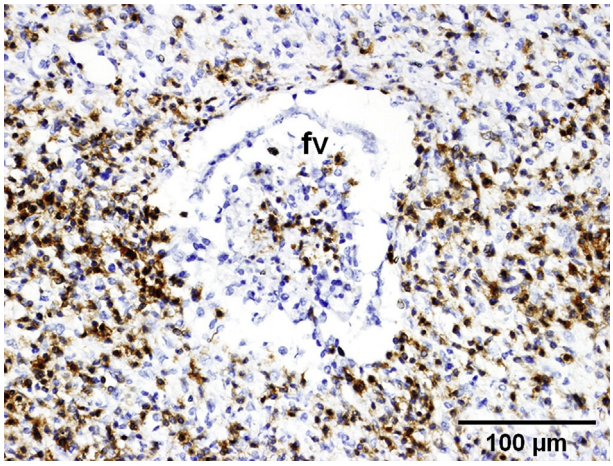

Fig. 1. Severe infiltration of $CD3^+$ T cells in the caruncle surrounding and within a necrotic fetal villus (fv) in a placentome collected from the group A water buffalo in which the fetus was non-viable. IHC.

Infiltrates of $CD8^+$ T cells in samples from all animals in groups A, B and C were sparse to mild. The group A dam carrying the dead fetus had a slightly higher $CD8^+$ T-cell infiltration score when compared with the other two animals in the group. The $CD8^+$ T-cell scores in group D animals were low.

In group A animals there was sparse to moderate infiltration of γδ T cells surrounding areas of necrosis in the caruncle. The score was higher in the dam with the dead fetus compared with the scores for the other two dams in the group. Similarly, in animals of group B, a sparse to moderate infiltration of γδ T cells was observed. In group C animals, some placentomes had a sparse infiltration of γδ T cells surrounding necrotic foci in the caruncle. Scores from group D animals were similar to those for animals in group C.

NK cell infiltration was sparse to mild in samples collected from animals in group A, with no detectable differences between the dams carrying live or dead fetuses. Similar results were seen in group B animals. In groups C and D animals there was sparse NK infiltration of the placentomes.

Cells expressing $CD79_{\alpha cy}$ morphologically and histologically resembled trophoblast cells rather than B cells. Mononuclear cuboidal cells and occasional binucleate cells were also labeled. Placentomes in group A animals contained $CD79^+_{\alpha cy}$ cells morphologically similar to trophoblast cells in the caruncle and FV, and these were not associated with pathological changes. In animals of groups B and C, $CD79^+_{\alpha cy}$ cells