The development and evaluation of a nested PCR assay for detection of *Neospora caninum* and *Hammondia heydorni* in feral mouse tissues

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1. Introduction

Neospora caninum is a tissue cyst forming apicomplexan parasite of subfamily *Toxoplasmatinae*. *Neospora caninum* is of particular importance to the beef and dairy industries due to its association with abortion resulting in significant economic loss (Dubey 1999; Trees et al. 1999; Reichel & Ellis 2006). Little is known about the epidemiology of neosporosis on cattle farms and there are few effective treatment options available for bovine neosporosis. A commercially available vaccine (Bovilis® Neoguard, Intervet) has been shown to decrease the incidence of abortion in a cattle herd to a degree, although the vaccine is not particularly efficacious (Barajas-Rojas et al. 2004; Heuer et al. 2004; Romero, Perez & Frankena 2004; Schetters et al. 2004). Moreover there are no drug treatments available for the tissue cyst stage of the disease. As a result, current research focuses on control and containment of the disease.

There is limited literature describing *N. caninum* in populations of feral rodents. A total of four studies exist describing natural infections of feral rodents and none of these studies were performed in Australia (Huang et al. 2004; Hughes et al. 2006; Ferroglio et al. 2007; Jenkins et al. 2007). This general lack of knowledge on feral rodents as reservoirs of *N. caninum* infection makes any knowledge relating to this particular area highly topical. The high rates of transplacental transmission observed in experimental infections of mice during pregnancy (Liddell, Jenkins & Dubey 1999; Quinn et al. 2002; Omata et al. 2004) and high rates of vertical transmission observed in naturally obtained rodent infections of *Toxoplasma gondii* (Owen & Trees 1998; Marshall et al. 2004), suggest that rodents may be an efficient reservoir of *N. caninum* infection and subsequently could play a major role in the epidemiology of farmyard neosporosis. The limited knowledge relating to natural infection in rodents implies the need for further research in the area.

Information relating to *Hammondia heydorni* is also scant. Dubey et al. (2002) suggests that all previous knowledge relating to *H. heydorni* is invalid since *N. caninum* was not ruled out in the parasite populations used in these studies. Consequently, the species *H. heydorni* requires further characterization including the identification of natural intermediate hosts. There is no information relating to *H. heydorni* infection in populations of feral rodents and the presence or absence of this parasite in feral rodent tissues will prove useful in further characterization of this species.

2. Materials and methods

2.1. Sourcing rodent tissues

One hundred and four feral house mice (*Mus musculus domesticus*) were trapped on a semi rural property in Sydney's outer west using humane, live catching traps. All mice were euthanized using carbon dioxide according to recommendations of the local Animal Care & Ethics Committee. The brains, livers and hearts were removed using scissors and tweezers. In between each individual mouse tissue, the implements were cleaned with water followed by ethanol and then flaming to prevent cross contamination between samples. Samples were stored at -20ºC until used.

2.2. Preparation of DNA from rodent tissues

Entire mouse tissues were snap frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. In between each tissue, the apparatus were cleaned with double distilled water followed by ethanol. This ethanol / water wash was repeated three times to prevent any carry over from previous samples. The resulting tissue powder was digested with 2ml of a digesting buffer containing 10mM Tris-Cl, 100mM EDTA, 40 Units of proteinase K [50mg/ml] and 1% SDS (pH 9). Digestion was carried out at 65ºC for between 4 and 24 hours depending on the time needed to fully digest the tissue. The resulting solution was extracted three times with phenol:chloroform (1:1) and once with chloroform. The DNA was precipitated by addition of 40µl of 5M NaCl and 2-3 volumes of cold absolute ethanol. The precipitated DNA was dialyzed against TE buffer [10mM Tris-Cl, 1mM EDTA (pH8)] overnight.

2.3. Nested PCR assay

An assay was developed with the potential to detect multiple members of *Toxoplasmatinae* with a minimal number of additional steps. To achieve this, external primers were selected with specificity for all members of *Toxoplasmatinae* while internal primers were selected on the basis of being species specific. The reaction using the external primers will amplify a product for all members of *Toxoplasmatinae*, while the secondary (species-specific) nested primers will differentiate which member of *Toxoplasmatinae* is present in a given sample. Consequently, the product of a single primary reaction can be aliquoted and placed in separate reactions with different internal nested primers which are specific for a given member of *Toxoplasmatinae*. This will enable detection and differentiation of infections from a given member of *Toxoplasmatinae* in a single sample, simultaneously.

Sixteen ribosomal DNA gene sequences for *Neospora caninum* (GenBank accession numbers AF338411, U16159, AY259039, AY259042, AY259037, AY259041, AY259040), *Neospora hughesi* (DQ997621) *Toxoplasma gondii* (X75453), *Hammondia hammondi* (AF096498, DQ022686), *Hammondia heydorni* (AF317282, AF317281, AF096501, AF096502) and *Besnoitia besnoiti* (DQ227420) were aligned using the default parameters of CLUSTALW (not shown). Regions conserved for members of *Toxoplasmatinae* were identified as were species-specific regions (Figure 1). Based on these alignments three new primer pairs were designed;

the JB, SF and HYD primer pairs (Table 1). The priming sites of each primer pair are shown in Figure 1.

Experiments involving changes in $MgCl_2$ concentration, annealing temperatures and primer concentration were carried out to obtain the optimum reaction conditions for detection of miniscule amounts of parasite DNA. These conditions were then used in subsequent JB-SF and JB-HYD reactions.

The external PCR reactions contained a $MgCl_2$ concentration of 2mM, 0.16mM dNTP, 500nM of primer JB1, 500nM of primer JB2, 1.4U of Taq polymerase (Fischer Biotec), 5µl of the accompanying Taq polymerase buffer (Fischer Biotec) and 500ng of sample DNA in a total volume of 50µl.

The secondary nested PCR reactions for detection of *N. caninum* contained a $MgCl_2$ concentration of 1.67mM, 0.267mM dNTP, 833nM of primer SF1, 833nM of primer SF2, 1.4 U of Taq polymerase (Fischer Biotec) and 5µl of the accompanying Taq polymerase buffer (Fischer Biotec) in a volume of 30µl. For template, 20µl of PCR product was removed from a corresponding JB PCR and placed into the mix in a total volume of 50µl. The same method was used for preparation of secondary polymerase chain reactions for detection of *H. heydorni* though in place of primer SF1, HYD forward was used and in place of SF2, HYD reverse was used.

All PCR reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) under the following reaction conditions. The primary reaction (with JB external primers) consisted of 25 cycles of 95ºC for 5 minutes, 94ºC for 1 minute, 57ºC for 1 minute and 72ºC for 1 minute followed by 72ºC for 5 minutes and holding at 4ºC. Secondary reactions (with either the HYD or SF internal primer pair) consisted of 30 cycles of 95ºC for 5 minutes, 94ºC for 1 minutes, 60ºC for 1 minute and 72ºC for 1 minute followed by 72ºC for 5 minutes and holding at 4ºC. Upon completion of each reaction, the PCR product of either the SF or HYD secondary reactions were subject to electrophoresis in a 2% agarose gel and the gel stained with ethidium bromide. Bands were visualised under UV light.

2.4. Sequencing

Four DNA bands corresponding to four positive mouse samples were excised from agarose gels with a fine scalpel blade. The PCR product was extracted from the agarose gel using a QIAquick gel extraction kit (QIAGEN) according to the protocol described by the manufacturer. Samples were prepared to contain 10 picomoles of either SF1 or SF2 and approximately 25ng of PCR product in a total volume of 16µl of ddH_2O. Sequencing of each sample was performed twice for the forward primer and twice for the reverse primer. All sequencing was performed by the service provider SUPAMAC.

2.5. Testing of the analytical specificity of primers

Seven PCR assays were prepared for each JB, SF and HYD primer sets. Each PCR was prepared to contain a $MgCl_2$ concentration of 2mM, 0.16mM dNTP, 500nM of forward primer, 500nM of reverse primer, 1.4U of Taq polymerase (Fischer Biotec) and 5µl of the accompanying buffer. For sample, 1ng of DNA from either: a Mouse, *T. gondii* ME 49 strain, one of 2 dog isolates of *H. heydorni*, one of 2 isolates of *Besnoitia spp*. or *N. caninum* were used as sample for each reaction. Each reaction was made to a total volume of 50µl. All reactions was carried out in a PTC-100 and subject to 30 cycles of 95ºC for 5 minutes, 94ºC for 1 minutes, 60ºC for 1 minute and 72ºC for 1 minute followed by 72ºC for 5 minutes and holding at 4ºC.

2.6. Determination of the analytical sensitivity of the assay

Seven PCR reactions were prepared according to the protocol in section 2.3. Each sample was prepared to contain 500ng/µl of Mouse DNA and concentrations of *N. caninum* or *H. heydorni* DNA ranging from 1ng/µl to 10^{-6}ng/µl. Mouse DNA was used as a negative control. Based on the level of detection obtained in this DNA titration and average DNA yields from mouse liver tissue, the minimum detectable level of parasitemia was calculated in terms of the number of parasites per liver.

2.7. Analysis of field samples

The DNA of the brain, liver, heart or any combination of these was extracted as described in section 2.2, for each of the 104 trapped mice. The DNA was diluted to 500ng/µl and tested using the nested PCR assay (as described in section 2.3), each set of reactions being accompanied by positive controls of *N. caninum* and *H. heydorni* DNA and a negative control of either mouse DNA or water.

3. Results

3.1. Specificity of primer pairs

The JB set of primers were shown to produce a PCR product of approximately 500bp for *N. caninum*, *T. gondii*, *H. heydorni* experimentally (Figure 2). A product of approximately 300bp was produced for *Besnoitia spp* (Figure 2). The *SF* primer set was shown to be specific for *N. caninum* only, producing a product of approximately 250bp (Figure 2). Similarly, the HYD set of primers only produced a product for *H. heydorni* of approximately 200bp (Figure 2). Based on sequence analysis, a PCR product is also expected for *H. hammondi* using the JB set of primers but no other primer set (data not shown).

3.2. Sensitivity of the assay

Based on serial dilutions of DNA, the JB-SF and JB-HYD PCR assays were able to detect as little as 10^{-5}ng of *N. caninum* and *H. heydorni* DNA (equivalent to 0.1 of a parasite) respectively in a background of 500ng of mouse DNA (Figure 3). The average mass of each rodent organ was determined to be 0.25g, 0.18g and 0.41g for brains, hearts and livers respectively. The average DNA yield of a liver was approximately 1mg. The sample size of each assay is 500ng (1/2000 of a liver). Given that DNA equivalent to 0.1 parasites is detectable in 1/2000 of a mouse liver, a single parasite is detectable in 1/200 of a liver. Thus a sensitivity of ~200 parasites in an entire mouse liver was achieved (approximately 488 parasites per gram of tissue). Assuming a similar tissue mass to DNA yield ratio for mouse hearts, brains and livers the JB-SF/ JB-HYD assay should detect as few as ~44 parasites per mouse heart and ~122 parasites in a mouse brain.

3.3. PCR analysis of the rodent samples

A total of 104 mice were tested for the presence of *N. caninum* DNA in at least one of three tissues (liver, heart or brain). A total prevalence of 26.92% (28/104) was obtained (Table 2). Additionally, 22.5% of livers, 22% of hearts and 17.3% of brains tested positive for the presence of *N. caninum* DNA (Table 2).

Of these 104 mice 31 had all three organs tested (brain, heart and liver). Nine of these thirty-one mouse brains were positive for *N. caninum*. Fourteen of thirty-one mice were positive in at least one of the three tissues. Hence, in this group of 31 mice there were 5 instances where the brain was negative while at least one other tissue was positive. Throughout the course of testing, there were also 3 other instances discovered where a mouse brain was negative while one other tissue was positive. Thus, a total of 8 instances were discovered where the brain was negative while at least one other tissue was positive.

Sequencing of PCR products obtained from four mouse brains indicate that the sequence amplified was *N. canium* specific (Figure 5). All sequences shared 100% identity to sequences from other strains of *N. caninum* present in GenBank. Alignment of sequences obtained in this study with ITS1 sequences from *N. caninum* and *N. hughesi* strongly suggest that the organism being detected is in fact *N. caninum*. See Figure 4 for examples of gels exhibiting positive PCR products.

4. Discussion

A novel PCR assay is described for the detection of DNA from the parasites *N. caninum* and *H. heydorni*. The assay has the potential to be adapted for other related species of parasites such as *Besnoitia* species and *H. hammondi* / *T. gondii* as a complex (due to the high similarity between their ITS1 regions), with minimum of effort. This study is also the first report of *N. caninum* in populations of feral rodents in Australia detected by the newly designed JB-SF nested PCR assay described here. Measurement of the masses of five brains, livers and hearts helped to further characterise the sensitivity of the JB-SF nested PCR assay in terms of levels of detectability per organ. The estimated minimum detection levels were calculated as ~200, ~122 and ~44 parasites in livers, brains and hearts respectively. Sequencing of the PCR products of four positive samples and subsequent alignments of these with the ITS1 sequences of NC Nowra and NC Liverpool confirmed that the JB-SF nested PCR assay amplified DNA from *N. caninum*. Furthermore, the sequencing data obtained from this study provides strong evidence for the presence of *N. caninum* in the tissues of the mice population tested. For definitive evidence of the presence of *N. caninum*, isolation of parasites from a naturally infected rodent of this population is required.

A final prevalence of 26.92% was obtained for *N. caninum* in the mouse population studied. This is the second highest prevalence recorded in this type of study, second only to the prevalence described in rats [39.7%] by Jenkins et al. (2007). This particular study utilised two different nested PCR assays for testing of the same sample. It should be noted however that this study only screened brains for the presence of *N. caninum*.

Hughes et al. (2006) described the use of a nested PCR assay based on the NC-5 region. The brains were the only tissue screened in this study. Low prevalence's of 3% (3/100) and 4.4% (2/45) were obtained for mice and rats respectively. A similarly low prevalence was obtained in the study performed by Huang et al. (2004) [2/55 brown rats]. Huang et al. (2004) made use of a conventional (non-nested) PCR, also based on the NC-5 region. Ferroglio et al. (2007) described prevalence's of 13.6%, 13.8% and 3.6% for rats, house mice and field mice respectively. The Ferroglio et al. (2007) study made use of a non-nested PCR assay also based on the NC-5 region. The brain, kidney and gluteal skeletal muscle of each rodent was screened. Ferroglio et al. (2007) suggest that the increased prevalence of *N. caninum* in this study is due to the testing of three tissues as apposed to one. Ferroglio et al. (2007) states that 21 out of 25 animals were brain tissue negative whilst being positive in at least one or both of the other tissues examined (either kidney or gluteal muscle).

The current study provides evidence to suggest that the values obtained in this study and previous studies are under-representations of the actual prevalence of *N. caninum* in feral rodents. Firstly, this study screened for the presence of *N. caninum* in more than one tissue though this was not performed for the whole test population. However, the current study reports 8 instances of the brain testing negative for *N. caninum* DNA while at least one other tissue (either the heart or liver) was positive. These findings are supported by the findings of Ferroglio et al. (2007) who report similar observations in their study. However, Ferroglio et al. (2007) did not make use of a nested PCR. In the current study, the comparatively poor sensitivity of conventional PCR approaches was compared to the high sensitivity of nested PCR. Of 54 mouse brain samples screened using the JB primer set only in a 45 cycle semi nested PCR, zero returned a positive result (data not shown). Subsequent screening of

these samples with the JB-SF nested PCR revealed that 10 of these samples were actually positive for the presence of *N. caninum* DNA. As mentioned previously, Huang et al. (2004) also only made use of a conventional PCR. The data obtained in the present study suggest that the prevalence's obtained by Huang et al. (2004) and Ferroglio et al. (2007) are underestimates of the true prevalence. This is particularly true for the study performed by Huang et al. (2004) who make use of a conventional PCR and only tested brain tissue. As screening of all three rodent tissues was not achieved in this study, it is also suggested that the prevalence of 28% described herein is also an under-representation.

The findings of Collantes-Fernandez et al. (2006) suggest that *N. caninum* is present in tissues other than (and including) the brain only during the early phases of infection, after which the host immune response clears parasites from tissues other than the brain. This could suggest that animals screened in field studies which returned positive results in tissues other than the brain were infected not long before capture and euthanasia. However, this does not explain the presence of *N. caninum* positive livers and/or hearts in the absence of a positive brain (the current study discovered 8 instances of such an event). It should also be noted that in this study, the prevalence of *N. caninum* DNA in hearts and livers was greater than the prevalence observed in brains (though the values obtained for hearts and livers are less significant). Consequently, it is probable that *N. caninum* will encyst in a range of tissues – not only the brain. For the purpose of field studies involving feral rodents, it is therefore suggested that more than one tissue be screened for the presence of *N. caninum.* It is also suggested that a nested PCR assay is employed. Although the studies described by Jenkins et al. (2007) and Hughes et al. (2006) did make use of nested PCR, only the brain was screened. Subsequently, it is suggested that prevalence values obtained for these two studies are also under-representations of the actual situation.

Hammondia heydorni was not detected in the brains of feral rodents. Of the 54 samples tested, zero of these returned a positive result. Considering the reasonable sample size and the sensitivity of the PCR assay used for detection, it is not unreasonable to hypothesis that *H. heydorni* rarely infects mice. Further information is required to either refute or support this hypothesis, including the study of other tissues. Feeding of wild type mice with tissue cysts or oocysts of *H. heydorni* would also determine whether the house mouse can be infected with *H. heydorni* via the oral route. This would provide an insight into the possibility of *M. musculus domesticus* being an intermediate host of *H. heydorni*.

In summary, based on the results of this study, it is evident that the JB-SF nested PCR assay can be successfully applied to field studies with a high level of fidelity. One hundred and eighty five individual mouse tissues were screened for the presence of *N. caninum* and *H. heydorni* and only 6 results were difficult to interpret (data not shown). *Neospora caninum* infection was therefore reasonably common in this population of feral mice. Data obtained from this study suggest that this and other studies on *N. caninum* in rodents are likely to provide under represented values for prevalence. This could hold important epidemiological implications regarding neosporosis on farms where feral rodents are often present.

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Figure Legends

Fig. 1. Alignment of rDNA sections for four members of subfamily *Toxoplasmatinae.*
A dash (-) represents a gap introduced to improve alignment. An asterisk (*) represents a nucleotide match for all species. NC: *N. caninum* Liverpool strain (Genbank Accession U16159), HHY: *H. heydorni* CZ1 isolate (AF317282), TG: *T. gondii* P Strain (X75453), BBES: *B. besnoiti* (DQ227420). The JB priming sites are shaded grey, SF priming sites are in bold font and HYD priming sites are in bold italic font. All forward sense priming sites are underlined. The JB priming sites are conserved for the four members of *Toxoplasmatinae* while the priming sites for the HYD and SF set of primers are present only for *H. heydorni* and *N. caninum* respectively. The priming site of JB1 (shaded/underlined) lies within the 18S ribosomal RNA gene while all other primers have priming sites within ITS1.

Fig. 2. Gel images showing the specificity of the JB (A), HYD (B) and SF (C) primer sets.
M: Molecular weight marker, Lane 1: *N. caninum* (NC- Liverpool strain), Lane 2: *T. gondii* (Me49 Strain), Lane 3: *H. heydorni* (dog isolate 1), Lane 4: *H. heydorni* (dog isolate 2), Lane 5: *Besnoitia sp.* (cow isolate), Lane 6: *Besnoitia sp.* (wildebeest isolate), Lane 7: Mouse DNA (negative control). The JB set of primers were shown to produce a ~ 500bp product for *N. caninum, T. gondii* and *H. heydorni* (A). A ~300 bp product is present for *Besnoitia sp*. (A). No product was produced for mouse DNA for all 3 primer sets. The HYD set of primers (B) show specificity for *H. heydorni* (producing a ~200bp product) while the SF set of primers (C) show specificity for *N. caninum* (producing ~250bp product).

Fig. 3. Gel image showing a test of sensitivity for the JB-SF nested PCR.
N: Negative control containing 500ng of mouse DNA, Lane 1: 1ng of *N. caninum* DNA, Lane 2: 10^{-1}ng of *N. caninum* DNA, Lane 3: 10^{-2} of *N. caninum* DNA, Lane 4: 10^{-3} of *N. caninum* DNA, Lane 5: 10^{-4} of *N. caninum* DNA, Lane 6: 10^{-5} of *N. caninum* DNA, Lane 7: 10^{-6}ng of *N. caninum* DNA. All *N. caninum* DNA samples also contained a background of 500ng of mouse DNA. Lanes 5 and 6 show the expected PCR band (~250bp). At concentrations of template above 10^{-3}ng, smearing and artefact formation becomes quite apparent (Lanes 1-3). This figure demonstrates that as little as 10^{-5}ng of mouse DNA can be detected in a large background of mouse DNA. The same level of sensitivity was achieved for the JB-HYD nested PCR assay.

Fig. 4. Examples of gel images obtained from testing of mouse brain samples.
M: Molecular weight marker, P: Positive control, N: negative control. Gel A is an example of a 15 well gel and gel B is an example of a 30 well gel. Tracks containing PCR products (a positive result) Positive results are marked with a spot. Note the two weak positive samples in gel A.

Fig. 5. Sequence alignment of PCR products from three mouse brain samples with segments of ITS1 from NC Liverpool, NC Nowra and *N. hughesi*.

A dash (-) represents a gap introduced to improve alignment. An asterisk (*) represents a nucleotide match for all sequences. This figure provides strong evidence of the presence of *N. caninum* in the brains of feral mice. This also demonstrates the first substantial evidence of the presence of *N. caninum* in populations of feral mice in Australia. All sequences obtained from these mouse brains share a 100% identity with a segment of ITS for NC Liverpool and NC Nowra (shown above) as well as all other strains. This figure shows that the organism in these samples was not *N. hughesi* (NH).

Tables

References

Barajas-Rojas, J., Mapes, G., Yanez, I., Morales, E. & Lastra, G. 2004, "Field efficacy of a vaccine against Neospora caninum in Mexico." Proceedings 23rd world Buiatrics Congress, p. 35.

Collantes-Fernandez, E., Lopez-Perez, I., Alvarez-Garcia, G. & Ortega-Mora, L.M. 2006, "Temporal distribution and parasite load kinetics in blood and tissues during Neospora caninum infection in mice", Infect Immun, vol. **74**, no. 4, pp. 2491-2494.

Dubey, J.P. 1999, "Neosporosis in cattle: biology and economic impact", J Am Vet Med Assoc, vol. **214**, no. 8, pp. 1160-1163.

Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkas, I., Bjorkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modry, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J. & Lindsay, D.S. 2002, "Redescription of Neospora caninum and its differentiation from related coccidia", Int J Parasitol, vol. **32**, no. 8, pp. 929-946.

Ferroglio, E., Pasino, M., Romano, A., Grande, D., Pregel, P. & Trisciuoglio, A. 2007, "Evidence of Neospora caninum DNA in wild rodents", Veterinary Parasitology, vol. **148**, no. 3-4, pp. 346-349.

Heuer, C., Nicholson, C., Muñoz Bielsa, J. & Weston, J.F. 2004, "Efficacy of a vaccine against Neospora caninum related abortions..." Proceedings 23rd world Buiatrics Congress, p. 29.

Huang, C.C., Yang, C.H., Watanabe, Y., Liao, Y.K. & Ooi, H.K. 2004, "Finding of Neospora caninum in the wild brown rat (Rattus norvegicus)", Vet Res, vol. **35**, no. 3, pp. 283-290.

Hughes, J.M., Williams, R.H., Morley, E.K., Cook, D.A., Terry, R.S., Murphy, R.G., Smith, J.E. & Hide, G. 2006, "The prevalence of Neospora caninum and co-infection with Toxoplasma gondii by PCR analysis in naturally occurring mammal populations", Parasitology, vol. **132**, no. Pt 1, pp. 29-36.

Jenkins, M.C., Parker, C., Hill, D., Pinckney, R.D., Dyer, R. & Dubey, J.P. 2007, "Neospora caninum detected in feral rodents", Veterinary Parasitology, vol. **143**, no. 2, pp. 161-165.

Liddell, S., Jenkins, M.C. & Dubey, J.P. 1999, "Vertical transmission of Neospora caninum in BALB/c mice determined by polymerase chain reaction detection", J Parasitol, vol. **85**, no. 3, pp. 550-555.

Marshall, P.A., Hughes, J.M., Williams, R.H., Smith, J.E., Murphy, R.G. & Hide, G. 2004, "Detection of high levels of congenital transmission of Toxoplasma gondii in natural urban populations of Mus domesticus", Parasitology, vol. **128**, no. Pt 1, pp. 39-42.

Omata, Y., Nidaira, M., Kano, R., Kobayashi, Y., Koyama, T., Furuoka, H., Maeda, R., Matsui, T. & Saito, A. 2004, "Vertical transmission of Neospora caninum in BALB/c mice in both acute and chronic infection", Vet Parasitol, vol. **121**, no. 3-4, pp. 323-328.

Owen, M.R. & Trees, A.J. 1998, "Vertical transmission of Toxoplasma gondii from chronically infected house (Mus musculus) and field (Apodemus sylvaticus) mice determined by polymerase chain reaction", Parasitology, vol. **116**, no. Pt 4, pp. 299-304.

Quinn, H.E., Miller, C.M., Ryce, C., Windsor, P.A. & Ellis, J.T. 2002, "Characterization of an outbred pregnant mouse model of Neospora caninum infection", J Parasitol, vol. **88**, no. 4, pp. 691-696.

Reichel, M.P. & Ellis, J.T. 2006, "If control of Neospora caninum infection is technically feasible does it make economic sense?" Vet Parasitol, vol. **142**, no. 1-2, pp. 23-34.

Romero, J.J., Perez, E. & Frankena, K. 2004, "Effect of a killed whole Neospora caninum tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions", Vet Parasitol, vol. **123**, no. 3-4, pp. 149-159.

Schetters, T., Dubey, J.P., A. Adrianarivo, Frankena, K., Romero, J.J., Pérez, E., C. Heuer, Nicholson, C., Russell, D. & Weston, J. 2004, "Intervet symposium: bovine neosporosis", Veterinary Parasitology, vol. **125**, pp. 137-146.

Trees, A.J., Davison, H.C., Innes, E.A. & Wastling, J.M. 1999, "Towards evaluating the economic impact of bovine neosporosis", Int J Parasitol, vol. **29**, no. 8, pp. 1195-1200.

Table 1
Newly designed primers for detection of *H. heydorni* and *N. caninum* DNA

Primer Name (orientation)	Sequence (5'-3')
JB1 (Forward)	AGGAAGGAGAAGTCGTAACAAGG
JB2(Reverse)	GAGCCAAGACATCCATTGC
SF1(Forward)	CCCTGTGAGTTGTATCGCCTTC
SF2(Reverse)	TCTCTTCCCTCAAACGCTATCC
HYD Forward	CTGCTGATATCCGGGAGTGG
HYD Reverse	CATCTTTGTTTCGTAAATAATCTGGCAT

Table 2

Results of screening mouse tissues with the JB-SF/JB-HYD nested PCR assays

	No. of mice tested[1]	Prevalence[2] (%)	No. of brains tested positive/total (%)	No. of livers tested positive/total (%)	No. of hearts tested positive/total (%)
For *N. caninum*:	104	28/104 (26.92)	18/104 (17.3)	9/40 (22.5)	9/41 (22)
For *H. heydorni*:	54	0/54 (0)	0/54	0/0	0/0

[1] This refers to the total number of mice for which at least one tissue (brain, heart, liver or any combination of these) was tested.

[2] This refers to the number of mice for which at least one tissue (brain, heart, liver or any combination of these) tested positive.

Figure 1

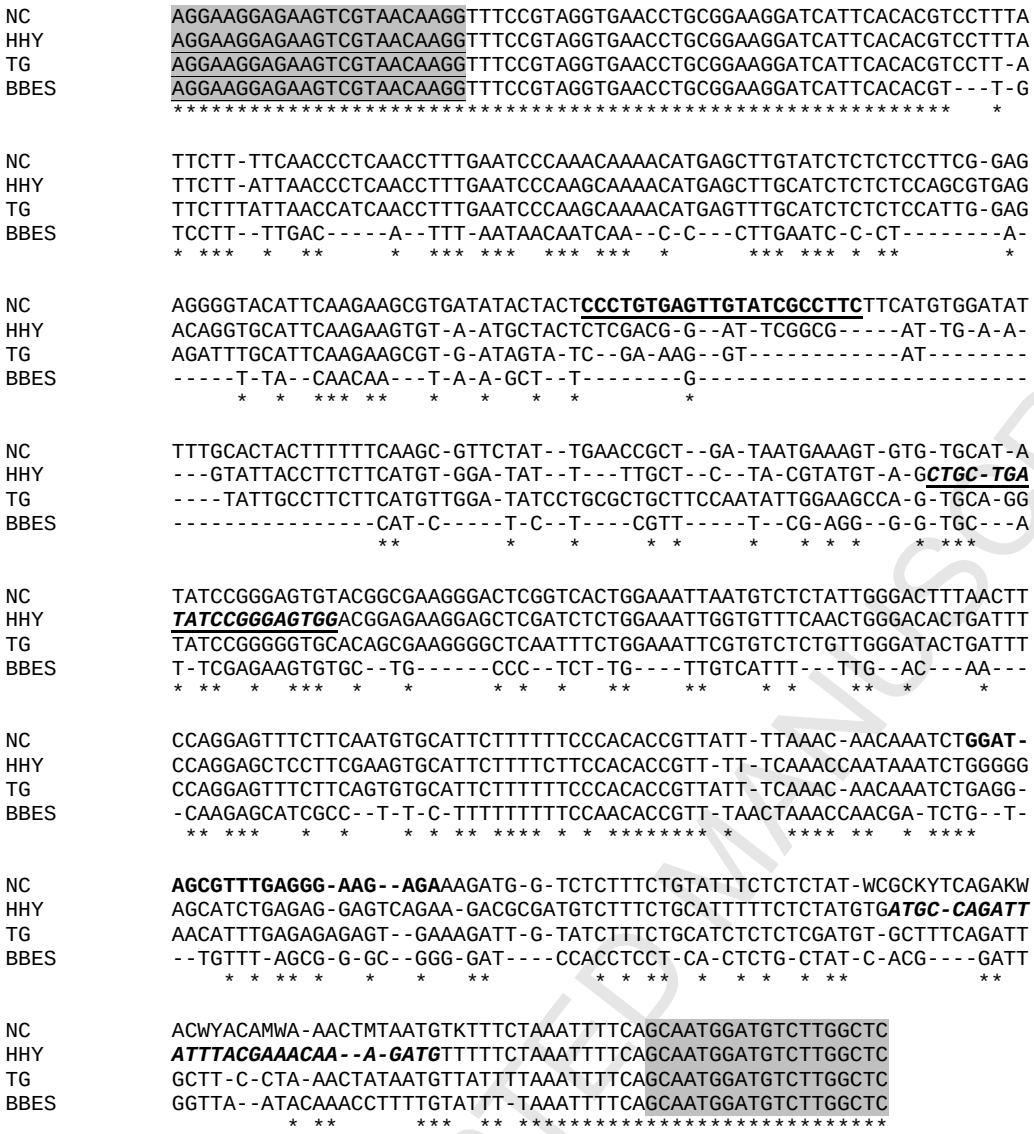

Figure 2

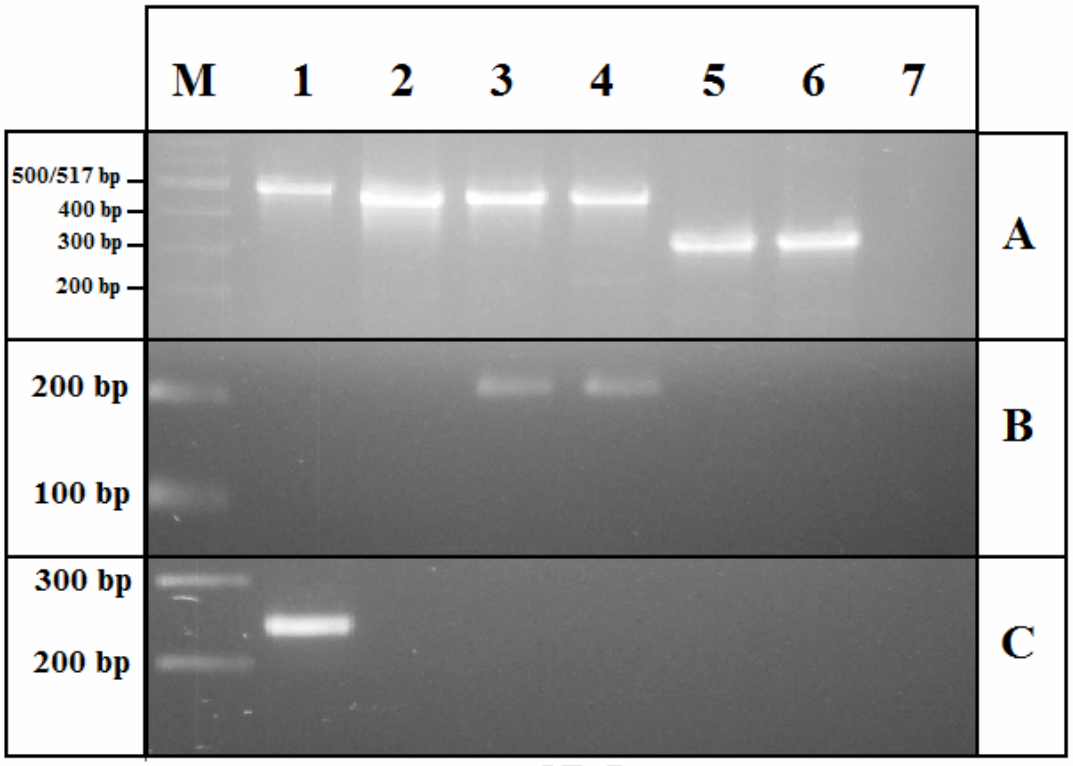

Figure 3

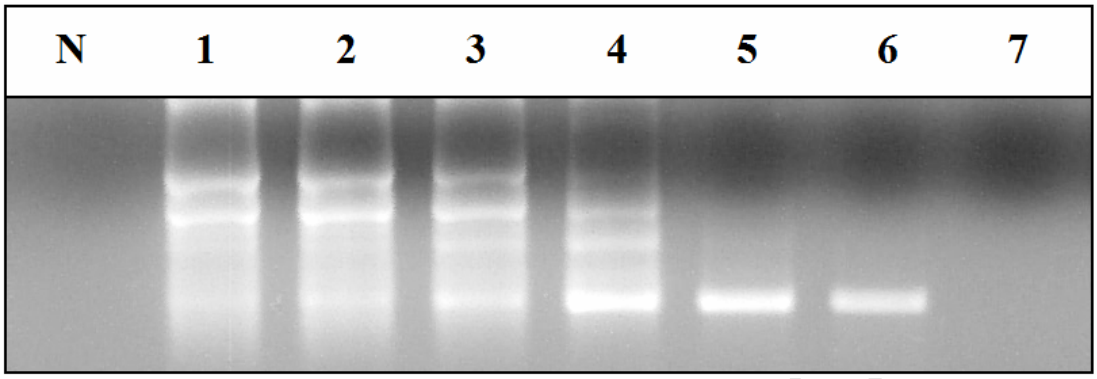

Figure 4