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Neospora caninum: Quantification of DNA in the blood of naturally infected aborted and pregnant cows using real-time PCR

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Abstract

This study quantified *Neospora caninum* DNA in the blood and brain of pregnant and aborted heifers by monitoring PCR product formation in real-time using SYBR Green I, a double-stranded DNA-binding dye. Primers were designed to amplify a 188 bp product specific to *N. caninum* from the Nc-5 gene fragment of *N. caninum*. Similarly, a 71 bp product was amplified from the 28S rRNA gene of bovine genomic DNA that served as a control. Agarose gel electrophoresis and analysis of the melting curve for PCR products showed that both primer pairs were specific to their targets. Standard curves were generated for both bovine and *N. caninum* genomic DNA, and were used to compute the relative concentration of parasite to bovine DNA in the test samples. The concentration of *N. caninum* DNA in 1 ng of bovine genomic DNA obtained from blood ranged between 0.097 ng at the 1st week of the observation and 0 ng at the 15th week in aborted cows. In pregnant cows, the values ranged between 0.080 ng at the 1st week and 0.155 ng at the 15th week of observation. There was a sustained decrease of DNA concentration in the aborted group after abortion and an increase in DNA concentration in the pregnant group. Comparison of parasite DNA in blood and brain of infected heifers showed a higher DNA concentration in brain than in blood. This study shows that *N. caninum* DNA can be quantified to obtain the relative concentration of parasite DNA to host genomic DNA in blood. This technique allows testing of a large number of samples at one time and can be done without the need for slaughter of tested animals.

Index Descriptors and Abbreviations: BLAST, basic local alignment search tool; Bp, base pairs; BVDV, bovine viral diarrhoea virus; DNA, deoxyribonucleic acid; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; IFN, interferon; IgE, immunoglobulin E; IgG, immunoglobulin G; IL, interleukin; $MgCl_2$, magnesium chloride; NeoF, *Neospora* forward; NeoR, *Neospora* reverse; PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid; TBE, Tris–borate–EDTA; Th, T helper; *T. gondii*, *Toxoplasma gondii*; TNF, tumour necrosis factor; UNG, uracil *N*-glycosylase; UV, ultraviolet

Keywords: *Neospora caninum*; Real-time PCR; Abortion; Pregnant; Parasitaemia

1. Introduction

Neospora caninum is an apicomplexan protozoan parasite that causes neonatal neuromuscular disease in dogs and abortions in cattle (Lindsay et al., 2001). *N. caninum*-induced abortion was first reported in dairy cows in New Mexico in 1989 (Thilsted and Dubey, 1989). Many factors predispose cattle to neosporosis. Some of these factors include congenital transmission; presence of possible intermediate host animals in the farm, e.g., the dog and domestic fowl, lactogenic transmission, immunosuppressive effect of bovine viral diarrhoea virus (BVDV), and pregnancy.

Pregnancy may trigger recrudescence of latent infection in a cow with chronic neosporosis. This may result to parasitaemia and foetal infection. In a naïve

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pregnant cow, infection with *N. caninum* is easily achieved because the immune regulation is suppressed (Quinn et al., 2002). It could be assumed that foetal infection follows a maternal parasitaemia although most infections occur in cattle already harbouring a persistent infection before pregnancy was established (Buxton et al., 2002). Infected cows may or may not abort their foetuses and most unaborted foetuses become congenitally infected and may or may not show clinical signs at birth (Dubey and Lindsay, 1996). The above outcome of neosporosis during pregnancy may depend on all or a combination of the following: the gestational age/age of the foetus at the time of infection, the virulent nature of the particular *N. caninum* including the magnitude and time of parasitaemia (Innes et al., 2002; Williams et al., 2000). During pregnancy, the body down-regulates the production of Th1 cytokines which may be incompatible with pregnancy (Quinn et al., 2002) since they promote foetal rejection. However, Th1 cells produce interleukin (IL)-2, tumour necrosis factor (TNF)-β, and interferon (IFN)-γ, and are the main effectors of phagocyte-mediated host defences (Mosmann and Coffman, 1989; Romagnani, 1991), which are highly protective against infections sustained by intracellular parasites. The down-regulation of Th1 cytokine during pregnancy might lead to parasitaemia, when parasite tachyzoites can be found in several organs (Barr et al., 1994; Buxton et al., 1998) and body fluids (Okeoma et al., 2004a; Ortega-Mora et al., 2003).

Diagnosis of neosporosis is usually based on serology which detects parasite-specific antibodies in live animals (Björkman and Uggla, 1999) or the visualisation of characteristic histopathological lesions in organs of dead animals and aborted materials. Foetal serology may also be used in diagnosis (Barr et al., 1995; Buxton et al., 1997; Paré et al., 1995). Most diagnostic analyses for neosporosis including some PCRs are done utilising materials from dead animals. We have shown that *N. caninum* DNA can be amplified in the blood of naturally infected cows allowing diagnosis of infection in live animals (Okeoma et al., 2004a).

In the present study, we quantified *N. caninum* tachyzoite DNA in the blood of naturally infected aborted and pregnant heifers by monitoring parasite DNA concentration in the animals. At the end of monitoring, we compared parasite DNA concentration in brain with that obtained from blood for infected aborted and pregnant heifers. We also compared the sensitivity of real-time and block PCRs. The quantification of *N. caninum* DNA in the blood can complement serology in determining infection intensities in live animals and thus offers a tool to significantly improve research into the epidemiology and pathogenesis of *N. caninum* infections.

2. Materials and methods

2.1. Animals and sample collection

Six rising 2-year-old heifers (5 from a group of 20 heifers from one farm and 1 from a group of 4 heifers from two other farms) were in their 5th month of pregnancy when tested on March 5, 2003, or later. Pregnancy tests were repeated on all heifers on April 16, 2003. The latter test showed that of the six heifers used in this study, three were still pregnant and the other three had aborted. The exact time of abortion was not known for those aborting and no aborted material from them was recovered.

Heifers included in this study were selected from the initial 24 in the following way. First, they were divided into two groups based on their initial serology, i.e., a positive or negative IDEXX ELISA *N. caninum* antibody result. Then, the positives were divided into pregnant and aborted groups. From these two groups, the DNA of one heifer in each group (that had the most obvious PCR band on an agarose gel) was cloned and sequenced; confirming that products were *N. caninum* sequences (Okeoma et al., 2004a). These heifers (heifers 172 and 133) were selected. The other four heifers, two each from the aborted (35 and 95) and pregnant (64 and 86) groups were randomly selected. After abortions were noticed, blood samples were obtained from all heifers initially weekly for 7 weeks and then biweekly for 8 weeks. DNA was isolated from blood as described by Okeoma et al. (2004a). At calving, precolostral blood was obtained from calves of heifers 64, 86, and 133. The samples were tested for presence of *N. caninum* antibodies using immunofluorescence antibody test (IFAT). The result (1:4000, 1:8000, and 1:2000, respectively) for these calves showed that they were congenitally infected. In addition, brain samples were collected for analysis from all animals at slaughter. *N. caninum* tachyzoites were isolated from the brains of heifer 133 and her calf (Okeoma et al., 2004b). PCR analysis of the brains of all calves confirmed that they were congenitally infected.

Total DNA was isolated from the brains of heifers using a Qiagen DNAeasy blood kit according to manufacturer's instructions. Electrophoresis of each DNA sample on a 2% agarose gel in 1× TBE buffer was undertaken to check integrity. The DNA samples were then diluted 10-fold and quantified using a Hoefer DNA Quant 200 Fluorometer. A total of 2 μl of sample DNA containing 1 ng/μl of bovine brain genomic DNA was used in a reaction volume of 20 μl for the PCR assay.

2.2. Block-PCR assays

Following DNA isolation, samples were used for block-PCR amplification of *N. caninum* DNA using