Comparison of the biological characteristics of two isolates of *Neospora caninum*

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SUMMARY

This study compared the biological and genetic properties of a bovine (NC-SweB1) and a canine (NC-Liverpool) isolate of *Neospora caninum*. A mouse model for CNS infection demonstrated marked differences in pathogenicity between the isolates. NC-Liverpool induced severe clinical signs of neosporosis in 57/58 mice including discoordinated movement, hindlimb paralysis and coat ruffling with severe weight loss. In contrast NC-SweB1 induced similar but less severe symptoms in a much smaller proportion of mice over the same time-period. Statistically significant differences were observed between the isolates in the response (mean weight loss) of mice through time to the different doses inoculated. Histopathological effects on brain tissue reflected the isolate-based differences described above. NC-Liverpool infection resulted in intense inflammatory infiltrates and highly necrotic lesions whereas NC-SweB1 induced a milder meningoencephalitis. Passage in cell-culture over a period of 14 months did not affect the pathogenicity of NC-Liverpool. Immunoblots showed that antibodies to *N. caninum* appeared earlier in mice inoculated with NC-Liverpool than with NC-SweB1. Finally, RAPD–PCR analysis of NC-Liverpool DNA generated profiles distinct from that observed with DNA from NC-SweB1 or *Toxoplasma gondii*. In summary this study provides evidence for significant biological and genetic differences between 2 isolates of *N. caninum*.

Key words: *Neospora caninum*, RAPD–PCR, pathogenicity.

INTRODUCTION

Neospora caninum is an apicomplexan protozoan which has emerged as a major cause of bovine abortion in many countries (Barr *et al*. 1997). Knowledge of the biology of this organism, in particular life-cycle data are limited, and a definitive host has only just been identified (McAllister *et al*. 1998). Isolates from cows and dogs have been described (Dubey *et al*. 1988; Conrad *et al*. 1993) and these appear to share essentially identical biological properties (Conrad *et al*. 1993; Stenlund *et al*. 1997). At the genetic level no differences in sequence of ribosomal DNA are detectable between *N. caninum* isolates (Marsh *et al*. 1995; Stenlund *et al*. 1997; Ellis *et al*. 1998). However, a number of researchers have shown that different populations of *Toxoplasma gondii*, a close relative of *N. caninum*, are biologically and genetically distinct (Sibley & Boothroyd, 1992; Howe & Sibley, 1995; Rinder *et al*. 1995; Meisel *et al*. 1996; Guo & Johnson, 1996) despite sharing identical rDNA sequences (Ellis *et al*. 1994; Luton, Gleeson & Johnson, 1995; Payne & Ellis, 1996; Homan *et al*. 1997; Ellis *et al*. 1998). We therefore hypothesized that isolates of *Neospora* may differ in their biological and genetic properties. This hypothesis was examined by investigating the course of infection of *Neospora* in mice and by RAPD–PCR.

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MATERIALS AND METHODS

Procedures were conducted in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Parasites

NC-Liverpool (Barber *et al*. 1993) and NC-SweB1 (Stenlund *et al*. 1997) were propagated *in vitro* by growth in Vero cells as described by Barber *et al*. (1993). Tachyzoites were harvested during late schizogony and purified from residual host cells by filtration through a 3 μm nucleopore filter in phosphate-buffered saline. Preparations were then concentrated by centrifugation and live tachyzoites counted using a haemocytometer.

Infection of mice

Female, in-bred Balb/C mice (sourced from ARC, Perth, Western Australia or Gore Hill Research

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Laboratories, NSW) approximately 20 g in body weight, were housed in groups of 6–12 in plastic box cages and provided feed and water *ad libitum*. Defined doses of tachyzoites as outlined in Table 1 were inoculated into mice using intraperitoneal or subcutaneous routes (Lindsay *et al*. 1995). Mice were weighed at regular time-intervals following inoculation. Representative mice were killed at all time-points and their tissues processed for pathological examination.

Pathology

Multiple sections of brain and portions of heart, lung, liver, spleen, small intestine, skeletal muscle and spinal cord were fixed in 10% formalin and processed using routine histological techniques, cut at 5 μm and stained with haematoxylin and eosin. Sections were coded and read without knowledge of mouse strain, *Neospora* isolate or dose of organisms. Brain lesions were graded according to severity of inflammation: nil = 0, slight = grade 1, mild = grade 2, moderate = grade 3, severe = grade 4, extreme = grade 5.

Analysis of body weights

A statistical analysis of the changes in mean body weight of infected mice was performed by an orthogonal repeated-measures analysis of variance, with Isolate and Dose as the factors and Time as the repeated measure. Only the first 4 sampling times were included in the analysis, as many mice began experiencing severe symptoms thereafter such as head tilting and limb paralysis which forced euthanasia where possible to limit unnecessary distress.

Immunoblot analysis

Purified tachyzoites prepared as described above were centrifuged at 3000 ***g***, snap-frozen in liquid nitrogen and stored at −80 °C until used. Pellets were then thawed and boiled in sodium dodecyl sulphate polyacrylamide gel electrophoresis buffer. Insoluble material was separated by centrifugation at 10000 ***g*** and the supernatant fraction containing the soluble fraction was aliquoted and stored at −80 °C until required. Protein from each isolate was revolved on non-reduced preparative gels (20 μg/isolate) containing 5% stacking and 12% separating gels using a BioRad minigel apparatus (200 V, 45 min, room temperature (RT)). Proteins were then transferred to a 0·45 micron PVDF membrane (Amersham) using a Novex transfer system (50 V, 45 min, RT). Membranes were then incubated in Tris-buffered saline with 5% skim milk to block non-specific antibody binding (45 min, RT). After blocking the membranes were placed in a BioRad multiscreen unit and isolated strips probed with a 1/250 dilution of screening serum (45 min, RT). Screening serum diluted in blocking solution consisted of serum from control mice ($n = 2$ pooled, day 12 post-inoculation) and serum collected from mice infected with either isolate (1×10^5 tachyzoites) at days 6, 9 and 12 post-inoculation ($n = 4$ pooled/group). After incubation of primary sera, membranes were washed (Tris-buffered saline plus Tween) and placed in a 1/2000 dilution of secondary antibody conjugate (anti-mouse IgG, alkaline phosphatase conjugate; Sigma) for 45 min at RT. Washing was repeated and membranes were placed in developing solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) for 20 min at RT.

RAPD–PCR

RAPD–PCR was performed according to the method described by Guo & Johnson (1995) with primers B4, B5 and B12. Genomic DNAs tested were from *N. caninum* (strains NC-Liverpool and NC-SweB1) and *T. gondii* (strains ME49 and RH). ME49 is avirulent in mice and readily forms brain cysts whereas RH replicates intraperitoneally and is considered as virulent. RAPD–PCR was formed in duplicate in order to monitor reproducibility which is frequently a problem associated with this technique.

Cross-protection

On day 0 and day 19 of this experiment, 10 in-bred Balb/C mice were inoculated with 1×10^6 tachyzoites of NC-SweB1 or normal saline (control group). On day 37, 2 of the mice in each group were dispatched for brain pathology. The remaining 8 mice in each group were then challenged with 1×10^7 tachyzoites of NC-Liverpool. Mouse weights, clinical symptoms and brain histopathology were assessed in both groups of animals until the end of the experiment on day 75.

RESULTS

Pathology

Mice inoculated intraperitoneally with *N. caninum* failed to yield clinical signs of disease (rough hair coats followed by head tilts and limb weakness or paralysis; Lindsay & Dubey, 1989; Lindsay *et al*. 1995). Furthermore, brains of sacrificed mice did not reveal the presence of typical brain lesions associated with neosporosis. The following results were obtained by subcutaneous injection into mice of *N. caninum* tachyzoites.

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Table 1. Lesion scores from brains of *Neospora*-infected mice

Isolate	Dose	Lesion score/number of mice 0	1	2	3	4	5
SweB1	1×10^5	4	7	1	—	—	—
Liverpool	1×10^5	—	1	2	5	2	1
SweB1	1×10^6	1	5	11	3	—	—
Liverpool	1×10^6	—	—	1	11	13	2
SweB1	1×10^7	—	1	7	11	5	—
Liverpool	1×10^7	—	—	—	7	9	3

NC-Liverpool

Brain lesions were present in all mice from this group, and varied from moderate to extreme (grades 3–5) in severity of inflammation. Lesions were characterized by multifocal meningoencephalitis with areas of necrosis, macrophage and gitter cell infiltration, astrocytosis and mononuclear perivascular cuffing, with frequent tachyzoites observed in and adjacent to lesions and occasional sponginess of the neuropile suggesting oedema. Rarely, large clumps of tachyzoites were observed that resembled cystic structures; however, a cyst wall was not identified surrounding such structures. Further, extreme infiltration of inflammatory cells in some cases gave the lesions the appearance of granulomatous inflammation. A summary of the morphological diagnoses for brain lesions from this group is moderate to extreme acute to subacute multifocal necrotizing to granulomatous meningoencephalitis, usually in association with the presence of tachyzoites (presumed *N. caninum*). Few lesions (occasional mild focal granuloma in livers, peribronchial cuff in lung) were observed in other organs and none consistently more than in control mice.

NC-SweB1

Brain lesions were present in the majority of mice from this group but not all, and varied in severity of inflammation from nil or slight to mild (grades 0–3). Lesions were characterized by multifocal meningoencephalitis as evidenced by infiltration of mononuclear inflammatory cells in meninges and perivascular cuffing in affected areas of the neuropile, often in associated with focal areas of necrosis which were accompanied by accumulation of gitter cells and astrocytes. Tachyzoites were occasionally seen both in association with lesions and in unaffected areas and, rarely, sponginess suggesting oedema and mineralization of necrotic areas was observed. A summary of the morphological diagnoses for brain lesions from this group is slight to moderate acute to subacute multifocal nonsuppurative to necrotizing meningoencephalitis, occasionally in association with the presence of tachyzoites (presumed *N. caninum*). Few lesions were observed in other organs apart from focal necrosis in the liver of 1 mouse, and lesions as observed in controls as described above.

There was clearly a difference in the severity of the brain lesions between the groups of mice inoculated with NC-Liverpool which induced more necrosis and a more florid inflammatory response than NC-SweB1. The nature of this difference is best summarized by comparison of the 2 morphological diagnoses, with NC-Liverpool producing a moderate to extreme necrogranulomatous response and NC-SweB1 inducing a nil to moderate nonsuppurative to necrotizing response. Table 1 summarizes the severity of the lesion scores found in mice during these experiments. The mean lesion scores for NC-Liverpool and NC-SweB1 were 3·6 ($n = 57$) and 2·0 ($n = 56$) respectively.

Observation of clinical symptoms and analysis of body weights

The significant 3-factor interaction ($P < 0{\cdot}001$ for Strain × Dose × Time) indicates that the 2 isolates differ significantly in their response through time to the different parasite doses given (Table 2). Specifically, NC-Liverpool shows an effect of dose (mouse weight decreases through time) whereas NC-SweB1 shows no such effect. Fig. 1 shows an example of the changes in mean mouse body weight with time during the course of *N. caninum* infection for the 2 isolates. Dramatic differences in the response of the mice to the 2 isolates are evident. Mouse weight increases slightly through time for the controls; mouse weight remains unchanged through time for NC-SweB1; mouse weight decreases significantly through time for NC-Liverpool. Weight losses of 18–35% were experienced by all but 1 mouse ($n = 57/58$) by 14–27 days post-inoculation (p.i.) with NC-Liverpool. Symptoms commenced at approximately 10 days p.i. with coat ruffling, and progressed to limb weakness and paralysis, immobility, conjunctivitis and discoordination at 16–27 days p.i. which was concurrent with the later stages of severe weight loss. In contrast, NC-SweB1 induced similar symptoms in a smaller proportion of mice ($n = 9/57$) over an equivalent time period. Cutaneous lesions developed around the inoculation site in mice for both isolates but could not be consistently related to a particular stage of infection.

Effect of culture

The effect of culture on the pathogenicity of NC-Liverpool was investigated by comparing weight loss and histopathology in mice ($n = 10$/group) inoculated with culture-derived tachyzoites. The two populations used differed in that one had been maintained in culture for 14 months longer than the other, which had been stored as a frozen stock (in

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Table 2. Analysis of variance for mouse weights

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Probability
Strain	141·735	1	141·735	5·656	0·020
Error	1679·078	67	25·061		
Dose	190·839	4	47·710	1·904	0·120
Error	1679·078	67	25·061		
Time	27·520	3	9·173	18·442	< 0·001
Error	99·983	201	0·497		
Strain × Dose	131·906	4	32·976	1·316	0·273
Error	1679·078	67	25·061		
Strain × Time	76·759	3	25·586	51·437	< 0·001
Error	99·983	201	0·497		
Dose × Time	99·530	12	8·294	16·674	< 0·001
Error	99·983	201	0·497		
Strain × Dose × Time	31·456	12	2·621	5·270	< 0·001
Error	99·983	201	0·497		

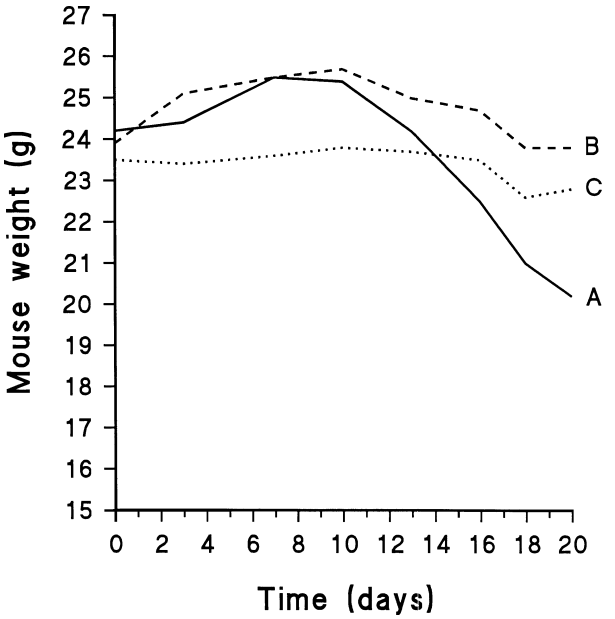

Fig. 1. Graph showing changes in mean body weight of infected mice with time post-infection. Mice ($n = 12$) were infected with 1×10^6 tachyzoites of either NC-Liverpool (——, A) or with NC-SweB1 (-----, B). (C) The uninfected control group ($n = 6$).

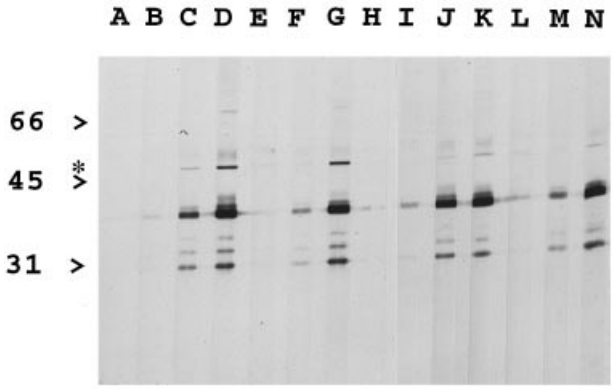

Fig. 2. Immunoblot analysis of *Neospora caninum* proteins using mouse sera. Proteins of NC-Liverpool (Lanes A–G) or NC-SweB1 (Lanes H–N) were probed with sera from mice infected with either NC-Liverpool (Lanes B–D, I–K) or NC-Sweb1 (Lanes E–G, L–N). The pooled sera were obtained from mice at 6 (Lanes B, E, I and L), 9 (Lanes C, F, J and M) or 12 (Lanes D, G, K and N) days p.i. Responses of control mice are shown in Lanes A and H. The marker sizes are in kDa. * Shows the location of the 50 kDa antigen referred to in the text.

liquid nitrogen) during this time. Analysis of mean weight loss, clinical symptoms and histopathology of the brains from these infected mice showed culture did not alter the pathogenicity of NC-Liverpool. Both populations induced identical effects on mice to that described above.

Immunoblot analysis

Fig. 2 shows an immunoblot where solubilized *N. caninum* proteins were probed with pooled antisera obtained from mice during the course of infection by either NC-Liverpool or NC-SweB1. The main immunodominant antigens of *N. caninum* (37, 30–36, 29/30 kDA), were all first detected at day 9 by antibodies from mice inoculated with NC-Liverpool. Antibodies from mice infected with NC-SweB1 showed a somewhat weaker response to these antigens at an equivalent day p.i. Fig. 2 also shows the results obtained using NC-SweB1 proteins probed with sera from mice infected with NC-Liverpool or NC-SweB1. Similar antigenic profiles were detected except that an antigen at approximately 50 kDa differed in immunoreactivity between the isolates during the course of infection.

RAPD–PCR

RAPD–PCR analyses of NC-Liverpool and NC-SweB1 genomic DNA using primers B4, B5 and B12 are shown in Fig. 3. All 3 primers generated RAPD–PCR profiles that contained several bands.

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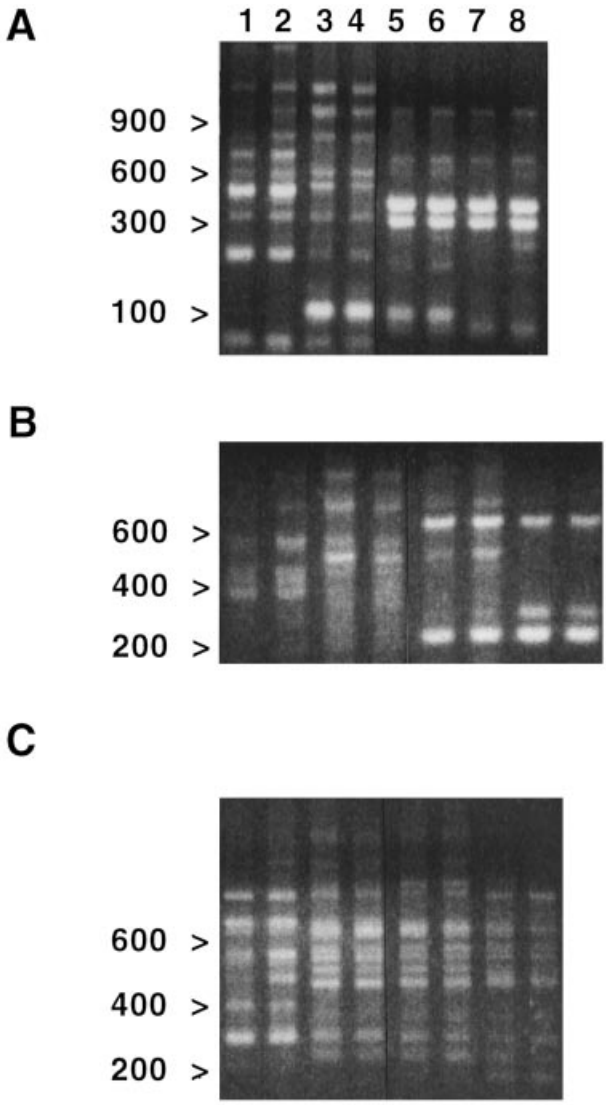

Fig. 3. RAPD–PCR analysis using primer (A) B12, (B) B5 and (C) B4. The genomic DNAs studied are (from left to right): Lanes 1 and 2, ME49; 3 and 4, RH; 5 and 6, NC-SweB1; 7 and 8, NC-Liverpool. The size markers are in bp.

In all instances, the NC-Liverpool DNA generated a profile that differed from NC-SweB1 and *T. gondii*. For example, primer B12 generated low molecular weight bands between 100 and 200 bp in size that differed in molecular weight between NC-Liverpool and NC-SweB1. Primer B5 produced a RAPD–PCR profile from NC-Liverpool which contained only 3 predominant bands, whereas NC-SweB1 supported the PCR of at least 4 bands, 2 of which were not present in the profile from NC-Liverpool. NC-Liverpool DNA generated a band of approx. 200 bp in RAPD–PCR with primer B4 whereas DNA from NC-SweB1 did not. Finally, we note that RAPD–PCR using primer B4 generated profiles from *N. caninum* (except for the above-mentioned discrepancy for NC-Liverpool) that were very similar to that generated for the RH strain of *T. gondii*.

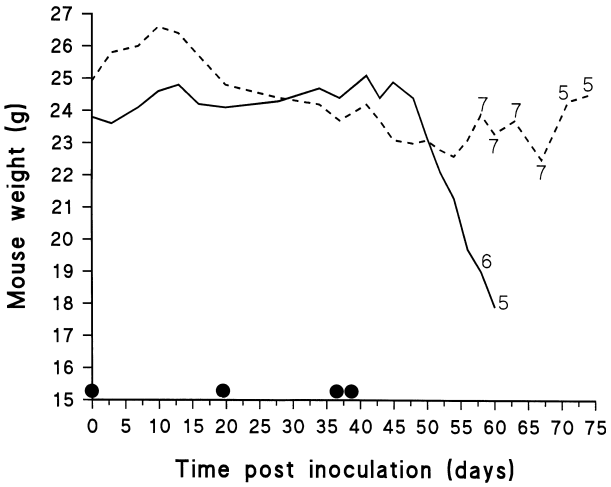

Fig. 4. Cross-protection experiment. Graph shows changes in mean body weight of mice with time (days p.i.). On days 0 and 19 mice were inoculated with 1×10^6 culture-derived tachyzoites of NC-SweB1 (-----) or normal saline (control group; ——). On day 37 mice were challenged with 1×10^7 culture-derived tachyzoites of NC-Liverpool. The numbers on the graph show the number of mice surviving at that time-point. (●) Time of inoculation with NC-SweB1 or saline; (●, ●) time of challenge by NC-Liverpool.

Cross-protection study

The evidence provided above indicates that NC-Liverpool and NC-SweB1 differ in their genetic and biological properties. In particular, NC-SweB1 appears remarkably attenuated in its ability to induce a marked pathological response in the inbred Balb/C mouse model. It therefore seemed appropriate to address whether infection by NC-SweB1 would protect against a lethal challenge by NC-Liverpool in this model. The dose chosen for NC-SweB1 was based on the consideration that higher doses may induce a mild pathological response in the mouse, which was considered as deleterious to the animal.

In the control group (inoculated with saline), 8 mice challenged with NC-Liverpool rapidly lost weight 16 days after challenge (Fig. 4), which correlated with the onset of clinical symptoms of coat ruffling, discoordinated movement and death. All mice in this group became unwell and were euthanased by day 60 of this experiment. Histopathological effects detected in the brains of these mice were typical of an NC-Liverpool infection.

In contrast 8 mice inoculated with NC-SweB1 lost weight slowly after infection (Fig. 4); 1 mouse appeared very slightly ruffled 25 days into the experiment; another mouse developed left side paralysis at 38 days. They continued to lose weight after the lethal challenge by NC-Liverpool. One mouse died of neosporosis at day 56 of the experiment; this mouse was a different animal to those 2 identified above; the remaining 7 mice became mildly ruffled and survived until day 70 when 2 of

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them deteriorated and were euthanased. The remaining 5 mice did not die and were euthanased at the end of the experiment on day 75. Histopathology of the brains of all these mice demonstrated a moderate acute multifocal non-suppurative meningoencephalitis that was characteristic of an NC-SweB1 infection.

DISCUSSION

In this study, biological and genetic characteristics of 2 isolates (NC-Liverpool, NC-SweB1) of *N. caninum* have been compared. Pathological examination of infections established in inbred Balb/C mice demonstrated a significant difference in the severity and character of the inflammatory response in the brains of mice inoculated with NC-Liverpool when compared to NC-SweB1. In the majority of cases NC-Liverpool induced necrotizing to granulomatous encephalitis whilst non-suppurative to necrotizing encephalitis was observed in mice infected with NC-SweB1. Furthermore, a statistical analysis of the changes in mean body weight of infected mice was performed by an orthogonal repeated-measures analysis of variance and this demonstrated that mice infected with NC-SweB1 induced little or no change in body weight; however, mice infected with NC-Liverpool were severely compromised; they rapidly lost weight and died or required euthanasing by 27 day p.i. Taken together these data indicate that these 2 isolates of *N. caninum* differ significantly in their pathogenicity towards the host organism, which in this model is the mouse. Previous studies have recorded differences between 2 canine isolates (NC-1 and NC-3) in their course and severity of infection in immunocompetent Balb/C mice (Lindsay *et al.* 1995). NC-3 when injected into mice did not induce clinical signs of neosporosis nor did it induce severe brain lesions. The behaviour of NC-SweB1 appears more pathogenic than NC-3 since severe brain lesions were detected (albeit few) in mice inoculated with this isolate compared to NC-3.

Johnson (1988) pointed out that mice infected with *Toxoplasma* succumbed when their body weight was between 20 and 30% of the original 3 day mean weight. The results presented here indicate that a 20–25% decrease in body weight resulting from infections due to *Neospora* is also lethal to the mouse. In an attempt to identify an humane end-point for these experiments we determined the average weight loss of mice that were dead at the next sample time-point. Weight losses of 20–25% were normally observed during the acute phase of infection prior to death, and therefore, in order to limit unnecessary suffering, may be recommended as a time to implement euthanasia in mice infected with *Neospora*.

The pathological differences induced by the 2 isolates probably do not result from differences in the time they have been cultured *in vitro*. Continuous culture of NC-Liverpool for just over 1 year did not affect its ability to induce marked weight loss or brain pathology in mice.

Minor antigenic differences between *N. caninum* isolates have been detected previously by Western blotting (e.g. Barber *et al.* 1995; Paré, Hietala & Thurmond, 1995). Therefore we investigated the IgG antibody response by mice to *N. caninum* antigens. The time-course of the antibody response differed between NC-Liverpool and NC-SweB1, in that NC-Liverpool induced an earlier IgG response. Additionally, in non-reducing gels minor antigenic differences were observed between NC-Liverpool and NC-SweB1. An antigen approximately 50 kDa in size appeared significantly more immunogenic in NC-Liverpool compared to NC-SweB1.

All of the molecular studies published to date have revealed no evidence for genetic differences among isolates of *Neospora*. However, all the genetic loci examined to date have been rDNA in nature and therefore may not be suitable markers for investigating the relationships between different strains of the same parasite species. Such is the case in *Toxoplasma* where 2 populations (virulent and non-virulent) of parasites are known to exist which differ significantly in their biological and genetic characteristics, yet share identical rDNA sequences. Consequently, given the prior knowledge reported by Guo & Johnson (1996) that RAPD–PCR can distinguish between virulent and non-virulent populations of *T. gondii*, we investigated whether RAPD–PCR could identify strain-specific markers for the canine and bovine isolates of *Neospora* studied here. Using primers previously described by Guo & Johnson (1995, 1996) we confirm in the first instance that RAPD–PCR easily distinguishes between virulent and avirulent populations of *T. gondii*. We also confirm that the RAPD–PCR profile of *N. caninum* differs from *T. gondii*. Intriguingly, however, we have observed with primer B4 that the RAPD–PCR profile of *N. caninum* is very similar to that observed for virulent strains of *T. gondii*. This observation corroborates extensive phylogenetic analysis of 18S rDNA which shows these taxa to be very closely related (Ellis *et al.* 1994; Morrison & Ellis, 1997). Of additional importance is the observation that isolates NC-Liverpool and NC-SweB1 appear genetically quite distinct from each other by RAPD–PCR. As far as we are aware this represents the first reported observation of significant genetic differences between isolates of *N. caninum*.

It is evident from the work presented here and elsewhere (e.g. Lindsay *et al.* 1995) that some isolates of *N. caninum* are less pathogenic to the murine host than are others. Experimental animal models have shown that attenuated strains of *T. gondii* protect

against lethal challenge in adult mice (Waldeland & Frenkel, 1983) and congenital toxoplasmosis in sheep (reviewed by Buxton, 1993). Data from our study indicates that inoculation of NC-SweB1 confers a degree of protection to a lethal challenge with NC-Liverpool. Consequently, naturally or experimentally attenuated isolates of *N. caninum* may form the basis of future vaccines against congenital neosporosis should vaccination be considered as a practical control measure for the disease. Experimental models, such as those developed in the mouse, will also aid in the investigation of the determinants governing virulence in *N. caninum*. In conclusion, this study has demonstrated the existence of biologically significant differences between 2 isolates of *N. caninum*.

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