Genetic and biological diversity among isolates of *Neospora caninum*

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SUMMARY

Neospora caninum is a protozoan parasite that causes bovine abortion. The epidemiology of *N.caninum* is poorly understood and little is known about the genetic diversity of the parasite, or whether individual isolates differ in virulence. Such diversity may, among other factors, underlie the range of pathologies seen in cattle. In this study we analysed biological and genetic variation in 6 isolates of *N. caninum* originating from canine and bovine hosts by measurement of growth rate *in vitro*, Western blotting and random amplification of polymorphic DNA (RAPD). This comparative analysis of intra-species diversity demonstrated that heterogeneity exists within the species. The relative growth rate *in vitro*, as assessed by ^{3}[H]uracil uptake, showed significant variation between isolates. However, no significant differences were detected between the antigenic profiles of each isolate by Western blotting. RAPD-PCR was performed on DNA from the 6 *Neospora* isolates; 3 strains of *Toxoplasma gondii*, *Sarcocystis* sp. and *Cryptosporidium parvum* were also analysed. Twenty-six RAPD primers gave rise to 434 markers of which 222 were conserved between all the *Neospora* isolates and distinguished them from the other Apicomplexa. An additional 54 markers were unique for *Neospora* but were polymorphic within the species and able to differentiate between the individual isolates. The RAPD data were subjected to pair-wise similarity and cluster analysis and showed that the *Neospora* isolates clustered together as a group, with *T. gondii* as their nearest neighbour. *N. caninum* isolates showed no clustering with respect either to host or geographical origin. The genetic similarity between *Neospora* isolates from cattle and dogs suggests that these hosts may be epidemiologically related, although further analysis of bovine and canine field samples are required. The genetic and biological diversity observed in this study may have important implications for our understanding of the pathology and epidemiology of neosporosis.

Key words: *Neospora caninum*, neosporosis, genetic diversity, RAPD analysis, molecular epidemiology, virulence, ^{3}[H]uracil uptake, Western blotting.

INTRODUCTION

Neospora caninum is a cyst-forming Apicomplexan parasite that causes neosporosis, notably in cattle (*Bos taurus*) and domestic dogs (*Canis familiaris*). Infection in cattle is relatively common with estimates of seroprevalence to *N. caninum* varying from 6% in dairy cattle in England and Wales (Davison, Otter & Trees, 1999), to 22% of Danish dairy cattle (Jensen *et al*. 1999) and 24% of beef cattle in the USA (Sanderson, Guy & Baszler, 2000). Although obvious clinical disease does not always accompany infection, *N. caninum* is thought to be an important cause of bovine abortion world-wide; in the United Kingdom it may account for around 12·5% of all bovine abortions (Davison *et al*. 1999). Neosporosis affects both dairy and beef production and is acknowledged to have a significant economic impact on the cattle industry (Trees *et al*. 1999). The dog has recently been identified as a definitive host of the parasite (McAllister *et al*. 1998), although only small numbers of oocysts appear to be produced under experimental conditions. Oocyst shedding by dogs under natural conditions has never been reported. In both dogs and cattle, the only proven route of transmission is from mother to offspring (Anderson *et al*. 1997; Barber & Trees, 1998). However, although vertical transmission is thought to account for the majority of bovine infections it is thought that some postnatal infection must occur to maintain the level of infection within the population (French *et al*. 1999). The epidemiology of *N. caninum* thus remains poorly defined.

N. caninum has a wide host range and has also been found in sheep (*Ovis aries*) (Dubey & Lindsay, 1990), goats (*Capra hircus*) (Dubey, Acland & Hamir, 1992), deer (*Cervis eldi*, *Odocoileus hemionus*) (Woods *et al*. 1994), foxes (*Vulpes vulpes*) (Buxton *et al*. 1997) and other carnivores (Barber *et al*. 1997).

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The parasite was first isolated in tissue culture from dogs (Dubey *et al*. 1988) and from cattle by Conrad *et al*. (1993). Although isolation is difficult and time-consuming, *N. caninum* has now been isolated from cattle and dogs in various countries including England (Barber *et al*. 1995; Davison *et al*. 1999), Sweden (Stenlund *et al*. 1997), Italy (Magnino *et al*. 1999), Japan (Yamane *et al*. 1997) and Korea (Kim *et al*. 2000).

A key question underlying our understanding of both the epidemiology and pathogenicity of *N. caninum* is the extent to which both biological and genetic diversity might exist within the species. Indeed, some isolates of *Neospora* have been reported to differ in their pathogenicity in murine models (Lindsay *et al*. 1995; Atkinson *et al*. 1999). Its large host range, wide geographical distribution and potential for sexual recombination might be expected to produce significant variation within the species. Such diversity may have an impact on the range of pathology associated with infection in both cattle and dogs and would be an important consideration in the design of vaccines to protect against neosporosis. Moreover, an understanding of the molecular epidemiology of *N. caninum* could be exploited to help determine the importance of various transmission routes and the relative roles of intermediate and definitive hosts in the spread of infection.

The phylogenetic position of *N. caninum* has been defined by ribosomal DNA sequencing and places it as a separate species within the Sarcocystidae, closely related to *Toxoplasma gondii* (Franzen *et al*. 2000). However, no differences have been reported between ribosomal DNA sequences of *N. caninum* isolates (Marsh *et al*. 1995; Stenlund *et al*. 1997; Ellis *et al*. 1998), although the conservation of ribosomal RNA genes means that these markers are unlikely to give a good indication of intra-species diversity. Atkinson *et al*. (1999) analysed 2 isolates, NC-Liverpool and NC-SweB1, using 3 RAPD-PCR primers and demonstrated variant banding patterns. However, the small number of primers and isolates used precludes wider interpretation of these data.

In this study we analysed genetic heterogeneity and biological diversity in 6 isolates of *N. caninum* by RAPD-PCR, by comparison of growth rates *in vitro* and by Western blot analysis. Isolates were selected from a range of geographical sources and included those originally derived from both canine and bovine infections. Genetic diversity was analysed by RAPD using up to 26 different primers, which gave rise to several hundred independent genetic markers. *Neospora* isolates were also compared with *T. gondii*, *Sarcocystis* sp. and *Cryptosporidium parvum* RAPD profiles. We demonstrate that significant biological and genetic diversity occurs within isolates of *N. caninum* and conclude that this may have important implications for our understanding of the pathology and epidemiology of the parasite.

MATERIALS AND METHODS

N. caninum *and* T. gondii *tachyzoites*

Six laboratory-maintained isolates of *N. caninum* were analysed in this study; details of their isolation and maintenance are summarized in Table 1. None of these isolates had been previously cloned. Isolate NC-1 and isolate NC-Liverpool originated from dogs and the remainder of the isolates from cattle. The highest number of tissue culture passages for each isolate ranged between 33 and 56 passages, except for NC-1, which had been maintained in tissue culture for significantly longer (212 passages). Parasites were maintained in African Green monkey kidney (VERO) cells seeded in 25 cm^2 vented flasks. Cells and parasites were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 5 % horse serum (Advanced Protein Products Ltd, UK) or 5 % foetal calf serum (Labtech International, UK) at 37 °C in a 5 % CO_2 humidified incubator. *T. gondii* tachyzoites (RH, S48 and M3 strains) were also maintained in tissue culture as above. For DNA and protein analysis of parasites, tachyzoites of *N. caninum* or *T. gondii* were isolated from host cells by filtration through 3 μm polycarbonate Nucleopore Track-Etch™ membranes (Whatman International, UK). Tachyzoites were then pelleted by centrifugation at 1500 ***g*** followed by 6 washes in phosphate buffered saline (PBS).

Growth characterization

The relative growth rate of *N. caninum* parasites in tissue culture was measured by the incorporation of ^{3}[H]uracil into tachyzoites as described by Innes *et al*. (995). For all growth comparisons the same batch of horse serum was used to supplement the IMDM medium. Briefly, VERO cells were seeded into 96-well flat bottom plates at 4×10^4 per well and incubated for 24 h. Tachyzoites of each isolate were then added to cell monolayers in quadruplicate at parasite to cell ratios of 1:1, 2:1 and 4:1 and the cultures pulsed with 5 μCi [^{3}H]uracil per well. After 24 h, cultures were harvested onto fibreglass filters (Canbarra Packard, Pangbourne, UK) and radioactivity measured using a β-scintillation counter (Matrix-96, Packard Bioscience Company, UK). The final counts were expressed in mean counts per minute (cpm) for each set of quadruplicate wells. Relative growth rate estimates were compiled from 5 replicate experiments for each isolate and the results evaluated statistically by Kruskal-Wallis analysis.

Antigen preparation and Western blot analysis

N. caninum, *T. gondii* and host cell antigen was prepared by freeze/thaw and sonication for 5 s on ice

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Table 1. Isolation and maintenance of *Neospora caninum* parasites

Isolate	Host of origin	Isolated and maintained by	Highest passage number	Country of origin	Reference
NC-1	Canine	Tissue culture	212	USA	Dubey *et al*. (1988)
NC-Liverpool	Canine	Tissue culture	41	UK	Barber *et al*. (1995)
BPA-1	Bovine	Tissue culture	36	USA	Conrad *et al*. (1993)
NC-SweB1	Bovine	Tissue culture	36	Sweden	Stenlund *et al*. (1997)
JPA-2	Bovine	Mouse inoculation/ tissue culture	33	Japan	Yamane *et al*. (1997)
NC-LivB1	Bovine	Tissue culture	56	UK	Davison *et al*. (1999)

Table 2. Primer sequences used for RAPD analysis

Name	Sequence (5′ to 3′)
OPBC-01	CCTTCGGCTC
OPBC-02	ACAGTAGCGG
OPBC-03	GGCTTGACCT
OPBC-04	CCACGTGCCA
OPBC-05	GAGGCGATTG
OPBC-06	GAAGGCGAGA
OPBC-07	TGTGCCTGAC
OPBC-08	GGTCTTCCCT
OPBC-09	GTCATGCGAC
OPBC-10	AACGTCGAGG
OPBC-11	TTTTGCCCCC
OPBC-12	CCTCCACCAG
OPBC-13	CCTGGCACAG
OPBC-14	GGTCCGACGA
OPBC-15	CCAGACTCCA
OPBC-16	CTGGTGCTCA
OPBC-17	CCGTTAGTCC
OPBC-18	GTGAAGGAGG
OPBC-19	ACAAGCGCGA
OPBC-20	AGCACTGGGG
APB 1	GGTGCGGGAA
APB 2	GTTTCGCTCC
APB 3	GTAGACCCGT
APB 4	AAGAGCCCGT
APB 5	AACGCGCAAC
APB 6	CCCGTCAGCA

using an Ultrasonic Processor (Jencons Scientific Ltd, UK). SDS (Sigma-Aldrich Chemie GmbH, Germany) (1 %) was added to the resulting crude lysate to help solubilize the protein and total protein concentration was assayed using the BCA™ reagent (Pierce, Rockford, USA). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli (1970) in a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA, USA) using a 12 % discontinuous linear gel run at a constant 175 V for 55 min. Approximately 5 μg of protein/well and two doubling dilutions were loaded into each well. Pre-stained markers (MultiMark™, Novex, USA) were used to determine relative molecular masses. Electrotransfer of the proteins from the gel to a Protan R nitrocellulose transfer membrane (Schleicher & Schuell, Germany) was carried out using a semidry transfer apparatus (Sigma-Aldrich, USA) at 0·9 mA/cm^2 of gel for 1 h with Tris–glycine buffer containing 20 % methanol. After transfer, the membrane was incubated for 30 min with 5 % Marvel/PBS to block non-specific binding and washed with PBS/Tween80. Blots were incubated overnight with the various test sera. After washing with PBS/Tween80, the membrane was then incubated with either rabbit anti-bovine IgG peroxidase conjugate at 1:10000 in PBS/Tween80 (Sigma, USA) or goat anti-rabbit peroxidase conjugate (Dako Ltd, UK) at 1:2000 in PBS/Tween80. The bound peroxidase was visualized using an ECL Western blotting detection kit (Amersham Pharmacia Biotech, UK).

DNA preparation

DNA from *N. caninum* and *T. gondii* parasites was extracted from a minimum of 2×10^7 filter-purified and washed tachyzoites using the GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. *C. parvum* DNA was extracted using the same method, but from sporozoites that had been excysted from Type II strain oocysts kindly provided by Professor H. W. Smith (Scottish Parasite Diagnostic Laboratory, UK). *Sarcosystis* sp. DNA was a gift from Mr S. Wright (Moredun Research Institute, UK) and was purified by caesium chloride centrifugation (Sambrook, Fritsch & Maniatis, 1989) from parasites purified from a sheep heart. All purified total genomic DNA was analysed and quantified by agarose gel electrophoresis and staining with ethidium bromide before analysis.

Random amplification of polymorphic DNA (RAPD)

RAPD analysis on DNA samples was performed using Ready-to-Go™ RAPD analysis beads containing Taq Polymerase (Amersham Pharmacia Biotech, UK). PCR was performed with the beads in 10 mM Tris, 30 mM KCl, 3 mM $MgCl_2$ (pH 8·3)

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buffer, 0·4 mM dNTPs (each) and bovine serum albumin (2·5 μg). DNA was amplified with 25 pmol of primer and water to a final volume of 25 μl with the addition of one RAPD analysis bead to approximately 10 ng of template DNA. The mixtures were subjected to 45 cycles of amplification (95 °C for 60 s, 36 °C for 60 s, and 72 °C for 120 s for each cycle) with an initial incubation step at 95 °C for 5 min, in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer). Twenty-six primers were used in the RAPD obtained from either Amersham Pharmacia Biotech, UK or from Operon (Operon Technologies Inc, CA, USA) (Table 2). Amplified fragments were separated by 2% agarose gel electrophoresis at 120 V and were visualized after staining with ethidium bromide (10 μg/ml) under UV illumination. Data were recorded using a gel imager (Appligene Oncor, France).

Statistical analysis

Each DNA band on the gel was defined as a marker and for each sample these were scored as present/absent in a binary matrix. Data were then analysed using a publicly available clustering calculator programme (http://www.biology.ualberta.ca/jbrzusto/cluster.html). The matrix was subjected to pair-wise similarity analysis.

The index of similarity (S) of samples i and j was estimated using Jaccard's coefficient, denoted by:

$$S = a/(a+b+c),$$

where a = bands present in both samples; b = bands present in sample i, absent in sample j; c = bands present in sample j, absent in sample i.

The un-weighted pairgroup arithmetic average (UPGMA) algorithm was used for clustering analysis and the output was used to construct a phenogram.

RESULTS

Growth rate in tissue culture

The relative rates of tachyzoite multiplication in tissue culture as assessed by incorporation of ^{3}[H]uracil over 24 h for all *Neospora* isolates are shown in Fig. 1. As expected, an increase in the parasite:cell ratio resulted in an increase in the overall incorporation of ^{3}[H]uracil ($P < 0·01$) for each isolate. Kruskal-Wallis analysis showed that overall, there was a significant difference ($P = 0·001$) in the incorporation rates of ^{3}[H]uracil between the 6 *N. caninum* isolates at all 3 parasite:cell ratios. Most strikingly, at all parasite:cell ratios, NC-Liverpool multiplied significantly faster when compared to the other isolates ($P < 0·001$) and at more than twice the rate of isolate NC-1. By contrast, isolate NC-SweB1 incorporated ^{3}[H]uracil at a significantly slower rate ($P < 0·01$) and at about 10 times less than that of the fastest growing isolate, NC-Liverpool. Isolates NC-LivB1, BPA-1, JPA-2 and NC-1 multiplied at intermediate rates, although NC-1 grew significantly faster than both NC-LivB1 ($P < 0·01$) and BPA-1 ($P < 0·04$), but not faster than JPA-2 ($P > 0·05$). As expected, uninfected VERO cells showed a constantly low background incorporation of ^{3}[H]uracil.

Western blot analysis

To determine whether any variation could be detected in the antigenic profile of the *N. caninum* isolates, protein was prepared from tachyzoites of each isolate and separated by SDS–PAGE. The corresponding protein was immunoblotted with (a) hyperimmune serum derived from calves experimentally infected with *N. caninum* (NC-1 isolate), (b) sera from a pool of naturally infected cattle and (c) hyperimmune serum produced by experimental infection in rabbits (NC-1 isolate). The 3 antisera all produced clear and reproducible banding profiles recognizing bands ranging from 14 to > 148 kDa (Fig. 2). However, no antigenic differences were detected by Western blotting between any of the 6 isolates using the above immune serum. To ensure that possible antigenic differences between the isolates were not being masked by variations in the amount of protein loaded onto each gel, all samples were compared as doubling dilutions; this further confirmed that there were no detectable differences in the antigenic profiles of the 6 isolates. The antigenic profiles given by serum from experimentally infected cattle and naturally infected cattle were identical (data not shown), but the profiles from blots probed with antisera from rabbits differed markedly from that of cattle (Fig. 2). Nonetheless, all 6 isolates gave identical patterns with the respective sources of antisera. As expected, *N. caninum* antisera showed only limited cross-reactivity with *T. gondii* antigen, except for a prominent band at just below 60 kDa in parasites probed with the rabbit hyperimmune serum and 2 or, possibly 3 weakly-staining diffuse bands with bovine immune serum.

RAPD analysis

RAPD analysis was performed using the primers in Table 2 on total genomic DNA from 6 laboratory isolates of *N. caninum*, 3 strains of *T. gondii*, 1 isolate of *Cryptosporidium parvum* and 1 isolate of *Sarcocystis* sp. All the primers gave clear and reproducible banding patterns. Representative gels for 4 of the primers are shown in Fig. 3. Some primers gave almost identical patterns with all 6 *Neospora* isolates (Fig. 3A, primer OPBC 04, samples A–F). However, in most cases primers gave rise to variant profiles

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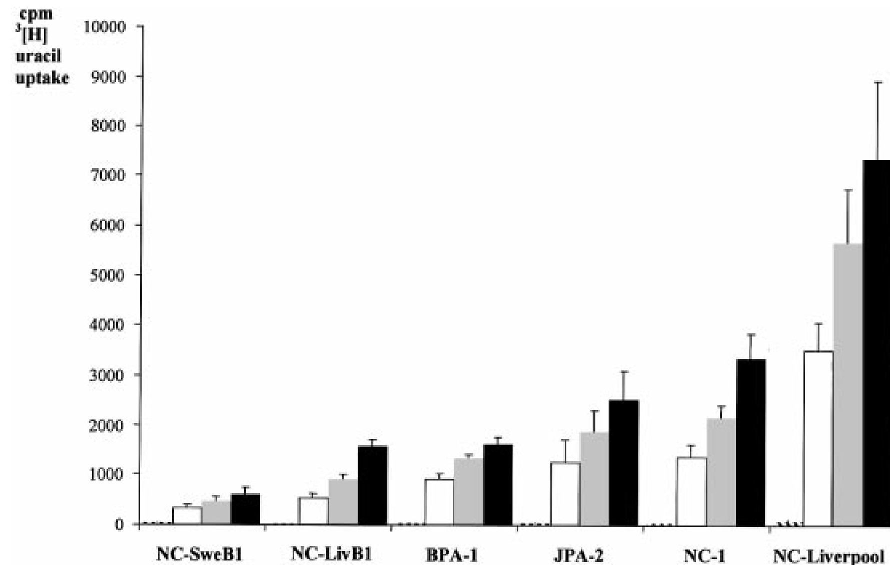

Fig. 1. Rate of multiplication of *Neospora caninum* isolates in tissue culture as assessed by the differential uptake of ^{3}[H]uracil. Uptake of radioisotope was measured over 24 h in both parasites and uninfected host cells (▨). VERO cells were infected in quadruplicate at parasite : cell ratios of 1 : 1 (□), 2 : 1 (▩), 4 : 1 (■). Each experiment was performed 5 times and data are expressed as mean ±S.E.

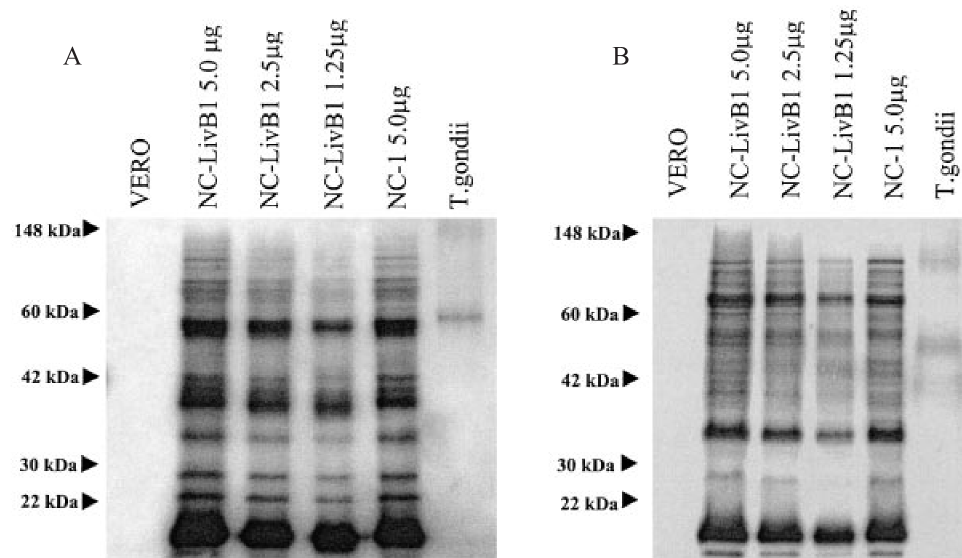

Fig. 2. (A) Representative Western blot analysis of antigen from isolate NC-1 compared with dilutions of protein from isolate NC-LivB1. The blot was probed with hyperimmune rabbit serum. *Toxoplasma gondii* strain (S48); M_r = relative molecular mass. (B) Western blot analysis of above samples except probed with sera from experimentally infected cattle. Data for other isolates not shown.

indicating that RAPD analysis was able to detect DNA polymorphisms between isolates of *N. caninum*. For example, primer APB04 gave an identical banding pattern for isolates BPA-1, JPA-2, NC-1, NC-LivB1 and NC-SweB1, but the profile for isolate NC-Liverpool showed clear polymorphic bands at approximately 360, 480 and 630 bp (Fig. 3A primer APB04, sample E). Similarly, primer OPBC16 gave a consistent profile for isolates BPA-1, JPA-2, NC-1, but showed a clear polymorphic band at approximately 470 bp in isolates NC-LivB1 and NC-SweB1 (Fig. 3B, primer OPBC16). Some primers gave very polymorphic banding profiles. For example, primer OPBC18 gave identical patterns only with isolates JPA-2, NC-Liverpool and NC-1, whereas isolates BPA-1, NC-LivB2 and NC-SweB1 showed highly variant patterns (Fig. 3B, primer OPBC18). All primers gave banding patterns for the *N. caninum* isolates that were markedly different from the 3 strains of *T. gondii*, and from *C. parvum* and *S. cruzi*.

Since RAPD analysis entails amplification of the total genomic DNA in each sample, contamination of parasite material with host cell DNA would give

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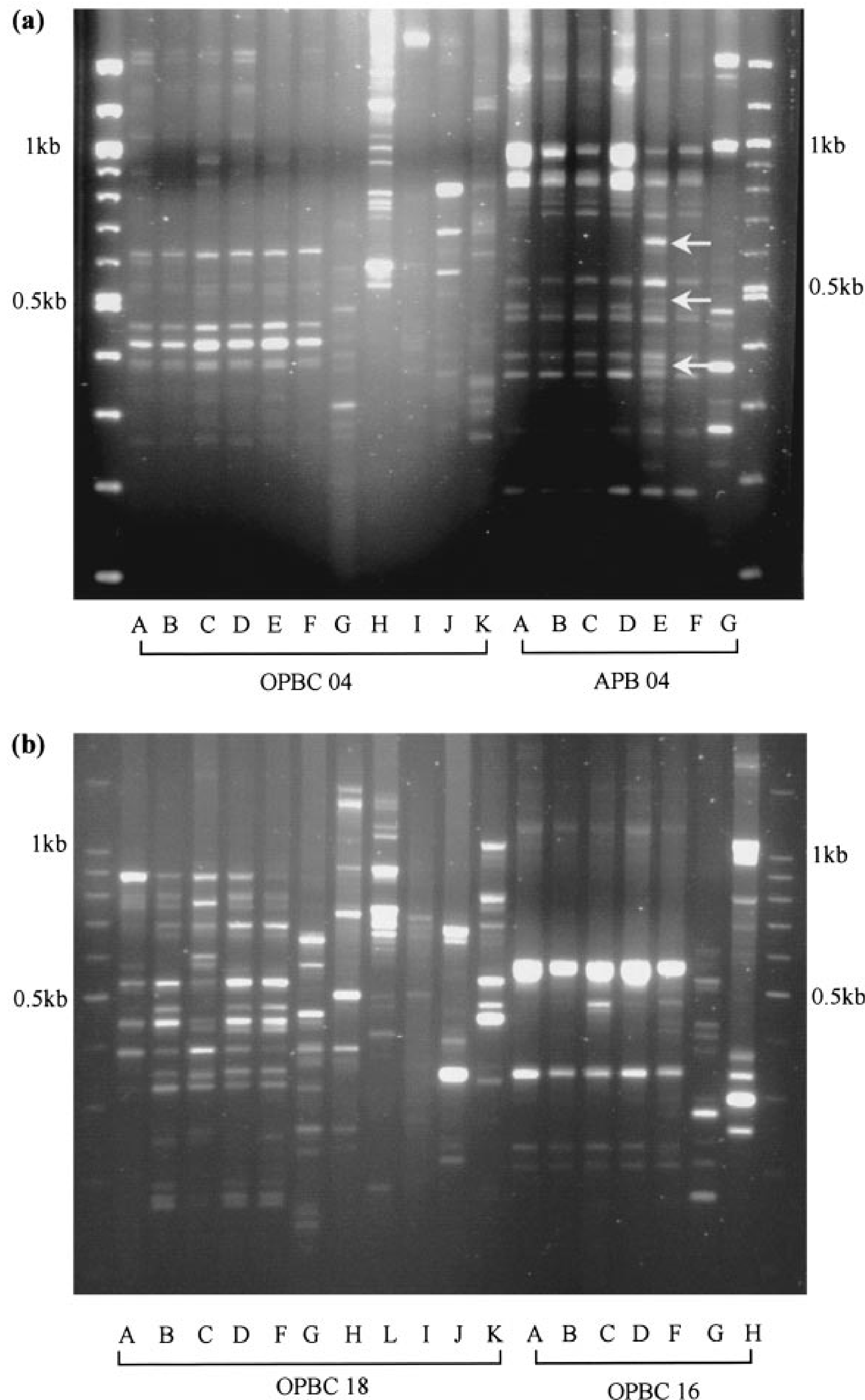

Fig. 3. RAPD analysis of *Neospora caninum* isolates (bovine and canine origin), *Toxoplasma gondii* (S48, RH and M3 strains), *Sarcocystis* sp. *Cryptosporidium parvum* and host (VERO) cell DNA. Representative amplifications from 4 primers (OPBC 04, APB 04, OPBC 18, OPBC 16) are shown. Codes for samples A–L are: A = BPA-1 *N. caninum*; B = JPA-2 *N. caninum*; C = LivB1 *N. caninum*; D = NC-1 *N. caninum*; E = NC Liverpool *N. caninum*; F = NC-SweB1 *N. caninum*; G = S48 *T. gondii*; H = RH *T. gondii*; I = *C. parvum*; J = *Sarcocystis* sp.; K = Host VERO DNA; L = M3 *T. gondii*. Typical polymorphic bands between *N. caninum* isolates are shown for primer APB04 (white arrows).

potentially misleading results. To establish whether our procedures for removal of host cell DNA were adequate and that there was no carry-over of host DNA into the parasite DNA samples, RAPD analysis was performed using primers on samples of both host cell and parasite DNA respectively.

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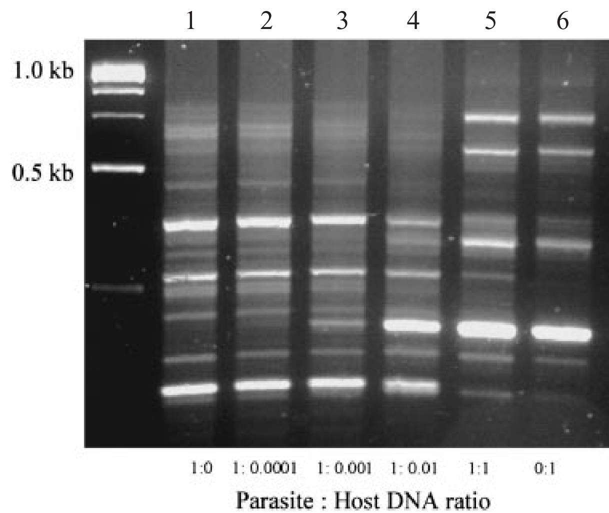

Fig. 4. RAPD analysis of titrated samples of parasite and host cell DNA using primer OPBC2. Lane 1, *Neospora caninum* (LivB1 isolate); Lane 6, VERO cell; Lanes 2–5, titration of parasite DNA with host cell DNA.

Samples of *Neospora* DNA were seeded with increasing concentrations of host cell DNA and RAPD analysis performed on both the mixtures and on the pure samples. Fig. 4 shows the typical outcome of such an experiment using primer OPBC2 and demonstrates that parasite RAPD profiles differed markedly from those given by host cell DNA alone. Moreover, where occasionally band sizes given by host and parasite DNA were similar (1 out of 6 bands for primer OPBC2), no titration effect was seen, confirming that these bands were unlikely to result from DNA cross-contamination.

Cluster analysis

To quantify the data from the RAPD analysis, the total number of bands produced by each primer were scored as present or absent and recorded in a binary matrix. Each band on a gel was defined as a marker and only clear and unambiguous bands were included in the analysis. RAPD analysis with the 26 primers gave rise to 434 markers of which 222 were conserved between all the *Neospora* isolates and distinguished them from the other Apicomplexa. An additional 54 markers were also unique for *Neospora* but were polymorphic within the species and able to differentiate between the individual isolates. The RAPD data were subjected to pair-wise similarity analysis using Jaccard's coefficient and using the UPGMA method for clustering. The resulting phenogram is shown in Fig. 5 and shows clearly that genetic diversity exists amongst the *N. caninum* isolates in this study. However, the *N. caninum* isolates clustered together and were clearly distinct from *T. gondii*, *Sarcocystis* sp. and *C. parvum*. *T. gondii* strains S48, RH and M3 clustered together and were the closest to the *N. caninum* cluster, followed by *Sarcocystis* sp. and then *C. parvum*. The phenogram indicates that *N. caninum* isolates originating from bovines (BPA-1, JPA-2, NC-Sweb1, NC-LivB1) did not cluster separately from those originally derived from dogs (NC-1, NC-Liverpool). Furthermore, isolates that had been adapted for tissue culture the longest, for example isolate NC-1, remained genetically close to isolates that had been adapted to tissue culture for less time, such as isolate BPA-1.

DISCUSSION

In this study we assessed biological characteristics and analysed the genetic diversity in 6 isolates of *N. caninum* of both bovine and canine origin using genetic markers derived from RAPD-PCR analysis. This represents the most comprehensive analysis of intra-species diversity in *N. caninum* and demonstrates clearly that heterogeneity exists within the species. These findings may have important implications for our understanding of the pathology and epidemiology of neosporosis.

Comparative analysis of the growth rates of the 6 isolates showed that some isolates grew significantly faster than others under identical tissue culture conditions. However, no correlation was seen between growth rate and the number of previous tissue culture passages undergone by each isolate. For example, based on its high number of tissue culture passages, isolate NC-1 might be expected to be the most 'tissue culture-adapted' and hence likely to multiply at the fastest rate. However, isolate NC-1 grew at only half the rate of isolate NC-Liverpool, which had undergone only one fifth the number of passages. This effect was reproducible at all 3 initial parasite to cell ratios. Similarly, the isolate that had undergone the second highest number of passages (NC-LivB1) showed the second slowest growth rate. The fact that the number of previous tissue culture passages did not appear to influence growth rate *in vitro* suggests that the variation seen in growth rate between the isolates may reflect genuine biological diversity. These biological observations are supported by the RAPD analysis in this study, which shows that there is no genetic clustering of isolates with respect to passage number and support data of Atkinson *et al*. (1999) that suggest that virulence in mice also occurred independently of passage number.

Atkinson *et al*. (1999) compared the pathogenicity of isolate NC-Liverpool and NC-SweB1 in mice inoculated subcutaneously with tachyzoites and found that brain lesions resulting from infection with NC-Liverpool were associated with more necrosis and a greater inflammatory response than those from NC-SweB1. Mice infected with NC-Liverpool also showed a greater and more rapid weight loss compared with NC-SweB1. Our *in vitro*

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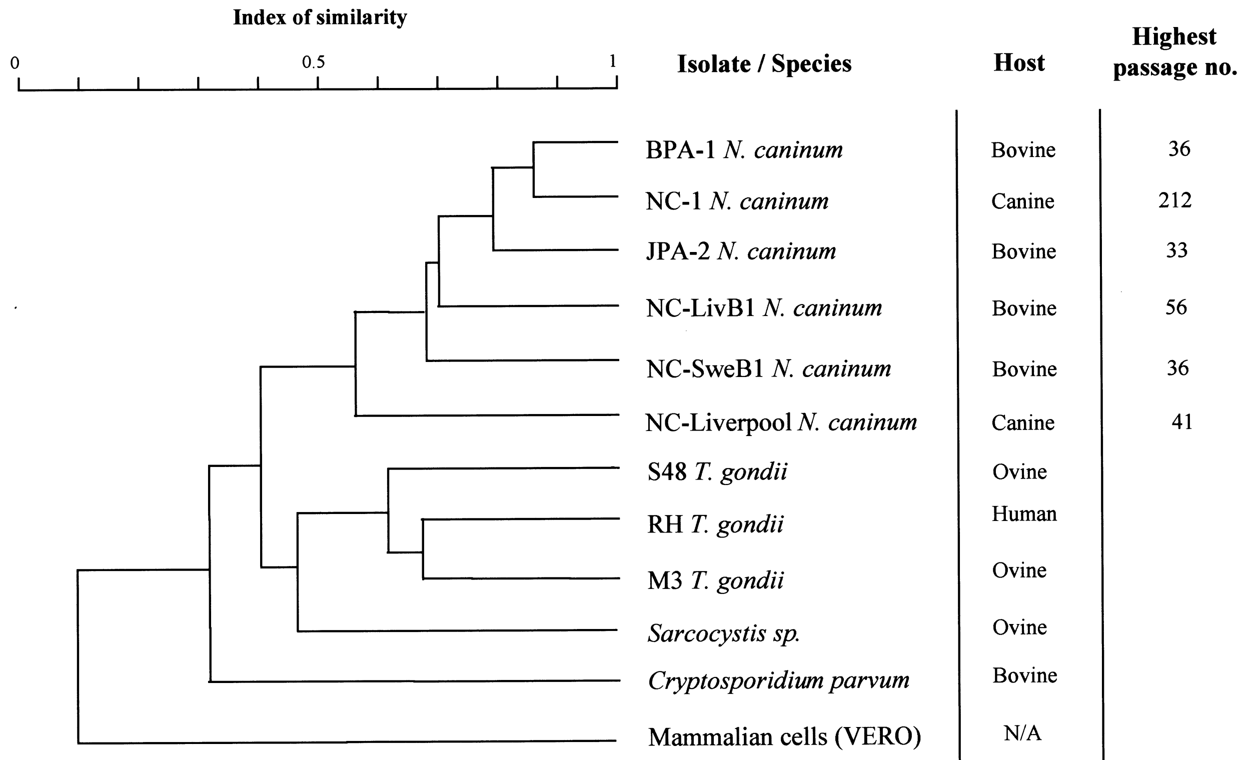

Fig. 5. Phenogram showing genetic relationships between *Neospora caninum* isolates (bovine and canine origin), *Toxoplasma gondii* (S48, RH and M3 strains), *Sarcocystis* sp. and *Cryptosporidium parvum* as estimated by cluster analysis of RAPD patterns obtained with 26 primers. The similarity data were derived from Jaccard's coefficient and subjected to cluster analysis.

results with the same isolates appear to reflect these *in vivo* observations. In this study, isolate NC-Liverpool grew at over 10 times the rate in tissue culture compared to NC-SweB1. Although these data demonstrate that different isolates of *N. caninum* grow at different rates *in vitro*, this does not imply a direct correlation with virulence in other hosts. Pathogenicity in cattle will be a combination of host (Williams *et al.* 2000) and parasite factors, as well as external factors such as the timing of infection and the immune status of the animal. Further studies to examine the interaction of host and parasite in determining pathogenicity are essential to our understanding of neosporosis and central to efforts to devise intervention strategies to minimize the impact of the disease.

In contrast to the growth rate data, the antigenic profiles recognized by the polyclonal sera between all isolates were identical, indicating that there was no substantial variation in the immunodominant proteins. These results are in keeping with those of Marsh *et al.* (1998) who also failed to detect substantial antigenic variation between the BPA-1 and NC-1 isolates and also of Atkinson *et al.* (1999) who showed only minor antigenic differences between the NC-Liverpool and NC-SweB1 isolates. The number of antigens that can be resolved by one-dimensional SDS–PAGE is limited and variation between isolates could easily have been missed by the low resolution of the technique. Immunoblotting of higher resolution separations, for example by two-dimensional electrophoresis, may prove a more useful technique for analysis of differential protein expression between isolates. However, translated gene products such as those detected by electrophoretic methods are always more likely to be conserved than DNA polymorphisms which may arise from non-coding stretches of DNA.

Genetic analysis of the *Neospora* isolates by RAPD-PCR produced a large number of polymorphic markers that revealed significant genetic variation in *N. caninum*. The decision to use RAPD-PCR to examine genetic variation was based on the fact that it is a quick and reliable method for the analysis of DNA samples obtained from cultured parasites. However, data from RAPD-PCR must be interpreted with caution. First, DNA samples contaminated with host material could give rise to misleading results. Substantial care was necessary to ensure that no cross-contamination occurred, as demonstrated by the titration experiments using parasite and host cell DNA. Second, co-migration of RAPD fragments may result from non-homologous genomic sequences that by chance have the same electrophoretic mobility. For this reason the data from RAPD analysis were used to draw a phenogram based on a similarity index since these data cannot strictly be used to measure genetic distance (van de

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Zande & Bijlsma, 1995). Nevertheless, RAPD analysis has been used both as a measure of genetic diversity and for determining phylogenetic lineages in other protozoa, most notably in trypanosomes (Brisse, Barnabe & Tibayrenc, 2000; Muller *et al*. 1997) and *Leishmania* (Banuls, Hide & Tibayrenc, 1999). An excellent correlation has also been shown between data obtained by RAPD analysis and that derived from other typing methods such as multilocus enzyme electrophoresis (MLEE) or pulsed-field gel electrophoresis in bacteria (Barbier *et al*. 1996).

In this study the RAPD data and subsequent cluster analysis showed that all the *Neospora* isolates clustered together independently from the other apicomplexan parasites and placed them closest to the *T. gondii* strains. *Sarcocystis* sp. was the next closest neighbour followed by *Cryptosporidium parvum* which appeared most distant from *N. caninum*. This pattern is consistent with the phylogenetic placing of *N. caninum* by analysis of both the small-subunit ribosomal RNA (Franzen *et al*. 2000) and the large subunit ribosomal RNA gene sequences (Mugridge *et al*. 1999).

Cluster analysis of the individual *Neospora* isolates indicated that there was no segregation of markers with respect to either host origin or geographical location. Thus isolates derived from dogs and cattle, such as NC-1 and BPA-1, appeared more similar than 2 isolates derived from dogs (NC-1 and NC-Liverpool). Although the number of isolates was limited, the lack of genetic clustering between dog and bovine isolates does suggest that there is likely to be an epidemiological link between these hosts. This does not imply that cattle necessarily become infected from oocysts passed in dogs faeces; but simply that there is likely to be an exchange of parasite material between the two so that parasites of a similar genotype are found in both hosts. Indeed, dogs could also become infected by ingestion of bovine material.

The RAPD data in this study show genetic diversity between the 3 strains of *T. gondii* and reflect that found in other studies (Guo & Johnson, 1995). However, although there are obvious similarities shown in this RAPD study, the extent to which the population structure of *N. caninum* mirrors that of *T. gondii* is not known and awaits more detailed analysis. In *T. gondii* a population structure exists in which there are 3 clonal lineages (Type I, Type II and Type III strains) (Howe & Sibley, 1995). These lineages are still able to undergo meiotic recombination (albeit rarely) thus demonstrating that they are not separate species (Sibley & Howe, 1996). The predominantly clonal population of *Toxoplasma* appears to result in a correlation between the development of disease and parasite genotype. Sibley & Howe (1996) analysed animal and human cases of toxoplasmosis and found a strong link between biological phenotype and specific parasite lineages. It is tempting to speculate that a similar clonal population structure may exist in *N. caninum*, particularly since the opportunity for sexual recombination might be even more limited due to the predominance of vertical transmission in cattle (French *et al*. 1999) and the still ambiguous role of the definitive host in the natural life-cycle of the parasite.

Further analysis of samples from both dogs and cattle are required before the population structure of the parasite can be determined with any certainty. Only then will hypotheses regarding the frequency of recombination and the implicit need for a sexual cycle be testable. Analysis of field isolates from defined outbreaks is essential since laboratory isolates are unlikely to be fully representative. We are currently generating PCR-based molecular markers for use on clinical samples to enable the rapid typing of individual field isolates and allow a thorough analysis of the population genetic structure of *Neospora* in both cattle and dogs. Such studies should yield valuable information not just about the epidemiological links between various hosts, but also provide crucial information on how genetic diversity might correlate with pathology and geographical distribution of the organism.

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