SHORT COMMUNICATION:

Investigating the prevalence of *Neospora caninum* infection in sheep in New South Wales following suspected ovine cerebral neosporosis

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Abstract

Presence of *Neospora caninum* DNA was detected in the brain and spinal cord of an adult Merino sheep suspected of dying with non-suppurative meningoencephalitis due to neosporosis. As this was the first known occurrence of disease in sheep in Australia caused

by *N. caninum* in sheep, we surveyed sera from five sheep properties in New South Wales (NSW) to obtain information on the likely prevalence of *N. caninum* infection in NSW sheep flocks. Serology using a commercial indirect enzyme-linked immunosorbent assay (ELISA) revealed no exposure of sheep to *N. caninum* (*n*=184). However an observed prevalence for *N. caninum* using a commercially available competitive ELISA was 2.2% (5/232). We conclude that although the diagnosis of fatal ovine cerebral neosporosis is of importance to our surveillance program for transmissible spongiform encephalopathy (TSE) exclusion, sheep in NSW are not commonly infected with *N. caninum* and this species likely plays only a minor role in the life cycle of this parasite in Australia.

Keyword: neosporosis, sheep, PCR, ELISA, *Neospora caninum*, prevalence, fatal neurological disease, epidemiology

Neospora caninum is an apicomplexan protozoan parasite capable of causing fatal neurological disease in dogs, and has emerged as a leading cause of cattle abortion worldwide (Dubey et al., 2007; Reichel et al., 2007; Wouda, 2000). While sheep are used as an animal model for neosporosis, the epidemiological role of sheep in *N. caninum* is less clear (Buxton et al., 1997; McAllister et al., 1996). Research reports of clinical manifestations of *N. caninum* in sheep is limited to a fatal congenital neosporosis in a newborn lamb (Dubey et al., 1990), and a ewe and her two foetuses (Kobayashi et al., 2001). Ovine abortion due to infection of *N. caninum* has been implicated in New Zealand and Switzerland (Hässig et al., 2003; West et al., 2006). Moreover, repeated abortions in *Neospora*-infected ewes have been reported under laboratory conditions and documented in an ewe on a farm in Switzerland (Hässig et al., 2003; Jolley et al., 1999).

Serological surveys for *N. caninum* in sheep have reported variable findings, with seroprevalence of below 2% to over 10% regardless of any history of abortion. A study from the United Kingdom found 0.45% (3/660, titre of ≥1:100 by immunofluorescent antibody test; IFAT) prevalence in eves that aborted (Helmick et al., 2002). A study from Switzerland found 10.3% (12/117, titre of ≥1:160 by IFAT) in sheep exhibiting a persistent abortion problem (Hässig et al., 2003). A study from New Zealand reported a seroprevalence of 0.63% (4/640, titre of ≥1:50 by IFAT and a commercial ELISA (IDEXX CHEKIT* *Neospora caninum* ELISA) in ram sera (Reichel et al., 2008). A survey in the Orobie Alps, Italy resulted in 2% (22/1010) apparent seroprevalence for *N. caninum* using CHEKIT* (Gaffuri et al., 2006). In an extensive study in Brazil, at least one sheep on each of the thirty farms tested positive for *N. caninum*; overall 9.2% (55/597, titre of ≥1:50 by IFAT) ewes tested positive for *N. caninum* (Figliuolo et al., 2004). Furthermore, a recent study found overall 11.5% (63/547, VMRD's *Neospora caninum* cELISA) seroprevalence across nine sheep farms in the Czech Republic (Bartova et al., 2009).

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62 Our study investigated an unusual occurrence of sporadic mortalities in late 2006 and
63 early 2007 involving 6 adult sheep on an 'index' farm at Mangoplah in south western NSW
64 following a period of 'hand' feeding during drought. The only case observed before death
65 was a 3-year-old Merino ewe that displayed neurological signs described as 'star gazing and
66 fits'. At necropsy there was evidence of prolonged recumbency and 'limb paddling'. A range
67 of tissues were submitted in 10% formalin for histology, including the brain for exclusion of
68 TSE. As non-suppurative meningoencephalitis was observed in the midbrain, a diagnosis of
69 neosporosis infection was suspected. This was supported by detection of the presence of
70 apicomplexan-like stages on transmission electron microscopy, positive
71 immunohistochemistry to *N. caninum*, and negative immunohistochemistry for *Toxoplasma*
72 *gondii* in the brain and medulla oblongata.

73 The aim of this study was to undertake PCR on paraffin-embedded brain tissue from
74 this case for definitive identification of *N. caninum*. We also performed a serological survey
75 for *N. caninum* at the affected property and several other sheep properties in NSW to
76 determine the likely prevalence of *N. caninum* infection in Australian sheep.

77 Histopathology of liver, lung, heart and kidney revealed a range of lesions including
78 occasional hepatic microabscesses, severe diffuse pulmonary congestion and moderate
79 multifocal non-suppurative myocarditis. Histology of the brain and spinal cord revealed
80 severe acute non-suppuratives meningoencephalitis and mild to moderate non-suppurative
81 myelitis. The most severe neurological lesions were found in the midbrain at the rostral
82 coliculi, consisting of multifocal oedema and necrosis accompanying moderate to severe
83 multifocal vasculitis and gliosis (Fig. 1). Large perivascular foci progressing to white matter
84 necrosis with axonal swelling and loss and karyorrhexis of glial cells were observed. Glial
85 nodules were numerous in the brain and throughout spinal cord gray and white matter.

DNA was successfully extracted from paraffin embedded, formalin fixed, brain tissues (PEFF) using a method that uses ammonium acetate to improve DNA yield (Santos et al., 2008). Sheep DNA was successfully amplified from all PEFF tissues (Fig 2A) by PCR. Sheep DNA was detected using primers OV-H (5'- TTA AAG ACT GAG AGC ATG ATA -3') and OV-L (5'- ATG AAA GAG GCA AAT AGA TTT TCG -3') which give a 120 bp specific PCR product (Santaclara et al., 2007). The DNA was then tested using *N. caninum* and *T. gondii* specific PCR (Homan et al., 2000; Müller et al., 1996). Species specific primers based on the Nc5 region of *N. caninum* were Np6plus (5'- CTC GCC AGT CAA CCT ACG TCT TCT -3') and Np21plus (5'-CCC AGT GCG TCC AAT CCT GTA AC -3') giving a 337 bp product. The species specific PCR confirmed the presence of *N. caninum* DNA in two of three PEFF tissues (Fig 2B). PCR for *T. gondii* targeting a repetitive fragment used primers TOX4 (5'- CGC TGC AGG GAG GAA GAC GAA AGT TG -3') and TOX5 (5'- CGC TGC AGA CAC AGT GCA TCT GGA TT -3') did not yield a specific 529 bp product in any of the three sheep samples (Fig 2C). Sera were not collected from the index case or from any animal in the same sheep flock at the time of the disease onset.

To examine the extent of the *N. caninum* infection in sheep in NSW, we investigated the seroprevalence of *N. caninum* on the affected property and 4 other NSW sheep farms (Table 1). We used an indirect ELISA (CHEKIT* *Neospora caninum* Antibody ELISA Test Kit, IDEXX Laboratories, Australia), that detects antibodies against *N. caninum* in samples of ruminants including sheep. Using the indirect ELISA all sere were *N. caninum* negative; none (0/184) of the investigated sheep sera had a value greater than 30% (a corrected sample to positive ratio and expressed in %), considered suspect positivity for *N. caninum*. Sera were also tested using a competitive ELISA (*Neospora caninum* Antibody Test Kit – cELISA, VMRD, Pullman, WA, USA). Unlike the indirect ELISA the competitive ELISA is not limited by the use of species-specific antisera, theoretically making it suitable for use in any

species of interest (Baszler et al., 2001). Currently this commercially available competitive ELISA is validated for cattle and goats. The formula for calculating percent inhibition (%I) equals 100 – [(sample OD × 100) / (mean negative control OD)]. Using the recommended %I cut-off greater that 30% for our sera we detected 2.2% (5/232; A#12, B#3147, C#4, C#5, C#28) *N. caninum* seroprevalence with positive sera originating from 3 different sheep flocks. Seroprevalence was ranging from 1.7-7.1% (Table 1); Property A (one serum %I: 35%), Property B (one serum %I: 32%), and Property C (3 sera %I: 47%, 49%, 38%). To further evaluate these results we have assumed Property B to be *N. caninum* negative and calculated their mean of the ELISA result in both assays. Then we used the negative mean plus three standard deviations (±3 S.D.) to calculate the negative to positive cut-off for each assay. This revised cut-off yielded a seroprevalence of 1.61% (2/184; C#3, C#22) and 0.58% (1/232; C#5) to *N. caninum* using the indirect and the competitive ELISA, respectively. All the seropositive sera were from Property C and did not overlap between the indirect and competitive ELISA. Only one serum (#5) was positive using both the manufacturer's and revised cut-off using the competitive ELISA. None of these sera tested positive for *N. canimum* by IFAT using ≥1:100 as threshold; moreover additional randomly selected sera from Property C and B tested *N. caninum* negative by IFAT. It is concluded, that these positive sera testing positive using IDEXX's and VMRD's ELISAs, respectively, may be false positive reactions as one expects the proportion of false positives will increase with a low prevalence of infection.

Comment [MPR1]: But what is it, name the percentage value

Recently, Reichel et al. (2008) evaluated the indirect ELISA (IDEXX) using sera from experimentally infected sheep. Using sera from pre-exposure sheep (negative) and sera from experimentally infected sheep with *N. caninum* isolates (NC-2 and NC-Liverpool), a negative to positive cut-off of 11.8% (mean of negative samples ±3 S.D.) was determined resulting in a specificity of 98.8% for known positive sera. Within our sheep serum data base, only two

sera from Property C (Table 1) were close (#3, 11.5%) or above the 11.8% cut-off (#22, 12.5%). Because neither of these sera were positive by IFAT (titre of ≥1:100), we conclude that lowering the cut-off value from the manufacturer's 30% was not needed~~. However, both cut-off values for detection of *N. caninum* infection are only presumptive, because neither ELISA nor IFAT has been validated by recovering viable parasites as the gold standard test.~~ Based on the reference test also used in this study, the IFAT, all sera were negative, not allowing us to evaluate the sensitivity of the assays under field conditions in Australia. Therefore, further larger sampling and re-evaluation of the specificity and sensitivity using sera from field-infected sheep is required.

Comment [MPR2]: You don't need to recover viable parasites, that's not the gold standard. Arguably we had good negative sera and established a threshold from there (the mean plus 3SD). That also seemed to work well with the exp infected sheep ser-converting, based on that cut-off at around 7-14 days, that works out okay.

The time between the initial disease event and subsequent bleed of the index flock (16 months) is not considered to have caused the finding of low seroprevalence in this flock. A study involving experimental infection of sheep with *N. caninum* showed mean antibody titres to increase the year following infection (Reichel et al., 2008). The negative seroprevalence in sheep (n = 60) from Mangoplah, NSW (Property A), where the fatal neosporosis occurred, resembles the flock (n = 50) from Japan which tested negative for *N. caninum*, despite the clinical neosporosis that occurred in the ewe from this property (Kobayashi et al., 2001).

At the index case farm, a kelpie puppy was brought onto the farm six months prior to the disease event. This animal was seropositive when it was tested by the competitive ELISA (VMRD) at the time the flock was bled. Horizontal *N. caninum* infection in cattle herds commonly manifests as an 'abortion storm' situation (Wouda et al., 1999a). The index case flock were unjoined ewes with no history of abortion prior to the fatal neosporosis. In dairy herds, the two most significant risk factors facing abortion storms due to *N. caninum* infection are dry feeding and the introduction of a dog onto the farm within 1.5 years of a disease event (Wouda et al., 2000). This is due to the increased chance of cattle exposure to

oocysts through ingestion of spoilt feed and oocyst shedding by a previously naive dog (Wouda et al., 1999b). Experimental models of oocyst shedding in dogs following primary exposure have shown that mean oocyst production is significantly higher in puppies (mean: 166,400 oocysts) than that of adult dogs (mean: 2,900 oocysts) (Gondim et al., 2002). As low as 1,000 oocysts were shown to induce seroconversion in sheep (O'Handley et al., 2002). The route of transmission by which the index case contracted *N. caninum* infection was likely postnatal transmission, through the ingestion of oocysts. In Australia, dogs and dingos are known to be infected with *N. caninum* and the ability of dingoes to shed oocysts has recently been demonstrated (King et al, unpublished).

The index case is the first study reporting *N. caninum* in an adult sheep with signs of neurological disease. Clinical signs of ataxia and opisthotonos in or index case are similar to resemble reported signs in congenitally *N. caninum* infected newborns calves and lamb (Dubey, 2003; O'Toole and Jeffrey, 1987). The severity of the lesions found by histopathology in our case is greater than that of an asymptomatic ewe reported in a study from Japan where *N. caninum* DNA was found in the brain of an affected dam in the absence of neurological disease (Kobayashi et al., 2001). Histopathological lesions of widespread necrotising encephalomyelitis and meningitis have been recorded in congenital *N. caninum* infection in bovines (Wouda et al., 1997) and in experimental adult mice (Atkinson et al., 1999) and share similarities with the index case ewe in our study.

In cattle vertical infection with *N. caninum* (congenital transmission) is considered the primary mode of transmission with horizontal infection considered capable of introducing new infections into naïve herds. Available experimental and field data clearly indicate a vertical transmission route for the parasite in sheep (Hässig et al., 2003; Jolley et al., 1999) and the role of horizontal transmission of infection in this species is unclear. Plausible scenarios for transmission in the index flock include both recrudescence of a chronic

infection or horizontal infection. Five days prior to the onset of the disease, the index case flock was shorn and placed on medium quality dry feed. The sporadic death of 6 ewes with confirmation of one ewe as suffering fatal neosporosis could suggest exposure of the flock to immunosuppressing mycotoxins leading to recrudescence of past infection (Bartels et al., 1999) although we have no evidence that mycotoxins were present in the feed. Rupture of *T. gondii* tissue cysts is know to occur in AIDS patients (Luft and Remington, 1992) and has been induced in mycotoxin and aflatoxin immunosuppressed mice suffering chronic toxoplasmosis (Venturini et al., 1996). This phenomenon is yet to be demonstrated in animals suffering neosporosis. Alternatively, the introduction of a young infected dog 6 months prior to the onset of the disease in the sheep with the possibility of contamination of the feed by *N. caninum* oocysts, does suggest that the disease in the sheep may have resulted from a 'point source' of infection and horizontal transmission.

In conclusion, our study confirms *N. caninum* is capable of causing fatal ovine neosporosis but our serostudy revealed negligible seroprevalence of the infection in sheep, even within the flock where fatal neosporosis was confirmed. The sheep properties studied represent typical Australian rural farms and are in the vicinity of cattle farms that in a recent study have been shown to sustain an overall *N. caninum* prevalence of 21.1% (Hall et al., 2006). Because disease in sheep can be readily induced experimentally and as demonstrated above does occur under Australian conditions, we conclude that environmental exposure to *N. caninum* rarely occurs in sheep. However, the environmental factors that prevent sheep from the exposure to *N. caninum* oocysts but facilitate cattle exposure remain to be identified.

Acknowledgements

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Table 1. ***Neospora caninum*** **serological survey across sheep in New South Wales, Australia**

	Indirect ELISA[1]			Competitive ELISA[2]	
Cut-off	30%	11%	Property B mean + 3 S.D.	30%	Property B mean - 3 S.D.
Property A	0% (0/50)	0% (0/50)	0% (0/50)	2.0% (1/50)	0% (0/50)
Property B	0% (0/60)	0% (0/60)	*n.a.*	1.7% (1/60)	*n.a.*
Property C	0% (0/39)	5.1% (2/39)	5.1% (2/39)	7.1% (3/42)	2.4% (1/42)
Property D	0% (0/14)	0% (0/14)	0% (0/14)	0% (0/23)	0% (0/23)
Property E	0% (0/21)	0% (0/21)	0% (0/21)	0% (0/57)	0% (0/57)
Total	0% (0/184)	1.1% (2/184)	1.6% (2/124)	2.2% (5/232)	0.6% (1/172)

Note: Values indicate percent above the cut-off (number of positive/total number of assayed sera). Property A - Mangoplah Station, Wagga Wagga, 4-yr-old, first-cross Merino ewes. Property B - Kemps Creek Farms, . Property C - Mayfarm, Camden, 5-month-old lambs. Property D - Cobbitty Sheep Research Unit, Sydney University, 3-year-old wethers. Property E - Arthursleigh Farms.

[1] CHEKIT* *Neospora caninum* Antibody ELISA Test Kit (IDEXX Laboratories, Australia). [2] *Neospora caninum* Antibody Test Kit, cELISA (VMRD, Inc., Pullman, WA, USA).

Legend to figures:

Figure 1. Histopathology of the midbrain of the index case displaying non-suppurative meningoencephalitis characterised by oedema, necrosis and gliosis.

Figure 2

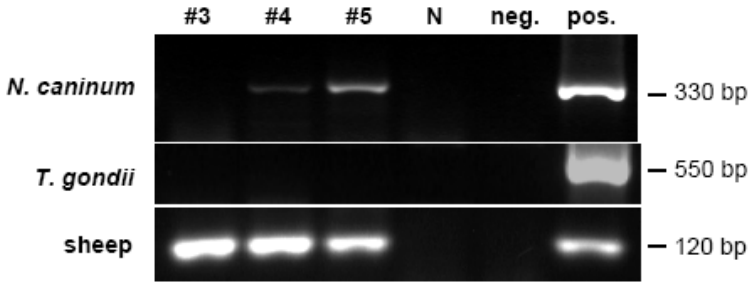

Figure 2. Detection of *Neospora caninum* DNA in paraffin-embedded formalin fixed brain tissue. *N. canimum* DNA was detected by PCR using Np6plus and Np21plus primers yielding ~340 bp specific product. *Toxoplasma gondii* DNA was detected using TOX4 and TOX5 primers yielding ~530 bp specific product. Presence of host DNA was verified using ovine specific primers OV-H and OV-L. As a positive control (pos.) a DNA from *N. caninum* Nc-Liverpool strain, *T. gondii* ME49 strain and sheep blood was used. As a negative control (neg.) a template DNA was replaced by nuclease-free water. As a control during DNA extraction from paraffin embedded formalin fixed sections a water control was processed alongside the sheep sections and run in all PCR (N).

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