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SHORT COMMUNICATION

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Comparative molecular investigation of Nc5-PCR amplicons from *Neospora caninum* NC-1 and *Hammondia heydorni*-Berlin-1996

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Abstract The clinical relevance of *Neospora caninum* as a cyst-forming coccidian parasite is increasingly acknowledged within veterinary medicine, although the pathways of transmission are far from being solved. The parasite is well known for causing diaplacental infections in cows associated with abortion and/or severe damage of the fetus. In addition, it may cause neuromuscular disease in dogs, which thus apparently act as intermediate hosts as well as final hosts. In our previous studies, we have demonstrated that molecular diagnosis of *N. caninum* infections has a high performance when a highly sensitive polymerase chain reaction (PCR) targeted to the Nc5 region of the parasite is used. The present study indicates that the high sensitivity of the PCR is the consequence of a target dose effect which reflects a high redundancy of Nc5-type sequences within the genome of the parasite. The PCR was shown to amplify a set of DNA molecules exhibiting significant sequence differences. A complex composition of Nc5-type sequences was observed in the parasite isolate *N. caninum* NC-1 but also in another isolate, designated *Hammondia heydorni*-Berlin-1996. Investigation of the infection pattern of this parasite in its intermediate and final canine hosts showed it to be indistinguishable from *N. caninum* NC-1.

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Neospora caninum is considered to be an important cause of infectious abortion and stillbirth in cattle worldwide. Infection in cattle is common and apparently may often be passed from mother to the fetus without introducing disease. The latter occurs when the parasite multiplies intensively in the developing calf and its placenta, and then causes sufficient damage to trigger abortion or stillbirth. At present, diagnosis of neosporosis in the context of an abortion problem can be achieved by examination of fetal tissues for parasite-specific lesions, detection of parasite antigens or DNA and, with a controversial diagnostic value, parasite-specific antibodies in fetal fluids. In the last few years, diagnosis of neosporosis has been much improved by the development of *N. caninum*-specific polymerase chain reactions (PCRs), which allow highly sensitive and specific detection of the parasite through the amplification, and subsequent demonstration, of parasite-specific DNA sequences (reviewed by Dubey 1999; Ellis 1998) in affected tissues. One of the most commonly used diag-

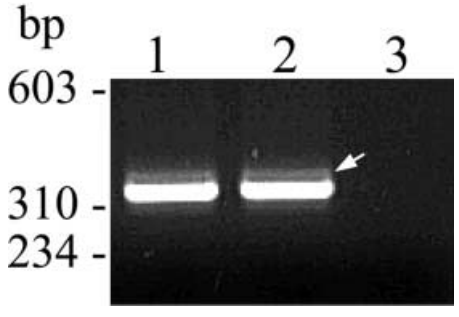

Fig. 1 Nc5-PCR amplification products from *Hammondia heydorni* Berlin-1996 (*lane 1*), positive control *Neospora caninum* NC-1 (*lane 2*), and negative control containing no DNA template (*lane 3*) were analysed by 2% agarose gel electrophoresis. The diagnostic amplification products are represented by a major lower and a minor higher (*arrow*) band migrating with sizes of approximately 337 bp. Size markers are indicated in bp on the leftmost side

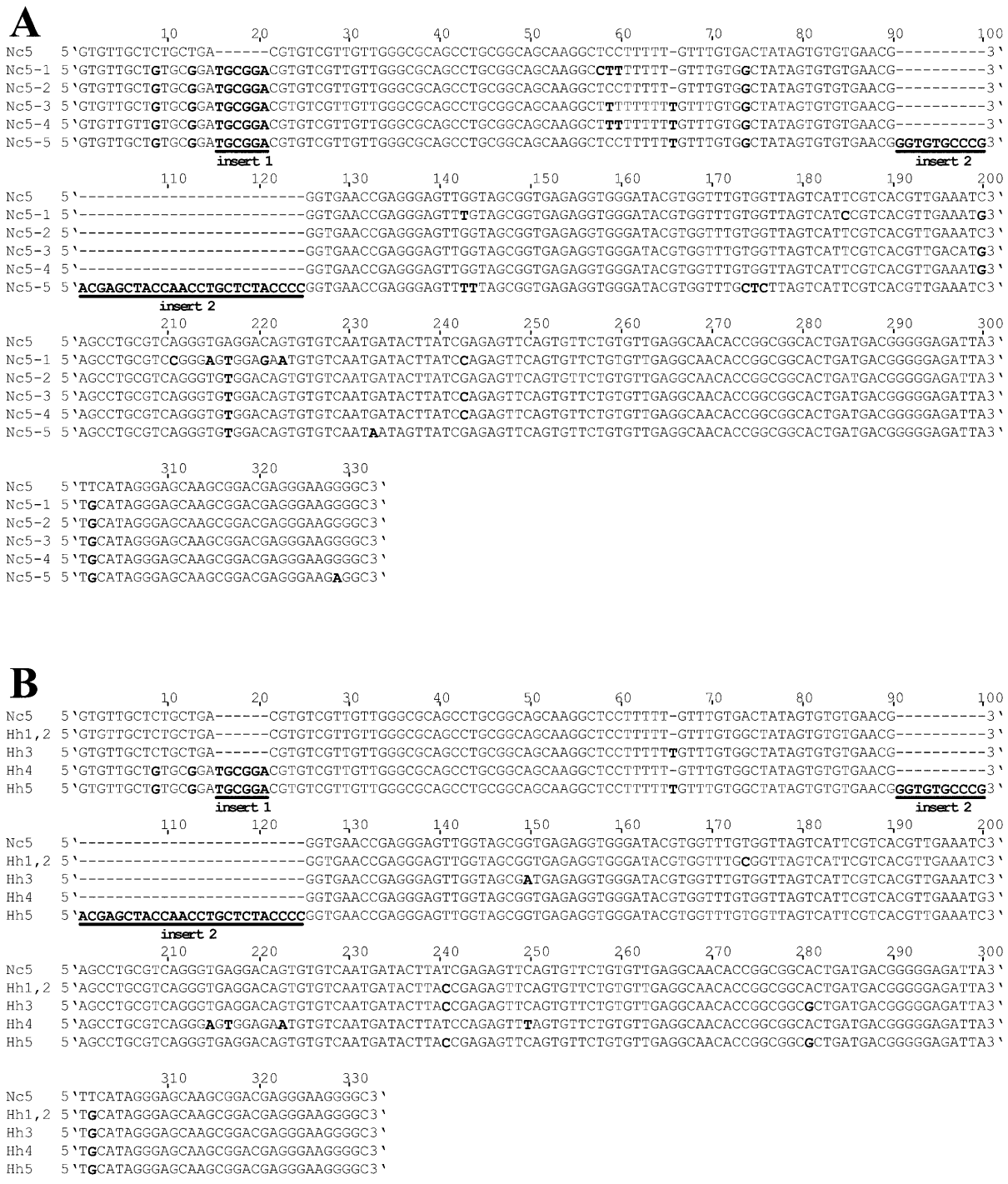

Fig. 2 Alignment of nucleotide (nt) sequences from *N. caninum* Nc5-sequence and cloned Nc5-PCR amplification products originating from **A** *N. caninum* NC-1 (clones Nc5–1 to 5–5) and **B** *H. heydorni*-Berlin-1996 (clones Hh1 to 5). *Bold characters* indicate an nt position different to the previously described Nc5 sequence (Yamage et al. 1996). *Dashes* within the sequences indicate alignment gaps. *Bars* mark positions of sequence inserts 1 and 2. Alignment and comparison of the corresponding nt sequences were done with the MultAlin computer package. Sequence targets of forward Np21plus and reverse Np6plus primers are not included in the alignment

nostic PCRs includes a set of primers targeted to the genomic region Nc5 (Kaufmann et al. 1996; Müller et al. 1996; Yamage et al. 1996). Both the diagnostic sensitivity and specificity of this PCR assay had been confirmed by testing the DNA amplification reaction on a panel of different apicomplexan parasites including *N. caninum*, *Hammondia hammondi*, *Toxoplasma gondii*, and various species of the genus *Sarcocystis* (Müller et al. 1996). In this investigation, the Nc5-PCR identified

DNA equivalents from only one *N. caninum* tachyzoite but did not amplify DNA from the other apicomplexan organisms.

In the present study, the evaluation of the Nc5-PCR was extended in that the reaction was additionally tested on an *N. caninum*-like organism, designated *Hammondia heydorni*-Berlin-1996 (Schares et al. 2001). This isolate had recently been examined by successfully infecting intermediate ruminant and rodent hosts as well as the final canine hosts. Analyses of the respective infection parameters indicated that the isolate was indistinguishable from *N. caninum* NC-1.

PCR analysis of isolate *H. heydorni*-Berlin-1996 was performed as previously described (Müller et al. 1996). The amplification reaction (25 µl reaction mix) was done on DNA from about three oocysts that had been prepared by using the DNAeasy Tissue Kit system (Qiagen, Basel, Switzerland) according to the protocol suggested for tissue samples. As shown in Fig. 1, the sample exhibited amplification products which in the agarose gel co-migrated with the amplicon of *N. caninum* NC-1 control DNA.

A careful inspection of the ethidium bromide-stained gel indicated that amplicons from both isolates separated into a predominant lower band and a minor higher band (Fig. 1). In order to obtain more information on the two apparent PCR products, amplicons from isolates NC-1 and *H. heydorni*-Berlin-1996 were cloned into plasmid vector pGEMT Easy I (Promega, Madison, Wis.) according to the manufacturer's instructions. Subsequently, five clones from the two experimental groups were obtained and investigated further. In both groups, cloned PCR products demonstrated a significant heterogeneity in sizes ranging approximately from 340 to 380 bp (data not shown). These size differences were also confirmed on the DNA sequence level. Sequence analyses (performed by Solvias, Basel, Switzerland) demonstrated that some of the cloned PCR products (clones Hh1, 2 and 3) were nearly identical to the corresponding sequence within the previously published Nc5 region (Fig. 2). However, others were strikingly different from Nc5. Clones Nc5–1, 5–2, 5–3, 5–4 and Hh4 contained one [6 nucleotide (nt) insert 1 between positions 16 and 21, see Fig. 2] and clones Nc5–5 and Hh5 two (insert 1 plus a 35 nt insert 2 between positions 91 and 125, see Fig. 2) additional sequence motifs.

Taken together, the present data indicate that isolates *N. caninum* NC-1 and *H. heydorni*-Berlin-1996 contain a complex set of Nc5-type sequences, which are simultaneously amplified in the respective PCRs. This target dose effect is most likely responsible for the extremely high diagnostic sensitivity of the Nc5-PCR which has been shown to detect trace amounts of parasite DNA (Müller et al. 1996). Furthermore, our study demonstrated that both isolates are characterized by a nearly identical complexity regarding their Nc5-type sequence composition. This finding further supports the data from the study of Schares et al. (2001) which – on the basis of infection-oriented aspects – classified the two isolates as indistinguishable. However, to allow a full phylogenetic or taxonomic interpretation of our results, a more detailed molecular investigation is required to confirm that the Nc5-type sequence family represents a suitable genetic marker for differentiating isolates/strains within the species *N. caninum* and other *Neospora*-like organisms.

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