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The epidemiology of *Neospora caninum*

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UNIVERSITY
of
GLASGOW

A thesis submitted for the degree of Doctor of Philosophy

Division of Infection and Immunity
Institute of Biomedical and Life Sciences
The University of Glasgow

One main factor in the upward trend of animal life has been the power of wandering.

Alfred North Whitehead

Declaration

I declare that this thesis consists entirely of my own work, unless specifically indicated. This thesis has not been accepted in any previous application for a degree.

A handwritten signature in black ink, appearing to read 'Sophia Latham', with a stylized, cursive script.

Sophia Latham

Abstract

A seroepidemiological study was undertaken in a pedigree dairy herd that had a history of abortions due to neosporosis. The infection in this closed herd was thought to have arisen from a point-source infection, after which sporadic abortions have occurred. All cattle were bled twice, once in the winter and again the following summer and antibodies to *N. caninum* measured using an ELISA. The overall seroprevalence of *Neospora* was found to be 18 %. Three data sets; age-prevalence data, dam-daughter pair analysis and family tree data showed vertical transmission to be an important route of transmission of neosporosis in this herd. Analysis of anti-*Neospora* antibody titres with respect to the stage in the breeding cycle of cows appeared to show no association on a herd level. Data was collected on the number of Artificial Insemination (AI) services per successful pregnancy which showed a significantly greater number of AI services in *Neospora*-seropositive cattle compared with *Neospora*-seronegative cattle. This is the first study to assess the effect of neosporosis on cattle fertility in a quantitative manner and suggests that a wider study is justified. *N. caninum* shares many similarities with *T. gondii* and has widely been assumed also to have a world-wide distribution. Two regions of Africa, Ghana in West Africa and Tanzania in East Africa, were studied in a cross-sectional survey of neosporosis in cattle indigenous to these areas. A prevalence of 8.1 % and 2 % was found in two different areas in cattle native to Tanzania. Despite sampling a significant number of cattle in all three ecological zones of Ghana and of several different breeds, no *Neospora*-seropositive cattle were found. Possible reasons for the apparent absence of *N. caninum* in West Africa are discussed. To determine the overall genetic diversity in laboratory isolates of *N. caninum*, RAPD and AFLP methods were used. Genetic diversity was found to be low amongst *Neospora* laboratory isolates, relative to *T. gondii*, but demonstrated that genetic heterogeneity does exist within the species. Both RAPD and AFLP data were subjected to pair-wise similarity and cluster analysis and showed that there was no clustering with respect to host or geographical origin. The genetic similarity between cattle and dog isolates suggests that these hosts are epidemiologically related. In order to exploit the genetic heterogeneity in *N. caninum* to analyse a wider range of clinical field samples, several methods were attempted to devise PCR-based sequence-specific typing approaches that could be used on infected bovine tissue. Microsatellite markers were identified in

N. caninum DNA sequences, however none of the microsatellite regions gave rise to detectable size differences, although they remain to be tested on a wider range of field samples. Laboratory isolates of *N. caninum* were also analysed for polymorphisms with two conserved minisatellite probes, 33.6 and 33.15, but although hybridisation occurred to digested parasite DNA, identical fingerprints were obtained for each isolate. In a final attempt to identify sequence-specific polymorphic markers, intron regions from two genes, actin and tubulin, were amplified and sequenced in both laboratory and field isolates. This approach revealed a number of single nucleotide polymorphisms (SNPs) that were able to differentiate between some isolates of *N. caninum* and might serve as useful molecular markers. SNPs were found more frequently in the clinical field samples, suggesting that the diversity of *N. caninum* is greater than that represented by current laboratory isolates. Further genotyping of field samples will enable the genetic population structure of *N. caninum* to be determined to facilitate molecular epidemiological studies.

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I would like to thank Dr. Jonathan Wastling for providing the opportunity to work on *Neospora*, a parasite that has caused many problems on our farm. I very much appreciate all his hard work and for supervising this project with such enthusiasm and patience, and especially all his support at difficult times. Many thanks also to Dr Lee Innes for her continued support and encouragement during my PhD.

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I thank all collaborators for isolates of *N. caninum* and other Apicomplexa, without which the work would not have been possible. *Cryptosporidium parvum* sporozoites were kindly provided by Professor H.W. Smith (Scottish Parasite Diagnostic Laboratory, Glasgow). *Sarcocystis* sp. DNA was a gift from Mr. S. Wright (Moredun Research Institute, Edinburgh). The following generously supplied the isolates of *Neospora caninum*. Dr Camilla Bjorkman (National Veterinary Institute, Uppsala, Sweden) isolate NC-SweB1; Dr Pat Conrad (School of Veterinary Medicine, University of California)

isolate BPA-1; Prof. AJ Trees (Liverpool School of Tropical Medicine, United Kingdom) isolates NC-Liverpool and NC-LivB1; Prof. Milton McAllister (University of Wyoming, USA) isolate NC-Beef; Dr Jan Slapeta (David Axelrod Institute for Public Health, USA) isolate CZ-4. Thank you to Prof. David Sibley (University of Washington State, USA), Dr. Annette Macleod (Wellcome Centre for Molecular Parasitology, Glasgow), Dr David Barrett (University of Glasgow Vet school) and Prof. Martin Shirley (Institute of Animal Health, Compton) for useful discussions.

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I have been very lucky to have wonderful flatmates (who I now hope to see more of) and hope they forgive some antisocial hours. Thank you especially to Jo and Marie.

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Lastly, I am indebted to my parents and brother, Martin for unlimited access to the source of inspiration for this work and specifically for help in sampling and searching herd records, but mainly for their love and unquestioning support over the years.

To my parents

Abbreviations

A	amperes
Ab	antibody
AFLP	Amplified Fragment Length Polymorphism
Ag	antigen
AI	Artificial insemination
APS	ammonium persulphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
cm ³	cubed centimetre
Ci	curie
dddH ₂ O	double-distilled de-ionised water
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESTs	Expressed Sequence Tags
FCS	foetal calf serum
g	gram
GRA	granule antigen
h	hour
HEPES acid]	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic
HFFF	Human foetal foreskin fibroblast
IFAT	Immuno-flourescent antibody test
IGS	Intergenic spacer
IMDM	Iscoe's modified Dulbeccos medium
ITS	Internal transcribed spacer
kb	Kilobase(s)
kDa	kilodalton(s)
l	litre
LB	Luria-Bertani
LSU	Large subunit
m	milli
M	Molar
min.	minute

MLEE	Multi-Locus Enzyme Electrophoresis
MLST	Multi-Locus Sequence Typing
Mt	Mitochondria
<i>N. caninum</i>	<i>Neospora caninum</i>
NTS	Non-transcribed spacer
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PV	parasitophorous vacuole
RAPD	Random Amplified Polymorphic DNA
RBC	red blood cell
rDNA	ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
s	second
SAG	surface antigen
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
SSU	small subunit
T25	25 cm ³ tissue culture flask
T75	75 cm ³ tissue culture flask
TBE	Tris-borate-EDTA
TCA	trichloroacetic acid
TE	Tris-HCl EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
Tris	tris(hydroxymethyl)aminomethane
μ	micro
UV	ultraviolet
V	volts
W	watts
°C	degrees centigrade

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List of common reagents

Acrylamide PAGE solution, 40 % (PlusOne™)	Amersham Pharmacia Biotech, Bucks, U.K.
Agarose, Seakem LE	BMA, ME, USA
Ammonium bicarbonate	BDH, Poole, U.K.
Ammonium persulphate (PlusOne™)	Amersham Pharmacia Biotech, Bucks, U.K.
n-Butanol	BDH, Poole, U.K.
Calcium chloride	BDH, Poole, U.K.
Ethanol (Anal ^R)	Bamford Laboratories, Rochdale, U.K.
Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific, Leics, U.K.
Foetal Calf Serum	Labtech, Sussex, U.K.
Formaldehyde	BDH, Poole, U.K.
Glycerol, 87 % (PlusOne™)	Amersham Pharmacia Biotech, Bucks, U.K.
HEPES	Fisher Scientific, Leics, U.K.
Iscove's modified Dulbeccos medium	Life Technologies, Paisley, U.K.
Magnesium chloride	Riedel de Haën, Germany
Magnesium sulphate	Sigma, Dorset, U.K.
Methanol (Anal ^R)	BDH, Poole, U.K.
Mineral oil (light)	Sigma, Dorset, U.K.
Penicillin G sodium / streptomycin sulphate (100 U/ml / 100 µg/ml)	Life Technologies, Paisley, U.K.
Phosphate Buffered Saline (tablets)	Sigma, Dorset, U.K.
Potassium chloride	BDH, Poole, U.K.
Sodium acetate	Sigma, Dorset, U.K.
Sodium chloride	Riedel de Haën, Germany
Sodium dodecyl sulphate (PlusOne™)	Amersham Pharmacia Biotech, Bucks, U.K.
Sodium hydrogen carbonate	Riedel de Haën, Germany
Sodium hydroxide	Riedel de Haën, Germany
Sodium thiosulphate	Sigma, Dorset, U.K.
TEMED (PlusOne™)	Amersham Pharmacia Biotech, Bucks, U.K.
Tris base (PlusOne™)	Amersham Pharmacia Biotech, Bucks, U.K.
Trypsin-EDTA (1x)	Life Technologies, Paisley, U.K.
Trypsin, sequence grade (modified)	Promega, Southampton, U.K.
Uracil	Sigma, Dorset, U.K.
Versine	Media Services, University of Glasgow, U.K.

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Chapter 1

1. 1 Introduction

Neospora caninum (Dubey *et al.*, 1988a) is an intracellular coccidian parasite that was first identified as a distinct organism in 1984 (Bjerkas *et al.*, 1984) and isolated into tissue culture in 1988 (Dubey *et al.*, 1988b). It is recognised as a major cause of abortion and congenital defects in cattle and dogs worldwide. Neosporosis is an important cause of abortion epidemics in cattle, but can also be responsible for endemic abortion problems. Vertical transplacental infection in cattle is an important transmission route for the parasite but it has also been shown that dogs can act as definitive hosts by the excretion of oocysts (McAllister *et al.*, 1998) . The extent to which this occurs naturally and the existence of other sylvatic hosts is unknown and many aspects of this disease, including basic biology, epidemiology and transmission routes remain unclear.

N. caninum was first reported as a cause of abortion in British cattle in 1994 (Trees *et al.*, 1994). The parasite has a wide host range and can cause abortion and congenital defects in cattle (Thilsted and Dubey, 1989), goats (Dubey *et al.*, 1992) horses (Dubey and Porterfield, 1990) and dogs (Dubey *et al.*, 1988b). It has been implicated in 43 % of bovine abortions in California, USA (Thurmond *et al.*, 1995), 16 % in Scotland (Buxton *et al.*, 1997a) and 12 % of bovine abortion in England and Wales (Trees *et al.*, 1999). The parasite has a wide geographical distribution and has been reported to be associated with bovine abortions in many countries.

It is difficult to quantify the economic losses involved due to neosporosis, but they are thought to be considerable (Trees *et al.*, 1999). Ellis *et al.* estimated that neosporosis infection cost the Australian dairy industry about Aus\$85 million per year (reviewed in Trees *et al.*, 1999). The main cost of natural infection with *Neospora* is loss of income to the farmer through abortion, lower milk yield, production of weak calves and the loss of highly valued genetic lines from pedigree herds. One of the difficulties with determining the impact of neosporosis is the lack of quantitative data on the reduction in cattle fertility caused by the infection.

Currently there are no clinically proven vaccines or drugs to treat or prevent neosporosis. Many infected dams produce congenitally infected calves, which are often clinically normal and may provide a reservoir of infection within the herd, although *Neospora* can cause repeat abortion in some animals. Accurate diagnosis of infected individuals to be culled or treated is extremely important for control of this disease. Diagnosis of neosporosis is largely achieved using the indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA), which detect *Neospora* specific antibody in sera from infected animals and by histological examination of the aborted foetus and placenta.

1.2 Diagnosis of *N. caninum* infection

It is important to be able to identify animals infected with *Neospora* and several diagnostic techniques are currently in use. As *N. caninum* tachyzoites, present in smears of cells taken from cerebro-spinal fluid (CSF), bronchial lavage and dermal sores, are not distinguishable morphologically from *T. gondii* tachyzoites by light

microscopy, more specific tests are essential for accurate diagnosis. Positive serology results are only indicative of exposure to *N. caninum*.

1.2.1 ELISA

Enzyme linked immunosorbant assays (ELISA) can provide consistent, objective and rapid results (Crowther, 1998), often with increased sensitivity and specificity when compared to IFAT (Frossling *et al.*, 2003). ELISA kits are currently commercially available for detection of *Neospora*-specific antibody in cattle (Williams *et al.*, 1997). The kit developed by Williams *et al.* (1997) used whole tachyzoites and very little cross reaction was observed with sera from cattle experimentally infected with other closely related coccidian parasites. Several ELISAs have been developed using sonicated tachyzoites of bovine and canine *Neospora* tachyzoites as antigen (Pare *et al.*, 1995; Osawa *et al.*, 1998). Incorporation of a range of antigens may overcome the potential problem of antigenic diversity of *Neospora* isolates giving greater sensitivity (Pare *et al.*, 1995).

More specific ELISAs using extracted tachyzoite proteins of *N. caninum* incorporated into immunostimulating complexes have been developed by Bjorkman *et al.* for diagnosis in dogs (1994) and cattle (1997). The major antigens incorporated into iscoms are amphipathic molecules such as membrane proteins (Lovgren *et al.*, 1987). The immunodominant antigens of *N. caninum* identified by Western blot are located in rhoptries, dense granules, micronemes and the parasitophorous vacuole (Bjerkas *et al.*, 1984; Barta and Dubey, 1992).

Bjerkas *et al.* (1984) showed consistent recognition of several dominant antigens of *Neospora* NC1 dog isolate with immune sera from domestic and wild animals, therefore a dog isolate can be used for diagnosis of natural infection in cattle.

An IgG avidity iscom ELISA has been developed that detects specific bovine antibodies (Bjorkman *et al.*, 1999). This diagnostic method is able to distinguish between recent and chronic *N. caninum* infections and has provided further evidence that endemic *N. caninum*-associated abortion predominantly affects herds where *N. caninum* is transmitted vertically. Similarly, abortion storms, presenting as an epidemic outbreak of abortions in a herd, tend to be associated with a recent infection that could be more indicative of a point-source infection.

In addition, the P-38 ELISA has been modified to examine bulk milk samples that can be used to assess regional prevalence of *N. caninum* (Schaes *et al.*, 2000). It was tested in the Rhineland-Palatinate state in Germany where the bulk milk sampling gave an overall prevalence of 10 %.

1.2.2 IFAT

The indirect fluorescent antibody test (IFAT) was the first diagnostic test developed for the detection of *N. caninum* infection and measures the *Neospora*-specific antibody titre in serum of infected animals (Conrad *et al.*, 1993b). This test is not accurate enough for diagnosis of individual cases, as clinically normal animals have been found to have titres greater than 1:800 (Cole *et al.*, 1993), but it can be used to give an indication of herd status. Antibody titres in infected animals can also decrease significantly over a prolonged period of time (Conrad *et al.*, 1993b). As whole *Neospora* tachyzoites are used in this test there is also some possibility of cross-reaction with antibody to closely related coccidian parasites. In all cases, care must be taken in the interpretation of serology results, as it is not possible to predict the relationship between a high antibody titre and the outcome of pregnancy.

1.2.3 Latex agglutination Test

A latex agglutination test (LAT) has been developed that can detect antibodies to *N. caninum* in serum from dogs, cattle and other species (Fitzpatrick *et al.*, 2000). It has the advantage that cross-reactivity to other coccidians, for example *T. gondii*, that can sometimes be a problem with IFAT/ELISA, is minimised.

1.2.4 Histology

It is possible for cattle that have previously aborted due to neosporosis to become antibody negative. Therefore, diagnosis should not be based on IFAT or ELISA results alone, but also on detection of the parasite in the tissues of the animals and the pathology of the disease in the aborted foetus. Diagnosis is usually confirmed where possible using immunohistochemical techniques to examine paraffin embedded tissue sections taken from the aborted foetus for the presence of *Neospora* tachyzoites (Barr *et al.*, 1994b). However, it should be noted that polyclonal *Neospora* serum from experimentally infected rabbits can occasionally cross react weakly with *T. gondii* antigen (Bjerkas *et al.*, 1994), cross reactions have occurred at dilutions of less than 1:50 (Cole *et al.*, 1993), and the fact that each laboratory produces its own polyclonal serum which may also cause a variation in results.

1.2.5 Polymerase chain reaction

As previously discussed, serology based on diagnostic IFAT and ELISA tests is not accurate enough for diagnosis of neosporosis in individual animals. Definitive diagnosis depends on the identification of parasite antigen in aborted foetal tissues,

amniotic fluid or blood. One method used to identify parasite antigen is by amplification of parasite-specific DNA in host tissues using the polymerase chain reaction (PCR) (Ho *et al.*, 1996; Holmdahl and Mattsson, 1996; Slapeta *et al.*, 2002b). PCR is a sensitive and specific method for the detection of *Neospora* parasites, but as *N. caninum* tachyzoites and tissue cysts are found infrequently in maternal and foetal tissues, the availability of suitable tissue could greatly affect diagnosis using this method. PCR should therefore be used as a supplementary method of diagnosis for confirming *Neospora* infection in a herd.

1.3 Epidemiology of *N. caninum*

1.3.1 Bovine neosporosis

As early as 1985 it was becoming clear that some previously unknown *Toxoplasma*-like infectious agent was responsible for numerous abortions in Californian dairy cattle (Ho *et al.*, 1996). A retrospective study begun in 1988 indicated the disease was the largest single identifiable cause of bovine abortion in Californian cattle (Anderson *et al.*, 1995). Development of an immunohistochemical test to identify *Neospora* organisms in tissues associated with these lesions (Dubey *et al.*, 1989a) led to the discovery of the parasite in aborted bovine foetuses.

Bovine neosporosis has since been reported in Argentina (Campero *et al.*, 1998), Australia (Boulton *et al.*, 1995), Brazil (Corbellini *et al.*, 2002), Canada (Duivenvoorden and Lusi, 1995), Costa Rica (Romero *et al.*, 2002), Ireland (Collery, 1995), Japan (Yamane *et al.*, 1997), Korea (Kim *et al.*, 2002), Mexico (Morales *et al.*, 2001), New Zealand (Thornton *et al.*, 1991), Sweden (Stenlund *et*

al., 1997), Tanzania (unpublished pers. obs, 1999), Thailand (Chanlun *et al.*, 2002), UK (Otter *et al.*, 1993) and the USA (Thurmond *et al.*, 1995).

Surveys of bovine neosporosis using IFAT (Conrad *et al.*, 1993b) and ELISA (Pare *et al.*, 1995) for antibody to *Neospora* in sera linked the disease to high rates of bovine abortion in Scotland, 16 % (Buxton *et al.*, 1997a), England and Wales, 4-10.5 % (Otter *et al.*, 1995) and California, 43 % (Thurmond *et al.*, 1995).

Vertical transmission was found to occur in naturally infected cattle with an efficiency of about 95 % (Conrad *et al.*, 1993b; Anderson *et al.*, 1997). Abortion due to *N. caninum* infection can occur repeatedly in some animals which is in contrast to the closely related protozoan *T. gondii*, which stimulates life-long immunity in the host following primary infection (Buxton *et al.*, 1997a). Transplacental transmission of *N. caninum* has been experimentally reproduced in cattle (Barr *et al.*, 1994b) providing a model for the study of infection in the natural host. The rate of repeat abortion is not yet known but has been estimated at approximately 5 % of cattle (Dubey *et al.*, 1996a). This is a particular problem in pedigree herds where a family line may be severely affected, or indeed lost. A number of risk factors have been identified for bovine neosporosis. Bartels *et al.* (1999) showed that the presence of dogs on a farm was the most important risk factor for the occurrence of *N. caninum*-associated abortions. In a study on Dutch dairy farms with an endemic *Neospora* problem, it was found that introducing a naïve dog was a risk factor for future point source infections (Dijkstra *et al.*, 2002)

1.3.2 Transmission in cattle

Abortion is defined as the expulsion of the foetus between 42 days after conception and approximately 260 days after gestation. Before day 42, embryonic death generally leads to resorption, or expulsion with no clinical signs in the cow.

Expulsion of the foetus after 260 days is regarded as early parturition. The definition of 'stillborn' is used if the calf is dead at birth or dies within 24 hours.

Neosporosis has been found in both beef and dairy cattle, but has been reported more frequently in dairy cattle (Woods *et al.*, 1994). Harvested feeds stored on or around the farm, often open to faecal contamination by farm dogs may be a source of infection by the oocyst stage of the parasite. If infected feed is then fed to the entire herd, this may explain why neosporosis induces clusters of abortions or 'abortion storms' (Anderson *et al.*, 1991; Dannatt *et al.*, 1995). Both of these factors would be conducive to the spread of disease among a large number of animals. Despite the efficiency of transplacental transmission of *N. caninum* in pregnant cows, it is apparent that post-natal routes of infection are essential to maintain parasite prevalence (French *et al.*, 1999). Interestingly, Innes and colleagues (2001) have recently demonstrated that it is possible to protect against vertical transmission in an experimental infection. They found that cows which had been inoculated with tachyzoites six weeks before mating and then challenged with *Neospora caninum* mid-gestation, gave birth to normal calves with no antibodies to *Neospora caninum*. In contrast, cows that were only challenged at mid-gestation had calves with high levels of specific antibody to *N. caninum* (Innes *et al.*, 2001). These results are encouraging and suggest it might be possible to develop a vaccine for cattle.

Basic epidemiological information on seasonal patterns of neosporosis may help studies identifying events that may contribute to transmission of disease or trigger abortion in infected cows. A study by Thurmond *et al.* (1995) of dairy cattle over a 6-year period indicated that there does seem to be an increased risk of abortion due to *Neospora* in the months of November, December, January and February. But as there is only 16 % difference between expected and observed figures, a substantial number of abortions may not be influenced by seasonally related factors.

Neospora was confirmed as a cause of abortion in cattle when Barr *et al.* (1994b) reproduced foetal infection and death by experimentally infecting pregnant cattle with a bovine isolate, BPA1, and re-isolating parasite from foetal tissues. Infection was found to be similar to that caused by natural infection as it did not cause clinical symptoms in the dams and immunohistochemical analysis of foetal neural tissue revealed clusters of tachyzoites, which reacted, positively to anti-*Neospora* sera. As with the closely related coccidian parasite *T. gondii*, the time of infection and therefore the immune competence of the foetus may be important in determining the outcome of infection. A mummified foetus was expelled at 67 days post infection from a dam infected at 95 days gestation. A second animal infected at 115 days gestation, when some foetal immune competence would have been attained, carried a clinically normal calf to full-term. This calf had raised pre-colostral antibody titre to *Neospora* and mild encephalomyelitis, although no parasites were found in tissues.

Neosporosis has also been reported to cause repeat infection of foetuses by the same dam. This was first reported in a study by Barr *et al.* (1993) in which four dairy cows were followed that had previously aborted due to neosporosis. All of the cows were

kept on their respective dairies, successfully re-bred, were monitored throughout pregnancy and all produced full-term calves. Two of the calves had marked neurological defects including decreased patellar reflexes and ataxia. Both were found to have mild non-suppurative encephalomyelitis and thick walled protozoal cysts were found randomly distributed in brain tissue and spinal cord. Tachyzoites and tissue cysts were found in the CNS of one other calf, although it was clinically normal. This contrasts with the closely related protozoan parasite *T. gondii* which stimulates a strong immune response in the host that provides life-long protection against repeat abortion. It has not been determined whether repeat foetal infection is due to recrudescence of infection or re-infection in the dam, but as repeat congenital infection is not characterised by widespread infection in other previously uninfected animals on the farms, the former is likely to be true.

A large scale study by Pare *et al.* (1996), which lasted over 2 years and monitored over 400 calves born on 2 dairies, showed that although a majority of seropositive cows (81 %) produced infected offspring, 5 % of seronegative cows also produced congenitally infected calves. Therefore the serological status of the dam will not always predict infection in the calf. Congenital infection was not associated with dam age, lactation number, history of abortion calf gender or length of gestation. High dam *Neospora* antibody levels at calving were significantly associated with the probability of congenital infection in the calf. From this study it was concluded that congenital transmission was likely to be the major mode of transmission of *N. caninum*. Ugglå and co-workers showed that calves may be infected orally by tachyzoites being added to milk (Ugglå *et al.*, 1998). Davison and colleagues also

found that calves could be infected lactogenically (Davison *et al.*, 2001). This would suggest that pooling of milk to feed a number of calves could be a method of transmitting the disease horizontally.

Maley and co-workers investigated the early pathogenesis of neosporosis in experimentally infected cattle and found that foetal death or survival depended on a number of factors: timing of infection, host stress factors (immunosuppression) and intensive management stress factors (Maley *et al.*, 2001). In addition, differences in the virulence of parasite isolates may also affect the outcome of infection supporting a strategy of identifying molecular markers for individual *Neospora* isolates. In addition, molecular markers should also help to elucidate the importance of particular transmission routes of the parasite.

1.3.3 Canine neosporosis

Neospora caninum was first described in dogs in 1984 by Bjerkas and colleagues who reported neurological disorders 2-6 months after birth in five of six boxer pups which were all offspring of the same bitch (Bjerkas *et al.*, 1984). Post mortem examination of all six animals identified necrosis and lesions in the central nervous system and skeletal muscles associated with cysts containing a parasite. Under light microscopy the parasites present in these lesions resembled *T. gondii*, but sera taken from the infected animals lacked specific antibodies to *Toxoplasma*. A further study by Dubey *et al.* (1988a) reviewed tissues from 23 cases of *Toxoplasma*-like illness in dogs and again found parasites associated with lesions in the CNS which did not cross react with anti-*T. gondii* antibody positive serum.

In 1988, the parasite associated with these lesions was isolated into tissue culture and mice for the first time by Dubey and co-workers who proposed the name, *Neospora caninum*, for the newly discovered parasite (Dubey *et al.*, 1988b).

Dubey *et al.* (1988b) isolated *N. caninum* from homogenates of canine tissues into tissue culture and experimental inoculation reproduced the disease in a dog. Dubey also suggested that *N. caninum* was congenitally transmitted in dogs after observing infection in two successive litters from the same bitch indicating reactivation of sub-clinical infection. Vertical transmission in dogs was observed in 1990 (Dubey *et al.*, 1990) by a case study in which successive litters of German shorthaired pointers from the same bitch developed limb paralysis. The other eight littermates of this bitch all had hind limb paralysis and died before six months of age. Four of her pups were found to have *N. caninum* tachyzoites, tissue cysts or both present in extra-ocular muscles. Transplacental transmission was later experimentally induced in dogs by Cole *et al.* (1995). Inoculation of tachyzoites on day 21 of gestation induced abortion in five pregnant bitches, and full-term pups from another bitch were born with clinical symptoms of neosporosis including proprioception deficits and spasticity in pelvic limbs.

1.3.4 Seroprevalence in dogs

There have been very few surveys on the incidence of neosporosis or *Neospora* antibody in the dog population and there is some debate over what level of antibody is considered positive for neosporosis in dogs. A study by Trees *et al.* (1993) showed 13 % of 163 randomly selected dogs tested at the Liverpool Small Animal Hospital had antibody titres of >1:200, with no correlation between antibody titre and

breed, sex, age, type of feeding or presence of other dogs in the household. Again there was no association between *Toxoplasma* and *Neospora* antibody in these samples, suggesting minimal serological cross reactivity between these parasites. None of the animals tested in the British study had clinical symptoms of neosporosis. A study in Kansas by Dubey and Lindsay (1990a) found only 2.2 % of dogs had titres > 1:50, and a small-scale survey of feral coyotes in Texas found 10 % had low antibody titres to *N. caninum*. A retrospective analysis of histopathology cases has however shown that *N. caninum* infection in dogs has occurred since 1957 in the USA (Dubey and Lindsay, 1996).

The dog has been identified as a definitive host of *N. caninum* (McAllister *et al.*, 1998). Oocysts morphologically similar to *T. gondii*, *Hammondia hammondi* and *Hammondia heydorni* were found in the faeces of three beagles fed mouse tissue infected with *N. caninum* tissue cysts. Mice had been inoculated with the canine NC2 (Hay *et al.*, 1990) and NC Liv (Barber *et al.*, 1995) and bovine NC Beef (McAllister *et al.*, 1998) strains of the parasite. *Neospora* tachyzoites were isolated from mice orally inoculated with infected faeces confirming that the oocysts were infective and caused clinical neosporosis.

The first report of oocysts being shed by a naturally infected dog was reported in Argentina (Basso *et al.*, 2001). Slapeta and co-workers also isolated and characterised oocysts from the faeces of a naturally infected 1-year old German Shepherd from Central Bohemia in Czech Republic (Slapeta *et al.* 2002b). PCR amplification of the NC-5 gene that is specific to *N. caninum* was successful and sequencing of the ITS1 rDNA and the D2 domain of the large subunit rDNA (D2

LSU) determined the isolate as *N. caninum*. This isolate from Czech Republic was named CZ-4.

These findings clearly have implications for the development of disease control strategies and further studies on the survival of oocysts, levels of contamination on pasture and shedding of oocysts by infected dogs will be important in the development of these strategies.

1.3.5 Transmission in dogs

Congenital transmission of *N. caninum* has been shown to occur in naturally infected puppies (Dubey *et al.*, 1990; Barber and Trees, 1998; Dubey *et al.*, 1998) and typically the first clinical signs appear 5-8 weeks after birth. Neosporosis is therefore often thought of as a disease of puppies although infection has been reported in dogs of all ages (Barber *et al.*, 1996). The disease may affect several littermates but more often than not involves an isolated case (Barber *et al.*, 1996). Transplacental transmission may also occur repeatedly in litters from the same bitch (Dubey *et al.*, 1990; Barber and Trees 1998). A serological study by Barber and Trees (1998) reports that the transmission rate to pups is low, as 80 % of pups born to seropositive dams were not infected. The frequency of vertical transmission was also found to vary between litters and may be too low to sustain infection alone. These findings suggest that postnatal infection must occur to maintain infection at seroprevalence rates reported in dog populations.

Vertical transmission of *N. caninum* has been reproduced in a canine model of infection by inoculation with NC1 tachyzoites (5×10^6) on day 21 of pregnancy (Cole

et al., 1995). Of six bitches infected, five aborted and *N. caninum* tachyzoites were isolated from pups in two of the aborted litters and from four infected bitches. A previous study by Lindsay and Dubey (1989a) reported that experimental infection on day 35 of pregnancy caused only a mild illness, although this may have been due to a lower dose (1.5×10^6) of tachyzoites. Experimental infection with *N. caninum* during pregnancy can therefore lead to foetal death, but whether *N. caninum* causes foetal death, abortion and sterility in naturally infected dogs is as yet unknown.

1.3.6 Neosporosis in horses

Neospora infections have been reported from an aborted foal, a congenitally infected foal and a 10-year old horse (Dubey and Porterfield, 1990; Lindsay *et al.*, 1996b; Gray *et al.*, 1996). *Neospora hughesi* was described and isolated for the first time from an 11-year old horse by Marsh and colleagues in 1998 (Marsh *et al.*, 1998). It was distinguished from *N. caninum* based on seven nucleotide differences in the ITS-1 sequences. No differences were found between the small subunit ribosomal RNA gene from the canine, bovine and equine isolates of *N. caninum*. *N. hughesi* tissue cysts and bradyzoites were smaller than those of *N. caninum* and also had a thinner cyst wall. However, only a few examples are available for comparison so these observed differences need to be confirmed. It is still unknown whether both *Neospora* species infect horses or if *N. hughesi* infections have previously been misdiagnosed as *N. caninum*. A study by Dubey and co-workers found that 21 % of 296 horses slaughtered in the United States were found to have antibodies to *N. caninum* (Dubey *et al.*, 1999). Cheadle and colleagues found that 11.5 % of horses (n=536) in Alabama, USA had positive IFAT titres to *Neospora* (Cheadle *et al.*, 1999a). A study by Walsh *et al.*, showed a marked difference in pathogenicity

between *N. caninum* (NC-1 and NC-Liverpool) and *N. hughesi* (Nh-A1 isolate) in gamma-IFNKO mice (Walsh *et al.*, 2000).

However, Mehlhorn and Heydorn (2000) suggested that these differences were too small to warrant a separate species. This is part of a much wider debate over the phylogeny of *Neospora*, *Toxoplasma*, *Isospora* and *Hammondia* and currently remains a matter of some controversy.

1.3.7 Neosporosis in other domestic animals

Natural infection with *N. caninum* has been reported in a wide range of animals and can cause abortion in goats (Barr *et al.*, 1992; Dubey *et al.*, 1992). *Neospora* has also been diagnosed in a full-term still-born deer, (Dubey *et al.*, 1996b).

Transplacental transmission has been experimentally induced in sheep (Dubey and Lindsay, 1990b; McAllister *et al.*, 1996, Buxton *et al.*, 1997c). However, a survey by Otter *et al.* (1997) did not find antibodies to *Neospora* in tissue samples from 281 aborted ovine foetuses submitted to veterinary investigation centres around England and Wales suggesting *Neospora* is not a major cause of abortion in sheep.

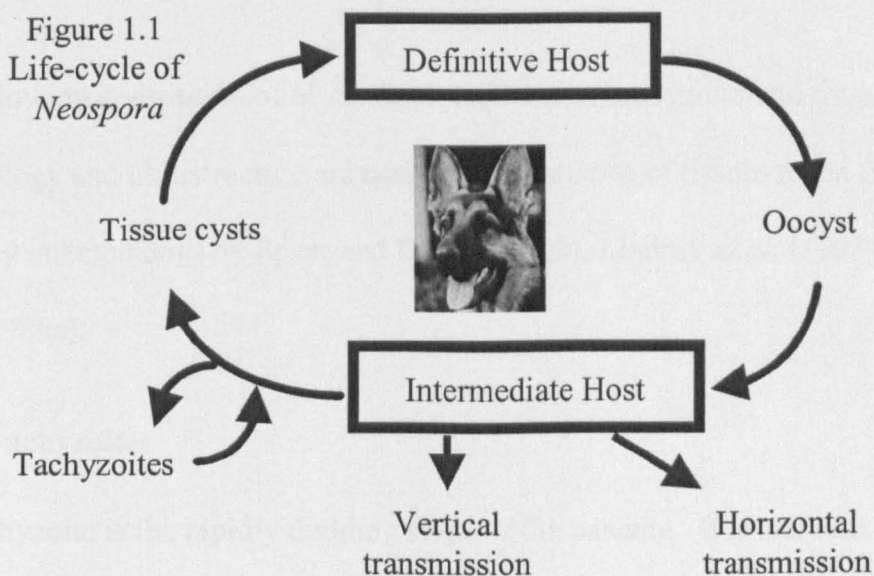
Transplacental transmission has been experimentally induced in cats (Dubey and Lindsay, 1989) but there have been no reports of the disease occurring naturally in this species. Further studies are needed, as the full host range for *N. caninum* is not yet known.

1.3.8 Neosporosis in Humans

Neosporosis has not been reported in humans, but no large-scale surveys have been undertaken. Experimentally infected primates (rhesus macaques) were susceptible to transplacental *Neospora* infection (Barr *et al.*, 1994a) and foetal lesions induced were found to be similar to those caused by transplacental *Toxoplasma* infections in primates. Infection was confirmed by re-isolation of the parasite *in vitro*.

1.4 Life-cycle of *N. caninum*

The complete life cycle of *N. caninum* has been demonstrated experimentally (McAllister *et al.*, 1998). This is shown in Figure 1.1.



Experimental infections, using tachyzoites, of other carnivorous potential hosts including coyotes (Lindsay *et al.*, 1996a) and racoons (Dubey *et al.*, 1993) have so far been unsuccessful in producing oocysts in the faeces of these species, but these studies have used only a maximum of 3 animals each. Infected coyotes and racoons did however sero-convert after infection. Another study to evaluate raptorial birds as

the definitive host for *N. caninum* (Baker *et al.*, 1995) used 2-3, mostly adult, birds of 4 different species which had been living in the wild before being submitted to public or wildlife agencies. No oocysts were found in the faeces of these animals.

1.5 Life-cycle stages of *N. caninum*

Several *Neospora* isolates have been grown in tissue culture from naturally infected bovine and canine tissues. A study by Jardine (1996) looked at the ultrastructure of bradyzoites and tissue cysts of these isolates and concluded that there are no distinct morphological differences between bovine and canine derived parasites at each stage of the parasite life cycle.

The following description of *N. caninum* tachyzoite, bradyzoite and tissue cyst morphology and ultrastructure are derived from studies of tissues taken from naturally infected dogs by Speer and Dubey (1989), Lindsay *et al.* (1993) and Dubey *et al.* (1988a).

1.5.1 Tachyzoites

The tachyzoite is the rapidly dividing stage of the parasite. It is crescent shaped, 6 x 2 µm in size with a single vesicular nucleus and has all the ultrastructural features that are characteristic of other closely related cyst forming coccidia. This stage in the life-cycle of the parasite replicates by endodyogeny and has a pellicle consisting of a plasmalemma and a single inner membrane. Twenty-two subpellicular microtubules were observed beneath the inner membrane complex and 2 elongated-branched tubular mitochondria were present, 1 anterior and 1 posterior to the nucleus.

Tachyzoites have several unusual characteristics including 8-12 anterior rhoptries,

electron-dense posterior rhoptries, many anterior and few posterior micronemes and lack micropores. As many as 50-100 tachyzoites may occur free or within a parasitophorous vacuole within a range of host cells including macrophages, neutrophils, neural cells, hepatocytes, fibroblasts and blood vascular endothelial cells. Tachyzoites of *N. caninum* are virtually indistinguishable from those of *T. gondii* by light microscopy.

1.5.2 Tissue cysts and bradyzoites

Tissue cysts are up to 107 μm long and are found in the CNS including the retina of naturally infected cattle, dogs and other infected hosts. The cyst wall is 1-4 μm thick, slightly thicker than that of *T. gondii*, and consists of a single primary wall which completely surrounds the cysts, and a thicker granular layer containing electron dense vesicles embedded in a matrix of fine granules. In contrast the primary wall of the *T. gondii* cyst is highly convoluted and does not have an electron dense matrix.

N. caninum bradyzoites found inside tissue cysts are approximately 7.3 x 1.5 μm in size. In addition to organelles and inclusion bodies typical of other coccidian parasites they contain numerous micronemes and amylopectin granules, and 6-12 anterior rhoptries. They are therefore similar in size and ultrastructure to those of *T. gondii*.

1.5.3 Oocysts

Oocyst shedding has been observed in naturally infected dogs in Argentina (Basso *et al.*, 2001) and the Czech Republic (Slapeta *et al.*, 2002b). The oocysts were colourless, almost spherical, 11.5 (10-13) x 10.8 (10-11) μm , with a thin (<1 μm)

single-layered oocyst wall. They contained two broadly oval tetrazoic sporocysts, 9.7 (9-10) x 6.6 (6-7) μm in size. A total of 10^6 oocysts were recovered using the sugar-concentration technique (Slapeta *et al.*, 2002). *N. caninum* oocysts have been identified in faeces from dogs fed *N. caninum* tissue cysts present in neural tissue of mice experimentally infected with NC2 (Hay *et al.*, 1990), NC Liv (Barber *et al.*, 1995) and NC Beef strains of the parasite (McAllister *et al.*, 1998). Oocysts experimentally produced were reported as unsporulated, spherical to sub-spherical in shape, measured 10-11 μm in diameter and contained a central sporont. The oocysts sporulated within 3 days, contained 2 sporocysts, each with 4 sporozoites, and were morphologically similar to *T. gondii*, *Hammondia hammondi* in cat faeces and *Hammondia heydorni* in dog faeces. At present, nothing is known about the frequency of shedding, their survival and if other canids are capable of acting as definitive hosts.

1.6 Isolation of *N. caninum* from infected tissues

The first successful isolation of *N. caninum* from cattle into tissue culture was by Conrad *et al.* (1993a). After several attempts in Sweden, *N. caninum* was successfully isolated from a stillborn calf, born to a *N. caninum* seropositive dam and the isolate was named NC-SweB1 (Stenlund *et al.*, 1997). The stillborn calf was delivered within 72 h of delivery and kept continuously at 2 °C. Parasitic growth was detected in the cell culture flasks after 56 days after the inoculation. This was longer than the 15-34 days observed by Conrad *et al.* (1993a) and NC-SweB1 has continued to exhibit slow growth compared to other isolates (Schock *et al.*, 2001). One reason for the long time period is the low numbers of parasite present in the

brains of infected animals. Autolysis is likely to further diminish the number of viable parasites.

1.7 Ribosomal DNA analysis of *Neospora* spp. and other Apicomplexa

There are currently about fifteen bovine and canine isolates of *N. caninum* used in studies around the world. Marsh *et al.* (1995) compared the nuclear small subunit ribosomal (nss-r) RNA sequences of bovine and canine *Neospora* isolates to that of *T. gondii*, *Cryptosporidium parvum* and *Sarcocystis muris*, three closely related coccidians. Although this sequence of approximately 400 nucleotides of the 5' region has been identified as being evolutionarily unstable, no differences were found between 4 different bovine isolates, BPA 1-4, isolated from aborted foetuses and congenitally infected calves.

Holmdahl and Mattsson (1996) also compared the internal transcribed spacer 1 (ITS-1) sequence of a bovine *Neospora* strain isolated from a stillborn calf in Sweden, named NC-SweB1 (Stenlund *et al.* 1997), with *N. caninum* NC1 canine isolate. This sequence is considered to be conserved within species, but to be more variable than individual rRNA genes between species. No differences were recorded between NC-SweB1 and NC1 isolates, in contrast to a large number of sequence differences between *N. caninum* and *T. gondii*. Comparison of the ITS-1 region of *N. caninum* NC1 and NC-Liverpool by Barber *et al.* (1995) also detected no differences in this region.

Slapeta *et al.* (2002a) sequenced the ITS-1 rDNA region and the D2 domain of the large subunit rDNA (D2 LSU) regions of *N. caninum* (isolate CZ-4) to distinguish between *N. caninum* and *H. heydorni*. Both regions have been used successfully to

distinguish between closely related coccidian genera, such as *Hammondia*, *Toxoplasma* and *Neospora* spp. (Ellis *et al.*, 1998; Mugridge *et al.*, 1999). The ITS-1 region has higher variability so is the preferred sequence for detailed species or isolate detection as even *N. caninum* and *N. hughesi* differ in this region (Dubey *et al.*, 2002). In contrast, D2 LSU rDNA is too conservative to distinguish between both *Neospora* species.

1.8 Taxonomic and phylogeny of *N. caninum*

The genus *Neospora* is positioned in the family Sarcocystidae as follows (Levine *et al.*, 1980; Marsh *et al.*, 1995; Holmdahl *et al.*, 1996; Mugridge *et al.*, 1999).

Superkingdom: Eukaryota

Phylum: Apicomplexa

Class: Coccida

Order: Eimeriida

Family: Sarcocystidae

Genus: *Neospora*

Species: *N. caninum*

N. hughesi

This taxonomic classification of *N. caninum* is open to some debate and is discussed further. Sequence analysis of the ss-rRNA gene showed only a few nucleotide differences between *N. caninum* and *T. gondii* (Ellis *et al.*, 1994; Luton *et al.*, 1995). Twenty-two percent nucleotide diversity was found between the two phyla when the ITS-1 region was compared (Homan *et al.*, 1997). Mugridge *et al.* (1999) used the full-length Large Subunit ribosomal DNA to show that *H. heydorni* is more closely

related to *N. caninum* than *T. gondii*. Mehlhorn and Heydorn (2000) believe there are only two valid species: *T. gondii* (including *H. hammondi* as a non-virulent strain) and the other being *T. heydorni* (including *N. caninum*). These conclusions are based on microscopy studies looking at the morphology of the parasites. This is refuted by a number of workers (Frenkel and Dubey, 2000) and Ellis *et al.* (1998) found that two species-specific primers for *N. caninum* failed to amplify any products with *H. heydorni*. In addition, Slapeta and co-workers, (2002b) found that *N. caninum* species-specific primers based on the NC-5 region produced a positive result when used with isolate CZ-4, which was isolated from a naturally infected dog, while primers specific to *H. heydorni* rDNA ITS-1 were negative. Three previously identified isolates, CZ-1, CZ-2 and CZ-3, also recovered from dog faeces in the Czech Republic, had been determined as *H. heydorni*, based on their ITS-1rDNA sequences (Slapeta *et al.*, 2002b). This data, along with analyses by (Dubey *et al.*, 2002), suggest that three distinct clades exist, namely the *N. caninum*-clade (*N. caninum* and *N. hughesi*), the *H. heydorni*-clade (*H. heydorni*) and the *T. gondii* – clade (*T. gondii* and *H. hammondi*).

There is some lively discussion in the literature regarding the phylogeny of *N. caninum*. Heydorn and Mehlhorn (2002) concluded that *N. caninum* represented a *nomen nudum* following a review of the available literature. In contrast Dubey *et al.* (2002) proposed that *N. caninum* and *H. heydorni* are separate species. Molecular diagnostic methods provide a rapid way of discriminating at a fine level and have been extremely useful in attempting to resolve this particular question since the oocysts from *N. caninum* and *H. heydorni* are morphologically indistinguishable.

1.9 Control

1.9.1 Chemotherapy

Toltrazuril has been used successfully to prevent any clinical signs of infection in wt-C57BL/6 mice that were experimentally infected with *N. caninum*. It also lowered the antibody concentration in the serum of treated animals compared to the non-treated ones (Gottstein *et al.*, 2001). Ponazuril chemotherapy of experimentally infected calves was found to reduce the symptoms (e.g. fever), decreased the humoral immune response and prevented lesion formation in the brain. These drugs appear to be acting against the tachyzoite stage of the parasite so it will be interesting to see if they have any efficacy against other parasite stages, particularly tissue cysts.

1.9.2 Vaccination

Several observations indicate that cattle may develop a certain level of protective immunity to *N. caninum*. Dijkstra *et al.* (2003) found that the proportion of congenital infections decreased with subsequent pregnancies from 80 % in heifers to 66 % in older cows, possibly due to an increased immunity to transplacental infection with increasing age of the dam. A similar phenomenon was seen in cows that had previously had a *N. caninum*-associated abortion. The risk of abortion in *N. caninum*-infected cattle was found to decrease with age in subsequent pregnancies (Thurmond and Hietala, 1997). However, protective immunity against *N. caninum* is less effective than with *T. gondii* in sheep, which if infected in pregnancy are resistant to new infections in subsequent pregnancies (Innes *et al.*, 2001). A polygenTM adjuvant-killed *N. caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following intravenous/intramuscular challenge with

tachyzoites (Andrianarivo *et al.*, 2000). In addition, the offspring of vertically infected dams were not protected after recrudescence of the infection in the dams. Also animals horizontally infected prior to mating gave birth to vertically infected offspring in field studies (Dijkstra *et al.*, 2002). However, seronegative cows experimentally infected before artificial insemination gave birth to seronegative offspring (Williams *et al.*, 2000; Innes *et al.*, 2001).

It is possible that intravenously or subcutaneously administered tachyzoites do not result in the development of bradyzoites in cattle, which are responsible for the recrudescence of infection in persistently infected animals (Williams *et al.*, 2000). Identification and characterisation of stage-specific (tachyzoites and bradyzoites) antigens may provide the answer. In one study, heifers infected before insemination and challenged at 20 weeks of gestation gave birth to seronegative offspring (Innes *et al.*, 2001).

Bovilis neoguard™ (Intervet International B.V.) has recently been licensed in the USA and claims to reduce abortion in cattle if administered in the first trimester of pregnancy. This vaccine consists of whole, killed *N. caninum* tachyzoite, however there is no published efficacy data to support these claims.

1.9.3 Farm management

Currently, the only methods of prevention and control that can be suggested to farmers and clinicians is to prevent domestic dogs (and any other animals) gaining access to feed stores in order to prevent potential oocyst contamination. In addition, placental material should be removed and destroyed after parturition and certainly

not fed to dogs as is the habit in some parts of the world. There has also been some speculation that horizontal transmission may occur by placentophagy by other cows but this is thought not to be a major transmission route (Schares and Conraths, 2001). Culling of infected individuals from herds has been recommended for some time, but this is obviously not possible if an abortion storm occurs when more than half the herd may be affected. If particularly valuable pedigree lines are in danger of being lost, or there is much difficulty in breeding from them, embryo transfer techniques can be used. It is also possible to breed high yielding cows, that have a history of *Neospora*-associated abortion, to beef breeds; thus maintaining milk production but stemming the introduction of infected individuals back into the herd.

1.10 Genetic Structure of *N. caninum*

The assumption is that, as with *T. gondii* and other Apicomplexa, there are three genomic components in *N. caninum*; the chromosomal genome as the major source of DNA, and two extrachromosomal DNA elements, the mitochondrial genome and the plastid genome (reviewed in Wilson and Williamson, 1997).

1.10.1 Chromosomal genome

No definitive account of the number of different chromosomes, genome size or number of genes that exist in *N. caninum* is available, but it is assumed that it closely resembles *T. gondii*. The chromosomal genome of *T. gondii* consists of at least 9 chromosomes and is estimated to be 80-90 Mb in size (Candolfi *et al.*, 1988; Sibley and Boothroyd, 1992). Parmley *et al.* (1994b) estimated that about 20, 000 genes exist in the genome of *Toxoplasma*. A few studies have been performed to characterise the chromosomes of *T. gondii*. Candolfi *et al.* (1988) isolated four

chromosomes from *T. gondii* using pulse field gel electrophoresis (PFGE). A major part of DNA was unresolved in this study, remaining at the top of the gel. In fact, PFGE has been performed on three different isolates of *N. caninum* during this study (results not shown) and a similar problem was encountered, despite five chromosomes being visualised; it is hoped to continue this work. Sibley and Boothroyd (1992) identified nine chromosomes sized between <2 Mb to 6 Mb (totalling about 40 Mb) during karyotype analysis using PFGE on *T. gondii*. The Guanine + Cytosine (G+C) content of *Toxoplasma* DNA is about 52 % and analysis of the available *Neospora* sequence data shows a similar G + C content in *N. caninum*.

1.10.2 Mitochondrial genome

Little is known of the mitochondrial genome of *N. caninum*. However, mitochondrial DNA sequences have regions with a high degree of diversity and are useful in phylogenetic analysis (Simon *et al.*, 1994) and thus could warrant future exploration.

1.10.3 Plastid genome

A large extrachromosomal DNA element of about 35 kb in size, was identified in the Apicomplexa and has been characterised during recent years. Gardner *et al.* (1991) found some sequence similarity of this genome and prokaryotic small subunit (SSU) rRNA genes. It was proposed to be a residual plastid genome of an ancient photosynthetic progenitor of this phylum (Wilson *et al.*, 1992). This extrachromosomal DNA was surrounded by a four membrane cryptic organelle and has been named 'apicoplast' (Kohler *et al.*, 1997). This apicoplast organelle seems to

have played a very important role in the biology of apicomplexan parasites including *T. gondii* and is considered to be a potential target for therapeutic agents (Soldati, 1999; Roos, 1999). Gleeson and Johnson (1999) used pulsed-field gel electrophoresis and Transmission Electron Microscopy (TEM) to show that the NC-Liverpool isolate of *N. caninum* does possess a plastid DNA. Four different plastid genes were detected in *N. caninum* by Southern blotting and probing FIGE (field inversion gel electrophoresis) gels.

1.11 Sequencing projects

At the beginning of this project, there were few sequences available on Genbank for *Neospora* but this has risen to 4861 sequences today. Of these, 263 are gene sequences and 4598 are expressed sequence tags (ESTs). A specific sequencing project has been set up for *T. gondii*, for which there is now 7 x genome sequence coverage (www.toxodb.org) and over 23,000 ESTs available. Due to the similarity of *N. caninum* and *T. gondii*, it is possible to exploit the resources available in the *T. gondii* sequencing project for studying *N. caninum*.

1.12 Molecular epidemiology of *N. caninum*

1.12.1 Molecular typing methods

A large variety of molecular based techniques have recently been developed that have extended knowledge into different areas of microbiology, including epidemiology, diagnosis and vaccine development. Strain characterisation is one of the basic applications of molecular biology for microorganisms.

1.12.1.1 Multi-locus enzyme electrophoresis (MLEE)

Differences in amino acid sequences can alter the net charge of amino acids, which in turn affects the electrophoretic mobility of enzymes. MLEE relies on this phenomenon to identify polymorphisms between samples (Selander *et al.*, 1986). A combination of structural enzymes are used in this method and each enzymatic pattern of polymorphism generated amongst strains is referred to as a 'zymodeme'. There are some disadvantages with this method, for example, only amino acid changes which affect the net charge of the protein molecule are detected and synonymous nucleotide substitutions are not recognised. MLEE relies on the availability of pure protein, which is impossible to obtain for clinical isolates of *N. caninum*. However, MLEE has been used successfully for population typing of *T. gondii* (Cristina *et al.*, 1995) and other Apicomplexa.

1.12.1.2 Pulse field gel electrophoresis (PFGE)

Pulse field gel electrophoresis (PFGE) is capable of separating large DNA molecules (Schwartz and Cantor, 1984) and has been used for the identification of genotypes in many organisms following digestion of genomic DNA. PFGE was used for chromosome identification in *T. gondii* (Candolfi *et al.*, 1988; Sibley and Boothroyd, 1992). There are several disadvantages to this technique in that the method is time consuming, not easy technically and requires a large amount of DNA as primary material. This is especially difficult with a parasite such as *N. caninum*, for which large tachyzoite yields from tissue culture are difficult to obtain.

1.12.1.3 PCR-based restriction fragment length polymorphisms (PCR-RFLP)

PCR-RFLP consists of amplification of a coding or non-coding DNA region being amplified and then the PCR products being digested using endonucleases and separation of the resulting restriction fragments by gel electrophoresis. This is a very simple technique that has been widely used for strain characterisation of organisms. Again there are difficulties as fragments with the same size co-migrate as one band in a gel, or some bands of a similar size may not be differentiated in agarose gels. Polyacrylamide gels can obtain better resolution but makes the method more difficult and time-consuming. Non-coding regions and non-transcribed spacers are more likely to be polymorphic but any polymorphisms generated by RFLPs which are chosen in regions of protein coding sequence are more likely to correlate with the phenotype of the strain (Parmley *et al.*, 1994a). *T. gondii* genotyping has been achieved by several researchers using the PCR-RFLP technique (Howe and Sibley, 1995; Howe *et al.*, 1997).

1.12.1.4 Random Amplification of Polymorphic DNA (RAPD)

Williams *et al.* (1990) introduced the Random Amplification of Polymorphic DNA (RAPD) technique and it has been used as a genetic analysis method on many organisms. The technique relies on the amplification of DNA regions annealed by single arbitrary primers of a short length at relatively low annealing temperatures. The reproducibility of this technique can be affected by several factors, for example, purity and concentration of DNA templates, different reagents and thermal cyclers (Ellsworth *et al.*, 1993). However, despite some debate on the validity of RAPD, this method has frequently been used for genotyping studies and has resulted in excellent correlation with results obtained using other methods (Monis and Andrews, 1998).

A major advantage with this method, if other factors can be controlled for, is that it relies on PCR thereby only requiring a small amount of starting material. In addition, RAPD is a fast, simple and relatively cheap technique.

1.12.1.5 Amplified Fragment Length Polymorphism (AFLP)

Amplified restriction fragment length polymorphism (AFLP) is also a PCR-based technique (Vos *et al.*, 1995). Selective amplification of digested genomic DNA is performed, so, as with RAPD analysis, pure DNA is required. It is technically difficult and more expensive than RAPD, but uses stringent reaction conditions that eliminate non-specific priming. The AFLP method has been used in the genetic analysis of plants and bacteria and is discussed further in Chapter 5.

1.12.1.6 Mini-/microsatellites

Mini- or microsatellites are arrays of repeat DNA sequence that vary in copy number between different genomes. Minisatellites, consisting of repeated units of ~ 6-20 nucleotides were first used to fingerprint the human genome (Jeffreys *et al.*, 1985). Microsatellites are short tandem repeats or multicopies of mono-, di-, tri- or tetra-nucleotides in genomic sequences and are even more highly variable than minisatellites. A CA repeat [(CA)₈] with purine-pyrimidine anchor in the 3' end was used as a single primer for genotyping *Trypanosoma cruzi*, *Leishmania braziliensis* and *Schistosoma mansoni* (Oliveira *et al.*, 1999).

1.12.1.7 DNA sequence analysis

DNA Sequencing methods have the advantage over other typing methods in that it can potentially investigate all alleles in the population of an organism.

Unfortunately, it is relatively expensive and time-consuming. Therefore, it is unsuitable for the rapid typing and phylogenetic analysis of a large number of strains. However, the sequencing of DNA targets from a limited number of representative strains may give sufficient information to allow designation of markers for an alternative method, such as PCR-RFLP.

1.13 Population biology

With the increasing availability of genome sequences, a wide range of molecular markers can be developed to address some of the outstanding questions and provide the means to track the sources of disease outbreaks. Molecular markers are extremely useful as tools to unravel the epidemiology and risk factors for a particular infectious organism. Key considerations are the population structure of the parasite undergoing investigation and the role of genetic exchange in generating variation. In turn, this has major implications for the application of chemotherapeutic- or vaccine-based control strategies.

Only one species of *Toxoplasma* has been identified despite an extremely large host range and geographical distribution. The lack of recombination and degree of clonality observed in *T. gondii* populations imply that sexual reproduction occurs rarely (Howe and Sibley, 1995). The two clonal lineages proposed by Howe and Sibley (1995) have been further supported in studies by Ajzenberg *et al.* (2001) and Grigg *et al.* (2001). Ajzenberg *et al.* (2001) used eight microsatellite markers to type 84 independent *T. gondii* isolates from humans and animals. The high discriminatory power of these markers showed that occasional genetic exchange did occur.

1.14 Aims

The epidemiology of *N. caninum* has received considerable attention in recent years, yet some key issues, particularly concerning the relative importance of the dog in horizontal transmission still remain unresolved. Moreover, there are no molecular epidemiological studies on *N. caninum* and nothing is known about its genetic diversity and population genetic structure. Such molecular epidemiological methods could help resolve unanswered questions concerning the transmission routes of *N. caninum* and the relationship between genotype and pathogenicity of *N. caninum*.

In chapter 2, a detailed case study of a *N. caninum*-infected Cheshire dairy herd is presented in which the sero-epidemiology of neosporosis is analysed and the role of vertical transmission in a closed herd examined. In this chapter the possibility of a link between cattle fertility and *Neospora* serostatus is also examined in a quantitative way for the first time. Such analysis is an essential component in determining the economic impact of the disease. It was also hoped that infected clinical material could be collected from this case study for subsequent genetic analysis.

N. caninum has been studied almost entirely in intensive Western farming systems and it is possible that results from these studies may have biased the overall picture of the epidemiology of this parasite. It is possible that the serostatus and genetic diversity of *N. caninum* could be very different in a more extensive farming system and in different breeds of cattle. Chapter 3 therefore describes a cross-sectional study of *N. caninum* in cattle from two diverse regions of Africa to test the assumption that *N. caninum*, like, *T. gondii*, has a worldwide distribution.

A major aim of this study was to assess the level of genetic diversity of *N. caninum* in both laboratory isolates and in clinical field samples. A variety of laboratory isolates were available for analysis. In addition, a small number of clinical field samples were also available from which it was hoped that *Neospora* DNA could be extracted and analysed. All previous studies have found the isolates of *N. caninum* to be identical at all loci, therefore there are no polymorphic markers available for molecular studies and consequently, nothing is known about the genetic diversity of *N. caninum*. For example, *N. caninum* could mimic the population structure of *C. parvum*, which is known to have very diverse lineages due to sexual recombination and has a variety of population structures. However, a population structure that follows *T. gondii* more closely might be expected, due to the closer taxonomic position of *N. caninum*, in which there are two clonal lineages but some recombinant genotypes still occur (Howe and Sibley, 1995). Chapters four and five describe the detailed analysis of the genetic diversity of laboratory isolates of *N. caninum*.

Tools for the analysis of genetic heterogeneity in clinical samples need to meet different criteria to those used for the analysis of laboratory stocks of parasites. Most importantly, clinical samples consist mainly of host DNA with very small amounts of parasite DNA. Thus the development of gene-specific typing methods using amplification of parasite DNA to maximise sensitivity are described in Chapter 6 and then are applied to a small number of clinical field samples that became available towards the end of this study.

To Susie (1976-2002)

Chapter 2: A case study of neosporosis in a Cheshire dairy herd

2.1 INTRODUCTION

During an 18-month period in 1990 and 1991, there was an outbreak of abortions in the Astonwich dairy herd located at Newton Hall farm, south Cheshire.

Subsequent investigation showed that the likely cause of these abortions was neosporosis. Since the initial outbreak, there have been on-going abortion problems on the farm, many of which have been diagnosed as being caused by *N. caninum*. In most cases diagnosis was made by a combination of serological tests on the dam and aborted foetus, and also immunohistological tests on the foetus to show the presence of *Neospora* antigen. These diagnostic tests were carried out by the Shrewsbury Veterinary Investigation Centre at the instigation of the Wilson McWilliam Veterinary group, Nantwich.

The Astonwich herd makes an excellent case study of neosporosis because it has been a closed herd for over 30 years and has well documented pedigree and production records. A closed herd is defined as a herd in which no cattle are bought in and all replacement stock are bred from the existing herd. In addition, the herd has been vaccinated for leptospirosis and brucellosis and until 2001/2002 was negative for Bovine Viral Diarrhoea Virus (BVDV). Thus, the most common causes of bovine abortion, other than *N. caninum*, were therefore considered not to be a problem in the herd. There have been few case studies in pedigree herds and there is to date no published account concerning how *Neospora* infection might affect fertility in cattle.

An increased number of abortions were first noted at the study farm in 1990 and this continued over an 18-month period, giving rise to an ‘abortion storm’ or epidemic outbreak. During this period nearly 50 % of adult cattle on the farm aborted. Fertility problems were also experienced and it was noticed that the calving index (the mean calving interval of all cows in a herd in a defined time) dramatically increased during this period. At the time there was very little knowledge of *N. caninum* amongst practising veterinary clinicians and therefore the infection went undiagnosed.

N. caninum infection in cattle can be diagnosed by serological testing (Bjorkman and Ugglå, 1999), although it is important to note that serological testing alone is not necessarily an indicator of the cause of abortion. To determine the serological status of individual animals and for example, to study the mode of transmission in a herd, the method of choice is to screen the entire herd at the same time for antibodies to *N. caninum*. To undertake this analysis on the Astonwich herd, all cattle were tested for the presence of antibodies to *N. caninum* by ELISA (Osawa *et al.*, 1998). Samples were taken from each animal in both January and August to give a more accurate picture of the serostatus of the herd. Stenlund *et al.* (1999) and Maley *et al.* (2001) have found that fluctuations in antibody titres can lead to misinterpretation of the serostatus of some individual animals. By sampling twice, it was hoped it would also be possible to discern whether the time of year had any effect on the outcome of serological testing for individual animals.

Dijkstra *et al.* (2002) showed that in 8 dairy herds studied in Holland, a point source exposure to *N. caninum* of the infected age-groups was found during a limited period of common housing and feeding. In the Dutch study a new dog had been introduced in all farms that were tested within a period of 1.5 years, before the first indication of *N. caninum* infection.

Three bitches were present at Newton Hall farm at the time of the *N. caninum* outbreak. Although none of these dogs were introduced less than 2 years prior to the abortion outbreak, several other dogs from surrounding farms often visited and it is possible that one or more of these other dogs may have been infected. It is possible that *N. caninum* may have been transmitted by the shedding of oocysts in dog faeces that contaminated feed (for example, in the haystack) that was subsequently fed to many cattle. In the summer, cows close to calving are kept in a paddock near to the farmhouse, where the dogs would have had access to placental material. It is conceivable that the farm dogs may then have become infected with *N. caninum* and subsequently transmitted the disease themselves (Dijkstra *et al.*, 2001). Several studies have shown that the prevalence of antibodies to *N. caninum* is higher in farm dogs than in urban dogs, possibly because of ingestion of bovine placental material on the farm (Sawada *et al.*, 1998; Basso *et al.*, 2001). A Brazilian study found that feral street dogs had a higher seroprevalence than owned dogs (Gennari *et al.*, 2002), again possibly because these animals were more likely to feed on uncooked meat. Unfortunately, the farm dogs at Newton Hall were no longer alive at the time of sampling in 2002, therefore were unavailable for serological testing or faecal sampling.

In this study, it was hoped to test the hypothesis that animals were initially infected by a point source infection and subsequent vertical transmission maintained the disease in the herd. Since an aim of this project was also to assess the genetic diversity of *N. caninum* it was hoped that clinical samples could be obtained from aborted foetuses from this farm for genetic analysis.

Cattle fertility is extremely important to the successful operation of a dairy farm and to date there is no published work on the potential effect of *N. caninum* infection on cattle fertility. This study also aimed to perform a preliminary analysis to determine whether a *N. caninum* seropositive status could adversely affect the fertility of a cow and whether a wider study could be justified.

In summary, the specific aims of this chapter were

- To determine the serostatus of individual cattle to *N. caninum* antibodies in the Astonwich herd and use these data to analyse probable transmission routes
- To analyse the Astonwich herd records for evidence of vertical or horizontal transmission of *N. caninum*
- To examine how neosporosis may have affected the fertility of individual animals within the Astonwich herd
- To obtain clinical samples of *N. caninum*-infected tissue for genetic analysis

2.2 MATERIALS AND METHODS

2.2.1 Farm and Animals

Sampling was carried out at Newton Hall farm which is situated south of Malpas in south Cheshire (OS reference SJ44, 468456) and home to the Astonwich herd (J. S. Latham and partners). All cattle on the farm are pedigree Holstein Friesian. Cows are split into two groups for management purposes – high and low yielders- depending on the time of lactation and are all housed separately from the heifers and calves. The cattle are kept on grass in the summer and inside in cubicles during the winter. They are fed on grass silage, brewer's grains and corn. There are about 125 cows, 30 heifers and a dozen calves at any one time. Year-round calving means that there are pregnant heifers and cows at all stages of gestation at any particular time of the year. Calves were defined as those less than 6 months of age. Heifers were 6 months or older that had not yet produced a calf. All animals were vaccinated against *Leptospira hardjo* and *Brucella abortus*. Cattle that had previously aborted were tested for Bovine Viral Diarrhoea virus (BVDV) and were found to be negative until serological testing in 2002. Milk from every lactating animal is tested monthly for quality, protein and fat content and the resulting National Milk Recording (NMR) programme records were available for analysis. In addition, the farm regularly takes part in a national pedigree scheme known as 'type classifying', in which cattle are scored for body conformation according to pedigree standards. Therefore, detailed pedigree records showing cow family relationships were also available for use in the analysis.

2.2.2 Sampling

A cross-sectional serological study was conducted on the herd on 30th January 2002 ('winter bleed') and 16th August 2002 ('summer bleed') (Plate 2.1). Blood samples were taken from all animals on the farm on these two dates by Susan Damodaran (BVMS, Cert CHP, MRCVS). The samples were collected from cows and heifers via the tail vein into a 10 ml vacutainer tube and from calves from the jugular vein. All animals sampled in January were sampled again in August. New calves (under 7 months), which were not born at the time of the first bleed, were only sampled in August.

All samples were centrifuged at approximately 1500 x g for 10 min and then the serum was removed and stored at -20 °C until it was used for serological testing.

2.2.3 ELISA

The ELISA to detect the presence of antibodies to *N. caninum* and BVDV were carried out at the Moredun Research Institute (MRI), Edinburgh.

2.2.3.1 *N. caninum* ELISA

Antibodies to *N. caninum* were measured using the ELISA of Osawa *et al.* (1998). To assess the validity of the ELISA, sensitivity, specificity and Spearman's correlation coefficient (r_s) were calculated based on the IFAT results using the same serum samples. Sensitivity was defined as the percentage of sera positive in the *Neospora* ELISA that had also been positive by the IFAT (Trees *et al.*, 1994) and specificity was defined as the percentage of IFAT-negative sera that were also negative in the ELISA. A cut-off value of 0.4 (OD value



Plate 2.1

Sample collection at the Astonwich herd, Malpas, Cheshire

otherwise referred to as 40 % OD) was selected resulting in the lowest number of diagnostic errors based on the IFAT results (Osawa *et al.*, 1998).

Briefly, polystyrene 96-well microtitre plates (F-Form of Immunolon, M-129A, Dynex Technologies, Billingham, UK) were coated with 150 µl/well of water-soluble *N. caninum* antigen diluted in 0.05 M carbonate- bicarbonate buffer (pH 9.6) and incubated for 24 h at 4 °C. Plates were given three washes in PBS containing 0.05 % Tween 20 (pH 7.4) and were shaken dry. A total of 150 µl of sera, diluted in PBS (pH 7.4)/0.05 % Tween 20 with blocking agent was added to each well. Plates were placed in a humidified chamber and incubated for 2 h at 37 °C and then washed and shaken dry as described above. A total of 150 µl of peroxidase-conjugated rabbit-anti-bovine whole molecule IgG (Sigma, Poole, UK), diluted in PBS/1 % Chicken ovalbumin (grade V, Sigma, Poole, UK)/0.05 % Tween 20 was added to each well, and incubated for 2 h at 37 °C. The plates were washed again three times and shaken dry before addition of 150 µl of the enzyme substrate [*o*-phenylenediamine dihydrochloride (OPD tablets, Sigma, Poole, UK) in phosphate-citrate buffer (0.4 mg/ml) containing 0.04 % of 30 % (v/v) hydrogen peroxide, pH 5.0] to each well. After incubation for 30 min in the dark the enzyme hydrolysis of substrate was arrested by the addition of 50 µl of 2.5 M sulphuric acid. The optical density (OD) at 492 nm was read in a microplate reader (Titertek® Multiskan, Type 312B, ICN Bio Medical, Thame, UK) and values over 40 % OD were considered to be positive (Osawa *et al.*, 1998). Positive and negative control sera and all test sera were tested in duplicate.

2.2.3.2 BVDV ELISA

Antibodies to BVDV were measured using an ELISA (Fenton *et al.*, 1991).

Irradiated 96-well ELISA plates (Dynatech) had 100 µl/well antigen added. The plates were covered, placed in a humidified sandwich box and incubated at 4 °C overnight. Dilution tubes were filled with 490 µl of ELISA diluent [PBST (Phosphate buffered saline, Tween) 10 % HS (Horse serum)] and were also incubated at 4 °C overnight. The diagnostic serum samples were diluted 1:50 and then 10 µl of test sample was added to the dilution tubes containing 490 µl PBSTH. Dilutions were also made of the BVD positive control serum (1965J) and the negative control foetal bovine serum (FBS) in PBSTH. Diluted positive and negative control sera (500 µl) were added to the appropriate dilution tubes. The plates were then washed four times in ELISA wash fluid (PBST) using the ELISA plate washer (Dynatech Ultrawash ELISA washer). After washing, 100 µl diluted diagnostic sera, positive and negative control dilutions were added to wells. The samples were thoroughly mixed by pipetting up and down. The plates were then put into a humid sandwich box and incubated at 37 °C for 1 h. Rabbit anti-bovine HRP (Sigma, UK) was diluted in ELISA Diluent (PBSTH). After washing the plates four times in ELISA wash fluid (PBSTH), 100 µl of conjugate was added to each well. The plates were again put inside the humid sandwich box and incubated at 37 °C for 1 h. The plates were again washed x 4 in PBSTH and 100 µl TMB substrate (Sureblue KPL) was added to every well. The plates were left at room temperature until the colour began to develop in the positive control wells. The reaction was stopped by adding 100 µl of 0.18 M sulphuric acid to each well. The OD value (absorbance) was read at 450 nm on

the ELISA reader (Dynex MRX ELISA reader). The cut-off point was defined as <0.1 OD is negative.

2.2.4 Statistical Analysis

Figures 2.1, 2.2, 2.3, 2.4 and 2.5 were constructed in the graphics program GraphPad Prism (Version 3.0 for Windows, GraphPad software, San Diego, California, USA). Linear regression was performed on all scatter graphs and r^2 values are given where appropriate.

Chi-squared analysis was performed on the pair-wise comparison of dams with their daughters with respect to *N. caninum* status. The following equation was used:

$$\chi^2 = \sum (O-E)^2/E$$

where O is the observed value and E is the expected value.

An F-test was performed to determine whether there was a significant difference between the variance of the mean numbers of Artificial Insemination (AI) services per successful pregnancy for *Neospora*-infected and uninfected cattle. The F-test showed that there was no significant difference between the variance of the means. Therefore, a two-tailed t-Test assuming equal variances was performed on the number of AI services per pregnancy in *Neospora* seropositive versus seronegative cattle.

2.3 RESULTS

2.3.1 *N. caninum* seroprevalence and age-prevalence

In the winter bleed, 16 % (24 out of 147) of animals sampled were found to be seropositive for *N. caninum*. This compared with 20 % (33 out of 166) found to be seropositive for *N. caninum* at the time of the summer bleed, giving an average *N. caninum* seroprevalence of 18 % in the Astonwich herd based on a 40 % cut-off in the ELISA.

To investigate the possibility of a relationship between the age of an animal and serostatus, the anti-*Neospora* antibody titres were plotted against the age (in days) for each animal and analysed by linear regression (Figures 2.1 a & b). The winter bleed results showed no association between increasing age and increasing seropositivity ($r^2 = 0.03$) and this was confirmed with the summer bleed results ($r^2 = 0.02$).

2.3.2 BVDV serology

The results from the BVDV ELISA showed that about one third of the herd were positive for the virus. The positive animals generally had high titres, which indicates a relatively recent infection. The herd has always been negative when previously tested for BVDV up until 2001/2002. Dr. Peter Nettleton, a veterinary clinician and senior researcher at the Moredun Research Institute, kindly interpreted the BVDV ELISA results. It is extremely unlikely that BVDV would have caused any of the abortions that have occurred on this farm as the BVDV infection appears too recent. It may, however pose a future risk and vaccination will be considered. However these results allowed us to exclude BVDV as a causative agent of abortion in this study.

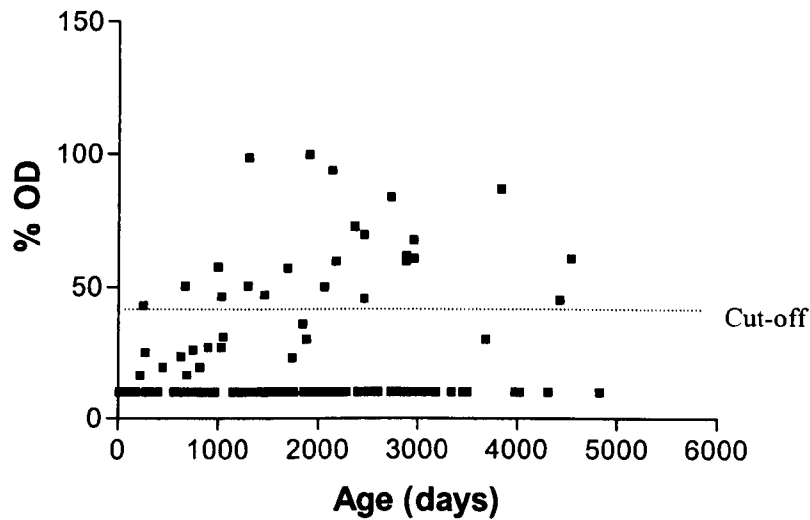


Figure 2.1a
Anti-*Neospora* antibody titres of the Astonwich herd plotted against age of each animal at the winter bleed (30/01/02) (n=147). Linear regression showed no association between age and increasing seropositivity ($r^2=0.03$).

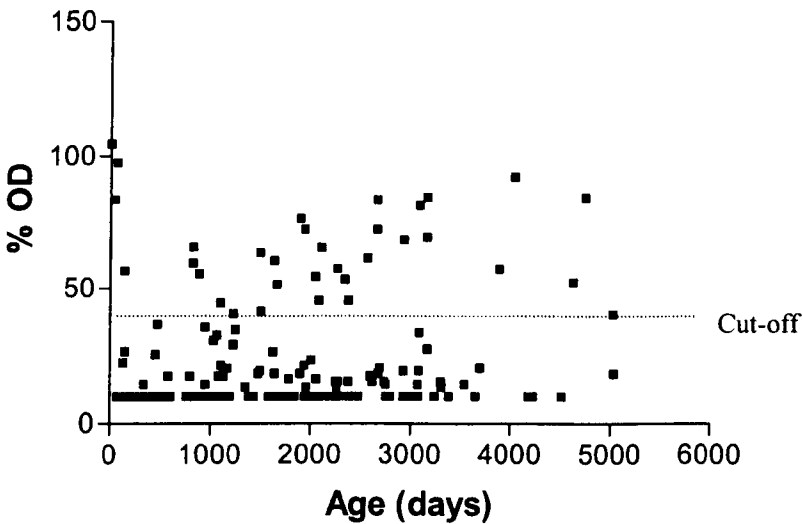


Figure 2.1b
Anti-*Neospora* antibody titres of the Astonwich herd plotted against age of each animal at the summer bleed (16/08/02) (n=166). Linear regression showed that there was no association between age and increasing seropositivity ($r^2=0.02$).

2.3.3 Seroconversion of cattle between winter and summer bleeds

In order to establish whether a single serological test accurately defines the serostatus of an individual animal, Tables 2.1a & b were constructed showing animals that changed serostatus between the first and second bleed. Nine animals that were seronegative to *N. caninum* at the winter bleed had positive antibody titres when they were re-tested in the summer (Table 2.1a). However, two of these were only just over the cut-off titre of 40 % OD. Only one animal that had previously tested seropositive to *N. caninum* was found to be seronegative at the second bleed (Table 2.1b). It was interesting to note that the higher titre results, from animals that had a different serostatus at each bleed, tended to coincide with their being in the latter half of gestation (generally in the final trimester). However, this was not the case for animals that were not pregnant at either bleed but had been served 3 or 5 times (Table 2.1a). Seroconversions only occurred in the cows, not the heifers or calves.

2.3.4 Serostatus and breeding cycle

To demonstrate whether there was a correlation between antibody titre and stage of the breeding cycle, anti-*Neospora* antibody titres of all animals were plotted against the stage in the breeding cycle at both bleeds (Figures 2.2a & b), but no correlation was noted. To test this further, anti-*Neospora* antibody titres of pregnant cows at the winter bleed (Figure 2.3a) were plotted against stage of gestation, but no association was found between increasing stage of gestation and antibody titre. The results from the summer bleed (Figure 2.4a) also showed no relationship between latter stages of gestation and increasing antibody titres. To ensure that the large number of seronegative cows had not skewed the results, the

Table 2.1a

Seroconversion of cows from *Neospora* negative status at the winter bleed (30/01/02) to *Neospora* positive status at the summer bleed (16/08/02) (cut-off at 40% OD).

Cow	January titre (%OD)	Notes	August titre (%OD)	Notes
4	30.0	Served x 3	57.5	8 months pregnant
31	27.0	Not pregnant (served x 1)	40.5	served x 3
61	16.5	3 months pregnant	55.5	calved 6 weeks ago
63	<10.0	Calved 6 weeks ago	40.5	6 months pregnant
70	27.0	Not pregnant (served x 1)	44.5	Served x 5
76	30.0	Not pregnant (served x 3)	45.5	5 months pregnant
81	36.0	Not pregnant (served x 3)	54.5	7 months pregnant
100	23.5	2 months pregnant	59.5	8.5 months pregnant
125	<10.0	3 months pregnant (served x3)	60.5	calved 5 weeks ago

Table 2.1b

Seroconversion of cows from *Neospora* positive status at the winter bleed (30/01/02) to *Neospora* negative status at the summer bleed (16/08/02) (cut-off at 40% OD).

Cow	January titre (%OD)	Notes	August titre (%OD)	Notes
96	59.5	6 months pregnant	33.5	Barren

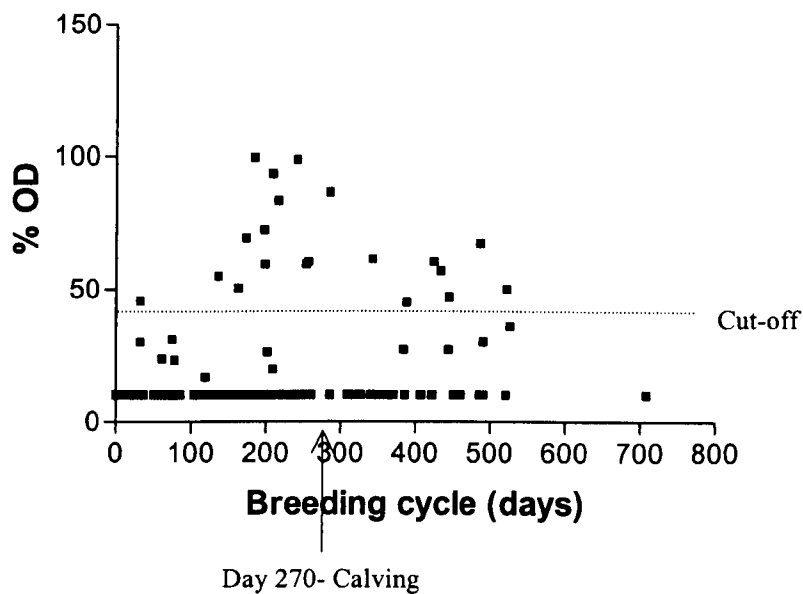


Figure 2.2a
Anti-*Neospora* antibody titres of Astonwich herd at the winter bleed (30/01/02) plotted against stage of breeding cycle. (Day 0 represents day of insemination)

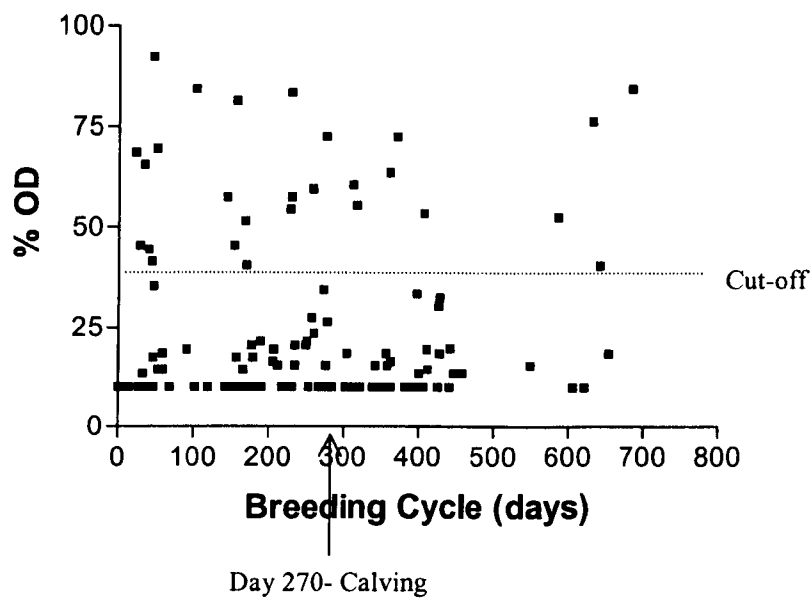


Figure 2.2b
Anti-*Neospora* antibody titres of Astonwich herd at the summer bleed (16/08/02) plotted against stage of breeding cycle. (Day 0 represents day of insemination)

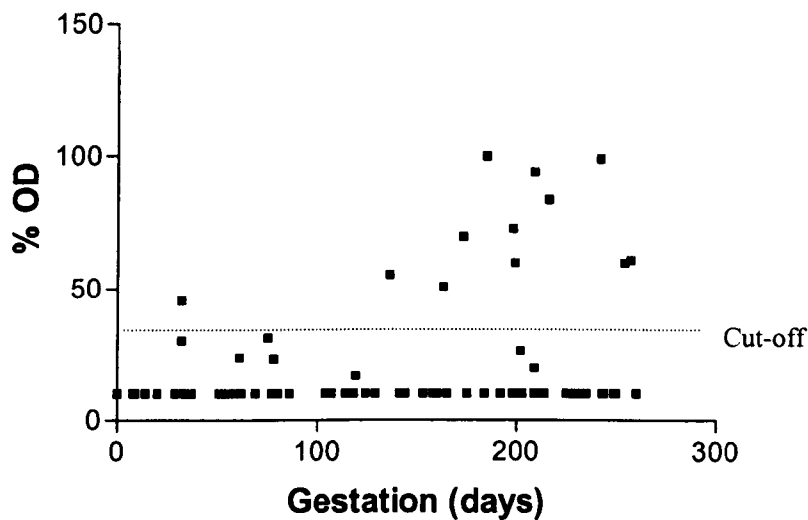


Figure 2.3a
Anti-*Neospora* antibody titres of pregnant cows in the Astonwich herd at the winter bleed (30/01/02) plotted against stage of gestation.

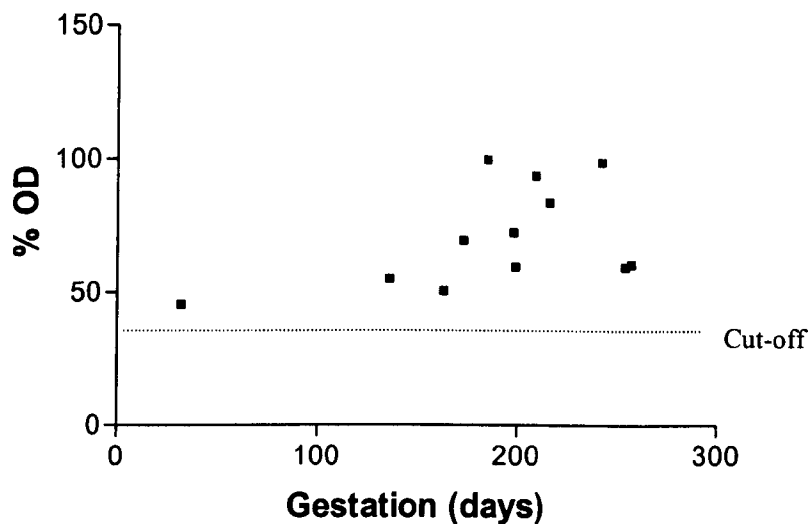


Figure 2.3b
Anti-*Neospora* antibody titres of *Neospora* seropositive pregnant cows in the Astonwich herd at the winter bleed (30/01/02) plotted against stage of gestation.

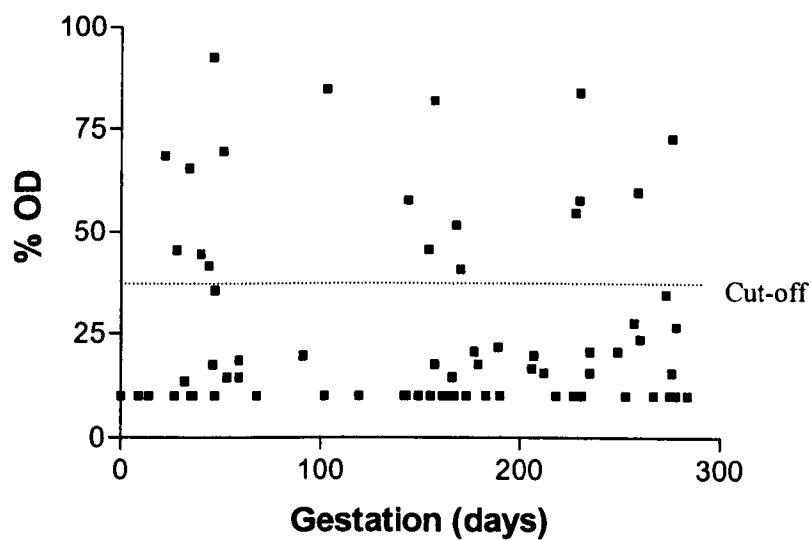


Figure 2.4a
Anti-*Neospora* antibody titres of pregnant cows in the Astonwich herd, at the summer bleed (16/08/02) plotted against stage of gestation.

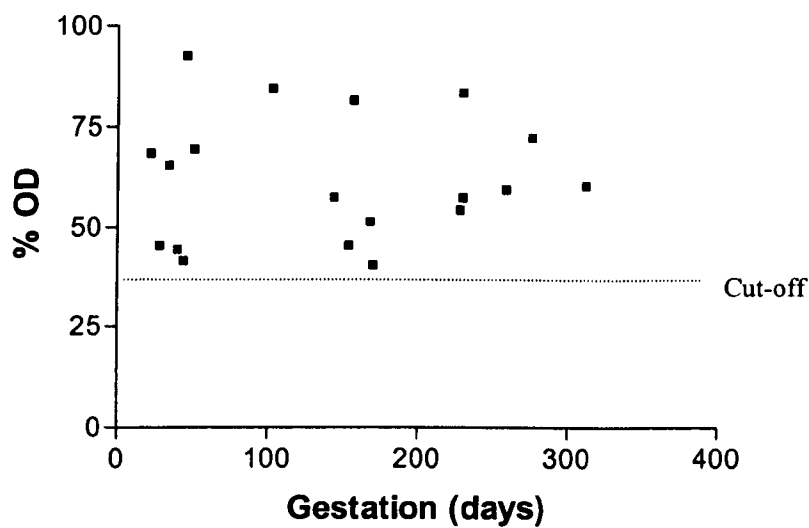


Figure 2.4b
Anti-*Neospora* antibody titres of *Neospora* seropositive pregnant cows in the Astonwich herd, at the summer bleed (16/08/02) plotted against stage of gestation.

anti-*Neospora* antibody titres of seropositive pregnant cows only, were plotted against the stage of gestation for both the winter and summer bleed (figures 2.3b and 2.4b). Again, no correlation was found although the number of data points were small in this analysis.

Tables 2.2a & b show the serostatus of pregnant and non-pregnant animals at the time of sampling. Sixty-nine percent of animals were pregnant at the winter bleed of which 14% were seropositive to *N. caninum*. Of the remaining 31 % of non-pregnant cattle at the winter bleed, 21 % were seropositive to *N. caninum* (Table 2.2a). In contrast, 50 % of the animals were pregnant at the summer bleed (Table 2.2b). Of the pregnant cattle, 26 % were seropositive to *N. caninum* compared to 18 % of the non-pregnant cattle.

To investigate further a possible relationship between stage of gestation and serostatus, the pregnant animals were again divided into the three trimesters of pregnancy for the winter and summer bleeds (Tables 2.3a & b). There were no consistent trends apparent with respect to the proportion of cattle positive for *N. caninum* in each category.

2.3.5 Pair-wise analysis of serostatus of dams and daughters

Vertical transmission is known to be an important mode of perpetuating *N. caninum* infection in a herd (Schaes *et al.*, 1998, Wouda *et al.*, 1998 and Davison *et al.*, 1999c). To test the role of vertical transmission in the Astonwich herd, animals were paired as dams and daughters and pair-wise analysis of the serostatus of dams and daughters was performed (Table 2.4). In

Table 2.2a

Serostatus of pregnant and non-pregnant adult cattle at the winter bleed

Winter status	Number	%	% <i>Neospora</i> +ve
Pregnant	85	69	14
Not pregnant	38	31	21

Table 2.2b

Serostatus of pregnant and non-pregnant adult cattle at the summer bleed

Summer status	Number	%	% <i>Neospora</i> +ve
Pregnant	61	50	26
Not pregnant	60	50	18

Table 2.3a

Percentage of cattle in each trimester of pregnancy seropositive to *N. caninum* at the winter bleed

Winter status	Number	%	% <i>Neospora</i> +ve
0-3 months	25	20	4
3-6 months	28	23	11
6-9 months	32	26	25
Not pregnant	38	31	21

Table 2.3b

Percentage of cattle in each trimester of pregnancy seropositive to *N. caninum* at the summer bleed

Summer status	Number	%	% <i>Neospora</i> +ve
0-3 months	21	17	33
3-6 months	21	17	29
6-9 months	19	16	16
Not pregnant	60	50	18

Table 2.4
Pair-wise analysis of serostatus of dams with the serostatus of their daughters
with respect to *N. caninum*. (Results taken from both bleeds)

	Daughter +ve	Daughter -ve
Dam +ve	12	5
Dam -ve	0	47

total, 64 pairs were found in the current herd. Animals were counted as being seropositive if they had been found to be seropositive to *N. caninum* antibodies at either bleed. Chi-squared analysis was performed where the expected value was calculated from the probability of any one animal in the Astonwich herd being positive (probability = 0.18) for *N. caninum*. The observed value was the actual number of positive animals in each group (Table 2.4). Chi-squared analysis showed that there was a significant increase in the likelihood of a seropositive daughter having a seropositive dam (accepted at the 5 % level with 1 degree of freedom) and that a seronegative dam was more likely to have a seronegative daughter.

2.3.6 Family trees

The Astonwich herd has 27 different cow families for which all of the family trees were constructed for this study. Two examples, the Astonwich Angie (Figure 2.5) and the Astonwich Collona (Figure 2.6) families are shown. These two families were chosen because they are large and contain *Neospora*-positive and negative lines within the same family. The family trees for all 27 families strongly indicated vertical transmission of *N. caninum* within the herd. The numbers following each cow name refer to the order in which they entered the herd. The % OD values for the winter and summer bleeds are shown under the names (the first referring to the winter bleed). The positive/ negative (+/-) signs following the *Neospora* serology results refer to the BVDV status of that animal as tested at the winter bleed. Animals that had aborted and for whom a

Astonwich Collona Family (1978 – present day)

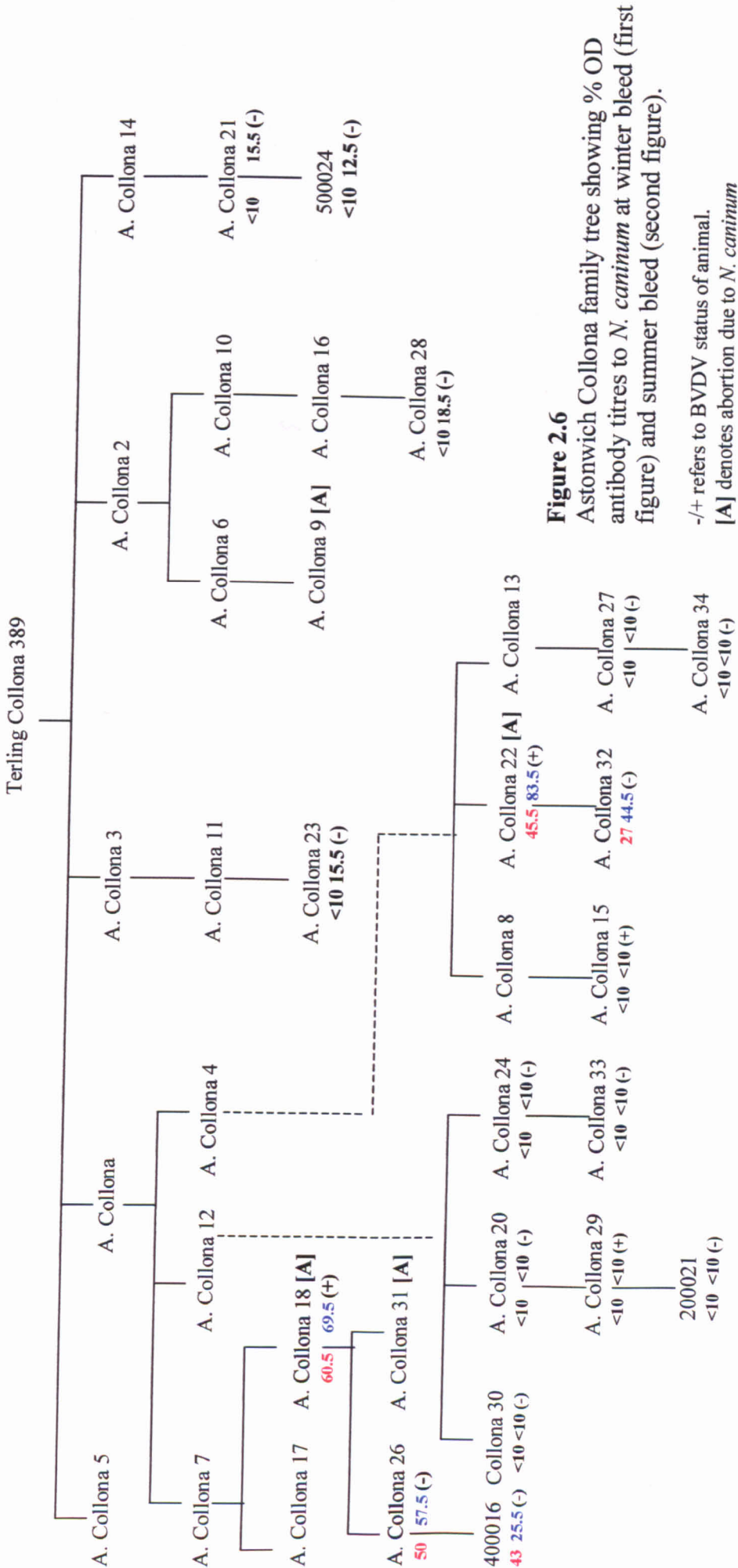


Figure 2.6
Astonwich Collona family tree showing % OD antibody titres to *N. caninum* at winter bleed (first figure) and summer bleed (second figure).

subsequent positive diagnosis found *N. caninum* to be the causative infectious agent are denoted by [A].

Figure 2.5 shows two highlighted lines involving three generations of the A. Angie family. The first family line shows three generations (A. Angie 20, 36 and 46) that gave a negative response to *N. caninum* antibodies and the second (A. Angie 22, 33 and 599) line that are all positive at one or both bleeds. It was interesting that A. Angie 22 aborted a five-month old foetus in 1998 due to neosporosis and her grand daughter, A. Angie 43 was culled from the herd due to fertility problems and had had several abortions due to *N. caninum*. A. Angie 34 aborted a five-month old foetus in 1999 and in June 2001, A. Angie 42 also aborted a 7.5 month-old foetus, both due to neosporosis.

Similarly, Figure 2.6 shows a positive antibody response to *N. caninum* in three generations of the Astonwich Collona family (A. Collona 18, A. Collona 26 and 400016 [eartag number as has not yet entered the herd]). This shows how efficiently vertical transmission can occur down family lines. In addition, A. Collona 18 had an abortion due to *N. caninum* infection in 1999. Her daughter, A. Collona 31 aborted twice in January and June 2001. Unfortunately, this cow had left the herd by January 2002 and so was unavailable for testing, but an inability to get her in calf was a major reason for culling. However, A. Collona 26, an older sister of A. Collona 31, was serologically tested twice and was found to be seropositive to *Neospora* antibodies both times. A. Collona 22 gave a positive response to *Neospora* antibodies and also aborted a five-month old foetus in 1998 where *N. caninum* was the designated abortifacient.

2.3.7 Fertility in the Astonwich herd

In order to determine whether the fertility of *Neospora*-infected animals (seropositive) had been impaired in comparison to the fertility of non-infected (seronegative) cattle, the number of Artificial Insemination (AI) services per successful pregnancy was recorded in two families from the Astonwich herd.

Tables 2.5 and 2.6 show the number of services per pregnancy in different lactations in the two study families, A. Angie and A. Collona. The number of lactations per animal differs due to age differences. In addition the number of services are not known in some cases (and so are omitted) at times when a bull was present on the farm and used to service maiden heifers. The mean number of AI services per pregnancy in seronegative cattle was 1.54 +/- 0.34 compared with 3.06 +/- 0.23 AI services per pregnancy in seropositive cattle. There was a significantly higher ($p < 0.001$) number of AI services per pregnancy in *N. caninum* seropositive cattle than in seronegative cattle in the two families analysed (Table 2.7).

The increased number of AI services per pregnancy indicates that *N. caninum* infection is likely to increase the calving interval (number of days from the birth of one calf to the birth of the next – ideally 365 days) of cattle in these families. The calving index is the mean calving interval of all cows in a herd at a defined point in time calculated retrospectively from their most recent calving at that time.

Table 2.5
Number of Artificial Insemination (AI) services per pregnancy in *N. caninum*
seropositive cattle in families A. Angie and A. Collona.

<i>N. caninum</i> serostatus	Cow	Lactation	AI Services	Average number services per lactation
Positive	A. Angie 22	10	1	2.90
		9	1	
		8	3	
		7	5	
		6	2	
		5	5	
Positive	A. Angie 33	5	4	2.75
		4	1	
		3	5	
		2	1	
Positive	A. Collona 22	5	3	3.00
		4	2	
		3	4	
Positive	A. Collona 18	7	1	2.50
		6	3	
		5	3	
		4	3	
Positive	A. Collona 32	2	4	3.00
		1	2	
Positive	A. Collona 26	3	5	3.33
		2	2	
		1	3	
Positive	A. Collona 31	2	4	4.00
Mean				3.06+/- 0.23

Table 2.6

Number of Artificial Insemination (AI) services per pregnancy in *N. caninum* seronegative cattle in families A. Angie and A. Collona.

<i>N. caninum</i> serostatus	Cow	Lactation	AI Services	Average no. services per lactation
Negative	A. Angie 20	11 10 9 8 7 6	1 1 1 1 1 1	1.00
Negative	A. Angie 30	7 6 5 4	2 4 1 1	2.00
Negative	A. Angie 36	4 3 2	1 1 1	1.00
Negative	A. Angie 37	5 4 3 2 1	3 1 2 1 1	1.60
Negative	A. Angie 38	4 3 2	1 1 1	1.00
Negative	A. Angie 45	2	1	1.00
Negative	A. Angie 46	2 1	2 1	1.50
Negative	A. Angie 47	2	1	1.00
Negative	A. Angie 48	1	2	2.00
Negative	A. Collona 20	6 5 4 3	4 3 2 1	2.50
Negative	A. Collona 21	4 3 2 1	3 3 3 1	2.50
Negative	A. Collona 23	6 5 4 3	1 1 5 3	2.50
Negative	A. Collona 27	4 3 2 1	3 1 2 1	1.75
Negative	A. Collona 28	4 3 2 1	1 1 1 1	1.00

Negative	A. Collona 29	3 2 1	2 1 1	1.30
Negative	A. Collona 30	4 3 2 1	1 1 1 1	1.00
Negative	A. Collona 33	2 1	2 2	2.00
Negative	A. Collona 34	1	1	1.00
Mean				1.54 +/- 0.34

Table 2.7
Two-tailed t-Test comparing the mean number of services per pregnancy for seropositive and seronegative groups of cattle.

	<i>Seropositive</i>	<i>Seronegative</i>
Mean	3.064286	1.536111
Variance	0.230595	0.343472
Observations	7	18
Pooled Variance	0.314026	
df	23	
P(T<=t) two-tail	3.03E-06	

2.3.8 Collection of *N. caninum*-infected tissues

Unfortunately it was not possible to obtain infected tissues during the course of the study. Foetuses were generally too autolysed when found.

2.4 DISCUSSION

This case study has shown that serological data is far more valuable if it can be analysed in conjunction with pedigree, production and reproductive records.

Newton Hall farm was an excellent case study because it has been a closed herd for more than 30 years and has detailed records for all cow family lines. It was known that *N. caninum* was endemic in the herd following an epidemic outbreak of abortions that began in 1990 and lasted for approximately 18 months. Cattle that aborted were always tested for antibodies to Bovine Viral Diarrhoea Virus (BVDV) but were consistently found to be negative. Two other common causes of bovine abortion, *Leptospira hardjo* and *Brucella abortus* were routinely vaccinated against and were therefore assumed not to be a problem in this herd. The entire herd was tested for BVDV serostatus during this study to ensure it was still not present in the Astonwich herd. Over 80 % of dairy herds in this area of Cheshire are known to be infected with BVDV (Dr. D. Barrett, University of Glasgow Veterinary school, UK, personal communication), so it was unusual that Newton Hall farm should have remained free for such a long time. This presumably reflects the benefit of having a closed herd because the cattle do not mix with other potentially infected animals. In fact, in 2002 at the time of sampling, the BVDV ELISA results showed that 31 % of the herd had a positive antibody titre. For those cattle infected, the titres were particularly high indicating a very recent infection in the herd. It has been postulated that a group of heifers from Newton Hall may have become infected in the summer of 2001 when they were summer-grazed at a neighbouring farm. Due to the recency of this infection and because all cattle that abort are tested for all abortifacients and have been found to be negative for BVDV, it was assumed that all abortions

experienced on the farm have been due to *N. caninum* and not BVDV.

Unfortunately BVDV may become a bigger problem in the future and a potential abortifacient on this farm, although its recent presence cannot account for the large numbers of abortions since 1990.

Evidence that vertical transmission was an important means of *N. caninum* transmission in this herd is based on several data sets: (1) the age prevalence data (where no relationship was found between increasing age and increasing antibody titre); (2) the pair-wise analysis of dam-daughter pairs (showing an increased likelihood of a daughter being seropositive if her dam was also seropositive); (3) the family tree data showing the transmission of *N. caninum* down generations.

The percentage of cattle in the Astonwich herd with a positive response to *N. caninum* of between 16 and 20 % is similar to the UK herd average of 17.1 % (Davison *et al.*, 1999d). Davison *et al.* (1999a) found the herd-specific prevalences to range from 7.3 % to 44 % in different UK herds. There was no convincing evidence in this case study of increasing seroprevalence with age of the cattle. This concurs with previous studies on *N. caninum* but these results are in stark contrast to the infection pattern found in sheep infected with the closely related apicomplexan, *T. gondii*. A positive age-prevalence correlation, as seen with *T. gondii* infections, indicates that environmental exposure to oocysts or tissue cysts is a dominant mode of transmission. In contrast, the *Neospora* data in this study agrees with other studies (Davison *et al.*, 1999c; Wouda *et al.*, 1999;

Osawa *et al.*, 2002) and implies that post-natally acquired neosporosis is not an important mode of transmission.

It was interesting that consecutive tests for the presence of *Neospora* antibody gave different results. Clearly, the definition of serostatus of an individual animal is dependent on the designated cut-off value, which represents a compromise between sensitivity and specificity. Here the cut-off was set at 40 % OD (Osawa *et al.*, 1998). In addition, 7 of the animals that changed to a positive serostatus had antibody titres that were much higher than the cut-off value. Maley *et al.* (2001) also found that antibody titres can fluctuate from one serological screening to another. Dijkstra *et al.* (2003) found that 95.3% of animals (n=1676) were assigned the same serostatus when tested three times and concluded that only a minor proportion of a herd would be incorrectly diagnosed if serological testing was used in conjunction with age distribution and pedigree data. However, it is very unusual to have information provided on the serostatus of an animal with respect to *N. caninum* at the time of a farm sale. For example, when re-stocking a herd, which has occurred in many parts of the UK since the outbreak of the Foot and Mouth epidemic in 2001, it is of great interest to the farmer to be able to identify accurately the serostatus of each animal with respect to *N. caninum*. This is especially important in dairy herds where the original cattle are heavily relied upon to produce replacement heifers and there is evidence that vertical transmission plays a strong role in disease transmission. It is possible that the immune status of cattle in the winter is different to their immune status in the summer due to different environmental stresses.

It might be expected that cattle antibody titres would increase during pregnancy due to the natural immunomodulation occurring in pregnancy, which causes down regulation of TH1-type responses and biases towards TH2-type responses (Innes *et al.*, 2001). This downregulation of TH1-type responses, that are known to be protective against the parasite, may account for recrudescence of a persistent infection, resulting in higher *Neospora* antibody titres later in pregnancy. Certainly 6 of the seroconversions observed in this study gave high titres in the second half of pregnancy, most in the final trimester. However the herd data showed no association between increasing stage of gestation and increasing antibody titres.

The antibody status of pregnant cattle was further explored by dividing them into trimesters, but no pattern between *Neospora* serostatus and stage of gestation could be found. It is possible that the chance distribution of *Neospora*-infected animals at different stages of gestation would preclude this and it highlights the need for a large number of cattle to be studied in order to identify trends.

Pair-wise analysis of the serostatus of dams with the serostatus of their daughters (Table 2.4) gave strong evidence that vertical transmission has played a large role in transmission of *N. caninum* in this herd. This correlates with previous observations by Thurmond and Hietala (1997) and Wouda *et al.* (1998), which gave robust evidence for congenital transmission by the presence of *N. caninum* antibodies in a high proportion of pre-colostral sera from the first and second generation descendants of cows aborted during an epidemic.

The family tree analysis highlights the vital role of vertical transmission in *Neospora* transmission. Occasionally an animal was found with *Neospora*-positive serostatus with no history of disease in that particular line (e.g. A. Collona 22). There are several other examples of this in other Astonwich families. This is also consistent with the fact that vertical transmission alone, although being a highly efficient mode of transmission, is not sufficient to maintain infection over time in a herd. This points to the involvement of horizontal transmission and has been postulated by several researchers (Pare *et al.*, 1997; Hietala and Thurmond, 1999). French *et al.* (1999) demonstrated by mathematical modelling that without horizontal transmission *N. caninum* would disappear from cattle herds. Horizontal infection may occur in a number of ways such as ingestion of tissue cysts in placenta (Dijkstra *et al.*, 2001), pooling of infected milk and then being fed to calves (Uggla *et al.*, 1998) or contamination of a food source with oocysts from a definitive host (McAllister *et al.*, 1998).

This case study demonstrates a statistically significant increased risk of abortion in seropositive dams compared to that of seronegative dams. However, a cross-sectional study by Wouda *et al.* (1998) found the risk to be unrelated to the serostatus of the dam, but this could be explained by the fact that *N. caninum*-infected dams may have been placed into the seronegative group, since antibodies to *N. caninum* may fluctuate below the threshold level (Conrad *et al.*, 1993b). A 1 to 2 % incidence of postnatal transmission versus 98 % by congenital transmission was estimated in dairy heifers before the first calving (Thurmond *et al.*, 1995). Bjorkman *et al.* (1996) found no evidence in the herd

they studied for postnatal infection rather, all infections could be traced back to two purchased infected cows. This highlights the importance of a closed herd (as in this case study), or careful testing several times prior to purchase of cattle to ensure that infected animals are not brought into a herd.

Cattle fertility is affected by many factors such as host immune status, nutrition, body scoring as well as possible presence of potential infectious agents. Due to the multifactorial basis of fertility, it is difficult to isolate one particular cause if problems are encountered in getting an animal pregnant. Therefore, this study analysed the service history for the cows in the two families, A. Angie and A. Collona and found a statistically significant increase in the number of AI services per successful pregnancy in seropositive cattle compared with seronegative cattle. This is the first study that has investigated and quantified the potential relationship between *Neospora* infection and fertility problems. However, care should be taken in extrapolating the data from this single case study.

There are many potential reasons for the increase in the average number of Artificial Insemination (AI) services per successful pregnancy, but it is possible that a recent *N. caninum* infection could cause animals to abort very early in their pregnancy before the presence of the foetus had actually been detected. This would result in a return to 'heat' three weeks later, in the same way that an unsuccessful AI service would. Williams *et al.* (2000) found that experimental intravenous challenge infection of cattle at day 70 caused resorption of the foetus.

Fertility in cattle can be measured in many ways, for example, by the number of services per successful pregnancy or the calving to first service index. Fertile cattle that are easy to get in calf are crucial to the success of dairy businesses because successful pregnancies are intrinsically linked to continual high milk yields. The calving index is used as one measure of the efficiency of the management and productivity of a farm. Poor fertility results in higher replacement rates, fewer calves per year, increased maintenance costs and higher insemination fees.

The milk yield of most cows increases up to the fifth lactation so any reduction in fertility, that reduces the number of calves the cow has in a given time span, will result in reduced milk yields (MAFF, 1984). A lower replacement rate would also enable the size of the heifer rearing enterprise to be reduced hence saving costs. Currently, over one-third of culls from dairy herds are due to poor fertility compared with 17 % due to low milk yields. This severely restricts the opportunity to select for production, a particular problem in a pedigree herd. A study by Royal *et al.* (2000) found that there has been an average decline in the fertility of British Holstein-Friesian cattle approaching 1% per year since 1975. This decline in fertility was also reflected in traditional measures of fertility such as the calving interval, which has lengthened from 370 to 390 days. One factor that may have contributed to the decline in fertility may be the breed substitution of North American Holstein for the British Friesian, the proportion of which has increased from 0 % to 80 % over the last 20 years (Royal *et al.*, 2000). Although the Holstein may have superior genetic merit in terms of milk yield compared with the Friesian, it may also carry undesirable genes, one of which may be

associated with subfertility. However, many other studies have suggested that the Holstein may inherently have high fertility but that in many cows reproductive performance is compromised by high milk production leading to negative energy balance during early lactation (Butler and Smith, 1989; Lucy *et al.*, 1992; Mcmillan *et al.*, 1996). Holmes *et al.* (1987) suggested that the high genetic merit cow produces more milk by having a greater propensity for losing body condition to support milk production. It would follow that the loss of body condition would probably lead to a reduction of immunity in the cow. The genetic gain in milk yield in the USA (Foote, 1996) has also been accompanied by a decline in pregnancy rates to first service.

Cattle fertility plays such a key role in determining the productivity of a farm that any factor that might reduce fertility, for example neosporosis, needs thorough investigation. This case study shows that *N. caninum* can reduce cattle fertility in a measurable way and suggests that a wider study is justified.

Chapter 3: Neosporosis in Ghana and Tanzania

3.1 INTRODUCTION

Neospora caninum has been reported in many countries and as with *T. gondii*, frequently referred to as having a worldwide distribution. However, there is in fact very little data concerning neosporosis in many parts of the world including Africa, and no studies to determine its seroprevalence or host range have been conducted in West Africa. African cattle breeds tend to be very different from European cattle. They belong to two species, *Bos taurus* and *Bos indicus*. Zebu cattle (*B. indicus*) can be visually differentiated from taurine cattle by a hump. African cattle are especially interesting to study since they are thought to be almost exclusively composed of a separate haplogroup, which is encountered only rarely elsewhere (Troy *et al.*, 2001).

During the course of this study, an opportunity arose to undertake sero-epidemiological studies in two contrasting sub-Saharan countries, Ghana in West Africa and Tanzania in East Africa. An existing British Council link between the University of Glasgow and the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana in Accra, gave the opportunity for collection of samples in Ghana for a two-week period in April 2002. The Tanzania study was designed as a pilot programme to obtain preliminary epidemiological data on both *N. caninum* and *Echinococcus granulosus* in north-western Tanzania. The investigation was conducted in two districts that differed in land-use characteristics, with

comparative data collected from hospital records of human cases, meat inspection records from government veterinary offices, coproantigen prevalence surveys in domestic dogs and wild carnivores (for studies on *E. granulosus*) and seroprevalence surveys in domestic dog, cattle and lion populations (for studies on *E. granulosus* and *N. caninum*).

There have been a very limited number of accounts of neosporosis in Africa. Barber *et al.* (1997) found that 22 % of dogs (n = 49) in Tanzania were seropositive to *N. caninum* using the IFAT test. In South Africa, 5.9 % of serum samples from non-domestic felids (n=68) were found to have antibodies against *N. caninum* although these animals were also seropositive to *T. gondii* so it is possible that some cross-reactivity may have occurred (Cheadle *et al.*, 1999b)

Any factor that limits productivity in cattle is particularly significant in African pastoral communities, which rely heavily upon livestock-derived foods and which currently suffer from high levels of poverty and malnutrition (McCabe *et al.*, 1997). There is a long history of cattle husbandry with the Masai people who live in Kenya and Tanzania. Indeed, the Masai tribe are a cattle people who believe all the cattle on earth belong to them and still occasionally go on raids to retrieve herds from other tribes (Saitoti, 1993). The entire way of life of the tribe is dictated by cattle and they therefore live a semi-nomadic way of life in order to follow the seasonal changes of grass and water for the herds.

Su *et al.* (2003) found that exotic isolates of *T. gondii* e.g. 'COUGAR' were particularly informative in molecular epidemiological and evolutionary studies of this parasite. It was hoped that if it were possible to obtain clinical material in either Ghana or Tanzania, exotic isolates of *N. caninum* could be cultured in the laboratory that would be similarly useful for genetic studies of *N. caninum*.

The aims of this chapter were

- To perform a cross-sectional serological study of cattle in Ghana and Tanzania to determine the prevalence of *N. caninum* in these regions.
- To collect clinical material to obtain exotic isolates of *N. caninum*.

3.2 MATERIALS AND METHODS

3.2.1 Study areas and animals

Ghana

The study was based at the Noguchi Memorial Institute for Medical Research (NMIMR) at the University of Ghana in Accra. Sampling was carried out in all three ecological zones in Ghana – coastal Savannah (Greater Accra region), forest belt (Ashanti region) and Northern Savannah (Northern, Upper East and Upper West regions). Three farms and one abbatoir were used for sample collection. Fifty-three cattle at the University of Ghana farm at Kpong were sampled. This farm lies in the coastal savannah region and the cattle are pure N'Dama (*Bos taurus*). N'Dama cattle are a beef breed that are resistant to trypanosomiasis and *Dermatophilus congolensis* infections. Twenty-one cattle were sampled at a government-owned dairy farm at Amrahia also located in the coastal savannah zone, north of Accra. The cattle are West African Shorthorn x Friesian/Jersey (*Bos taurus*) and have been crossed for two generations, as part of a dairy improvement programme. The third farm situated at Somanya, 150 Km north of Accra and within the coastal savannah was a private farm where 30 cattle of the White Fulani breed (*Bos indicus*) were sampled.

The Kumasi abbatoir is located in the forest zone of Ghana. This abbatoir draws cattle from a wide area, particularly the northern savannah regions (Northern, Upper East and Upper West regions) of Ghana and cattle are even brought from as far as the Sahelian regions of Burkina Faso, Togo and Niger. Blood samples were taken

from ninety-two cattle, of the White Fulani and N'Dama breeds, presented for slaughter in the Kumasi abattoir. Therefore sampling was comprehensive in that both the three ecological zones and three main breeds of cattle in Ghana were sampled in this study. In addition a number of cattle originated from other West African countries. Figure 3.1 shows the areas sampled highlighted with a red circle (map of Ghana from www.MapZones.com).

Tanzania

The study area was the Serengeti ecological region of North-western Tanzania (35° to 36° E, $1^{\circ} 30'$ to $3^{\circ} 7'$ S) with study villages located within Ngorongoro District and Serengeti District, adjacent to the Serengeti National Park.

The Ngorongoro District, comprising the Loliondo Game Controlled Area and the Ngorongoro Conservation Area, is a multiple-use controlled wildlife area, inhabited predominantly by Maasai people who practice traditional pastoralism, and recently, limited cultivation. The estimated total human population in 1999 was 31,500 with a density of 4.4 people/km^2 , based on 1988 human census data and projected population growth rate of 3.4 % per annum (Bureau of Statistics, 1991). Estimates of domestic dog densities, determined from the number of dogs per Maasai boma or household varied between 0.38 and 0.46 dogs/km^2 from 1992 to 1994 (Cleaveland, 1996). Figure 3.2 shows the area in Tanzania that was sampled highlighted with a red box (map also taken from www.MapZones.com).

Figure 3.1 Map of Ghana showing the origin of cattle sampled.





 denotes the areas from which samples were collected

Figure 3.2 Map of Tanzania showing the origin of cattle sampled



 denotes the area from which samples were collected

3.2.2 Collection of sera samples

Blood samples were usually taken from the middle coccygeal vein of cattle at the farms but from the carotid artery at the time of slaughter in the abbatoir.

All blood samples were collected into 5 ml, plain vacutainer tubes (Becton Dickinson, Plymouth, UK) and transported to the Immunology Unit of the Noguchi Memorial Institute for Medical Research. The samples were centrifuged at 800 x g for 10 min and then the sera (supernatant) were removed from the top of the tubes and put into three separate 1 ml eppendorf tubes for storage at – 80 °C. Samples were heat-treated by being placed in a water bath at 55 °C for 1 h and the tubes thoroughly disinfected prior to transportation of samples to the UK.

Samples were transported back under licence to the UK still frozen in dry ice in cool boxes. They were placed at – 20 °C storage on arrival until required for testing.

3.2.3 ELISA

An ELISA (Osawa *et al.*, 1998) was used to test bovine (cattle and buffalo) sera for the presence of antibodies to *N. caninum* and was performed exactly as described in Section 2.2.3 at the Moredun Research Institute, Edinburgh.

3.3 RESULTS

3.3.1 Collection of sera samples

Ghana

Serum samples were collected from cattle over a two-week period in April 2002 (Table 3.1). Several farms in the Greater Accra region were sampled including a commercial farm at Somanya; the University farm at Kpong; a Government farm at Amrahia. Prof. B.D. Akanmori, Mr. J. Otchere (Noguchi Memorial Institute for Medical Research, University of Ghana) and Dr. F.B. Awumbilla (Department of Animal Science, University of Ghana) collected blood samples. Samples were also taken from cattle at the time of slaughter in the Kumasi abbatoir. This abbatoir is the most modern in Ghana and has a high-throughput of animals. Cattle are brought from many different areas in the northern regions of Ghana (Northern, Upper East and Upper West regions) as well as from Burkina Faso, Niger and Togo. The collection of samples is shown in Plate 3.1.

Tanzania

Samples were collected from two intermediate host species (cattle and buffalo) and two potential definitive host species (domestic dog and lion). Collection of domestic animal samples was conducted in seven villages in the Serengeti district: Mugumu, Bwitengi, Bonchugu, Kisangura, Ngarawani, Nyamoko and Burunga between June and August 1999. Individual households were selected following discussions with community leaders.

Table 3.1

Origin of cattle sampled for antibodies to *N. caninum* in Ghana and surrounding West African countries.

Sample location	Date	Origin of cattle	Ecological zone	Number
Somanya	24/04/02	Somanya	Coastal savannah	30
University of Ghana Farm, Kpong	25/04/02	Kpong	Costal savannah	53
Government dairy farm, Amrahia	26/04/02	Amrahia	Coastal savannah	21
Kumasi abbatoir	29/04/02	Upper East	Northern savannah	4
Kumasi abbatoir	29/04/02	Upper West	Northern savannah	15
Kumasi abbatoir	29/04/02	Northern Region	Northern savannah	8
Kumasi abbatoir	29/04/02	Kumasi	Middle Forest	2
Kumasi abbatoir	29/04/02	Paga	Northern savannah	14
Kumasi abbatoir	29/04/02	Tamale	Northern savannah	3
Kumasi abbatoir	29/04/02	Bawku	Northern savannah	40
Kumasi abbatoir	29/04/02	Sandema	Northern savannah	4
Kumasi abbatoir	29/04/02	Burkina Faso	Sahel	2
Total				196



Plate 3.1

Ghana: Collection of
samples



Due to the remoteness of many villages in Ngorongoro District, sample collection was combined with on-going rabies and Contagious Bovine Pleuropneumonia (CBPP) vaccination programmes. Between June and August 1999, six villages were visited: Wasso, Sakala, Loliondo, Mgongo, Ng'arwa and Olemishiri.

Cattle and buffalo serum samples collected as part of other on-going disease surveys were also analysed in this study. Buffalo sera, collected opportunistically (Plate 3.2) also for rinderpest surveillance as part of the Programme for Pan-African Control of Epizootics, were obtained from the Veterinary Department, Tanzania National Parks (Dr. T. Mlengeya). Cattle samples that were collected in Ngorongoro District in 1998, as part of a disease prevalence survey, were provided by Prof. R. Kazwala (Sokoine University of Agriculture).

3.3.2 Serological survey for *N. caninum*

Ghana

None of the 196 serum samples collected from cattle gave a positive antibody response to *N. caninum*. The cut-off value for this ELISA is set at 40 % OD at or above which an individual animal would be designated as being seropositive to *N. caninum*. Seventy four percent of the samples gave a <10 % OD value. The highest OD value recorded was 24 % with 98 % of cattle giving a value of less than 19 %.



Plate 3.2
Tanzania: collection of samples

Tanzania

Sera from 246 cattle were collected from the Serengeti region during this study. Of these, 8.1 % of cattle were found to be seropositive to *N. caninum*. Fourteen of these positive samples gave very high OD values of >80 %. Table 3.2 shows the percentage of seropositive cattle in the seven villages tested in Serengeti District and the six villages sampled in Ngorongoro District of north-western Tanzania. Each area had approximately the same percentage of cattle seropositive to *N. caninum*.

Serological analyses of 130 cattle from Ngorongoro (collected by Prof. R. Kazwala) and 39 buffalo from the Serengeti National Park previously found the seroprevalence to *N. caninum* to be 0.8 % in Ngorongoro cattle and 2 % in Serengeti buffalo.

Unfortunately it was not possible to collect clinical material from any of the Tanzanian animals that were found to be positive for *N. caninum*.

Table 3.2

Prevalence of *N. caninum* infection in cattle in seven areas of Serengeti district and six areas of Ngorongoro District, Tanzania.

Village	Number of cattle sampled	Number of cattle seropositive	% cattle seropositive to <i>N. caninum</i>
Mugumu	8	0	0
Bwitengi	39	5	12.8
Bonchugu	20	2	10.0
Kisangura	20	3	15.0
Ngarawani	33	2	6.0
Nyamoko	20	2	10.0
Burunga	10	0	0
Wasso	0	-	-
Sakala	24	1	4.2
Loliondo	0	-	-
Ng'arwa	10	1	10.0
Mgongo	51	4	7.8
Olemishiri	11	0	0
Total	246	20	8.1

3.4 DISCUSSION

This study represents the first comprehensive epidemiological survey of *N. caninum* in cattle in West Africa. There was no evidence of the presence of *N. caninum* in the 196 cattle sampled. Moreover, cattle sampled at the Kumasi abattoir are drawn from a wide geographical area extending to Burkina Faso, Togo and Niger suggesting that the absence of *N. caninum* infection may extend to at least part of the wider West African region.

A large number of samples were also collected in East Africa (n=284). In contrast to the data from West Africa, there was a significant level of *Neospora* infection in Tanzania, although the prevalence in the two areas of East Africa was markedly different. The presence of *N. caninum* in domestic cattle and Serengeti buffalo represents the first reporting of this parasite in Tanzanian cattle. It is important to note that although the number of animals sampled in Ghana was lower than in Tanzania, the numbers would still have been sufficient to detect a prevalence as low as 0.8 % (based on 196 samples at the 95 % confidence level).

It is difficult to draw firm conclusions as to why the prevalence at the two sampling areas in East Africa was different. The prevalence of 8.1 % infection in Serengeti District is comparable with many other serological surveys in the UK at 12 % (Trees *et al.*, 1999), Paraguay at 29.8 % (Osawa *et al.*, 2002). Interestingly, the cattle in East Africa are of the N'Dama breed (*Bos taurus*), showing that this breed is also

susceptible to *N. caninum* infection. Most other studies have focused predominantly on European breeds.

The surprising aspect of this study is the apparent complete absence of *N. caninum* in the West African survey. Sampling covered both old animals (breeders) and animals presented for slaughter, as well as young ones. One possible explanation for this is that West African cattle are genetically diverse from those of East Africa and European breeds, rendering them resistant to *Neospora*. For example, substantial differences were found at 20 microsatellite loci tested between zebu and taurine cattle in a study of 20 different cattle populations in Africa (MacHugh *et al.*, 1997). The results from this study suggested that some West African populations of taurine cattle that are disease-tolerant are under threat of genetic absorption by migrating zebu herds. However, Troy *et al.* (2001) suggest that African cattle diversity is almost exclusively composed of a separate haplogroup which is encountered only rarely elsewhere. However, the N'Dama breed of cattle in Tanzania were found to be infected with *N. caninum* showing that the absence of the disease in Ghana is unlikely to be accounted for simply by host incompatibility due to breed differences. The N'Dama breed belongs to the *Bos taurus* species to which European cattle e.g. Holstein-Friesian also belong. The other cattle species tested in West Africa was *Bos indicus*, in which no evidence of *N. caninum* infection was found either.

If breed differences cannot account for the absence of *N. caninum* in West Africa, then a second possible explanation could be the hostility of the environment to

coccidian oocysts, due to desiccating conditions in some regions. It is known that oocysts of *T. gondii*, for example, survive optimally in cool moist conditions (Frenkel *et al.*, 1975). However, it would then follow that other coccidians such as *T. gondii*, which is spread by oocyst shedding, would also be absent from this region. Several studies have shown that this is not the case and that *T. gondii* prevalence in West Africa is similar in sheep and goats as it is in the UK. Van der Puije *et al.* (2000) found that 33.2 % of sheep tested (n = 732) in Ghana had anti-*T. gondii* antibodies. In addition, this study found 26.8 % of goats (out of n=526) to be seropositive for *T. gondii*. Moreover, dry desiccating conditions also persist in East Africa where *N. caninum* was found.

Another possibility for the absence of *Neospora* in Ghana could be that neosporosis is a facet of intensive cattle farming systems in which relentless genetic selection for productivity may increase susceptibility to infection. There is certainly a complete absence of intensive cattle farming in West Africa, where genetic selection for high milk production has been severely hampered by the increased susceptibility to disease in cattle improvement programmes. However, this theory is not supported by data from the Tanzanian survey where the farming systems are also extensive, although the volume of cattle rearing in East Africa is considerably greater than that in Ghana.

A final possible explanation for the absence of *N. caninum* in the West African region may be that the parasite has simply not been introduced to this area because

historically, there has been very little importation of European cattle into West Africa and therefore limited opportunity for importing the parasite also. There are several accounts of attempts to introduce European cattle to Ghana, the first recorded was in 1908 (Annual Report on the Northern Territories, 1908, cited in Oppong, 1998) when four Aberdeen Angus bulls were sent from Britain but died very quickly on arrival in Ghana. A similar outcome befell three young Hereford bulls that were delivered by train to Kumasi in 1911 (Oppong, 1998). All three bulls died on the walk north to Tamale thus tragically ending one of the first attempts to introduce temperate cattle from the UK to Ghana. In the early 1930s it became obvious that European breeds and even their crosses were unsuitable to the environment owing to their susceptibility to tick-borne disease in particular. A six-month tour of the north of Ghana (then Gold Coast) in 1911 by a Captain Beal, the first British veterinarian to visit the country, had already concluded that the increase in cattle production, in order to meet the requirements of the country, was fraught with difficulties such as disease, the small size of local cattle breeds, acute water shortage in the dry season, and except in the Fulani herdsman, a lack of knowledge on livestock breeding and improvement (Beal, 1973).

Therefore it seems that the most likely explanation is that *N. caninum* was never introduced into West Africa via infected cattle. Despite the presence of cross-bred cattle arising from the importation of a small number of Holstein-Friesian cattle from the UK, the probability of the exported animals being infected with *Neospora* would be quite low. Even if the parasite did reach West Africa perhaps it was not

sustained in the extensive farming systems of the region. It is possible that vertical transmission may occur more efficiently in intensive Western farming systems due to regular calvings that may annually lower immunity in cattle, allowing recrudescence of infection.

Historically, West Africa was not colonised in the same manner as East Africa where by 1920, 10,000 Europeans had arrived in Kenya (Lawrence, 1998) and this number increased to 80,000 by the 1950's. It is possible that European dogs, which commonly accompanied their owners to East Africa, carried *N. caninum* with them and subsequently established the *Neospora* life-cycle. By contrast, Ghana was colonised by a relatively small number of Europeans and principally acted as a coastal trading route.

Cattle in sub-Saharan Africa have to cope with a hostile environment ranging from extreme climatic conditions to a multitude of exotic diseases, such as trypanosomiasis, rinderpest and tick-borne fever. Therefore, *N. caninum* would rank as a relatively benign infection and would be unlikely to be the first priority for veterinary research in Africa. This may account for the lack of data on *N. caninum* prevalence from this region, but it is also a facet of samples being difficult to obtain. The infra-structure in these countries in terms of management of cattle, condition of roads and ability to access remote areas obviously makes sample collection much more difficult than in many other parts of the world. Nevertheless, it would be extremely interesting to collect further samples from these regions – perhaps

including samples from a wider host range and more countries. Whereas neosporosis may not be the most important cattle disease in Africa, further study of the disease on this continent, particularly for molecular analysis would be especially important for the study of the evolution of *N. caninum*. For example, if *N. caninum* in East Africa arrived via importation of European cattle or dogs, then we would expect close genetic similarities with isolates found in Europe.

Chapter 4: Analysis of genetic diversity in *Neospora caninum* using Random Amplification of Polymorphic DNA (RAPD)

4.1 INTRODUCTION

A key question underlying an 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. Some isolates of *Neospora* have been reported to differ in their pathogenicity in murine models (Lindsay *et al.*, 1995; Atkinson *et al.*, 1999) although it is not known how these data might relate to the disease in cattle. Serology data is extremely informative in many areas of epidemiology, for example in terms of prevalence of infection and defining host range (chapters 2 and 3) but it has little use in determining the extent of genetic variation amongst different isolates of a species. Schock *et al.* (2001) found that the antigenic profiles of six *Neospora* isolates recognised by polyclonal sera were all identical, indicating that there is no substantial variation in the immunodominant proteins. This re-enforces the results of Marsh *et al.* (1998) and Atkinson *et al.* (1999) who also failed to detect antigenic variation between two isolates of *N. caninum*. Genetic methods are therefore much more appropriate tools for the study of intra-specific variation and the broader field of molecular epidemiology and the majority of this study now focuses on the application of molecular techniques.

N. caninum has a wide host range and antibodies to the parasite have been found in sheep (*Ovis aries*) (Dubey and Lindsay, 1990b), goats (*Capra hircus*) (Dubey *et al.*, 1992), deer (*Cervis eldi*, *Odocoileus hemionus*) (Woods *et al.*, 1994), foxes (*Vulpes vulpes*) (Buxton *et al.*, 1997b) and other carnivores (Barber *et al.*, 1997). Due to its

wide host range, wide geographical distribution and potential for sexual recombination, *N. caninum* 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 *T. 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 two isolates, NC-Liverpool and NC-SweB1, using three RAPD-PCR primers and demonstrated variant banding patterns. However, the small number of primers and isolates used precludes wider interpretation of these data.

Genetic diversity can be analysed by comparing genes between different isolates of a species. The more genes that are analysed, the more powerful the measure of diversity. Selection of genes has an important influence on the outcome of this measure so need to be carefully chosen. Thus, studies which seek to measure diversity based on a few loci are open to the criticism that the results are biased by the genes selected for study. Techniques have therefore been developed that enable heterogeneity over a large

number of genes to be analysed simultaneously. One example of such a technique is Random Amplification of Polymorphic DNA (RAPD).

The RAPD technique allows the rapid detection of genomic polymorphism using a single short oligonucleotide primer of arbitrary sequence in a PCR reaction. It was first described by Williams *et al.* (1990) and has since been used to analyse genetic diversity in many organisms. The PCR reaction is carried out under low stringency conditions producing strain-specific fragments that are subsequently analysed by agarose gel electrophoresis. This method allows detection of polymorphisms at different loci simultaneously, using very small quantities of genomic DNA. In addition it is a very useful technique for organisms for which there is little genome information, such as *N. caninum*. The only requirement to perform this method successfully is that relatively pure genomic DNA can be isolated (section 4.2.3) because any contaminants (DNA or inhibitors) could cause spurious banding patterns. Thus, it is especially important that any heterogeneity found can be attributed to the different isolates rather than contaminant DNA. Several steps were taken in the study to ensure that the DNA used was very pure and these are described later (section 4.3.2).

The aims of this chapter were:

- To measure the extent of genetic diversity that exists amongst the available laboratory isolates of *Neospora caninum*.
- To analyse any specific genetic clustering of isolates, for example, to determine whether canine isolates cluster separately from those originating from cattle

4.2 MATERIALS AND METHODS

4.2.1 Parasite material

Six *Neospora caninum* isolates were used for RAPD analysis and are listed in Table 4.1 along with details of their isolation and maintenance. All isolates were maintained routinely by tissue culture and none of these isolates had previously been cloned. The highest number of tissue culture passages for each isolate before analysis by RAPD ranged from 33 and 56 passages, except for NC-1, which had been maintained in tissue culture for considerably longer (212 passages). The following colleagues generously supplied the isolates of *N. caninum*. Dr. Camilla Bjorkman (National Veterinary Institute, Uppsala, Sweden) isolate NC-SweB1; Prof. Pat Conrad (School of Veterinary Medicine, University of California) isolate BPA-1; Prof. AJ Trees (Liverpool School of Tropical Medicine, United Kingdom) isolates NC-Liverpool and NC-LivB1. The M3 strain of *T. gondii* was kindly supplied by Dr. Lee Innes (Moredun Research Institute, UK).

The *T. gondii* strains (RH, S48 and M3) were also maintained in tissue culture in the same way as described below for *N. caninum*. *Cryptosporidium parvum* sporozoites were obtained from Type II strain (Iowa) oocysts kindly provided by Prof. H.W. Smith (Scottish Parasite Diagnostic Laboratory, UK) and were excysted from oocysts using 1.5 % taurocholic acid (Sigma, UK) in PBS (pH 7.2) for 1.5 h at 37 °C. *Sarcocystis* sp. DNA was obtained from parasites purified from a sheep heart and was a gift from Mr. S. Wright (Moredun Research Institute, UK).

Table 4.1 Isolation and maintenance of *N. caninum* parasites

Isolate	Origin of host	Isolated and maintained by	Highest passage number	Country of origin	Reference
NC-1	Canine	tissue culture	212	USA	Dubey <i>et al.</i> 1988
NC-Liverpool	Canine	tissue culture	41	UK	Barber <i>et al.</i> 1995
BPA-1	Bovine	tissue culture	36	USA	Conrad <i>et al.</i> 1993
NC-SweB1	Bovine	tissue culture	36	Sweden	Stenlund <i>et al.</i> 1997
JPA-2	Bovine	mouse inoculation/ tissue culture	33	Japan	Yamane <i>et al.</i> 1997
NC-LivB1	Bovine	tissue culture	56	UK	Davison <i>et al.</i> 1999a

4.2.2 Parasite maintenance and tissue culture

4.2.2.1 Culture medium and Solutions

- (i) Iscove's Modified Dulbecco's Medium (Life Technologies, Paisley, U.K.) was used for cell culture and was supplemented with 5 % (v/v) foetal calf serum (FCS) and 100 U/ml penicillin and 100 µg/ml streptomycin sulphate. Culture medium was filter sterilised using a 0.2 µm membrane pore-size Sartolab® V500 filter (Sartorius, Surrey, U.K.) connected to a diaphragm vacuum pump.
- (ii) HEPES buffered balanced salt solution was used for washing cell monolayers prior to trypsinisation and was made as follows: 140 mM sodium chloride, 5 mM potassium chloride, 5 mM *D*-glucose, 10 mM HEPES and 0.001 % phenol red. The buffer was adjusted to pH 7.5 using 5 M sodium hydroxide and filter sterilised as described above.
- (iii) Suspension medium for the cryopreservation of *N. caninum* tachyzoites and host cells consisted of Iscove's Modified Dulbecco's Medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate and 12.5 % (v/v) DMSO. Suspension medium was filter sterilised using syringe filters. All solutions were stored at 4 °C.

4.2.2.2 Culture of host cells (Vero) and *N. caninum*

Parasite tachyzoites were maintained by twice-weekly serial passage using African Green Monkey kidney fibroblasts (Vero cells) as host cells. Cells and parasites were grown in filter sterilised IMDM in 25 cm³ (T25) or 75 cm³ (T75) tissue culture flasks

(Greiner, Germany) at 37 °C in a 5 % CO₂ humidified incubator. Confluent uninfected host cells were detached from the surface of culture flasks by washing the cell monolayer twice in HEPES saline for 1 min (5 ml for a T25, 10 ml for a T75) and incubating the cells in versine : trypsin (4:1) for approximately 5 min at 37 °C. Gentle tapping of the flask aided the removal of cells from the flask surface. Cells were centrifuged at 1500 x g for 5 min at 4°C and resuspended at a density of 2×10^6 cells/ml IMDM. Cells were re-seeded at 1×10^5 in 5 ml IMDM for a T25 flask and 4×10^5 in 15 ml medium for a T75 flask. Vero cells were infected 24 h later at a parasite-to-cell ratio of 3:1 and 4:1 for a T25 and T75, respectively. Tachyzoites were harvested 3 or 4 days later using a sterile cell scraper (Greiner Ltd., UK). Cells and parasites were counted using a Neubauer hemacytometer (Weber Scientific Ltd, U.K.).

4.2.2.3 Cryopreservation

Parasite tachyzoites and Vero cells were cryopreserved in liquid nitrogen using dimethylsulphoxide (DMSO) as a cryoprotectant. Following the same protocol as above (section 4.2.3.2), tachyzoites were harvested from flasks using a sterile cell scraper and counted with a haemocytometer. After washing in IMDM containing 10 % (v/v) FCS, the tachyzoites and host cells were centrifuged at 1500 x g for 10 min at 4 °C and resuspended in 1 ml suspension medium (Iscove's Modified Dulbecco's Medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate and 12.5 % (v/v) DMSO). Aliquots (1ml) were pipetted into cryotubes, which were placed in a polystyrene box insulated with cotton wool and cooled slowly to -80 °C. Vials were catalogued and immersed in liquid nitrogen for long-term storage. All

isolates were held at both the Division of Infection & Immunity, University of Glasgow and at the Moredun Research Institute, Edinburgh.

4.2.2.4 Preparation of parasite tachyzoites for DNA extraction

N. caninum and *T. gondii* tachyzoites were separated from host cells by filtration through 47 mm diameter 3 µm pore-size Nuclepore® polycarbonate filters (Whatman, U.K.). Filtered tachyzoites were washed twice in PBS (pH 7.4) by centrifugation at $1500 \times g$ for 20 min at 4 °C. Tachyzoites were given a final wash in PBS (pH 7.4) and pelleted by centrifugation at $13000 \times g$ for 3 min at 4 °C.

4.2.3 DNA extraction

The GenomicPrep™ Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech, UK) was used to extract DNA from *N. caninum* and *T. gondii* cultured parasites, *C. parvum* sporozoites and Vero cells. By using the same kit, all DNA preparations were standardised. The following method was calibrated for use with $3-5 \times 10^6$ cells but could be adjusted depending on the number of cells harvested. Cells were resuspended in a final volume of 40 µl PBS by vigorous vortexing. Cell Lysis Solution (600 µl) was added and the solution mixed by pipetting to lyse the cells. RNase A solution (3 µl) was then added to the cell lysate and mixed by inverting 25 times followed by incubation at 37 °C for 60 min. The samples were cooled on ice for 2-3 min to bring them down to room temperature, before adding 200 µl of Protein Precipitation Solution and vortexing vigorously to mix uniformly with the cell lysate. After centrifugation at $13000 \times g$ for 3 min, the supernatant (containing the DNA) was decanted into a clean 1.5 ml tube containing 600 µl of 100 % isopropanol and the original tube containing the precipitated protein pellet was discarded. The isopropanol

tube was inverted 50 times followed by centrifugation at 13000 x g for 1 min, leaving the DNA visible as a small white pellet. The DNA was washed with 70 % ethanol followed again by centrifugation at 13000 x g for 1 min. The tube was then drained onto absorbent paper and left to air dry for 15 min. The DNA was rehydrated by adding 100 μ l water to the pellet and was left overnight at room temperature. The DNA was subsequently stored at 4 °C.

4.2.4 DNA integrity and concentration

All DNA that was extracted and purified was run on an ethidium bromide stained gel to check that the DNA was intact. Five μ l of DNA was mixed with 1 μ l of loading buffer (0.25 % Bromophenol blue, 0.25 % xylene cyanol FF, 40 % w/v sucrose in water) and loaded onto a 1 % agarose gel in 0.5 x TBE buffer (pH 8.3). A spectrophotometer was also used to quantify DNA so that equivalent amounts of each isolate could be used in RAPD reactions.

4.2.5 Random Amplification of Polymorphic DNA (RAPD)

4.2.5.1 RAPD method

RAPD analysis on DNA samples was performed using Ready-to-Go™ RAPD analysis beads (Amersham Pharmacia Biotech, UK). The beads were stable at room temperature and contained AmpliTaq and Stoffel fragment, 0.4 mM dNTPs (each), 2.5 μ g bovine serum albumin, 10 mM Tris (pH 8.3), 30 mM KCL and 3 mM MgCl₂ [pH 8.3] buffer. DNA was amplified with 25 pmol of primer and water to a final volume of 25 μ l 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 analysis obtained from either Amersham Pharmacia Biotech, UK or from Operon (Operon Technologies Inc, CA, USA) (Table 4.2). To ensure that amplifications were reliable, lyophilised DNA from the BL21 and C1a strains of *E. coli* (included in the kit) was also tested.

4.2.5.2 Gel electrophoresis of PCR products

Five µl of each PCR amplicon was mixed with 1 µl of loading buffer (0.25 % Bromophenol blue, 0.25 % xylene cyanol FF, 40 % w/v sucrose in water) and loaded into 2 % agarose gel in 0.5 x TBE buffer (pH 8.3). The gel electrophoresis was run at 120 V for 1 h. A 100-base pair DNA molecular weight marker (Gibco BRL, UK) was used for size determination of amplicon fragments. The gels were stained in ethidium bromide (10µg/ml) in TBE buffer for 30 min and visualised under UV illumination. The gel images were photographed using a gel imager (Appligene Oncor, France).

4.2.6 Cluster 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.

Table 4.2

Primer sequences used in 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	TTTGTCCCCC
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

OPBC primers obtained from Operon Technologies Inc, CA, USA

APB primers from Amersham Pharmacia Biotech, UK

The index of similarity (S) of samples i and j was estimated using Jaccard's co-efficient, 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 pair group arithmetic average (UPGMA) algorithm was used for clustering analysis and the output was used to construct a phenogram.

RESULTS

4.3.1 Analysis of genomic DNA

The integrity of extracted and purified DNA was checked by running a small quantity on an ethidium bromide-stained gel. This was performed for each isolate prior to the RAPD study to check that the DNA was not sheared. Figure 4.1 is an example showing NC-LivB1, JPA-2, S48 and Vero cell DNA. The bands are distinct showing intact genomic DNA with no smearing present that would indicate shearing of the DNA has occurred.

4.3.2 Control experiments

E. coli DNA was used as a control to ensure the amplifications were working correctly. Figure 4.2 demonstrates the different banding pattern of the *E. coli* (BL21 or C1a) compared with *Neospora* and *Sarcocystis* sp. isolates. The banding pattern for the control DNA corresponded exactly to that published by the manufacturers and so ensured the method was working correctly.

Since RAPD analysis entails amplification of the total genomic DNA in each sample, contamination of parasite material with host cell DNA would give 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. 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. Figure 4.3 shows the typical outcome of such an experiment using primer OPBC2 and demonstrates that parasite RAPD profiles differed markedly

Figure 4.1

Genomic DNA of four *N. caninum* isolates run on a 1 % agarose gel

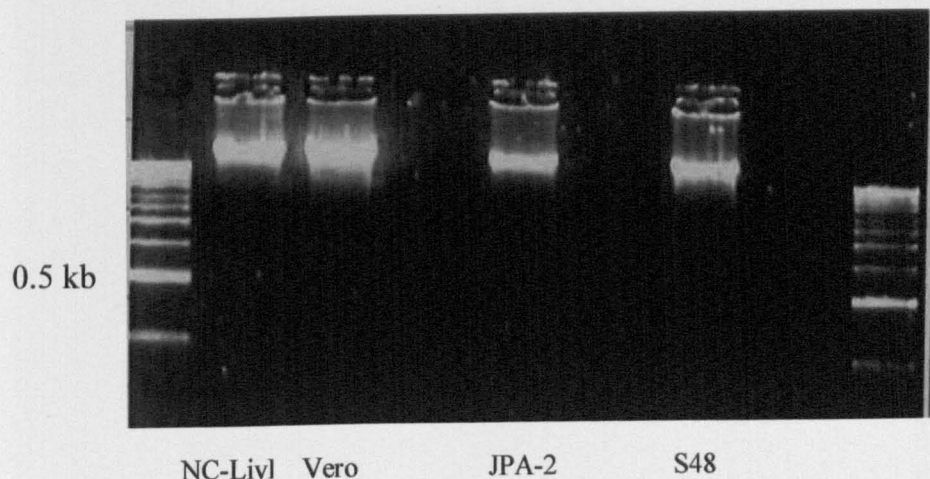


Figure 4.2

RAPD gel showing amplification with Primer 1 on a 1 % agarose gel

(1=1kb ladder; 2=BPA; 3=JPA-2; 4=NC-LivB1; 5=NC-1; 6=NC-LivC; 7= NC-Sweb1; 8=*Sarcocystis* sp.; 9= *E. coli* BL21; 10=*E. coli* C1a)

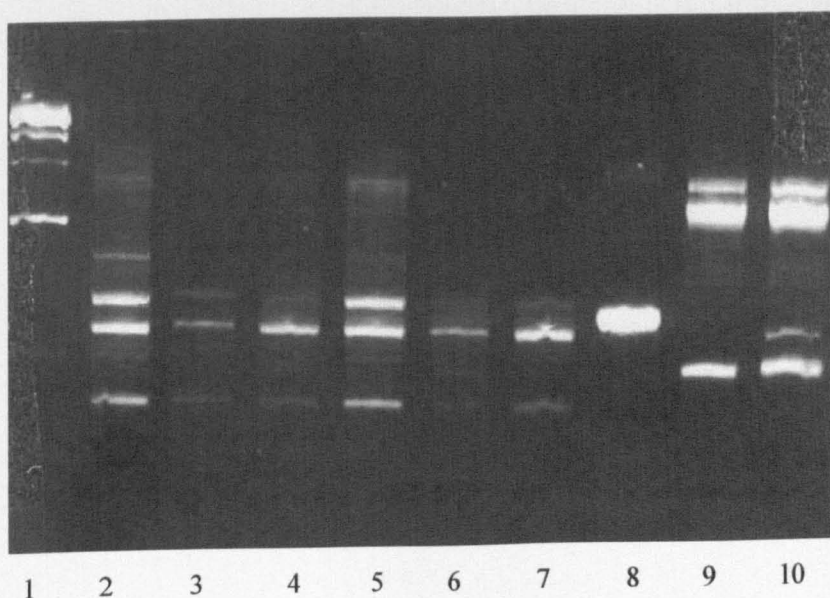
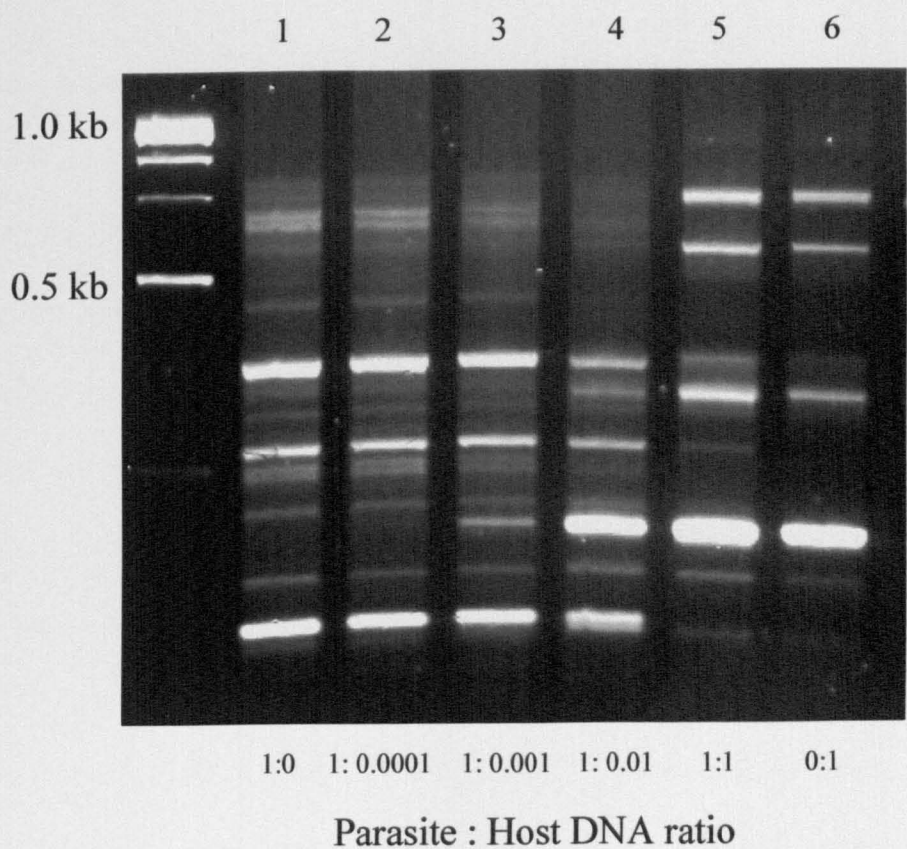


Figure 4.3
RAPD analysis of titrated samples of parasite and host cell DNA using primer OPBC2. Lane 1 = *N. caninum* (LivB1 isolate); Lane 6 = Vero cell; Lanes 2-5 = titration of parasite DNA with host cell DNA.



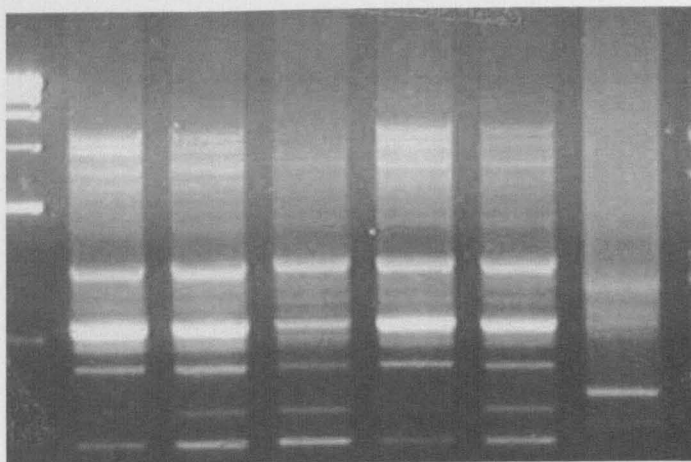
from those given by host cell DNA alone. Moreover, where occasional band sizes given by host and parasite DNA were similar (one out of 6 bands for primer OPBC2), no titration effect was seen, confirming that these bands were unlikely to result from DNA cross contamination.

4.3.3 Analysis of parasite DNA

RAPD analysis was performed using the primers in Table 4.2 on total genomic DNA from six laboratory isolates of *N. caninum*, three strains of *T. gondii*, one isolate of *C. parvum* and one isolate of *Sarcocystis sp.* All the primers gave clear and reproducible banding patterns. Representative gels for 6 of the primers are shown in Figures 4.4 and 4.5, 4.6 and 4.7. The majority of primers gave rise to banding patterns such as those seen in Figures 4.4 and 4.5, with relatively little variation in banding pattern between *Neospora* isolates. Figure 4.6 also shows almost identical fingerprint patterns with all six *Neospora* isolates (primer OPBC 04, samples A-F). However, in most cases primers gave rise to variant profiles 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 (Figure 4.6, primer APB04, sample E). Similarly, primer OPBC16 gave a consistent profile for isolates BPA-1, JPA-2 and NC-1, but showed a clear polymorphic band at approximately 470 bp in isolates NC-LivB1 and NC-SweB1 (Figure 4.7, primer OPBC16). Some primers gave particularly 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 quite variant patterns (Figure 4.7, primer OPBC18).

Figure 4.4

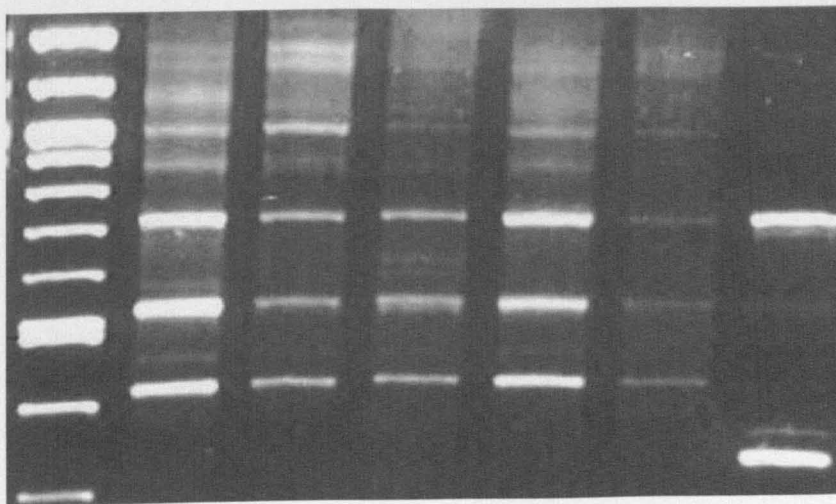
RAPD analysis of 5 *N. caninum* isolates (BPA, JPA-2, NC-LivB1, NC-1 and NC-SweB1) and *Sarcocystis* sp. RAPD profile produced using Primer OPBC 2.



1 kb BPA JPA-2 NC-LivB1 NC-1 NC-SweB1 *Sarcocystis* sp.

Figure 4.5

RAPD analysis of 5 *N. caninum* isolates (BPA, JPA-2, NC-LivB1, NC-1 and NC-SweB1) and *C. parvum*. RAPD profile produced using Primer OPBC 17.



1kb BPA JPA-2 NC-LivB1 NC-1 NC-SweB1 *C. parvum*

Figure 4.6

RAPD analysis of *N. caninum* isolates (bovine and canine origin), *T. gondii* (S48, RH and M3 strains), *Sarcocystis* sp., *C. parvum* and host (VERO) cell DNA. Representative amplifications from two primers (OPBC 04, APB 04) 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*.

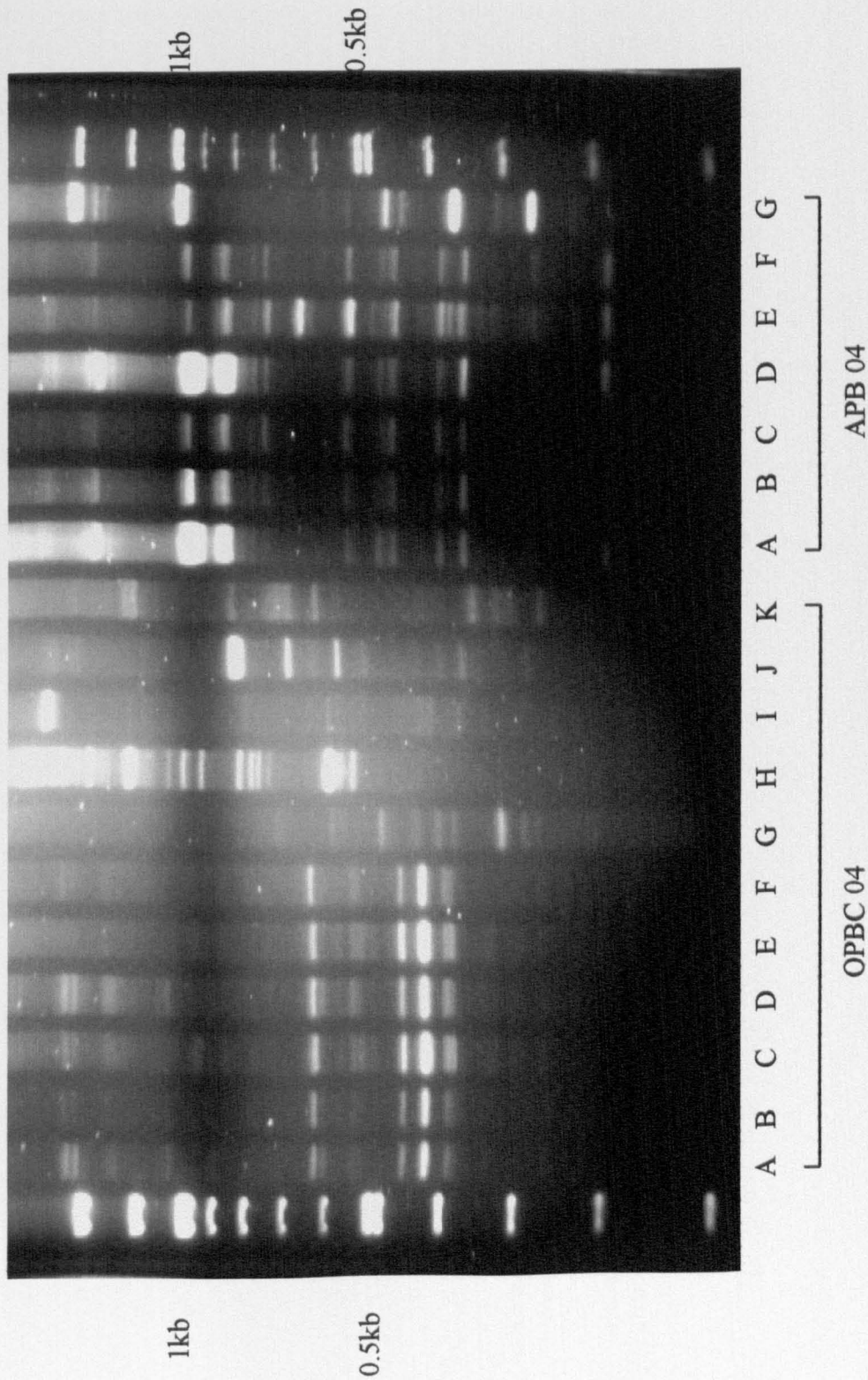
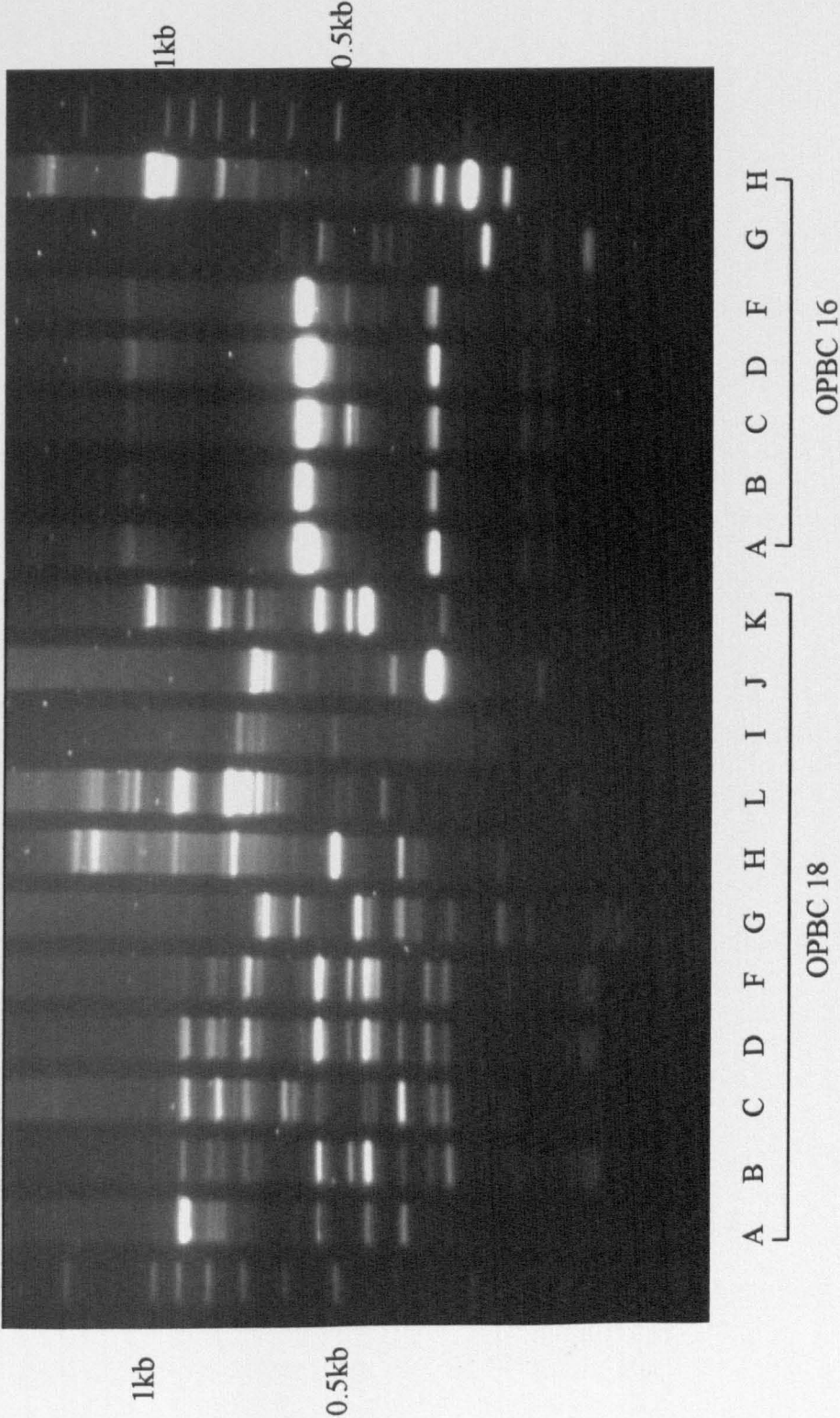


Figure 4.7 RAPD analysis of *N. caninum* isolates (bovine and canine origin), *T. gondii* (S48, RH and M3 strains), *Sarcocystis* sp., *C. parvum* and host (Vero) cell DNA. Representative amplifications from two primers (OPBC 18, OPBC 16) are shown. Codes for samples A-L are: A = BPA-1 *N. caninum*; 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*.



All primers gave banding patterns for the *N. caninum* isolates that were markedly different from the three strains of *T. gondii*, and from *C. parvum* and *Sarcocystis* sp.

4.3.4 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. An example of a binary matrix and subsequent cluster analysis is shown in Table 4.3. 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 was subjected to pair-wise similarity analysis using Jaccard's co-efficient and used the UPGMA method for clustering. The resulting phenogram is shown in Figure 4.8 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.

COMBINED PRIMERS 2, 3 & 4

65

	1	2	3	4	5	6	7	8	9
A	1	1	1	1	1	1	0	0	0
B	1	1	1	1	1	1	1	0	0
C	0	0	0	0	0	0	0	1	0
D	0	0	0	0	0	0	0	0	1
E	0	0	0	0	0	0	0	1	0
F	0	0	0	0	1	0	0	0	0
G	0	0	0	0	0	0	0	1	0
H	0	0	0	0	0	0	0	0	1
I	0	0	0	0	0	0	1	0	0
J	1	1	1	1	1	1	0	0	0
K	0	0	0	0	0	0	0	0	1
L	1	1	1	1	1	1	0	0	0
M	0	0	0	0	0	0	1	0	0
N	0	1	1	0	1	1	0	0	0
O	1	1	1	1	1	1	0	0	0
P	0	0	0	0	0	0	1	0	0
Q	0	0	0	0	1	0	0	0	0
R	0	0	0	0	0	0	1	0	0
A	0	0	0	0	0	0	0	0	1
B	1	1	1	1	0	1	1	0	0
C	0	0	0	0	0	0	0	0	1
D	0	0	0	0	0	0	0	1	0
E	0	0	0	0	0	0	0	0	1
F	1	0	1	0	0	0	0	0	0
G	1	0	1	1	1	1	0	0	0
H	0	0	0	0	0	0	0	0	1
I	0	0	0	0	0	0	0	1	1
J	0	0	0	0	0	0	0	0	1
K	1	1	1	1	1	1	0	0	0
L	0	0	0	0	0	0	1	0	0
M	0	0	0	0	0	0	0	1	0
N	1	1	1	1	1	1	0	0	0
O	0	0	0	0	0	0	0	1	0
P	0	0	0	0	0	0	1	0	0
Q	1	1	1	1	1	1	0	0	0
R	0	0	0	0	0	0	1	0	0
S	0	0	0	0	0	0	0	1	0
T	0	0	0	0	0	0	0	0	1
U	1	1	1	1	1	1	1	1	0
A	0	0	0	0	0	0	0	0	1
B	0	0	0	0	0	0	1	0	0
C	1	1	0	1	0	0	0	0	0
D	0	0	0	0	0	0	0	1	0
E	0	0	0	0	0	0	1	0	0
F	1	1	1	1	1	1	0	0	0
G	0	0	0	0	0	0	0	0	1
H	1	1	1	1	1	1	0	1	0
I	0	0	0	0	0	0	0	1	1
J	1	1	1	1	1	1	0	0	0
K	0	0	0	0	0	0	0	1	0
L	0	0	0	0	0	0	0	0	1
M	0	0	0	0	1	0	0	0	0
N	0	0	0	0	0	0	0	1	0
O	1	1	1	1	1	1	0	0	0
P	0	0	0	0	0	0	0	0	1
Q	1	1	1	1	1	1	1	0	0
R	1	1	1	1	1	1	1	0	0
S	0	0	0	0	0	0	0	1	1
T	0	0	0	0	0	0	0	1	0
U	1	1	1	1	1	1	0	0	1
V	0	0	0	0	0	0	1	0	0

Table 4.3

Binary table constructed for three primers showing typical cluster analysis output

Numbers refer to parasite isolates as follows:

- 1 = BPA
- 2 = JPA-2
- 3 = Nc-LIVB1
- 4 = NC-1
- 5 = Nc-LIVC
- 6 = NC-SWEB1
- 7 = *C. parvum*
- 8 = *Sarcocystis* sp.
- 9 = Vero cells

1 designates the presence of a band
0 designates the absence of a band
Letters denote bands on the gel

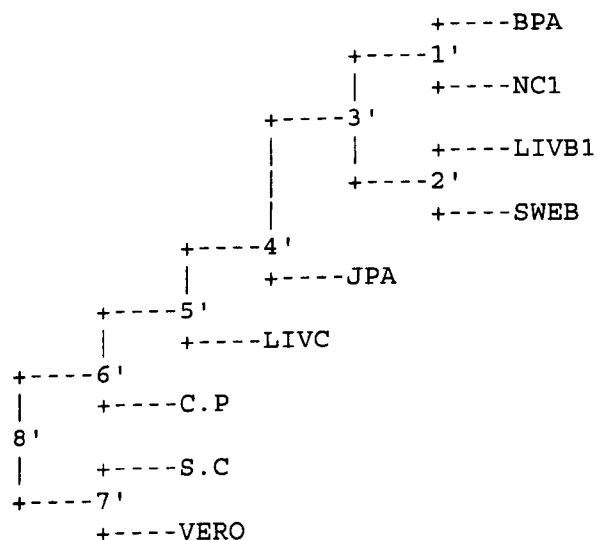
W	1	1	1	1	1	1	0	0	0
X	0	0	0	0	0	0	0	1	0
Y	0	0	0	0	0	0	1	0	0
Z	1	1	1	1	1	1	0	0	0

Cluster V0.1

Using Unweighted Arithmetic Average
The Distance/Similarity measure is Jaccard Similarity.
There are 9 samples.
Clustering procedure:

Step	A	B	Distance
1	NC1 BPA	0.045454545454545456	
2	SWEB LIVB1	0.045454545454545456	
3	2' 1'	0.10968379446640317	
4	3' JPA	0.11067193675889328	
5	4' LIVC	0.22317948717948716	
6	C.P 5'	0.8523223304473305	
7	VERO S.C	0.9032258064516129	
8	7' 6'	0.9629992829005987	

Tree Topology (edge lengths ignored)



From	To	Length
8' 6'	0.05533847622663407	
6' 5'	0.3145714216339217	
5' 4'	0.05625377521029694	
4' 3'	4.940711462450564E-4	
3' 1'	0.032114624505928856	
1' BPA	0.022727272727272728	
1' NC1	0.022727272727272728	
3' 2'	0.032114624505928856	
2' LIVB1	0.022727272727272728	
2' SWEB	0.022727272727272728	
4' JPA	0.05533596837944664	
5' LIVC	0.11158974358974358	
6' C.P	0.42616116522366526	
8' 7'	0.029886738224492893	
7' S.C	0.45161290322580644	
7' VERO	0.45161290322580644	

About to do stability analysis...

Results of stability analysis:

Runs: 65. New data generated by Jackknife

Node Count

1' 63
 2' 62
 3' 61
 4' 61
 5' 65
 6' 65
 7' 65
 8' 65

For each (interior) node in the original tree, the table says how many of the generated trees had an equivalent interior node.

Two interior nodes (in different trees) are equivalent if they contain the same samples AND they split those samples into the same two groups.

Cluster V0.1

Using Unweighted Arithmetic Average

The Distance/Similarity measure is Jaccard Similarity.

There are 9 samples.

Clustering procedure:

Step	A	B	Distance
1	NC1	BPA	0.045454545454545456
2	SWEB	LIVB1	0.045454545454545456
3	2' 1'		0.10968379446640317
4	3' JPA		0.11067193675889328
5	4' LIVC		0.22317948717948716
6	C.P 5'		0.8523223304473305
7	VERO S.C		0.9032258064516129
8	7' 6'		0.9629992829005987

Tree Topology (edge lengths ignored)

```

      +----BPA
      |
      +----1'
      |
      +----NC1
      |
      +----3'
      |
      +----LIVB1
      |
      +----2'
      |
      +----SWEB
      |
      +----4'
      |
      +----JPA
      |
      +----5'
      |
      +----LIVC
      |
      +----6'
      |
      +----C.P
      |
      +----8'
      |
      +----S.C
      |
      +----7'
      |
      +----VERO

```

From	To	Length
8' 6'		0.05533847622663407
6' 5'		0.3145714216339217
5' 4'		0.05625377521029694
4' 3'		4.940711462450564E-4

```

3' 1' 0.032114624505928856
1' BPA 0.022727272727272728
1' NC1 0.022727272727272728
3' 2' 0.032114624505928856
2' LIVB1 0.022727272727272728
2' SWEB 0.022727272727272728
4' JPA 0.05533596837944664
5' LIVC 0.11158974358974358
6' C.P 0.42616116522366526
8' 7' 0.029886738224492893
7' S.C 0.45161290322580644
7' VERO 0.45161290322580644

```

About to do stability analysis...

Results of stability analysis:

Runs: 100. New data generated by Bootstrap

Node Count

```

1' 53
2' 50
3' 6
4' 8
5' 74
6' 92
7' 72
8' 70

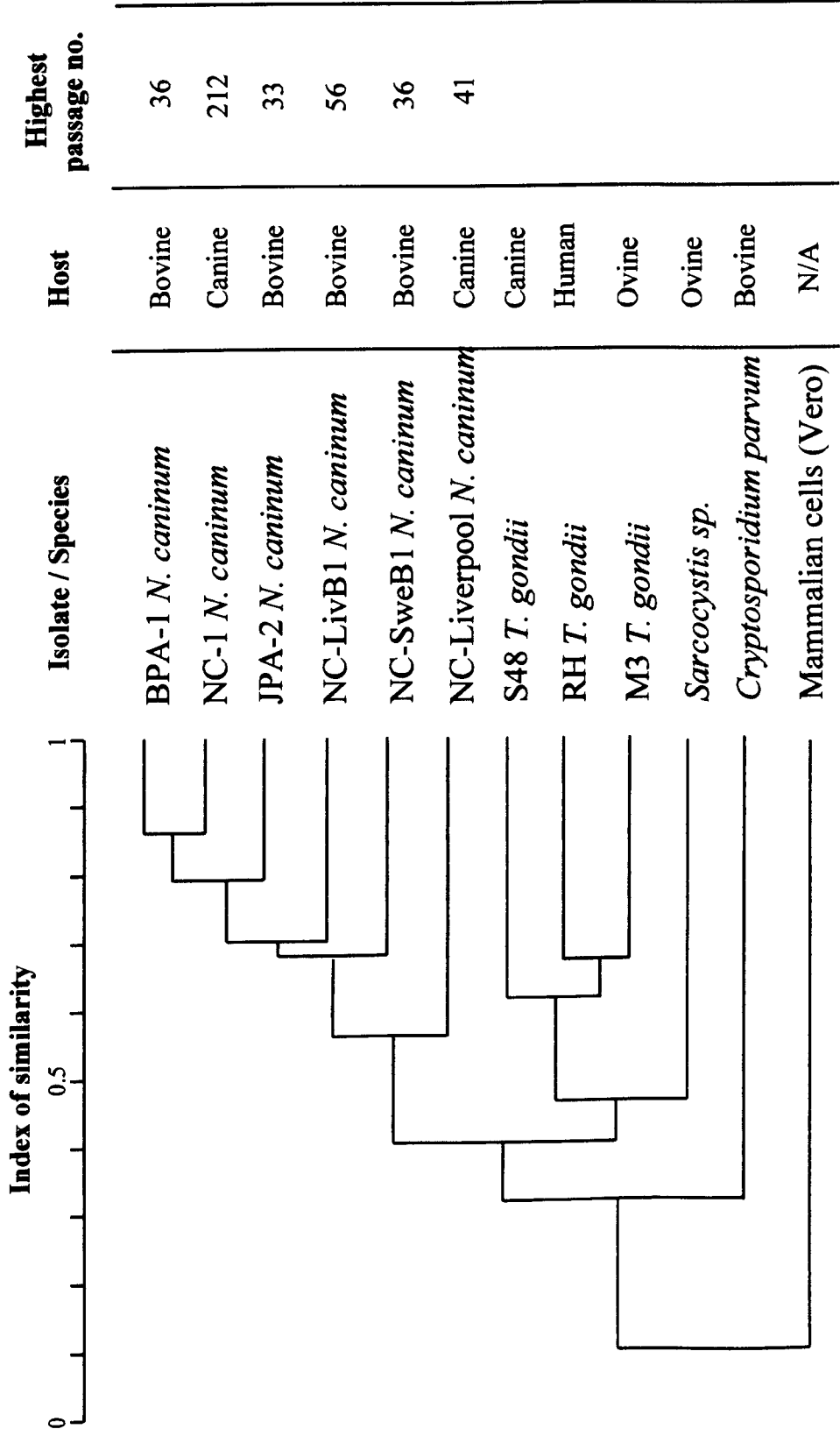
```

For each (interior) node in the original tree, the table says how many of the generated trees had an equivalent interior node.

Two interior nodes (in different trees) are equivalent if they contain the same samples AND they split those samples into the same two groups.

Figure 4.8

Phenogram showing genetic relationships between *N. caninum* isolates (bovine and canine origin), *T. gondii* (S48, RH and M3 strains), *Sarcocystis* sp. and *C. parvum* as estimated by cluster analysis of RAPD patterns obtained with 26 primers. The similarity data was derived from Jaccard's co-efficient and subjected to cluster analysis.



4.4 DISCUSSION

In this chapter the genetic diversity in six isolates of *N. caninum* of both bovine and canine origin was analysed using genetic markers derived from RAPD-PCR analysis. These data demonstrate that genetic heterogeneity exists within the species and may have important implications for our understanding of the pathology and epidemiology of neosporosis.

Genetic analysis of the *Neospora* isolates by RAPD-PCR produced a large number of polymorphic markers that revealed 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 the 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 Zande and 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 (Muller *et al.*, 1997; Brisse *et al.*, 2000) and *Leishmania* (Banuls *et al.*, 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 *C. 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 sub-unit 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 two 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 for example, cattle placenta.

Schock *et al.* (2001) compared the growth rates of the six *N. caninum* isolates analysed in this chapter and 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 three 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 occurs 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. Schock *et al.* (2001) showed that isolate NC-Liverpool grew at over ten 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.

The RAPD data in this study show genetic diversity between the three strains of *T. gondii* and reflects that found in other studies (Guo and 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 three clonal lineages (Type I, Type II and Type III strains) (Howe and Sibley, 1995). These lineages are still able to undergo meiotic recombination (albeit rarely) thus demonstrating that they are not separate species (Sibley and Howe, 1996). The predominantly clonal population of *Toxoplasma* appears to result in a correlation between the development of disease and parasite genotype. Sibley and 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.

The RAPD technique has proved to be an extremely useful and quick method of obtaining a preliminary measure of the extent of genetic heterogeneity within a species. Nevertheless, the number of genetic markers obtained by RAPD analysis was relatively low for this parasite. It would be very useful to be able to utilise a method that is even more discriminatory and can obtain a larger number of genetic markers that could be

adapted for use on clinical samples. Analysis of field isolates from defined outbreaks is essential since laboratory isolates are unlikely to be fully representative. Amplified fragment length polymorphism (AFLP) is an extremely sensitive and specific whole genome-typing technique and is ideal for this type of study.

Chapter 5: Analysis of genetic diversity in *Neospora caninum* using Amplified Fragment Length Polymorphism (AFLP) techniques

5.1 INTRODUCTION

Amplified Fragment Length Polymorphism techniques provide a sophisticated method of fingerprinting genomic DNA (Vos *et al.*, 1995) that has the distinct advantage of not requiring any prior genome sequence information. Like RAPD, AFLP surveys the entire genome of the species being studied but is considered to be far more reproducible because it uses high annealing temperatures at all PCR steps to ensure specific primer binding. AFLP is based on the selective amplification of a subset of genomic restriction fragments produced by restriction enzyme digestion followed by PCR. Initially genomic DNA is digested with restriction endonucleases and double-stranded DNA adapters are ligated to the ends of the DNA fragments to produce templates for subsequent amplification. Selective nucleotides are then added to the 3' ends of PCR primers so that only a subset of the fragments are amplified i.e. only those restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides being amplified. The amplified fragments are then analysed using denaturing polyacrylamide gel electrophoresis.

AFLP was initially used exclusively in the plant science field specifically to generate polymorphic markers for genetic linkage mapping (Simons *et al.*, 1997). Subsequently it has proved a valuable tool for the analysis of both prokaryote and eukaryote genomes in a range of species. Janssen *et al.* (1996) found the technique suitable for typing bacteria where it enabled the differentiation of

highly related *Xanthomonas* strains and even biovars. The technique has also been employed in studying the genetic diversity in cattle (Ajmone-Marsan *et al.*, 2002)

Masiga *et al.* (2000) describe the application of AFLP in the field of parasite genetics and Grech *et al.* (2002) showed the technique to be ideal in identifying distinct genetic markers between clones of *Plasmodium c. chabaudi*. A genetic linkage map for *Eimeria tenella* has also been constructed using AFLP (Prof. Martin Shirley, pers.comm.).

AFLP is an extremely powerful technique for detecting genetic polymorphisms because a typical single reaction gives rise to between fifty and one hundred markers that are detected by gel electrophoresis. Eventhough RAPD analysis showed that there was genetic heterogeneity amongst *Neospora* isolates (Chapter 4) it was felt that a technique such as AFLP would be more sensitive, more reproducible and reveal more polymorphisms between isolates. The greater discriminatory power of AFLP was considered important because polymorphic diversity in *N. caninum* was shown to be relatively low by RAPD analysis. For example, many RAPD primers showed few or no polymorphisms suggesting potentially low genetic diversity in the *N. caninum* isolates. Moreover, it was not known whether our laboratory stocks of *N. caninum* consisted of effectively “cloned lines” or whether heterogeneity existed within these stocks. This would have important implications for the future development of genetic markers for these stocks of parasites. The highly discriminatory nature of AFLP made it the method of choice for this analysis.

Both AFLP and RAPD are techniques that are poor at analysing mixtures of DNA, hence the initial requirement for highly purified DNA. This also explains the suitability of both methods for use with laboratory isolates. Parasite tissue culture allows us to grow large quantities of the organism for study. AFLP would not be suitable for clinical samples collected directly from the field since these samples contain both host and parasite DNA. Therefore it would be necessary to identify specific markers from which direct PCR amplification from mixed DNA samples is possible. Moreover, analysis is made easier since tachyzoites are haploid and there is therefore only one allele per gene. In principle, it is possible to use AFLP to isolate genes, or gene fragments. These could then be used to develop PCR-based typing methods such as PCR-RFLP and it was hoped that it would be possible to extract genetic markers from AFLP gels that could later be used to ascertain the degree of genetic diversity between clinical samples.

The aims of this chapter were

- To define the extent of genetic variation that exists amongst different isolates of *N. caninum* using AFLP and to compare results with RAPD analysis.
- To determine whether genetic heterogeneity existed within individual stocks of laboratory isolates by comparing the genotype of cloned lines of *N. caninum*.

- To identify genetic markers that could be developed as PCR-based markers for clinical isolates.

5.2 MATERIALS AND METHODS

5.2.1 Parasite isolates and DNA

Eight laboratory isolates of *N. caninum* were used in this study and are shown in Table 5.1. Two isolates, NC-1 and NC-Liverpool were isolated from dogs and the others were isolated from cattle. The geographical origin of the isolates are also given. Table 5.2 shows other apicomplexan parasites – *T. gondii*, *C. parvum* and *Sarcocystis* sp. that were used in the study for comparison purposes. Seven isolates of *T. gondii* originating from different locations around the world were used, as this parasite is considered most closely related to *N. caninum*. The *C. parvum* and *Sarcocystis* sp. samples were kindly donated as detailed in section 4.2.1. DNA from the *T. gondii* strains was generously given by Dr. Ashgar Fazaeli (Department of Medical Microbiology, University of Aberdeen). Tachyzoites and genomic DNA were prepared from tissue culture lysates as detailed in section 4.2.4. Since the outcome of each AFLP reaction is very much dependent on complete restriction digestion it was important that the DNA that was isolated was free of nucleases or inhibitors that might lead to incomplete DNA digestion.

5.2.2 Cloning *N. caninum* isolates

5.2.2.1 Culture of HFFF cells

Human Foetal Foreskin Fibroblast (HFFF-2) cells were used for cloning the parasites. They are more suitable for cloning than Vero cells because HFFF cells grow at a slower rate and can be maintained as a monolayer until required for use. HFFF cells were generally passaged once a week as required, as soon as a confluent monolayer had formed. HFFF cells also have a finite life-span as they

Isolate (abbreviation)	Reference	Host	Country
NC-1	Dubey et al. (1998)	dog	USA
JPA-2	not published	calf	Japan
NC-SweB1	Stenlund et al. (1997)	calf	Sweden
NC-Liverpool	Barber et al. (1993)	dog	UK
BPA-1	Conrad et al. (1993)	calf	USA
NC-LivB1	Davison et al. (1997)	calf	UK
NC-LivB2	not published	calf	UK
NC-Beef	not published	calf	USA

Table 5.1 *N. caninum* isolates used in the AFLP study

Isolate	Host	Country
sRH	Human	USA
RHW	Human	USA
S48	Sheep	New Zealand
PRU	Human	USA
JLO	Human	UK
LGE 96-1	Human	France
NED	Human	France
<i>Sarcocystis</i> sp.	Sheep	UK
<i>C. parvum</i>	Type I	UK
<i>C. parvum</i>	Type II	USA

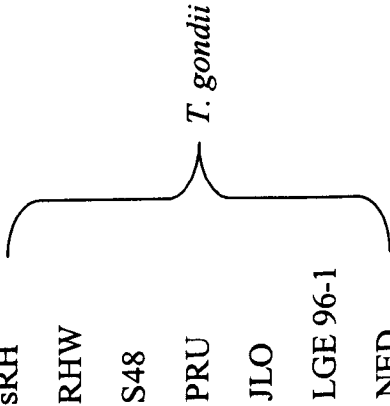


Table 5.2 Other species of the Apicomplexa used in the AFLP study

do not grow beyond twenty passages, unlike Vero cells that can grow indefinitely. Therefore, HFFF cells were not used for routine production of parasite stocks.

HFFF cells were detached from the bottom of culture flasks in the same way as described for Vero cells (section 4.2.3.2). The cells were resuspended in 10 ml of IMDM growth medium supplemented with penicillin, streptomycin and 10 % FBS. One ml of this resuspension was added to 4 ml (25 cm² flask) or 14 ml (75 cm² flask) of IMDM growth medium. The cells were incubated at 37 °C in a humidified 5 % CO₂ incubator.

5.2.2.2 Cloning by limiting dilution

Individual parasite clones of *N. caninum* isolates NC-Beef, NC-1, NC-SweB1 and NC-Liverpool were isolated by limiting dilution in microtitre 96-well plates containing a confluent monolayer of HFFF cells. It was assumed that 20-40 % of extracellular tachyzoites would be viable, so inoculation of a 96-well plate with an average of 0.25 tachyzoites per well should yield a predicted frequency of 5-9 wells containing a single parasite clone, and <0.5 wells containing more than one parasite. An inverted microscope (Zeiss Axiovert 25) was used to identify single parasite plaques, which were then removed to T25 flasks for normal growth. Single parasite plaques were observed between 7 and 10 days after initial inoculation of the plates. Flasks were ready for harvest after 2-3 weeks.

5.2.3 AFLP technique

The main steps involved in the AFLP technique are summarised in Figure 5.1.

These are:

- Restriction endonuclease digestion of the DNA using two enzymes
- Ligation of the adapters to generate template DNA for amplification
- Amplification of the restriction fragments in two consecutive reactions
- Gel analysis of the amplified fragments on a denaturing polyacrylamide gel

AFLP Analysis System II (GibcoBRL, Life Technologies, Paisley, UK) was suitable for the estimated size of the *Neospora* genome (80 MB, based on that of the *T. gondii* genome) and was used throughout this study. The reagents were from the AFLP Core Reagent Kit (Gibco BRL) and the AFLP Small Genome Primer Kit (Gibco BRL) and are shown in Table 5.3 and Table 5.4.

5.2.3.1 Restriction Endonuclease Digestion of genomic DNA

Genomic DNA was isolated and digested with two restriction endonuclease enzymes simultaneously. The enzymes used in this study were *EcoR* I and *Mse* I. *EcoR* I had a six base pair recognition site and *Mse* I had a four base pair recognition site. This generated DNA fragments that were smaller than one kilobase in size and were therefore ideal for separation on a denaturing polyacrylamide gel. Three types of fragments were produced (Figure 5.1) but primer design ensured that the *EcoR* I–*Mse* I fragments were preferentially amplified. Fragments that resulted from digestion only with *EcoR* I were likely to be too big to be resolved on a standard sequencing gel since there is a restriction site approximately every 4096 (4^6) bp (assuming random distribution

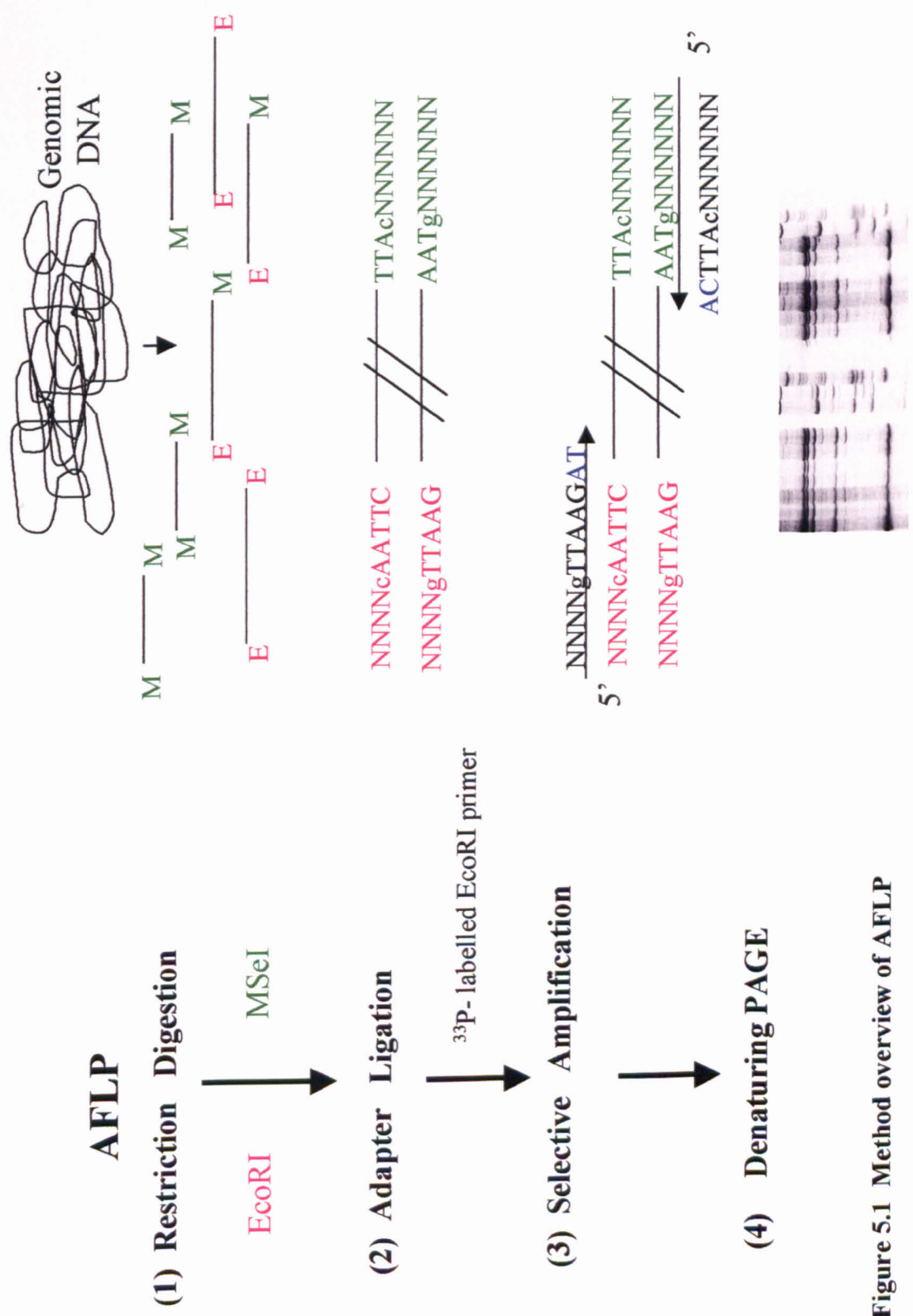


Figure 5.1 Method overview of AFLP

Table 5.3
AFLP Core Reagent Kit

Component	Volume
EcoRI/ MseI [1.25 units/μl each in 10mM Tris-HCl (pH 7.5), 50mM NaCl, 0.1 mM EDTA, 1mM DTT, 0.1mg/ml BSA, 50% (v/v) glycerol, 0.1% Triton® X-100]	100 μl
5X reaction buffer [50 mM Tris-HCl (pH 7.5), 50mM Mg-acetate, 250 mM K-acetate]	250 μl
Distilled water	1.25 ml
Adapter-ligation solution [EcoRI/ MseI adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate]	1.2 ml
T4 DNA ligase [1 unit/μl in 10 mM Tris- HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50 % glycerol (v/v)]	50 μl
TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]	4.5 ml
Arabidopsis DNA (100ng/μl)	10 μl
Tomato DNA (100ng/μl)	10 μl

Table 5.4**AFLP Small Genome Primer Kit**

Component	Volume
Pre-amp primer mix	2 ml
T4 kinase [10 units/ μ l in 50 mM Tris-HCl (pH 7.6), 25mM KCl, 1 mM 2-mercaptoethanol, 0.1 μ l ATP, 50% (v/v) glycerol]	32 μ l
5X kinase buffer [350 mM Tris-HCl (pH 7.6), 50 mM MgCl ₂ , 500 mM KCl, 5 mM 2-mercaptoethanol]	160 μ l
EcoRI primers (27.8 ng/ μ l):	
Primer E-AA	46 μ l
Primer E-AC	46 μ l
Primer E-AG	46 μ l
Primer E-AT	46 μ l
Primer E-TA	46 μ l
Primer E-TC	46 μ l
Primer E-TG	46 μ l
Primer E-TT	46 μ l
Mse I primers (6.7 ng/ μ l, dNTPs):	
Primer M-CAA	900 μ l
Primer M-CAC	900 μ l
Primer M-CAG	900 μ l
Primer M-CAT	900 μ l
Primer M-CTA	900 μ l
Primer M-CTC	900 μ l
Primer M-CTG	900 μ l
Primer M-CTT	900 μ l
10X PCR buffer plus Mg [200 mM Tris-HCl (pH 8.4), 15 mM MgCl ₂ , 500 mM KCl]	3.5 ml
TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]	8 ml
Distilled water	1.25 ml
Preamplified <i>Arabidopsis</i> DNA (control for selective amplification)	10 μ l

of the four bases in DNA. If these fragments were small enough to enter the gel they would still be detected. Restriction fragments flanked only with *Mse* I sites only will occur more frequently, every 256 (4^4) bp, on average but as they are unlabelled will not be detected. The vast majority of bands recognised on a gel are fragments flanked by an *Eco* RI and an *Mse* I restriction site.

For restriction endonuclease digestion, the following components were added to a 1.5 ml microcentrifuge tube: 5 μ l 5X reaction buffer, 2 μ l *Eco*R I /*Mse* I mix, 250 ng sample DNA in < 18 μ l and distilled water to a volume of 25 μ l. The reagents were gently mixed by pipetting and then collected at the bottom of the tube by brief centrifugation. The mixture was incubated at 37 °C on a heated block for 2 h. This DNA digestion was followed by incubation at 70 °C for 15 min to inactivate the restriction endonucleases. The tube was subsequently placed on ice and the contents were then collected by brief centrifugation.

5.2.3.2 Ligation of Adapters

To halt activity of the endonucleases the samples were heat inactivated and the DNA fragments were ligated to *Eco*R I and *Mse* I adapters to generate template DNA for amplification. The variable genomic DNA sequences were therefore flanked by common adapter sequences, which served as primary binding sites. This step allows the amplification of genomic DNA fragments for which there was no prior sequence knowledge.

To perform the ligation, the following mixture was added to the digested DNA: 24 μ l adapter ligation solution and 1 μ l T4 ligase solution. The resulting solution

was gently mixed at room temperature, briefly centrifuged and incubated at 20 °C for 2 h. Following ligation, a 1:10 dilution was performed by removing 10 µl of the reaction mixture to a clean 1.5 ml microcentrifuge tube and adding 90 µl TE buffer. After mixing well, 5 µl of the solution was used in the pre-amplification stage and the unused portion was stored at –20 °C.

5.2.3.3 Preamplification reaction

In the primary amplification reaction, genomic DNAs were amplified with an *Mse* I primer containing one selective nucleotide (N+1) and an *EcoR* I primer containing no selective nucleotides (N+0). The resulting PCR products were then diluted and used as a template for the selective amplification.

The preamplification reaction was set up to a volume of 51 µl by adding the following to a 0.5 ml microcentrifuge tube: 5 µl diluted template DNA (from ligation step, section 5.2.2.2), 40 µl pre-amp primer mix, 10X PCR buffer mix plus Mg, 1 µl *Taq* DNA polymerase (1unit/µl). The reagents were gently mixed by pipetting up and down and briefly centrifuged to collect the reaction. Twenty cycles of the following PCR reaction were performed in an MJ Dyad DNA engine (Cambridge, UK): 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s followed by 4 °C resting temperature. (Note that the samples were not denatured prior to PCR because this would reduce the annealing efficiency of the primer).

A 1:50 dilution was performed on the resulting reaction by transferring 3 µl to a 1.5 ml microcentrifuge tube containing 147 µl TE buffer. Up to 30 selective

AFLP amplification reactions could be performed on this diluted. The unused diluted and undiluted preamplification reactions were stored at -20°C .

5.2.3.4 Primer Labelling

The *EcoR* I primer in the selective amplification was labelled by phosphorylating the 5' end of the primer with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ and T4 kinase. The *EcoR* I primer was labelled by adding the following to a 1.5 ml microcentrifuge tube: 18 μl *EcoR* I primer, 10 μl 5X kinase buffer, 10 μl distilled water, 10 μl $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (2,000 Ci/mmol) and 2 μl T4 kinase to a total volume of 50 μl . After gentle mixing by pipetting and brief centrifugation to collect the contents, the reaction was incubated at 37°C for 1 h on a heated block. The T4 kinase was inactivated by incubating the tube at 70°C for 10 min followed by brief centrifugation.

5.2.3.5 Selective AFLP amplification

The second amplification employed an *Mse* I primer containing three selective nucleotides (N+3) and an *EcoR* I primer containing two selective nucleotides (N+2) that extend into the restriction fragments (Table 5.5). Therefore only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides would be amplified. This subset was then analysed by denaturing polyacrylamide gel electrophoresis. The complexity of AFLP patterns would be reduced four-fold with each additional selective nucleotide.

The two-stage amplification process results in clean results and reproducible fingerprints.

Table 5.5 Primer pair selection for AFLP using Gibco Analysis System II

Mse I primer

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AA	+	+	+	+	+	+	+	+
E-AC	+	+	+	+	+	+	+	+
E-AG	+	+	+	+	+	+	+	+
E-AT	+	+	+	+	+	+	+	+
E-TA	+	+	+	+	+	+	+	+
E-TC	+	+	+	+	+	+	+	+
E-TG	+	+	+	+	+	+	+	+
E-TT	+	+	+	+	+	+	+	+

EcoR I primer

EcoR I + 2 selective nucleotides

Red bases refer to variable nucleotides

Mse I + 3 selective nucleotides

Selective amplification was performed as follows: two 1.5-ml microcentrifuge tubes were labelled 'Mix 1' and 'Mix 2' and were made up in the following way. Mix 1 consisted of 5 µl of labelled *EcoR* I primer (from section 5.2.2.4) being added to 45 µl *Mse* I primer (contained dNTPs) to a volume of 50 µl which was sufficient for ten reactions. Mix 2 was made up by adding 79 µl distilled water to 20 µl PCR buffer plus Mg^{2+} and 1 µl *Taq* DNA polymerase (5 units/µl) to a final volume of 100 µl (sufficient for ten reactions). The amplification reaction was then set-up by adding 5 µl Mix 1 (primers/dNTPs) and 10 µl Mix 2 (*Taq* DNA polymerase/buffer) to 5 µl diluted template DNA (from section 5.2.2.3) in a 0.5-ml thin-walled microcentrifuge tube to a total volume of 20 µl. The reagents were gently mixed and centrifuged. Mineral oil (20 µl) was overlaid onto the PCR reaction to prevent evaporation of the sample. The PCR was started at a very high annealing temperature to obtain optimal primer selectivity. This annealing temperature was then gradually lowered to a temperature at which efficient primer binding occurred and then maintained at this throughout the remainder of the cycles.

The PCR reaction was run as described below.

One cycle at 94 °C for 30 s followed by 65 °C for 30 s and then 72 °C for 60 s. The annealing temperature was then lowered by 0.7 °C each cycle over twelve cycles giving a touch down phase of thirteen cycles. This was followed by 23 cycles at: 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. PCRs were performed on a Perkin Elmer 2500 PCR machine (Perkin Elmer,UK).

5.2.3.6 Denaturing polyacrylamide gel analysis

The PCR products from the selective amplification were separated on a 6% denaturing polyacrylamide (sequencing) gel. Following the PCR reaction, an equal volume (20 μ l) of formamide dye (98 % formamide, 10 mM EDTA, bromophenol blue, xylene cyanol) was added to each reaction. The samples were denatured by being heated for 3 min at 90 °C on a heated block and then immediately placed on ice.

A 6 % polyacrylamide gel (80 ml SequaGel® XR monomer solution [National Diagnostics, Hull, UK], 20 ml SequaGel buffer [5X TBE and TEMED] with 800 μ l 10 % Ammonium Persulphate [1 g Ammonium Persulphate in 10 ml water]) was poured using 0.4 mm spacers and sharkstooth combs. Gels were poured at an angle of 20 ° using a 50 ml syringe to prevent the formation of bubbles. The gel was left flat to set for at least 1 h at room temperature. Once set, the sharkstooth comb was carefully removed from the gel, washed and then replaced into the gel with the teeth pointing into the gel, thus forming the wells. The tank was then filled at the bottom and top using 1 X TBE.

Gels were pre-electrophoresed at constant power (~55 W) for about 20 min.

Samples (3 μ l) were then loaded into the gel and the gel was electrophoresed at constant power until the xylene cyanol was two-thirds of the way down the length of the gel (approximately 2 h).

After electrophoresis, gels were removed from the plates and placed onto filter paper, covered in cling film and dried using a Biorad vacuum drier for 2 h.

Dried gels were then placed in a cassette and exposed to x-ray film (Kodak Biomax™ MR) in a dark room. The cassette was placed in a -70°C freezer for several days. The autoradiograph was processed using an X-Ograph Compact X4 Imaging system.

5.2.4 Cluster Analysis

Each band on individual gels was scored as present or absent in a binary table and cluster analysis was performed exactly as described in Section 4.2.7.

5.2.5 Dendrograms

The dendrograms were drawn using results obtained from the cluster analysis UPGMA algorithm as in section 4.2.8.

5.2.5 Cloning polyacrylamide gel products

In order to obtain genetic markers for each isolate, an attempt was made to clone unique gel products from the polyacrylamide gels. Briefly, the autoradiograph was lined up exactly over the polyacrylamide gel by aligning the Stratagene markers (Stratagene, UK) stuck on the original gel. The polymorphic bands were then cut from the autoradiograph and the underlying polyacrylamide gel. The acrylamide gel fragment was then rehydrated overnight in distilled water and PCR was performed on the rehydrated gel fragment using the selective primers. PCR products were then analysed on 1% agarose gels.

5.3 RESULTS

The AFLP technique was carried out on 8 *Neospora* laboratory isolates, 7 *Toxoplasma* isolates, 2 *Cryptosporidium* isolates and a *Sarcocystis cruzi* isolate (Tables 5.1 and 5.2). All possible combinations of the selective *EcoR* I and *Mse* I primer pairs (Table 5.5) supplied in the AFLP Small Genome Primer Kit were tested (64 in total). The number of bands seen on each gel varied depending on the primer combination used. The average number was about 50 bands per sample per primer set. Figures 5.2, 5.3, 5.4 and 5.5 are examples of AFLP gels generated using the above primer combinations.

Figure 5.2 shows a gel resulting from the selective amplification of 8 *Neospora* isolates using primer pair E-AT and M-CAG. The fingerprint profiles obtained from the isolates are very similar but also reveal polymorphisms. Samples 5 and 6 show two different cloned lines of NC-Beef, which appear identical.

Figure 5.3 shows the AFLP profile of 6 *Neospora* isolates, a *Toxoplasma* isolate and a *Cryptosporidium* isolate obtained using primer pair E-AC and M-CAC. Although the 6 *Neospora* isolates had similar AFLP profiles, there were clear examples of additional bands present in some *Neospora* isolates but absent in others. The profiles for the RH strain of *Toxoplasma* and the Iowa strain of *Cryptosporidium* are completely different to all other samples tested.

The primer pair, E-AA and M-CAT, gave the gel seen in Figure 5.4. The similarity of the results for a particular genera, for example *Neospora* (samples 1 to 9) is clear. *Toxoplasma* isolates also gave very conserved banding patterns

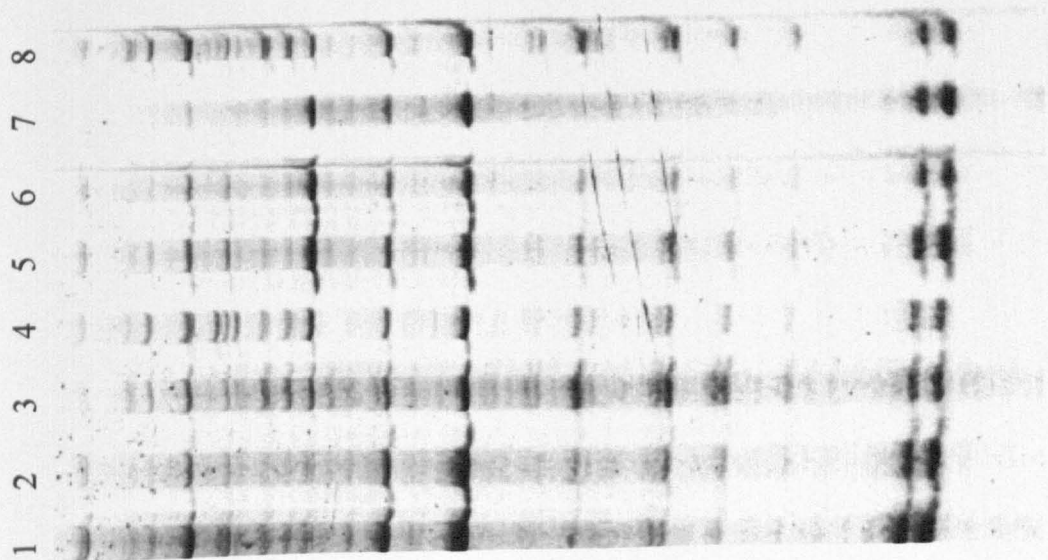


Figure 5.2

AFLP analysis of *Neospora* isolates using primer pair E-AT and M-CAG

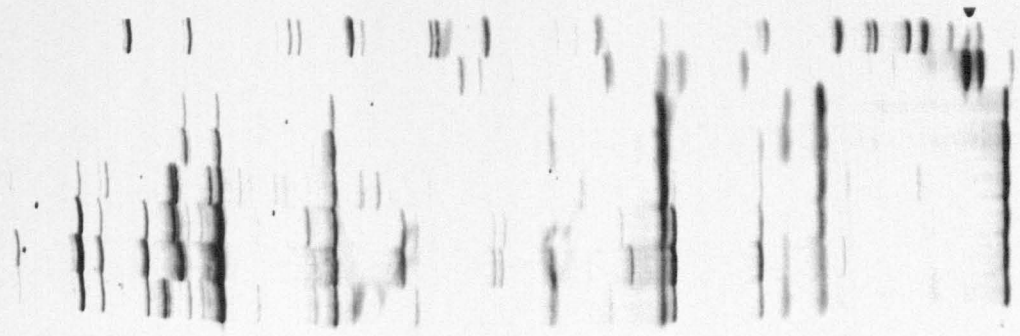
1. BPA-1
2. JPA-2
3. NC-LivB1
4. NC-Liverpool
5. NC-Beef
6. NC-Beef
7. NC-LivB2
8. NC-SweB1

1 2 3 4 5 6 7 8

Figure 5.3

AFLP analysis of 6 *Neospora* isolates, a *Toxoplasma* isolate and a *Cryptosporidium* isolate, using primer pair E-AC and M-CAC.

- 1. BPA-1
 - 2. JPA-2
 - 3. NC-LivB1
 - 4. NC-LivC
 - 5. NC-SweB1
 - 6. NC-SweB1
 - 7. RH - *Toxoplasma gondii*
 - 8. Iowa - *Cryptosporidium parvum*
- } *Neospora caninum*



1. BPA
 2. JPA
 3. & 4. LIVB1
 5. LIVB2
 6. LVC
 7. SweB-1
 8. NC-1
 9. NC-Beef
 10. sRH
 11. S48
 12. Pru
 13. JLO
 14. LGE
 15. NED
 16. RHW
 17. *Sarcocystis* spp.
 18. Iowa *C. parvum*
- N. caninum*
T. gondii

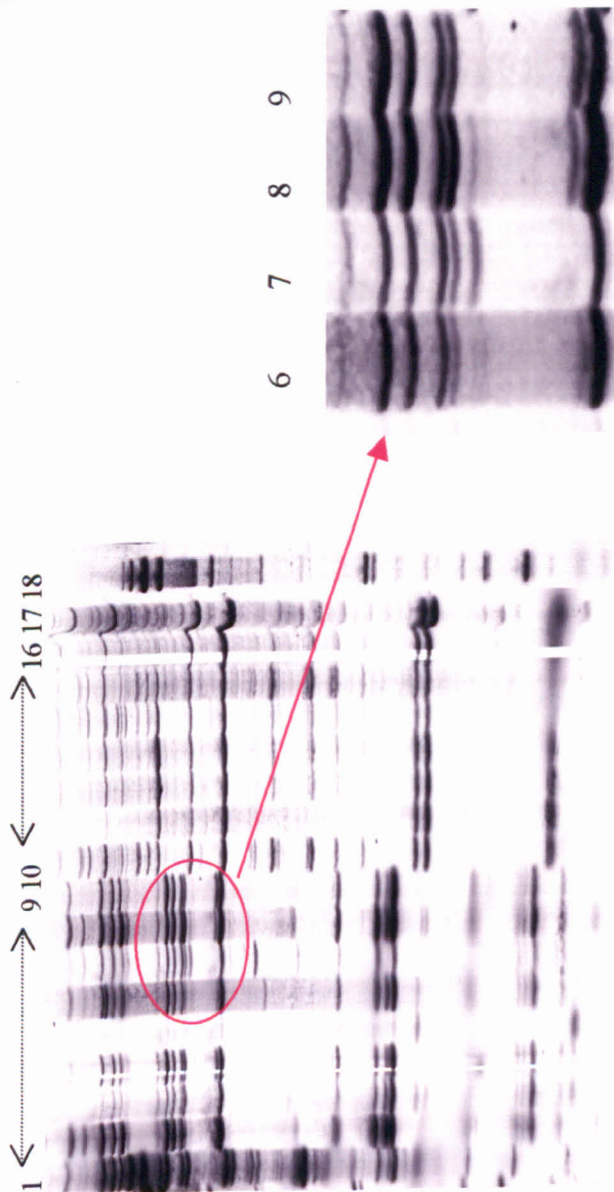


Figure 5.4
 AFLP analysis of *Neospora*,
Toxoplasma, *Sarcocystis* and
Cryptosporidium isolates using
 primer pair E-AA and M-CAT

(samples 10 to 16), but these were markedly different from those for *Neospora*.

The enlarged area demonstrates the resolution and clarity of the AFLP images obtained.

Figure 5.5 shows the amplification from Primers E-AG and M-CAT. Two separate selective amplifications were performed using these primers on different dates. This was carried out with a further 10 combinations of primers to check that the method was reproducible. Identical banding patterns were obtained for each analysis confirming the reproducibility of the AFLP technique.

Preparations of pure DNA from Vero cells were also amplified as a control to ensure that Vero DNA was not contaminating the parasite AFLP banding patterns.

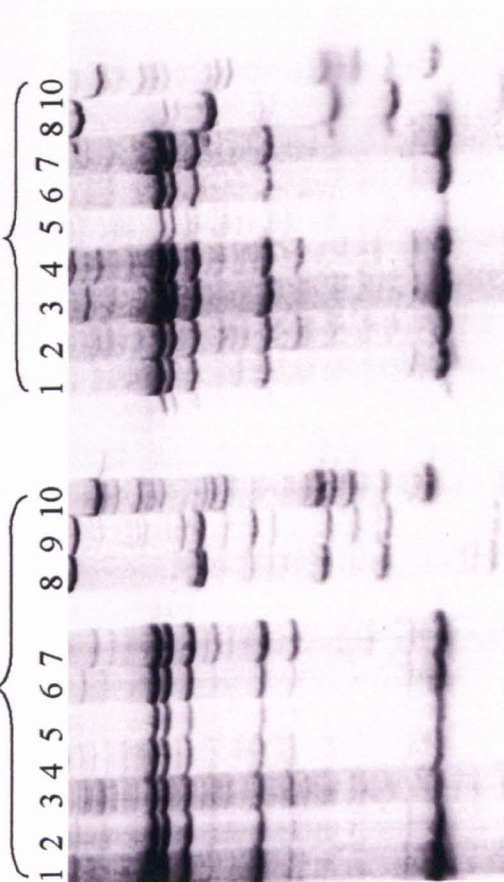
It was immediately apparent that very few bands were shared between the genera tested i.e. *Neospora* isolates shared almost no bands with the *Cryptosporidium* isolates. Likewise, *Toxoplasma* isolates shared very few bands with *Sarcocystis* sp. There were some shared bands between *Neospora* isolates and *Toxoplasma* isolates.

To determine whether the *Neospora* laboratory isolates consisted of homogeneous or heterogeneous stocks, parasites were cloned by limiting dilution and individual cloned lines were analysed by AFLP. Different cloned isolate samples of *N. caninum*, such as NC-Beef, gave the same AFLP fingerprints (Figure 5.2) showing that these particular stocks were likely to be homogeneous. Five cloned lines were grown and tested for each isolate.

Amplification date:

03/02/01

14/11/00



N. caninum *T. gondii*
 1 = BPA-1 8 = sRH
 2 = JPA-2 9 = S48
 3 = NC-LivB1
 4 = NC-LivB2
 5 = NC-LivC 10 = Vero cell
 6 = NC-SweB1
 7 = NC-1

Figure 5.5

AFLP analysis of *Neospora* and *Toxoplasma* isolates
 using selective primers E-AG and M-CAT

Dendrograms were drawn to show the similarity of isolates as determined by the AFLP analysis. The term dendrogram is commonly used in the literature interchangeably with phenogram, but it was decided to use the term dendrogram with the AFLP analysis here, because the results refer to fragments that have been amplified specifically i.e. size differences on the gel reflect unique sequence differences. The term phenogram was used in the RAPD analysis, since bands of the same size could also have resulted by chance via co-migrating fragments of different sequence composition.

The dendrograms resulting from UPGMA cluster analysis are shown in Figure 5.6. There are three dendrograms, one for each genera tested (only one sample of *Sarcosystis* spp. was tested so no dendrogram could be drawn). It was not possible to combine all the species and isolates tested on a single dendrogram because so few bands were conserved across the genera. The *Neospora* isolates tested showed an overall genetic diversity of 3 %. The diversity seen amongst the *Toxoplasma* isolates tested was slightly greater at 4.2 %. There was a much greater difference (11.5 %) seen between the two types of *Cryptosporidium* isolates tested.

The dendrograms formed for *Toxoplasma* spp. and *Cryptosporidium* spp. showed the isolates clustering into groups according to types that have been previously described by Sibley & Howe (1996). The *Neospora* isolates tested also clustered into three main groups. The cloned isolates (NC-Beef, NC-1 and NC-Sweb1) are shown in red and show that individual clones of each isolate were identical

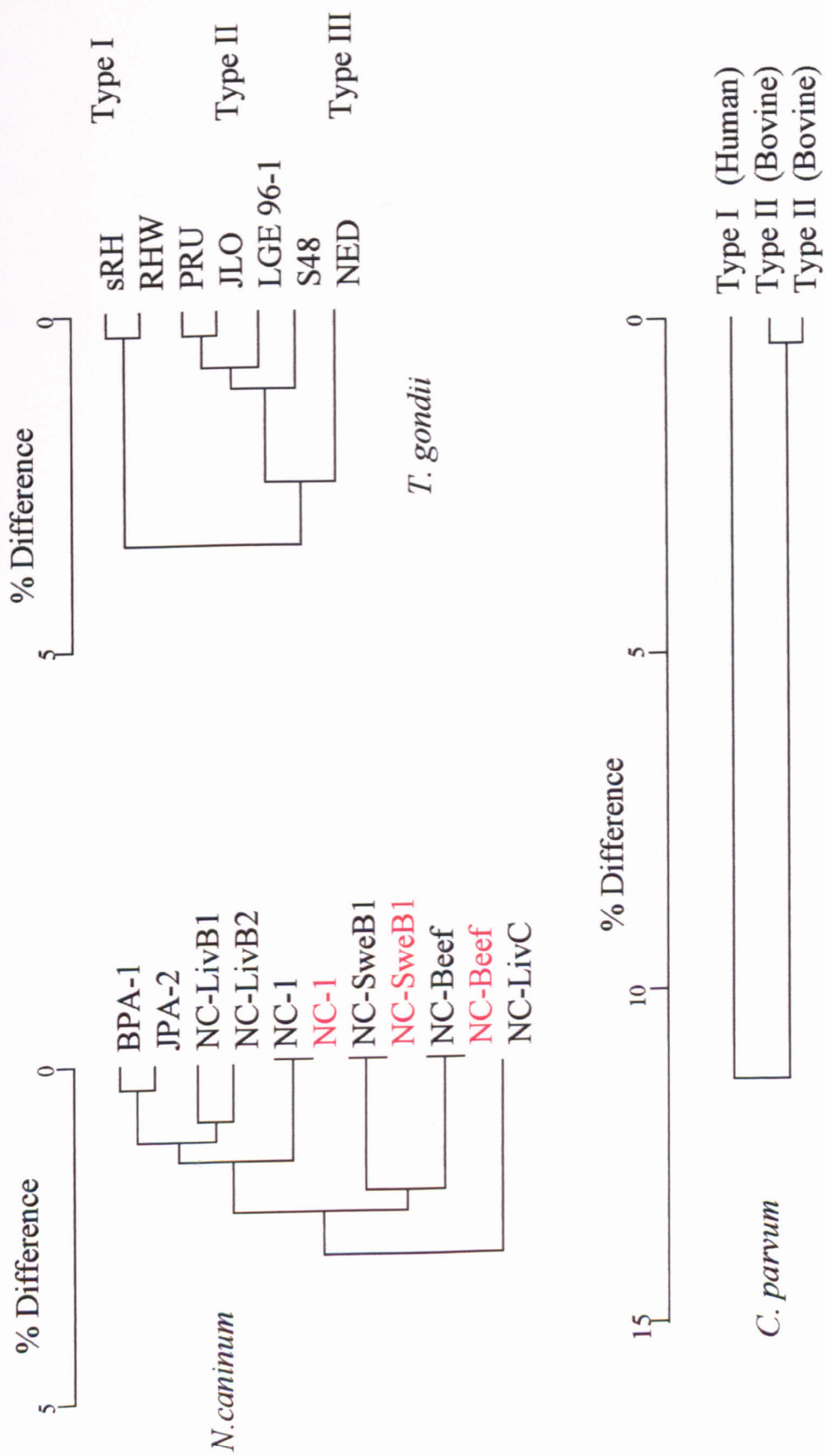


Figure 5.6
Dendrograms formed as a result of AFLP and cluster analysis for isolates for *N. caninum*, *T. gondii* and *C. parvum*.
Cloned *N. caninum* isolates are shown in red.

(denoted by straight line). The canine isolates did not cluster together and there was no clustering with respect to geographic origin.

Despite several attempts, it proved extremely difficult to extract bands from the polyacrylamide gels. Consequently it was not possible to amplify any sequences from the rehydrated gel fragments and so attempts to obtain markers, for subsequent PCR-RFLP analysis, from AFLP gels were abandoned.

5.3 DISCUSSION

AFLP has been found to be an excellent whole genome scanning method because genome information on the species, *N. caninum*, is sparse. The highly reproducible results obtained in this analysis are due to the use of high stringency PCR conditions that allowed specific primer binding.

The dendrogram obtained from the analysis of AFLP profiles of *Toxoplasma* isolates gave similar results (in terms of genetic distance or relatedness) to those obtained using other techniques such as MLEE and MLST by Howe and Sibley (1995). It has also been shown by Mallon *et al.* (2003) that the two strain types (Type I and Type II) of *C. parvum* are quite different as shown in Figure 5.5.

AFLP has previously been used to differentiate between the two *C. parvum* genotypes (Type I and Type II) by Blears *et al.* (2000). This study showed that by using more selective nucleotides, it should be possible to discriminate successfully between *C. parvum* sub-genotypes even of the same type. This could have potential importance in tracking the source of a *Cryptosporidium* outbreak in water supplies.

The congruence of the *T. gondii* dendrogram obtained by AFLP with other studies employing different approaches gave extra confidence in the output for the *Neospora* isolates obtained in this chapter. Overall, there was slightly more genetic diversity amongst the *Toxoplasma* isolates tested than the *Neospora* isolates tested. However, as the *Neospora* isolates consisted entirely of

laboratory stocks, this may well not reflect the actual diversity that exists in the field.

There was a slight difference in the phenogram obtained for RAPD compared with the dendrogram obtained using AFLP for *Neospora*. The results from both techniques are dependent on the number of primers (for RAPD) or primer pairs (AFLP) used and the results obtained are likely to be more reliable the greater the number of primers used. It is difficult to define a gold standard in terms of methods, but AFLP is an intrinsically more discriminatory and reproducible technique because of high stringency and the large number of primers used in AFLP.

The cloned lines of *Neospora* isolates gave identical fingerprint profiles showing that the tissue culture stocks of these isolates were homogeneous. Unfortunately, it was only possible to look at about 5 cloned lines for each of the three isolates cloned out due to time constraints. The identical fingerprints obtained suggested that the stocks are homogeneous. This could result from two situations. The clones could be identical in the host, or it is possible that pre-selection occurs in tissue culture where some lines out-compete others.

The role of the definitive host (i.e. dog) in the transmission of *N. caninum* has been shown experimentally but it is not known if sexual recombination can occur in the host. A study attempting to show sexual recombination had occurred between a cross of different isolate clones could use AFLP to type the progeny. Cloned lines of two different isolates would firstly be typed by AFLP, so the

isolates could be distinguished, prior to infection of the dog. Oocysts from the dog would then be fed to gerbils to propagate the parasite, and cloned lines of the parasite typed again by AFLP. If sexual recombination occurred between the two different isolates it would be possible to see this in the banding patterns for each isolate. This parallels the formal demonstration of recombination in *T. gondii* by Sibley, although AFLP was not used to genotype *T. gondii*.

It is important to recognise the limitations of AFLP analysis. For the optimum interpretation of relationships between genera it may be best to use AFLP in conjunction with other methods. Attempts to draw dendrograms linking all 4 genera were unsuccessful because of insufficient shared markers. From these data, AFLP has been shown to be a very good method for inferring relationships between strains, but is too discriminative for wider phylogenetic relationships, for example, within a phylum. The use of less selective nucleotides may help, but the aim here was to distinguish at an intra-species level in an organism suspected to have low genetic diversity. Some studies have used fluorescently labelled primers in AFLP studies, which then provide automated readouts of results. This type of analysis would ensure objectivity and would be less time consuming.

AFLP is a complex technique, which is highly reliant on the quality and purity of the DNA used, so the technique has limited use in mixed DNA samples (host and parasite) from clinical samples. Thus the extraction of polymorphic bands from the gels, which could subsequently be used as genetic markers, was an important step. Unfortunately this proved to be very difficult and it was not possible to

reamplify and sequence the gel fragments. These difficulties probably arose due to the labelled bands co-migrating with unlabelled fragments. This has also been the experience of other colleagues (Masiga *et al.*, 2000) and Prof. M. Shirley, Institute of Animal Health, Compton (personal communication).

Polymorphisms detected by AFLP are thought to result from single nucleotide changes in the restriction sites, or adjacent to these sites in the bases complementary to the selective nucleotides, or from insertion/deletion events between the cut sites. Polymorphisms created by single base changes appear to be the most frequent (Vos *et al.*, 1995). This suggested that searching for single nucleotide polymorphisms (SNPs) may be successful for *N. caninum*, having demonstrated that polymorphisms do exist between isolates. This led directly to work with SNP identification in polymorphic genes in chapter 6.

Chapter 6: Multi-locus genotyping of *Neospora caninum*

6.1 INTRODUCTION

The RAPD and AFLP approaches used in chapters 4 and 5 were successful in determining the extent to which genetic heterogeneity exists among laboratory isolates of *N. caninum*. However, the question arises as to whether the laboratory isolates that are currently available for *N. caninum* are a true reflection of the diversity of *N. caninum* in the field. The answer is probably not; they are unlikely to represent the full genetic diversity that exists for several reasons. First there are a relatively small number of laboratory isolates, reflecting the fact that isolation from infected tissues is difficult. Second, the process of isolation by tissue culture is in itself selective and may be more successful with some isolates rather than others. Thus to obtain a fuller picture of the level of *Neospora* diversity in the field, it is necessary and much more informative to analyse clinical samples.

RAPD and AFLP are methods that measure genetic diversity using a ‘whole-genome’ approach, in which the nature of the polymorphic loci are unknown. Since it proved impossible to extract and clone PCR fragments from AFLP gels to obtain genetic markers, these methods were not amenable to further exploitation with field samples. Unfortunately a primary requirement of the RAPD and AFLP techniques is that the DNA sample is not contaminated with other DNA, a condition that is impossible to meet with field tissue samples, which will consist mainly of contaminant host DNA. The sequence specificity of PCR-based methods of genotyping overcomes this constraint and are therefore

the preferred systems to genotype *N. caninum* tissue samples, providing of course that polymorphic genes can be identified.

A common genotyping approach is the use of DNA mini or microsatellites. These are 2-6 bp (microsatellites) or ~ 6-20 bp (minisatellites) repeats respectively that appear in DNA sequence and evolve due to strand slippage during replication. The number of times strand slippage occurs can vary between strains of a species and can lead to a variable number of DNA repeats. Mini/microsatellites can be very useful in differentiating isolates or strains of a species because the differing number of repeats can be visualised on agarose or polyacrylamide gels by different band sizes without the time and expense of full gene sequencing. This is a potentially rapid method of differentiating isolates and has been very successful for other protozoan parasites such as *C. parvum* (Mallon *et al.*, 2003); *T. brucei* (Barrett *et al.*, 1997; MacLeod *et al.*, 2000; MacLeod *et al.*, 2001) and *T. gondii* (Ajzenberg *et al.*, 2002).

A study by Anderson *et al.* (2000) using 12 microsatellite loci revealed a variety of population structures in *Plasmodium falciparum*. A total of 465 samples were collected from 9 locations worldwide and analysed to show that in areas of intense transmission, such as Africa and Papua New Guinea, there was a high degree of genetic diversity in the parasite, whereas in regions of low transmission for example, Colombia, low genetic diversity amongst parasite isolates was observed. This is an example of the intrinsic links between patterns of transmission or the epidemiology and the genetic structure of an organism.

Before the availability of extensive genome sequence information, mini- and microsatellites were found by the use of relatively long molecular techniques, for example in the case of *T. brucei* (Barrett *et al.*, 1997). The increasing amount of sequence data has made it possible to screen molecular databases using computer programs such as Tandem Repeats Finder (TRF) (Benson, 1999) to find mini- and microsatellite sequences. Primers can subsequently be designed to amplify these repeated regions and the PCR products from different isolates run on gels to see if there are any size differences. The development of a suitable panel of mini/microsatellite markers is a two-stage process. Initially it is necessary to identify the location of these repeated regions and ensure that it is possible to amplify them through careful primer design. Second, it is necessary to establish whether the regions are polymorphic between different isolates of the species to be studied. This approach was used successfully by Mallon *et al.* (2003) for the identification of a large number of polymorphic mini and microsatellites for *C. parvum*, which were subsequently used to study the population biology of this parasite. Mallon and co-authors (2003) used 3 minisatellite and 4 microsatellite markers to genotype 180 *C. parvum* isolates from both humans and cattle collected within a set time period from one area in Northeast Scotland. Four different population structures were defined by the 38 multi-locus genotypes identified, which helped establish the role of genetic exchange in these parasites.

It was hoped in this chapter that by using the available sequence data for *N. caninum*, it would be possible to identify sufficient mini- and microsatellites. These would then be assessed for polymorphisms, which would give rise to size differences on agarose gels and could be used as a rapid multi-locus genotyping

method for *N. caninum*. Importantly, this method could also be readily used with clinical field samples.

In addition to searching for microsatellites, it was hoped that isolates could also be differentiated by minisatellite DNA regions. Probes based on a tandem repeat of a 'core' minisatellite sequence have been used to produce DNA fingerprints in human genetic analysis (Gill *et al.*, 1985; Jeffreys *et al.*, 1990). If genetic heterogeneity exists in this conserved minisatellite sequence in *N. caninum* then this could be exploited to obtain DNA fingerprints of the parasites, or more carefully, devise PCR-based minisatellite typing methods adaptable to clinical field samples.

When working with clinical samples, it is essential to know that *N. caninum* DNA is actually present in the sample, especially when the amount of host DNA present will far outweigh that of the parasite. Moreover, cattle could potentially be infected with a wide-range of other tissue dwelling coccidian such as *T. gondii*, *Sarcocystis* spp. and *Hammondia* spp. It would be important to ensure that DNA from these parasites or bovine DNA were not being preferentially amplified. To check that was not the case, a region of the ITS-1 gene of *N. caninum* was amplified and sequenced from all isolates and clinical samples used in the analysis.

A second example of a PCR-based genotyping approach is multi-locus sequence typing (MLST) which has been used very successfully to genotype bacterial pathogens. For example, Maiden *et al.* (1998) determined the sequences of 470bp fragments from 6 housekeeping genes in a reference set of 107 isolates of *Neisseria meningitidis* from invasive disease and healthy carriers. Housekeeping genes are thought not to be subject to unusual selective forces, and diversify slowly by the random accumulation of neutral or nearly-neutral variation. The MLST approach allowed the reliable identification of the major meningococcal lineages associated with invasive disease to be identified. The advantage with MLST is that it can be applied to any haploid organism, since there is only one allele present and is therefore an ideal method for typing *N. caninum*, which exists in a haploid state in all but its definitive host. In addition, sequence data is generally unambiguous and can be readily compared with other laboratories across the world.

In this chapter, sequence diversity of the introns of two genes, actin and tubulin, was examined across a panel of laboratory isolates of *N. caninum* as well as a small number of clinical tissue samples obtained from the field. To maximise the likelihood of detecting polymorphisms, regions of these genes spanning introns were selected for PCR amplification and sequence analysis. Since introns are non-coding regions of DNA, the frequency of polymorphisms was expected to be higher than in the exons of these genes.

The aims of this chapter were

- To identify mini/microsatellite regions of DNA in *N. caninum* and determine whether these were polymorphic using a panel of laboratory isolates and field samples.
- To determine the sequence diversity of intron regions of the actin and tubulin genes in *N. caninum* and assess the suitability of these loci as genetic markers for the parasite.

6.2 MATERIALS AND METHODS

6.2.1 Mini/Microsatellite search

6.2.1.1 Expressed Sequence Tag (EST) searches

Genbank (www.ncbi.nlm.nih.gov) was searched on a regular basis for *Neospora* spp. gene sequences and ESTs. At the start of the project, no EST sequences were available, but this has increased to about 4000 by January 2003.

6.2.1.2 Tandem Repeat Finder

To identify microsatellite sequences in the *Neospora* genome, all *N. caninum* full or partial length genes (~ 250) and EST sequences (~ 3750) were analysed using the computer programme Tandem Repeats Finder (TRF) (Version 2.02) found at <http://c3.biomath.mssm.edu/trf>, and by Benson (1999).

6.2.1.3 Southern blotting

Southern analysis was carried out with the assistance of Ms. Janice Brock.

6.2.1.3.1 Restriction endonuclease digestion

Approximately 1-2 µg of *N. caninum* DNA was digested overnight at 37 °C with 2 Units of restriction enzyme Hinf I. A further 1 Unit of enzyme was added the following day for 1 h to ensure complete digestion. Digested DNA was then run on a 1 % agarose gel (Seakem) in 0.5 x TBE buffer at 120 V for 2.5 h. The gel was visualised under UV to establish whether DNA digestion was complete and to assess the amount of DNA present. The gel was then blotted using the following method.

6.2.1.3.2 Southern blotting

DNA gels were submerged in Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) for 45 min. After rinsing in double distilled water, the gel was transferred to Neutralisation Solution (1 M Tris-HCl (pH 7.4), 1.5 M NaCl) for 30 min at room temperature. The gel was then placed on top of a 3 M filter paper wick (Whatman, UK) soaked in 20 x SSC transfer buffer (3 M NaCl, 300 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$). Hybond nylon membrane was placed on the gel with a sheet of 3 M filter paper on top. A weighted pile of paper was added to the stack, to enable the buffer to rise through the gel, membrane and filter paper by capillary action and facilitate the transfer of DNA. The apparatus was left overnight to ensure complete transfer of the DNA.

The DNA was cross-linked onto the nylon membrane by UV before being incubated at 50°C in Rapid-hyb Prehybridisation Solution (Amersham Life Science, UK) for at least 2 h.

6.2.1.3.3 Probe

Minisatellite probes 33.6 and 33.15 (Gill *et al.*, 1985) were kindly provided by Dr. Annette McLeod, Wellcome Centre for Molecular Parasitology, University of Glasgow. Probes were labelled by adding 25 ng DNA (12.5 µl) to 9 µl H_2O and 10 µl random oligonucleotide primers. This mix was placed in boiling water for 5 min. Following this, 10 µl 5X primer buffer was added along with 5 µl of labelled nucleotide (3000 Ci/mmol) and the solution gently mixed by pipetting. Exo 1-1 Klenow enzyme (1 µl) (5 U/µl) was mixed into the solution and then incubated at 37 °C for 10 min. The reaction was halted by adding 2 µl of stop

mix. The probe was boiled for 5 min before hybridisation and then put on ice to maintain denaturation.

Unincorporated radio-nucleotide was removed from the probe mix using G25 Spin Columns (Amersham, UK).

6.2.1.3.4 Hybridisation

The radio-labelled probe was added to the Prehybridisation Solution and incubated with the membrane overnight at 42 °C. After this, the membranes were removed from the probe and incubated with Wash Buffer A (4 x SSC, 0.1 % SDS) at 50 °C for 30 min, with one buffer change, then for 30 min at room temperature with Wash Buffer B (0.1 x SSC, 0.1 % SDS), again with one buffer change. These washes removed any probe that had not been hybridised to the DNA on the membrane. The membranes were exposed to X-ray film for 1-2 days and developed using an X-Ograph Compact X4 Imaging system.

The blot was subsequently stripped in boiling 10 % SDS and re-probed as necessary.

6.2.2 DNA extraction from tissue samples

6.2.2.1 Reagents and stock solutions

The following stock solutions and reagents were prepared for the extraction of tissue samples.

(i) Stock solutions**(a) Tris buffer (1 M)**

121.1 g of Tris base (Sigma, UK) was dissolved in 800ml of reagent grade water.

The pH was adjusted to the desired value by adding concentrated HCl. The volume was then adjusted to 1000 ml with H₂O followed by dispensing into aliquots and sterilisation by autoclaving.

(b) Magnesium chloride (MgCl₂, 1 M)

203.3 g of MgCl₂ was dissolved in 800 ml of reagent grade H₂O. The volume was adjusted to 1000 ml with H₂O and dispensed into aliquots and sterilised by autoclaving.

(c) Potassium chloride (KCl, 1 M)

74.55 g KCl was dissolved in 800 ml of reagent grade H₂O and the volume subsequently adjusted to 1000 ml using H₂O. The stock solution was then dispensed into aliquots and sterilised by autoclaving.

(d) Proteinase K (20 mg/ml)

250 µl of reagent grade H₂O was added to a 25 mg vial of Proteinase K. After dissolving it was dispensed into 25 µl volumes and stored at -20 °C.

(e) Ammonium chloride (NH₄Cl, 0.16 M)

8.568 g NH₄Cl was dissolved in 1000 ml of reagent grade H₂O and was sterilised by autoclaving.

(f) Tris Buffer (0.17 M)

20.587 g Tris buffer was dissolved in 800 ml H₂O. The pH was then adjusted to 7.65 with HCl and made up to 1000 ml with H₂O.

(g) Phosphate buffered saline (PBS, 0.01 M)

The following were dissolved in 800 ml of reagent grade H₂O: 8 g of NaCl (Sodium Chloride, 0.137 M); 0.2 g KCl (Potassium Chloride, 0.0027 M); 1.44 g Na₂HPO₄ (Di-Sodium Hydrogen Orthophosphate; 0.0101M); 0.24 g KH₂PO₄ (Potassium di-Hydrogen Orthophosphate, 0.0017 M). The pH was then adjusted to 7.4 with HCl and made up to 1000 ml with H₂O. As usual this was then dispensed into aliquots and sterilised by autoclaving.

(h) TE buffer

TE buffer was made up using sterile stocks of 10 mM Tris buffer (pH 8.0) and 1 mM EDTA (pH 8.0). In preparation of 100 ml TE buffer, 1 ml of 1 M Tris was added to 200 µl of 0.5 M EDTA and 98.8 ml of reagent grade H₂O. This was dispensed into aliquots and sterilised by autoclaving.

(ii) Reagents

(a) DNA extraction buffer

DNA extraction buffer was prepared in 100 ml volumes and then stored as 1 or 5 ml aliquots at -20 °C until required. Proteinase K was added at a final concentration of 200 U/g/ml immediately before use (i.e. 50 µl stock solution to 5 ml buffer).

10 ml of 1 M Tris (pH 8.0) is added to 2 ml 1 M MgCl₂ and 40 ml of 1 M KCl followed by 500 µl of Tween 20 and then made up to 100 ml with 47.5 µl reagent grade H₂O.

(b) Red Blood Cell lysis buffer

200 ml of RBC lysis buffer was prepared by adding 20 ml Tris (0.17 M, pH 7.6) to 180 ml Ammonium chloride (0.16 M).

6.2.2.2 Extraction protocol

Approximately 1 g samples of tissue were sliced from frozen tissues using a scalpel and transferred to a sterile petri dish. Samples were finely chopped using a criss-cross action using the scalpel. The tissue was then transferred to a 1.5 ml screw-cap micro-centrifuge tube and washed with 1 ml of PBS. Following vortexing, the sample was centrifuged at 6000 x g for at least 2 min and the supernatant was then discarded.

If red blood cells (RBCs) were present at this stage, the following protocol was followed: 1 ml RBC lysis buffer was added to the tissue pellet and resuspended by vortexing. It was then mixed on the rotary mixer for 15 min. Following centrifugation at 6000 x g for 2 min the supernatant was discarded. If RBCs still remained in the sample at this stage, RBC lysis buffer was again added, mixed and centrifuged and the supernatant removed. This process was repeated until no RBCs were present.

DNA extraction buffer (500-1000 μ l) was then added to the tissue pellet, mixed and then left to digest overnight at 37 °C or for 2-3 h at 55 °C, with occasional vortexing. The proteinase K was then inactivated by boiling for 5 min.

6.2.2.3 Phenol/ Chloroform extraction of DNA from digested tissue sample

The supernate from tissue extraction was transferred to a fresh micro-centrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) added, vortexed and then centrifuged at 12000 x g for 2 min. The aqueous layer (usually top) was removed to a fresh tube and 2 volumes of cold (-20 °C) absolute ethanol

was added. The DNA was then precipitated at -20°C overnight or at -70°C for 1-2 h. Centrifugation at $12000 \times g$ at 4°C for 15 min was performed to pellet the DNA. After the supernatant had been removed carefully (so the pellet was not disturbed), the tube was left 10 min to air dry and then dissolved in 50 μl of TE buffer or reagent grade H_2O . 5-10 μl of DNA solution was used in PCR reactions.

6.2.3 PCR amplification

6.2.3.1 PCR reaction and conditions

PCR reactions were set up in 50 μl reactions as follows: 2 μl template DNA was added to 5 μl dNTPs (2 nM), 5 μl buffer, 3 μl MgCl_2 , 2 μl primers (1 μl of forward and 1 μl of reverse at 10 pmol/ μl), 0.5 μl Taq polymerase and 32.5 μl H_2O . All PCR reactions were performed using the Dyad DNA engine (MJ Research, UK). The conditions for each PCR were normally as follows, unless otherwise stated: 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 45 s followed by 72°C for 5 min.

Agarose gels (1 %) were run to visualise the PCR products as follows. Seakem LE agarose (1 g) was placed in a conical flask and diluted in 100ml 0.5 x TBE buffer by heating in a microwave for 1 min and then being poured into a gel cassette and being left to set for 40 min. 0.5 x TBE (200 ml) was used as running buffer and the gel placed inside the cassette. PCR product (5 μl) was mixed with 1 μl bromophenol blue dye and loaded into each lane. 100 bp ladder (5 μl) was also loaded into the end lane. The gel was then run at 100 V for 2 h.

6.2.4 Cloning PCR products

Cloning of PCR products was carried out with the assistance of Ms. Janice Brock. The pGEM®-T Easy Vector system (Promega, UK) was used to clone PCR products.

6.2.4.1 Ligation using the pGEM®-T Easy Vectors

Ligation reactions were set up as follows: PCR product (1 µl) was added to 5 µl of 2 X Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector (50 ng), 1 µl T4 DNA Ligase and 2 µl ddd H₂O in a 0.5 ml tube. The reaction was mixed by pipetting and was then incubated for 1 h at room temperature.

6.2.4.2 Transformation using the pGEM®-T Easy Ligation reactions

Two LB/ampicillin /IPTG/X-Gal plates were prepared for each ligation reaction as well as one plate to determine transformation efficiency. The 0.5 ml tubes containing the ligation reactions were centrifuged to collect the contents at the bottom of the tube. Two µl of each ligation reaction was added to a sterile 1.5 ml microcentrifuge tube on ice. An extra microcentrifuge tube was set up on ice with 0.1 ng of uncut plasmid to determine the efficiency of the transformation. Frozen tubes containing JM109 High Efficiency Competent cells were removed from -70 °C storage and were thawed in an ice bath for approximately 5 min. The cells were mixed by gently flicking the tubes. Competent cells (50 µl) were transferred into each ligation tube (100 µl cells for determination of transformation efficiency) and subsequent to gently flicking in order to mix the tubes, were left on ice for 20 min. The tubes were then placed in a water bath at 42 °C for 45 s to heat-shock the cells and immediately returned to ice for 2 min.

LB broth (950 μ l) (room temperature) was added to the tubes containing transformed ligation reactions and 900 μ l to the tube containing cells transformed with uncut plasmid. The tubes were then incubated for 1.5 h at 37 °C with shaking (~150 rpm). Each transformation culture (100 μ l) was plated onto duplicate LB/ampicillin /IPTG/X-Gal plates. A 1:10 dilution with LB broth was used for plating the transformation control. The plates were then incubated overnight at 37 °C. White colonies were picked and grown overnight in 10 ml cultures for preparation of plasmid DNA.

6.2.4.3 Purification of plasmid DNA

The QIAprep® Miniprep kit was used to purify plasmid DNA. This method is based on an alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure is briefly described.

Pelleted bacterial cells were resuspended in 250 μ l of Buffer P1 and were then transferred to a microcentrifuge tube. Buffer P2 (250 μ l) was then added and the tube was gently (to avoid DNA shearing) inverted 5 times to mix the contents. 350 μ l Buffer N3 was then added to the tube and it was immediately gently inverted 5 times. The tube was then centrifuged at 13000 x g for 10 min when a compact white pellet will form. The supernatant from each tube was then decanted to QIAprep columns. After 60 s centrifugation at 13000 x g the flow-through was discarded. The QIAprep spin column was washed by adding 0.5 ml Buffer PB and centrifuging at 13000 x g for 60 s to remove trace nuclease activity. Again the flow-through was discarded. The spin column was washed by adding 0.75 ml of Buffer PE and centrifuging at 13000 x g for 60 s. The flow-through was discarded and the column then centrifuged for another 1 min in

order to remove residual wash buffer. The QIAprep column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of each column. The tube was left to stand for 1 min and then centrifuged for 1 min.

6.2.5 Sequencing

PCR products were sequenced either after cloning into PGEMT vector using M13 primers, or directly from PCR products. All sequencing was performed by the Molecular Biology Sequencing Unit (MBSU), IBLS, University of Glasgow.

6.2.6 Phylogenetic analysis

The phylogenetic analysis was performed in AlignX, a component of the VectorNTI suite, v5.5, InforMax Inc. (1999). The Neighbour Joining method was used in which a matrix of distances between all the pairs of sequences is analysed. These distances are related to the degree of divergence between the sequences. The phylogenetic trees were constructed following the analysis of genetic distances, using the ClustalX programme and drawn using the TREEVIEW program.

6.3 RESULTS

6.3.1 Microsatellites

All *N. caninum* sequences including ~250 genes and 3750 expressed sequence tags (ESTs) were searched for micro- and minisatellites using the TRF program. Table 6.1 shows the microsatellite consensus sequences and the percent nucleotide matches for each one. Some of the microsatellites unfortunately had a relatively low nucleotide match e.g. Trap. Primers were designed around the microsatellite sequences found. These primers were intended to find length polymorphisms between isolates of *N. caninum* that could be visualised on gels (either agarose or polyacrylamide). Table 6.2 shows the primers used to amplify the microsatellites found using the Tandem Repeats Finder computer program. Primers were designed for these and they were tested using 7 laboratory isolates of *N. caninum*, NC-1, BPA, NC-Livc, NC-Beef, NC-Ger, NC-SweB1 and NC-LivB1. Figure 6.1 is an example of an agarose gel showing the amplification of the 'Trap' microsatellite with 4 isolates, BPA, Nc-Beef, Nc-Liverpool and Nc-SweB1. All isolates tested with this microsatellite gave the expected band size of 560 bp indicating that no size polymorphisms were detectable with this region of DNA even when gels were run-out for several hours. Figure 6.2 shows the amplification of another microsatellite, MS-1, with 4 isolates of *N. caninum*. As in the previous example all isolates gave the expected band size of 196 bp.

Analysis of all 6 of the *N. caninum* microsatellites (Table 6.1) revealed no detectable size polymorphisms for each laboratory isolate tested and were run out on both agarose and 6 % polyacrylamide gels.

Table 6.1

Microsatellite consensus sequences obtained from the Tandem Repeat Finder (TRF) program.

Microsatellite primer name	Copy Number	Consensus sequence	% Match
Trap	6	TGTTG	52
AA274	12.5	TA	82
BF824	8.2	TCGCTC	62
BF249	7.1	TCTCTTC	77
MS-1	10.8	TTCTC	75
MS-2	15.5	TA	93
MS-3	10.2	TACA	95

Table 6.2

Primers used to amplify microsatellites found by TRF analysis of *N. caninum* DNA sequences.

Primer name	Region	Length (bp)	Sequence 5' → 3'
TrapF	Trap gene	560	GCTTGTGGTCAGAGTGGAGCTCG
TrapR	Trap gene	560	CATTCTTCCCAATCCCCTGGCTC
AA274F	microsatellite	340	GCGAACAAACAGAAATGAGTC
AA274R	microsatellite	340	GAAGATGCCAGGATGGAGGGA
BF824F	microsatellite	326	CACGGACAGACCGAGTGGGTA
BF824R	microsatellite	326	AAAACAAACAAATTAGCCAAG
BF249	microsatellite	294	ATTCGTTGTTTGAAGAACCAA
BF249	microsatellite	294	AGGCGCACTGCCAGAACGATG
MS-1F	microsatellite	196	CACGAAGGGGATGTGTCAGAA
MS-1R	microsatellite	196	GTACCTTCACTAAATGAGAAC
MS-2F	microsatellite	441	CTCAAACACTGAAGATCGGAA
MS-2R	microsatellite	441	ATGCGTGTGTGAACATACATGC
MS-3F	microsatellite	326	CTCCTGGATGGTGAGACGCTTGA
MS-3R	microsatellite	326	GCCTAATCGACAGCAGATGAAGG

Figure 6.1

Example of ‘Trap’ microsatellite amplification of 4 isolates of *N. caninum*.
Identical results were obtained for all samples analysed.

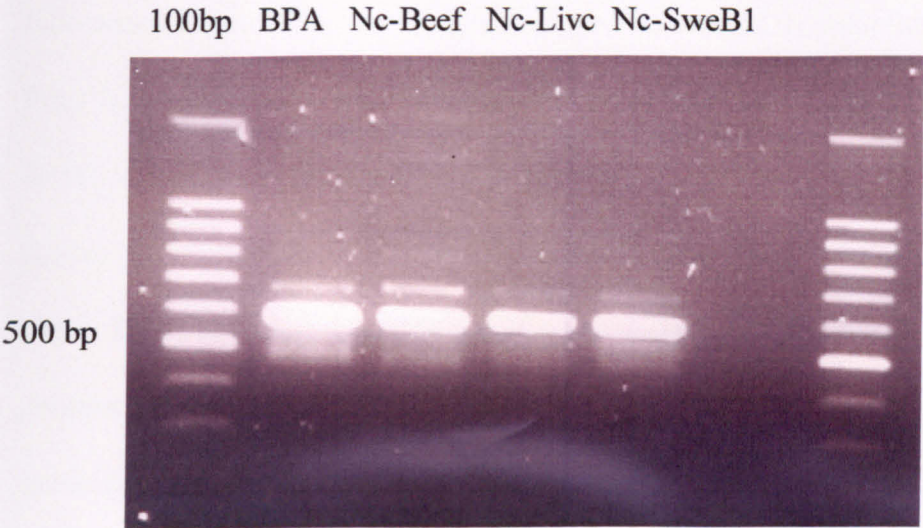
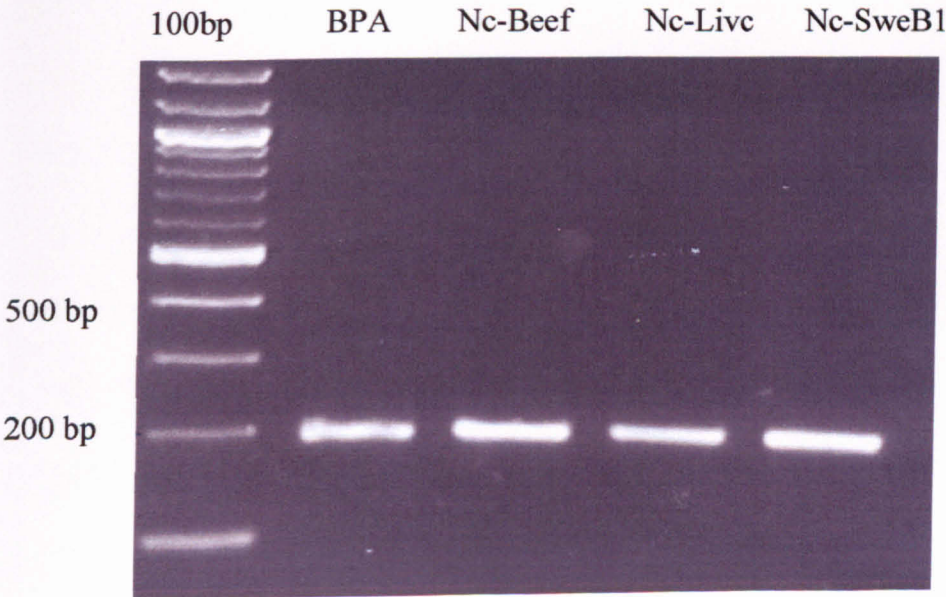


Figure 6.2

Example of ‘MS-1’ microsatellite amplification of 4 isolates of *N. caninum*.
Identical results were obtained for all samples analysed.



6.3.2 Southern blotting

In an attempt to determine whether polymorphisms might be present in minisatellite regions of *N. caninum* DNA, digested DNA from different laboratory isolates were hybridised with two conserved minisatellite probes. Four *N. caninum* and two *T. gondii* laboratory isolates were digested using the *Hinf*I restriction enzyme and probed probe 33.6 and probe 33.15. Figure 6.3 shows the southern blot of probe 33.6 following exposure to X-ray film for 2 days. Probe 33.6 hybridised to a single strong band at > 3 Kb revealing the presence of the 33.6 minisatellite region in *N. caninum*. However, careful examination of the blot revealed an identical banding pattern for each isolate.

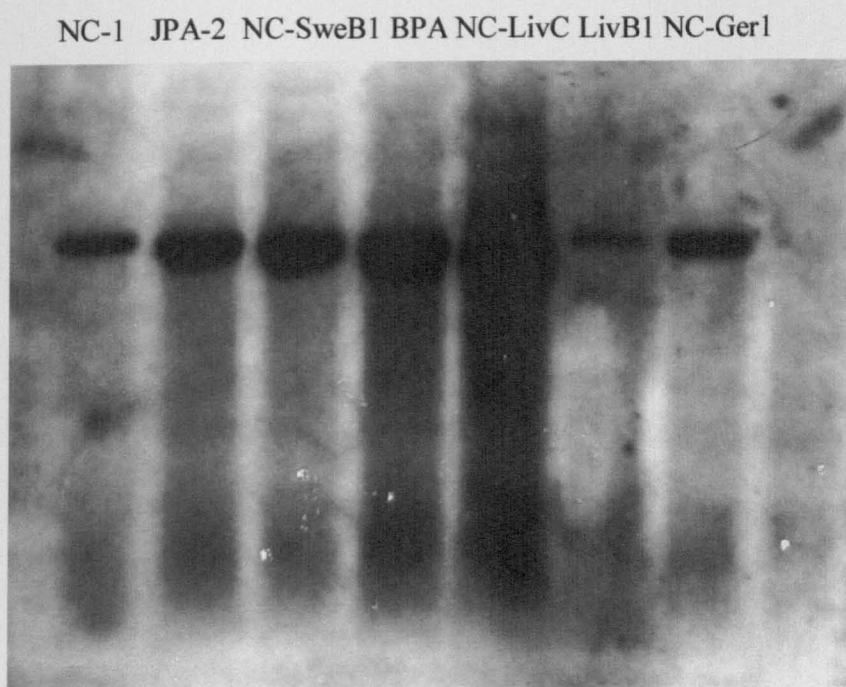
The blot was stripped and re-probed with probe 33.15. Figure 6.4 shows the autoradiograph following a 2-day exposure. Probe 33.15 hybridised to 3 bands. Again, the banding pattern obtained was again the same for each isolate, indicating that there were no detectable polymorphisms using this minisatellite probe.

6.3.3 Extraction of DNA from clinical samples

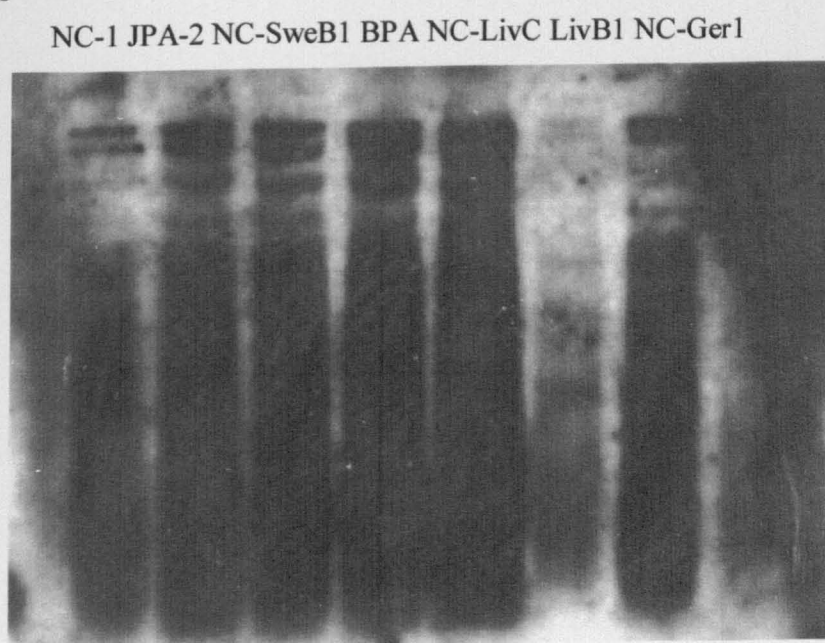
Clinical samples collected in Scotland, England, Switzerland and Sweden were extracted as described in Section 6.2.5. Unfortunately, of the 46 originally collected only a small proportion of these samples were suitable for PCR amplification. Although DNA was successfully extracted many of the *Neospora*-specific PCR amplifications failed suggesting there was insufficient amplifiable *Neospora* DNA in the sample. The following bovine clinical isolates were used: C-560, C919, 61 and 62 (Scotland); CH-2 (Switzerland) and SR-3 (Sweden).

Figure 6.3

Hybridisation of minisatellite probe 33.6 with *N. caninum* genomic DNA from 7 laboratory isolates. Approximately 1-2 µg DNA was digested with Hinf I overnight.

**Figure 6.4**

Hybridisation of minisatellite probe 33.15 with *N. caninum* genomic DNA from 7 laboratory isolates. Approximately 1-2 µg DNA was digested with Hinf I overnight.



6.3.4 PCR amplification of the actin and tubulin genes

The actin and tubulin intron regions were amplified using primers shown in Table 6.3 for all 7 laboratory isolates, the clinical isolates and DNA from *Hammondia heydorni*. Primer sequences were kindly suggested by Prof. David Sibley (Washington State University) based on intron 1 of the actin and tubulin genes of *N. caninum* (AY143096 and AY143097). Intron region primers in the *N. caninum* genes were designed by homology to the introns of the corresponding genes in *T. gondii*. All samples gave the expected 407 bp fragment when amplified with the actin intron primers and the 510 bp product when amplified with the tubulin intron primers.

6.3.4.1 Sequence analysis of actin, tubulin and ITS-1 genes of *N. caninum*

In order to identify any differences in the sequences of the introns of the actin and tubulin genes and the ITS-1 region, each gene was amplified by PCR, sequenced and the resulting data lined-up in Genedoc for sequence analysis.

6.3.4.1.1 Sequence analysis of actin intron 1

Eight laboratory isolates of *N. caninum* (BPA, NC-1, NC-Livc, NC-Beef, NC-Ger, NC-SweB1, NC-LivB1, NC-Drachten), *H. heydorni* and a clinical sample from Thurso in Scotland, C-560 were successfully amplified using intron 1 actin gene primers and then cloned and sequenced. Direct PCR sequencing was also performed on laboratory isolate BPA (denoted R2 and F2). The lined-up sequence is shown in Figure 6.5. The 407 bp intron sequence, published in Genbank (AY143096) is shown at the top denoted by ACT1-1, although extended sequence was obtained for all the isolates tested (up to 500 bp).

Table 6.3

Primers used to amplify actin and tubulin-1 intron-1 gene sequences from *N. caninum* and *H. heydorni*.

Primer name	Region	Length (bp)	Sequence 5' → 3'
Act-1F	Actin	407	GCTACATCGCCCTCGACTT
Act-1R	Actin	407	CATCGGGCAATTCATAGGAC
Tub-1F	Tubulin	510	CCGGTATCCAAATCGGTAAC
Tub-1R	Tubulin	510	ACCATGTTCCAGGCAGAAGA
ITS-1F	ITS-1 region	480	CCGCTGCAGAGGTGAACCTGCGG AAGGATC
ITS-1R	ITS-1 region	480	CACTGAAACAGACGTACC

Figure 6.5

Sequence alignment of intron-1 of the actin gene of *N. caninum*.

Eight laboratory isolates of *N. caninum* (BPA, NC-1, NC-Livc, NC-Beef, NC-Ger, NC-SweB1, NC-LivB1, NC-Drachten), *H. heydorni* and a clinical sample from Thurso in Scotland, C-560. Forward and reverse sequences are denoted by 'F' and 'R' respectively. ACT-1 represents the sequence from Genbank entry AY143096. The bottom sequence is the consensus sequence. Single nucleotide polymorphisms confirmed by both forward and reverse sequencing are highlighted. Dashed lines (---) represent areas where good sequence information was not obtained and do not infer deletions in the sequence.

		*	20	*	40	*	60	*	
ACT1-1	:	----	----	----	----	----	----	----	:
BPA-F	:	----	----	----	----	----	----	CATCGG	:
BPA-R	:	----	----	----	----	----	----	CATCGG	:
BPA-F2	:	----	----	----	----	----	----	CCATCGG	:
BPA-R2	:	----	----	----	----	----	----	----	:
NC-1-F	:	----	----	----	----	----	----	CATCGG	:
NC-1-R	:	----	----	----	----	----	----	CATCGG	:
LIV-C-F	:	----	----	----	----	----	----	CATCGG	:
LIV-C-R	:	----	----	----	----	----	----	CATCGG	:
H.Hey-R	:	----	----	----	----	----	----	CATCGG	:
H.Hey-F	:	----	----	----	----	----	----	CATCGG	:
Beef-F	:	----	----	----	----	----	----	CATCGG	:
Beef-R	:	----	----	----	----	----	----	CATCGG	:
NC-Ger-R	:	----	----	----	----	----	----	----	:
NC-Ger-F	:	----	----	----	----	----	----	CATCGG	:
Sweb-F	:	----	----	----	----	----	----	CATCGG	:
Sweb-R	:	----	----	----	----	----	----	CATCGG	:
LIVB1-F	:	----	----	----	----	----	----	CATCGG	:
LIVB1-R	:	----	----	----	----	----	----	GG	:
Drach-F	:	----	----	----	----	----	----	----	:
Drach-R	:	----	----	----	----	----	----	----	:
C560-R	:	----	----	----	----	----	----	CATCGG	:
C560-F	:	----	----	----	----	----	----	CATCGG	:
								CCATCGG	

	80	*	100	*	120	*	140	
ACT1-1	----	-----	-----	-----	-----	-----	-----	:
BPA-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	ctgaaacagaaacccgacgycgaacagggcca						31
BPA-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	GAACAGAAACCGACGGCAACAGGCCA						80
BPA-F2	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	CAACAGAAACCGACGGCAACAGGCCA						80
BPA-R2	-----	-----	-----	-----	-----	-----	-----	:
NC-1-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						35
NC-1-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
LIV-C-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
LIV-C-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
H.Hey-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
H.Hey-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
Beef-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
Beef-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
NC-Ger-R	-----	-----	-----	-----	-----	-----	-----	:
NC-Ger-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						48
Sweb-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
Sweb-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
LIVB1-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						76
LIVB1-R	-----	-----	-----	-----	-----	-----	-----	:
Drach-F	-----	-----	-----	-----	-----	-----	-----	:
Drach-R	-----	-----	-----	-----	-----	-----	-----	:
C560-R	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80
C560-F	GCAATTCATAGGACTTCTCGATGTCGCTGGAGTCCCTCAGCTGCCCTGA	AAACAGAAACCGACGGCAACAGGCCA						80

		160	*	180	*	200	*	220	
ACT1-1	:	aaaccgatgtgcgggacagtcctggcgccatagcagtccttccgctcaacgcagcagccacagaacttaaaaaa	:		:		:		105
BPA-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
BPA-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
BPA-F2	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		155
BPA-R2	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		109
NC-1-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
NC-1-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
LIV-C-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
LIV-C-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
H.Hey-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
H.Hey-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
Beef-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
Beef-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
NC-Ger-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		122
NC-Ger-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
Sweb-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
Sweb-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
LIVB1-F	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		150
LIVB1-R	:	AAACCCGATGTGCGGACAGTCTGGCCCATAGCAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		115
Drach-F	:	-----	:		:		:		-
Drach-R	:	-----GGAACAGTCTGGCGCCATAGCAAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		60
C560-R	:	AAACCCGATGTGCGGACAGTCTGGCGCCATAGCAAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
C560-F	:	AAACCCGATGTGCGGACAGTCTGGCGCCATAGCAAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		154
	:	AAACCCGATGTGCGGACAGTCTGGCGCCATAGCAAGTCTTCCGCTCAACGCAGCAGCCACAGAACTTAAAAAA	:		:		:		

	*	240	*	260	*	280	*
ACT1-1	:	aaccttcaggacaccacgcggaacaacgaac	:	tttataagcacattctgtctgcacaaagcca	:	aaacacagatttgt	:
BPA-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
BPA-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
BPA-F2	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
BPA-R2	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
NC-1-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
NC-1-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
LIV-C-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
LIV-C-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
H.Hey-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
H.Hey-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
Beef-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
Beef-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
NC-Ger-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
NC-Ger-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
Sweb-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
Sweb-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
LIVB1-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
LIVB1-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	ATTCTGCTGCACAAAGCAAAACAGATT	:
Drach-F	:	-----CGGAAACAACGAACCTTTTATAGCA	:	CATTCTGCTGCACAAAGCAAAACAGATT	:	TGTTGT	:
Drach-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	CATTCTGCTGCACAAAGCAAAACAGATT	:
C560-R	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	CATTCTGCTGCACAAAGCAAAACAGATT	:
C560-F	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	CATTCTGCTGCACAAAGCAAAACAGATT	:
	:	AACTTCAGGACACCA	:	CGGAAACAACGAACCTTTTATAGCA	:	CATTCTGCTGCACAAAGCAAAACAGATT	:

ACT1-1	:	acacaatgcatggtctaacaaatgtgacctaacgacgagcacggtccctccgcyaggtctggaaactccgcgtgcgc	:	253
BPA-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
BPA-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
BPA-F2	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	303
BPA-R2	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	257
NC-1-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
NC-1-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
LIV-C-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
LIV-C-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
H.Hey-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
H.Hey-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
Beef-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
Beef-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
NC-Ger-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	270
NC-Ger-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
Sweb-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
Sweb-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
LIVB1-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	298
LIVB1-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	263
Drach-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	132
Drach-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	208
C560-R	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302
C560-F	:	ACACAATGCATGTCTAACAAATGTGCGCTAACGCGACGACGCGCCCTCCGCGAGGGCTGGGAACTCCGCGTGCGC	:	302

	380	*	400	*	420	*	440	
ACT1-1	ttgcttctta	gagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
BPA-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
BPA-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
BPA-F2	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
BPA-R2	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
NC-1-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
NC-1-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
LIV-C-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
LIV-C-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
H.Hey-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
H.Hey-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
Beef-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
Beef-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
NC-Ger-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
NC-Ger-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
Sweb-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
Sweb-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
LIVB1-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
LIVB1-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
Drach-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
Drach-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
C560-R	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
C560-F	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat
	ttgcttctta	gagagatatg	caaaaaac	ggaatttc	gtccacc	aggaacct	gcgggata	ttgat

	460	480	500	5
ACT1-1	catggtactccttatccaccccttatacaaaaatacagatcctaaglttcggytaactagctccggyctgtgct			401
BPA-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
BPA-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
BPA-F2	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			449
BPA-R2	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			405
NC-1-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
NC-1-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
LIV-C-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
LIV-C-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
H.Hey-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
H.Hey-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
Beef-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
Beef-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
NC-Ger-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			418
NC-Ger-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
Swab-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
Swab-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
LIV1-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			446
LIV1-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			411
Drach-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			278
Drach-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			356
C560-R	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450
C560-F	CATGGCTACTCCTTTATCCACCTCTTTACAATAATACGATCTAAAGTTTGGGTAACCTAGCTCCGGGCTGTGCT			450

	20	*	540	*	560	
ACT1-1	tcgcgac	-----		-----		407
BPA-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
BPA-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
BPA-F2	-----	-----	-----	-----	-----	-
BPA-R2	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCAA	448
NC-1-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
NC-1-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
LIV-C-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
LIV-C-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
H.Hey-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGAA	492
H.Hey-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGAA	492
Beef-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGAA	491
Beef-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGAA	491
NC-Ger-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGAA	460
NC-Ger-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGAA	491
Sweb-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
Sweb-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	492
LIVB1-F	-----	-----	-----	-----	-----	-
LIVB1-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCA	453
Drach-F	-----	-----	-----	-----	-----	-
Drach-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCAAT	400
C560-R	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCAATCGAATT	500
C560-F	TCCGACCTTCA	TTTCCCTCGT	CGAAGTCGA	GGCCGATG	TAGCAATCGAATT	500

Forward and reverse sequence was obtained for the majority of the isolates tested. There was good consensus for the majority of the sequence, but some polymorphisms were found between the isolates and these are summarised in Table 6.4. However, the discrepancy between the cloned BPA-1 sequence (BPA-F and BPA-R) and the consensus sequence at nucleotide 122 appears to have been due to a *Taq* polymerase error as the direct sequence for BPA-1 (R2 and F2) agrees with the consensus sequence.

6.3.4.1.2 Sequence analysis of tubulin intron 1

Eight laboratory isolates of *N. caninum* (BPA, NC-1, NC-Livc, NC-Beef, NC-Ger, NC-SweB1, NC-LivB1, NC-Drachten), *H. heydorni* and three clinical samples from Scotland, 61, 62 and C919 were successfully amplified using the tubulin intron-1 primers. Following amplification, some isolates, NC-Ger and *H. heydorni*, were sequenced by direct PCR sequencing, but the others were cloned and then sequenced. Figure 6.6 shows the alignment of the tubulin intron-1 region with polymorphisms highlighted. Differences between sequences and the consensus sequence are shown in Table 6.4. There were 23 single nucleotide polymorphisms found in the intron of the tubulin gene in total. There were 5 SNPs from clinical isolates 61 and 62. Interestingly, some of the SNPs were conserved between these two samples, which were obtained from a similar geographical area. There were 6 SNPs in the sequence of sample C919, 1 in NC-Beef, 2 in BPA (although only the reverse sequence was successfully amplified), 1 in NC-1 and 3 in NC-LivC.

Table 6.4

Single Nucleotide Polymorphisms (SNPs) in the actin intron-1 of *N. caninum* and *H. heydorni*.

Isolate	bp	Base	Consensus
C560	103	T	G
BPA	122	G	A
C560	257	C	T
NC-Beef	287	C	A
C560	437	A	G
NC-Sweb	463	T	A
NC-Ger	520	C	T
H. Heydorni	560	A	C
Nc-Beef	560	A	C
NC-Ger	560	A	C

Table 6.5

Single Nucleotide Polymorphisms (SNPs) in the tubulin intron-1 of *N. caninum*

Isolate	bp	Base	Consensus
61	83	A	C
C919	121	C	A
62	148	A	G
61	186	G	A
62	186	G	A
NC-Beef	201	G	A
C919	219	G	A
C919	226	A	G
BPA-F*	234	A	T
61	262	C	T
62	262	C	T
C919	284	T	A
C919	292	C	T
61	321	T	C
62	321	T	C
61	330	T	A
62	330	T	A
NC-1	397	T	A
NC-LivC	397	T	A
C919	397	T	A
BPA-F*	431	C	T
NC-LivC	437	T	A
NC-LivC	593	G	A

* Only 1 sequence for BPA

Figure 6.6

Sequence alignment of intron-1 of the tubulin gene of *N. caninum*.

Eight laboratory isolates of *N. caninum* (BPA, NC-1, NC-Livc, NC-Beef, NC-Ger, NC-Sweb1, NC-LivB1, NC-Drachten), *H. heydorni* and three clinical samples from Scotland, 61, 62 and C919. Forward and reverse sequences are denoted by 'F' and 'R' respectively. TUB1-1 represents the sequence from Genbank entry AY143097. The bottom sequence is the consensus sequence. Single nucleotide polymorphisms confirmed by both forward and reverse sequencing are highlighted. Dashed lines (---) represent areas where good sequence information was not obtained and do not infer deletions in the sequence.

	80	*	100	*	120	*	140	
TUB1-1	gacacacaccccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				79
BPA-F	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
NC-1-F	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
NC-1-R	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
LIV-C-R	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
LIV-C-F	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
NC-Sweb-F	-----	-----	-----	-----	-----	-----	TGGCTC	6
NC-Sweb-R	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
NC-Beef-F	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
NC-Beef-R	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
NC-Ger-R2	gacacacaccccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				107
NC-Ger-F2	-----	-----	-----	-----	cctgtcctgggtccccgc	gctgatgatcggccgtt	tttccgcgtggctc	47
NC-Ger-R	-----	-----	-----	-----	-----	-----	-----	-
H.Hey-F	-----	-----	-----	-----	-----	-----	-----	26
H.Hey-F2	-----	-----	-----	-----	-----	-----	-----	66
H.Hey-R2	gacacacaccccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				107
H.Hey-R	-----	-----	-----	-----	GATCGGCCGTTTTC	CGCGTGGCTC		25
LivB-1-R	gacacacaccccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				102
LivB-1-F	-----	-----	-----	-----	-----	-----	-----	54
NC-Drach-R	-----	-----	-----	-----	-----	-----	-----	-
NC-Drach-F	-----	-----	-----	-----	-----	-----	-----	-
61-R	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				47
61-F	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				80
62-R	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				108
62-F	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				123
C919-F	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				107
C919-R	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				100
	gacacacacacacccgacagaa	cgctgtcctgtcctgtg	ccccgcgtgatgatc	cgccgttctccgcgtgc				

	220	*	240	*	260	*	280	
TUB1-1	acatgtggtgcatggaattgtcgaatcaagagctcaagactcgggtgtagataaaccctcacctggcaacta	:		:		:		223
BPA-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
NC-1-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
NC-1-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
LIV-C-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
LIV-C-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
NC-Sweb-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		150
NC-Sweb-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
NC-Beef-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
NC-Beef-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
NC-Ger-R2	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		251
NC-Ger-F2	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		191
NC-Ger-R	-----	:		:		:		-
H.Hey-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		170
H.Hey-F2	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		210
H.Hey-R2	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		251
H.Hey-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		169
LivB-1-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		246
LivB-1-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		198
NC-Drach-R	-----	:		:		:		-
NC-Drach-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		191
61-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		224
61-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		252
62-R	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		252
62-F	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		267
C919-F	ACGTGTGTTACATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		251
C919-R	ACGTGTGTTACATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		244
	ACATGTGTCATGGAATGTCGAATCAAGAGTCTAAAGACTCGGGTGTAGATAACCCCTCACCTGGCAACTA	:		:		:		

	300	*	320	*	340	*	360
TUB1-1	gtttcgctaaccgtgcatccggcgacatccgltcttagcacgtcaccggtatgttctctctagttctc	:	295				
BPA-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-1-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-1-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
LIV-C-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
LIV-C-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-Sweb-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-Sweb-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	222				
NC-Beef-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-Beef-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-Ger-R2	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
NC-Ger-F2	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	263				
NC-Ger-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	34				
H.Hey-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	242				
H.Hey-F2	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	282				
H.Hey-R2	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	323				
H.Hey-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	241				
LIVB-1-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	318				
LIVB-1-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	270				
NC-Drach-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	39				
NC-Drach-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	263				
61-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	296				
61-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	324				
62-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	324				
62-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	339				
C919-F	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	323				
C919-R	gtttcgctaaccgtgcatccggcgacatccgtcttagcacgtcaccggtatgttctctgtagtttctc	:	316				

	440	*	460	*	480	*	500	
TUB1-1	agtgaagaaacccgccgaccacaactt	gtga	tgaagagaagta	ag	tlaacgctga	aagatgt	gtggtgcggtag	439
BPA-F	AGTGAGAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
NC-1-F	AGTGAGAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
NC-1-R	AGTGAGAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
LIV-C-R	AGTGTGAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
LIV-C-F	AGTGTGAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
NC-Sweb-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	366
NC-Sweb-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
NC-Beef-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
NC-Beef-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
NC-Ger-R2	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	467
NC-Ger-F2	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	407
NC-Ger-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	178
H.Hey-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	386
H.Hey-F2	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	426
H.Hey-R2	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	467
H.Hey-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	385
LivB-1-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	462
LivB-1-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	414
NC-Drach-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	183
NC-Drach-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	407
61-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	440
61-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	468
62-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	468
62-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	483
C919-F	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	467
C919-R	AGTGAAGAACCGCCGACCAACACTTT	GGATGA	AGAGAAAGTA	GTAA	CGCTGA	AAAGATGT	GTTGCGCGGTAG	460

		*	520	*	540	*	560	*	
TUB1-1	:	ggtgcgctgcccgttttgcgctcacgctgtgtccccctgcagttctctcgcggtttgtacgctgcgtcttctctcatttag-	:	510					
BPA-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-1-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-1-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
LIV-C-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
LIV-C-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-Sweb-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-Sweb-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	438					
NC-Beef-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-Beef-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-Ger-R2	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	457					
NC-Ger-F2	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	539					
NC-Ger-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	478					
H.Hey-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	250					
H.Hey-F2	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	458					
H.Hey-R2	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	498					
H.Hey-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	509					
LivB-1-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	410					
LivB-1-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	504					
NC-Drach-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	483					
NC-Drach-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	225					
61-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	479					
61-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	512					
62-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	540					
62-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	540					
C919-F	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	555					
C919-R	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	539					
	:	GGTGCCTGCCGTTTGGCGTCAAGTGTCCCCCTGCAGTTCTCGCGGTTTGTACGTCGGTCTTTTCTCTATTYAGG	:	532					

	580	*	600	
TUB1-1	----	----	----	-
BPA-F	GAGCTCTTCTGCCTG	----	----	: 472
NC-1-F	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 481
NC-1-R	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 481
LIV-C-R	GAGCTCTTCTGCCTGGACATGGT	----	----	: 481
LIV-C-F	GAGCTCTTCTGCCTGGACATGGT	----	----	: 481
NC-Sweb-F	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 462
NC-Sweb-R	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 481
NC-Beef-F	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 481
NC-Beef-R	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 481
NC-Ger-R2	GAGCTCTTCTGCCTGGAACATGG	----	----	: 562
NC-Ger-F2	----	----	----	-
NC-Ger-R	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 274
H.Hey-F	GAGCTCTTCTGCCTGGAACATGGT	----	----	: 482
H.Hey-F2	GAGCTCTTCTGCCTGGAACATGGTAA	----	----	: 524
H.Hey-R2	----	----	----	-
H.Hey-R	----	----	----	-
LivB-1-R	----	----	----	-
LivB-1-F	----	----	----	-
NC-Drach-R	----	----	----	-
NC-Drach-F	GAGCTCTTCTGCCTGGAACATGG	----	----	: 502
61-R	GAGCTCTTCTGCCTGGAACATGGTAA	----	----	: 538
61-F	GAGCTCTTCTGCCTGGAACATGGTAATC	----	----	: 568
62-R	GAGCTCTTCTGCCTGGAACATGGTAATCT	----	----	: 569
62-F	GAGCTCTTCTGCCTGGAACATGGTAATC	----	----	: 583
C919-F	GAGCTCTTCTGCCTGGAACATGGTAAT	----	----	: 566
C919-R	GAGCTCTTCTGCCTGGAACATGGTAATC	----	----	: 560
	GAGCTCTTCTGCCTGGAACATGGTAATCT			

6.3.4.1.3 Sequence analysis of *N. caninum* ITS-1

Five laboratory isolates of *N. caninum* (BPA, LIVC, NC-Ger, NC-LivB1, NC-Drachten), *H. heydorni* and two clinical samples, CH-2 and SR-3 from Switzerland and Sweden respectively were amplified with primers for the ITS-1 region of *N. caninum*. Unfortunately, the ITS-1 region was not amplified successfully from all clinical isolates due to the lack of material available. Figure 6.7 shows the sequence comparison of the ITS-1 region for these isolates and includes the published sequence for 9 other isolates of *N. caninum* and also those for *N. hughesi* and NC-Oregon. Three different sequences are published on Genbank for laboratory isolate NC-Liverpool, NC-LIV, NC-1b and NC-Liv. All *N. caninum* isolates and the clinical samples gave identical sequences for the ITS-1 gene. The sequencing results for isolate NC-Liverpool found in this study agreed with the consensus sequence.

6.3.4.2 Phylogenetic trees

Phylogenetic analysis was performed using neighbour-joining trees drawn from the sequence data results for the introns of the actin and the tubulin genes of *N. caninum* and *H. heydorni* and the ITS-1 sequence. The scales refer to the number of substitutions per nucleotide site.

The phylogenetic tree for the intron of the actin gene sequence of *N. caninum* is shown in Figure 6.8. The sequence diversity was greatest with the clinical sample, C-560. It was interesting that *H. heydorni* clustered amongst *N. caninum* isolates.

Figure 6.9 shows the phylogenetic tree for the tubulin gene sequence. The

Figure 6.7

Sequence alignment of the Internal Transcribed Spacer (ITS-1) region.

Five laboratory isolates of *N. caninum* (BPA, LIVC, NC-Ger, NC-LivB1, NC-Drachten), *H. heydorni* and two clinical samples, CH-2 and SR-3 from Switzerland and Sweden respectively were amplified with primers for the ITS-1 region of *N. caninum*. Forward and reverse sequences are denoted by 'F' and 'R' respectively. In addition, 9 other published sequences for isolates of *N. caninum* [NC-BPA-1 (AF038860), NC-1 (U16160), NC-5 (AF249970), NC-Beef (AF249968), NC-2 (AF249969), NC-CN1 (AF038861), NC-Sweb-1 (AF029702), CZ-4 (AF432123), NC-Nowra (AF338411), NC-Liv (NCU16159), NC-1b (U16160) and NC-LIV (L49389)] and also the published sequences for *N. hughesi* (AF038859) and NC-Oregon (AF249967) were included in the alignment.

	80	*	100	*	120	*	140	
NE-Hughesi	:	CCTCAACCTTTGAATCCCAAGCAAAACATGAGCTTGTATCCCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-Oregon	:	CCTCAACCTTTGAATCCCAAGCAAAACATGAGCTTGTATCCCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-BPA-1	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-1	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-5	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-Beef	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-2	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-CN1	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	101				
NC-Sweb-1	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	112				
CZ-4	:	cctcaaccttgaatcccaacaacaacatgagcttgtatctctccttcggaagggtacattcaagaag	:	96				
NC-Nowra	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	95				
NC-1b	:	cctcaaccttgaatcccaacaacaacatgagcttgtatctctccttcggaagggtacattcaagaag	:	96				
NC-Liv	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	96				
NC-Liv	:	cctcaaccttgaatcccaacaacaacatgagcttgtatctctccttcggaagggtacattcaagaag	:	33				
LIVC-F	:	-----CTCTCTCCTTGGAGAGGGGTACATTCAAGAAG	:	61				
LIVC-R	:	-----GAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	76				
CH-2-F	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	-				
CH-2-R	:	-----	:	-				
LIV-B1-R	:	-----	:	-				
LIV-B1-F	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	75				
SR-3-R	:	-----CAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	69				
SR-3-F	:	-----CCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	66				
NC-Drach-R	:	-----CCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	66				
NC-Drach-F	:	-----CCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	66				
BPA-R	:	-----CCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	66				
BPA-F	:	-----CCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	66				
NC-Ger-F	:	-----CCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	94				
H.Hey-R	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:	92				
H.Hey-F	:	CCTCAACCTTTGAATCCCAACAAACATGAGCTTGTATCTCTCCCTTGGAGAGGGGTACATTCAAGAAG	:					

	220	*	240	*	260	*	280	
NE-Hughesi	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-Oregon	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-BPA-1	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-1	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-5	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-Beef	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-2	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-CN1	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 239
NC-Sweb-1	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 245
CZ-4	: Tctattgaacgcctgataatgaaagtgtgtgcataatccggagtgtaacggcgaagggaactcggtcactgg							: 256
NC-Nowra	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 240
NC-1b	: Tctattgaacgcctgataatgaaagtgtgtgcataatccggagtgtaacggcgaagggaactcggtcactgg							: 239
NC-Liv	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 240
NC-Liv	: Tctattgaacgcctgataatgaaagtgtgtgcataatccggagtgtaacggcgaagggaactcggtcactgg							: 240
LIVC-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 240
LIVC-F	: Tctattgaacgcctgataatgaaagtgtgtgcataatccggagtgtaacggcgaagggaactcggtcactgg							: 240
LIVC-R	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 177
CH-2-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 205
CH-2-R	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 220
	: -----AGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 49
LIV-B1-R	: -----AGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 49
LIV-B1-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 219
SR-3-R	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 213
SR-3-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
NC-Drach-R	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
NC-Drach-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
BPA-R	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
BPA-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
NC-Ger-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
H.Hey-R	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 210
H.Hey-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 238
H.Hey-F	: TCTATTGAACGCGCTGATAATGAAAGTGTGTGCATATATCCGGAGTGTACGGCGAAGGACTCGGTCACTGG							: 236

NE-Hughesi	300	*	320	*	340	*	360
NC-Oregon	AAATTAACGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-BPA-1	AAATTAACGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-1	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-5	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-Beef	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-2	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-CN1	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-Sweb-1	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
CZ-4	aaatlaatgctctctaatgggactttaactccaggagttcttcaatgtgcattcttttttc						ccacaccg
NC-Nowra	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-1b	aaatlaatgctctctaatgggactttaactccaggagttcttcaatgtgcattcttttttc						ccacaccg
NC-Liv	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-Liv	aaatlaatgctctctaatgggactttaactccaggagttcttcaatgtgcattcttttttc						ccacaccg
LIVC-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
LIVC-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
CH-2-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
CH-2-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
LIV-B1-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
LIV-B1-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
SR-3-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
SR-3-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-Drach-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-Drach-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
BPA-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
BPA-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
NC-Ger-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
H. Hey-R	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
H. Hey-F	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG
	AAATTAATGCTCTCTAATGGGACTTTAACTTCCAGAGTTTCTTCAATGTGCATTCTTTTTC						CCACACCG

	440	*	460	*	480	*	500	
NE-Hughesi	TCGCTTTGAGATTACCTAATAAACTATAATGTTTTTCTAATTTTCAGCAATGGA							438
NC-Oregon	TCGCTTTGAGATTACCTAATAAACTATAATGTTTTTCT							423
NC-BPA-1	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCTAATTTTCAGCAATGGA							438
NC-1	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCTAATTTTCAGCAATGGA							438
NC-5	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							421
NC-Beef	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							421
NC-2	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							421
NC-CNI	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCTAATTTTCAGCAATGGA							438
NC-Sweb-1	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCTAATTTTCAGCAATGGA							450
CZ-4	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							461
NC-Nowra	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							422
NC-1b	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							444
NC-Liv	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							445
NC-Liv	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							445
LIVC-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							419
LIVC-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							419
CH-2-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							411
CH-2-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							263
LIV-B1-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							263
LIV-B1-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							404
SR-3-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							427
SR-3-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							424
NC-Drach-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							424
NC-Drach-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							424
BPA-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							424
BPA-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							424
NC-Ger-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							356
H.Hey-R	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							452
H.Hey-F	TCGCTTTGAGATTACTTAATAAACTATAATGTTTTTCT							382

NE-Hughesi	:	580	*	:	600	*	:	620	:	:
NC-Oregon	:	-	-	-	-	-	-	-	:	-
NC-BPA-1	:	-	-	-	-	-	-	-	:	-
NC-1	:	-	-	-	-	-	-	-	:	-
NC-5	:	-	-	-	-	-	-	-	:	-
NC-Beef	:	-	-	-	-	-	-	-	:	-
NC-2	:	-	-	-	-	-	-	-	:	-
NC-CN1	:	-	-	-	-	-	-	-	:	-
NC-Sweb-1	:	-	-	-	-	-	-	-	:	-
CZ-4	:	-	-	-	-	-	-	-	:	-
NC-Nowra	:	-	-	-	-	-	-	-	:	-
NC-1b	:	-	-	-	-	-	-	-	:	-
NC-Liv	:	-	-	-	-	-	-	-	:	-
NC-Liv	:	-	-	-	-	-	-	-	:	-
LIVC-F	:	ACGC	-	-	-	-	-	-	:	467
LIVC-R	:	-	-	-	-	-	-	-	:	-
CH-2-F	:	-	-	-	-	-	-	-	:	-
CH-2-R	:	-	-	-	-	-	-	-	:	-
LIV-B1-R	:	-	-	-	-	-	-	-	:	-
LIV-B1-F	:	-	-	-	-	-	-	-	:	-
SR-3-R	:	-	-	-	-	-	-	-	:	-
SR-3-F	:	ACGCCA	-	-	-	-	-	-	:	502
NC-Drach-R	:	-	-	-	-	-	-	-	:	-
NC-Drach-F	:	-	-	-	-	-	-	-	:	-
BPA-R	:	-	-	-	-	-	-	-	:	-
BPA-F	:	-	-	-	-	-	-	-	:	-
NC-Ger-F	:	-	-	-	-	-	-	-	:	-
H.Hey-R	:	-	-	-	-	-	-	-	:	-
H.Hey-F	:	-	-	-	-	-	-	-	:	-
ACGCCA	:	-	-	-	-	-	-	-	:	-

Figure 6.8

Phylogenetic tree constructed using sequences of intron-1 of the actin gene of *N. caninum*.

Eight laboratory isolates of *N. caninum* (BPA, NC-1, NC-LIV-C, NC-Beef, NC-Ger, NC-Sweb1, NC-LivB1, NC-Drachten), *H. heydorni* and a clinical sample from Thurso in Scotland, C-560, were included in the analysis along with the published sequence, ACT1-1 (AY143096). BPA-R2 refers to a direct sequence.

Scale refers to rate of substitution per base.

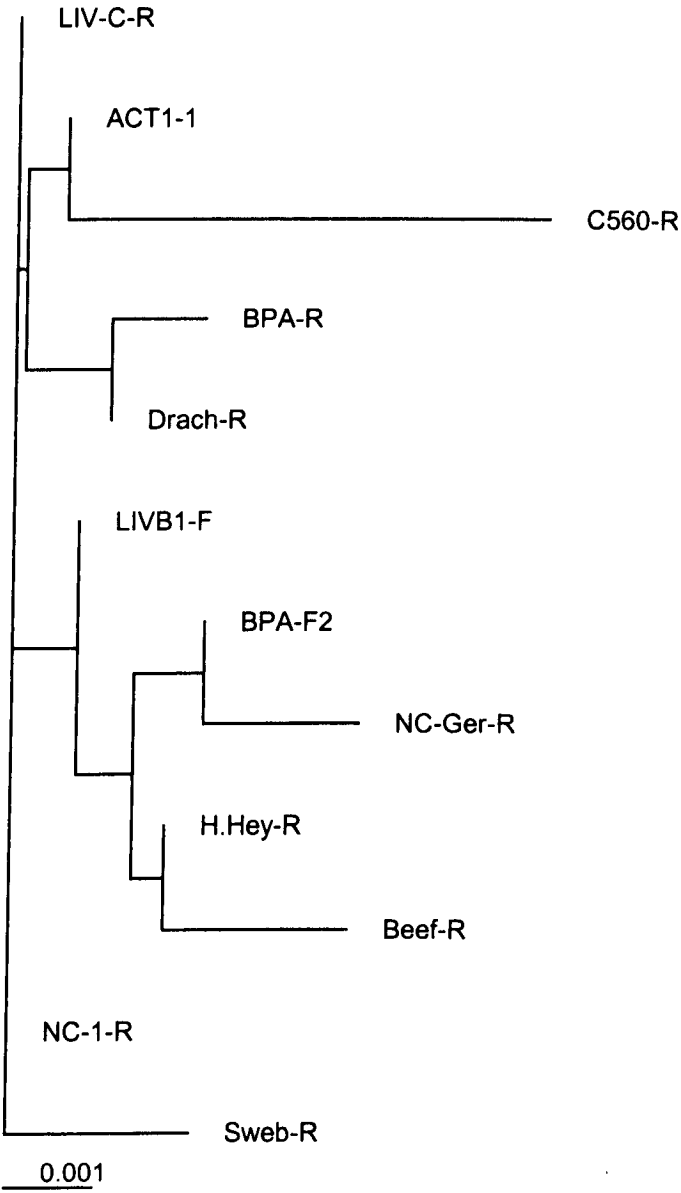
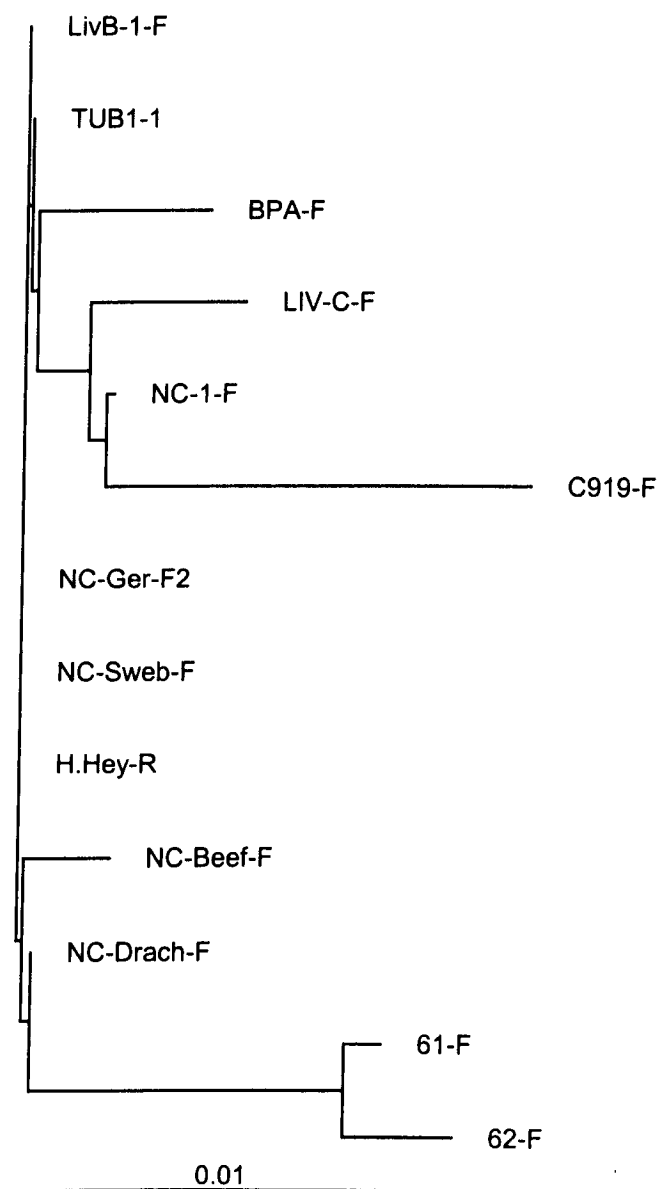


Figure 6.9

Phylogenetic tree constructed using intron-1 of the tubulin gene of *N. caninum*.

Eight laboratory isolates of *N. caninum* (BPA, NC-1, NC-Livc, NC-Beef, NC-Ger, NC-Sweb1, NC-LivB1, NC-Drachten), *H. heydorni* and three clinical samples from Scotland, 61, 62 and C919 were included in the analysis along with the published sequence, TUB1-1 gene, intron 1 (AY143097).

Scale refers to rate of substitution per base.



clinical samples, C919 and 61 and 62 seem to be most diverse from the other laboratory isolates tested. There were no SNPs with many of the laboratory isolates. Interestingly, the *H. heydorni* isolate clustered with the *N. caninum* consensus sequence for the tubulin intron.

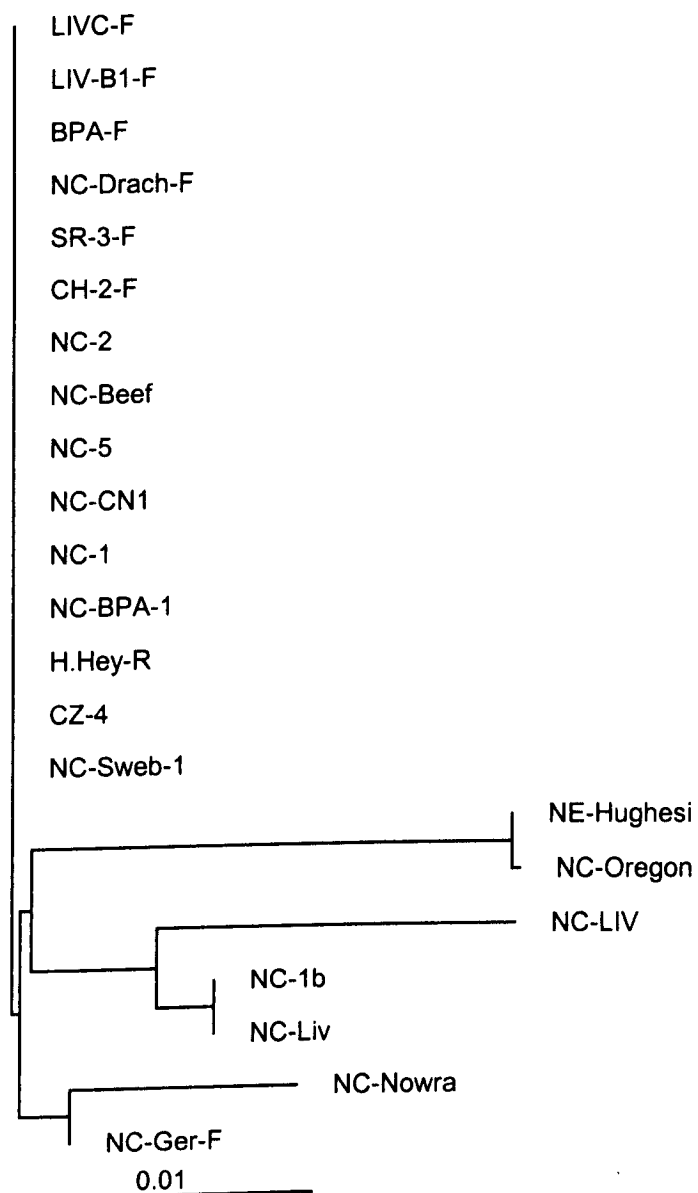
The phylogenetic tree constructed (using the Neighbour-Joining method) from the ITS-1 sequences is shown in Figure 6.10. It was interesting that the sequencing results from the laboratory isolate NC-Liverpool (LIVC) gave identical sequence to all others including most published results. There are three different published sequences for NC-Liverpool, shown on the tree as NC-LIV, NC-1b and NC-Liv. From these results, it seems that these published sequences for NC-Liverpool may contain errors. As expected, *N. hughesi* and *N. caninum* Oregon isolates were quite different to the other published results for *N. caninum*. It was surprising that *H. heydorni* clustered amongst *N. caninum* isolates at the actin and tubulin intron loci (Figures 6.8 & 6.9) and was identical with respect to the ITS-1 loci (Figure 6.10). In addition, two clinical isolates, SR-3 and CH-2 were found to have identical ITS-1 sequences to all the other laboratory isolates.

Figure 6.10

Phylogenetic tree constructed using the Internal Transcribed Spacer (ITS-1) region of *N. caninum*.

Five laboratory isolates of *N. caninum* (BPA, LIVC, NC-Ger, NC-LivB1, NC-Drachten), *H. heydorni* and two clinical samples, CH-2 and SR-3 from Switzerland and Sweden respectively were amplified with primers for the ITS-1 region of *N. caninum*. Forward and reverse sequences are denoted by 'F' and 'R' respectively but only one sequence is shown on the tree. In addition, 9 other published sequences for isolates of *N. caninum* [NC-BPA-1 (AF038860), NC-1 (U16160), NC-5 (AF249970), NC-Beef (AF249968), NC-2 (AF249969), NC-CN1 (AF038861), NC-Sweb-1 (AF029702), CZ-4 (AF432123), NC-Nowra (AF338411), NC-Liv (NCU16159), NC-1b (U16160) and NC-LIV (L49389)] and also the published sequences for *N. hughesi* (AF038859) and NC-Oregon (AF249967) were included in the line-up.

Scale refers to rate of substitution per base.



6.4 DISCUSSION

The development of molecular epidemiological tools is dependent on two factors: (1) the quality and scope of the panel of isolates that are available to develop them (2) the ability to identify polymorphic DNA sequences. This latter requirement can be aided greatly by the availability of genome sequence data that exists for the organism in question.

In this chapter, micro/minisatellite primers were designed using *Neospora* ESTs, but unfortunately no detectable length polymorphisms were found between the *Neospora* laboratory isolates tested. By their very definition, ESTs are expressed sequences, so it is perhaps less likely that they will produce polymorphisms since these may alter the expression or function of a gene. Within these microsatellites, it is possible that differences still exist between samples, as a single, di- or trinucleotide repeat for example, may not be detectable on an agarose or even polyacrylamide gel. However, even if sequence differences do exist, these microsatellites would be less useful for rapid genotyping as differentiation could only be made by sequencing, rather than size variation on a gel.

To determine the overall level of minisatellite diversity in laboratory isolates of *N. caninum*, a number of isolates were probed with minisatellite probes. All isolates were found to have very similar patterns, suggesting that minisatellite diversity is also low amongst these isolates. Clinical samples could not be screened for minisatellites in this way because it is not possible to obtain *Neospora* DNA independently from host DNA within field samples. The results

from this experiment show that the level of genetic diversity in the laboratory isolates of *N. caninum* currently available is not high enough for polymorphisms to be detected using hybridisation with these microsatellite probes under the conditions used. Jeffreys *et al.* (1990) showed that the use of lower stringency conditions with probes 33.6. and 33.15 increases the complexity of the banding pattern so this approach could be adopted for *N. caninum*. However, even at high stringency, Jeffreys and colleagues were able to detect minisatellite heterogeneity between different human placental samples.

Single nucleotide polymorphisms (SNPs) are unlikely to be detected by techniques such as AFLP, unless the SNP occurs in a restriction site. To detect possible SNPs a gene sequencing approach was therefore adopted. Sequencing approaches to genotyping such as MLST methods have been very successful in typing bacterial pathogens. However, the rate of recombination in bacterial species such as *N. meningitidis* is high, meaning that clones are unstable and diversify over a few decades (Enright and Spratt 1999). From the RAPD and AFLP results and the identity of ITS-1 sequences between *Neospora* isolates, it appears that the genetic diversity of *N. caninum* is much lower than in some bacterial species. Therefore, it may not be appropriate to devise a typing system for *N. caninum* using entirely conserved DNA regions such as housekeeping genes. Primers were previously designed to amplify coding regions of the tubulin gene in *N. caninum*, but these regions revealed no polymorphisms between isolates (this study, data not shown). Fortunately the intron sequences to the tubulin and actin genes became available at the end of this project and enabled the unambiguous identification of SNPs between both laboratory isolates and clinical field samples of *N. caninum*. This demonstration of heterogeneity

supports that shown using RAPD and AFLP analysis, but had hitherto eluded detection. To devise a full MLST analysis for *N. caninum*, the introns of other genes, that may also have polymorphic regions, should be investigated in the future. The increased number of *N. caninum* sequences becoming available will greatly aid the design of primers for potentially polymorphic genes. As direct sequencing was only successful for a small number of isolates, cloning prior to sequencing was necessary with many samples. Saiki *et al.* (1988) estimated that *Taq* polymerase errors occur every 1×10^4 nucleotide bases in PCR amplifications. Errors are more likely to occur in sequences obtained via cloning compared to direct sequencing of PCR products and an example of this was seen at nucleotide 122 in Figure 6.5. In future, care should be taken to sequence more than one clone and to use a high fidelity *Taq* polymerase such as *Pfu* to eliminate *Taq* polymerase-induced sequence errors such as these. The majority of the SNPs found should be correct and strong evidence for this is seen when two isolates vary at the same nucleotide, for example at nucleotide 262 in the tubulin intron when both clinical sample 61 and 62 have a cytosine base instead of a thymine base that is present in the consensus sequence. The probability of a *Taq*-induced error occurring at the same position in two independent samples is extremely low.

Of the 23 single nucleotide polymorphisms observed in the tubulin intron sequence comparison (Figure 6.8), 16 of them were from the three clinical samples, 61, 62 and C919 which were all from Scotland. Some of these SNPs were conserved between isolates from a similar geographical region, but the number of isolates was far too small for any analysis. However, it was interesting that the *N. caninum* sequences analysed did not fall immediately into

two clusters as might be expected with *T. gondii* whose population structure consists of two main clonal lineages. It was unfortunate that clinical samples from different geographic locations became available only at the end of the project. It is hoped that future studies will incorporate many more clinical samples from a wide geographic area.

Unfortunately it was only possible to amplify successfully from a small number of the clinical samples originally obtained. Failure to amplify efficiently from clinical isolates could jeopardise a full analysis of the population structure of *N. caninum* by reducing the number of DNA samples for analysis. Further development on the more efficient processing of DNA clinical samples and increasing the sensitivity of PCR detection, for example through the use of nested primers, is essential.

The phylogenetic trees shown (Figures 6.8, 6.9 & 6.10) reveal some interesting associations but only when further clinical samples are analysed and SNPs confirmed by either re-sequencing or the use of high fidelity *Taq* polymerase, can more robust trees be constructed.

Ajzenberg and colleagues (2002) used 8 microsatellite markers to type 84 independent isolates of *T. gondii*, taken from humans and animals, which subsequently identified 72 different multi-locus genotypes. This study represented a significant advancement in the ability to detect mixed infections and advance an understanding of the population structure of *T. gondii*. Very few other genetic studies of *T. gondii* have used large numbers of clinical samples, with the exception of Howe & Sibley (1995), thereby precluding any substantial

conclusions on the population structure of this parasite. This shows the importance of building a clinical isolate bank for *N. caninum* to enable a similar level of analysis.

To discriminate successfully between isolates of *Neospora*, it has been necessary to adopt a gene sequencing approach. Single Nucleotide Polymorphisms (SNPs) can only be identified by sequencing and in the majority of cases it was necessary firstly to clone the PCR amplicons, since direct PCR sequencing had a high failure rate due to the difficulty of obtaining sufficient clean amplicons from clinical samples. This will always be essential for clinical material and means that differentiating field samples will initially be expensive. Cloning PCR products before sequencing however makes the task of detecting mixed infections in a single sample difficult. If two different alleles are present in a sample, then several clones must be sequenced to detect each allele (since *N. caninum* is haploid, the discovery of two different alleles must arise from a mixed infection). It will be necessary to amplify many more sequences from a diverse range of *N. caninum* clinical samples until a more defined population structure begins to emerge.

Sequencing of the ITS-1 gene confirmed that no sequence differences exist between isolates of *N. caninum*. This might be expected since analysis of the ITS-1 region from all three clonal lineages of *T. gondii* found it to be identical (Su *et al.*, 2003). The NC-Oregon isolate of *N. hughesi* clusters with the other *N. hughesi* isolate, but separately from *N. caninum*. Apparent sequence discrepancies between published sequences of NC-Liverpool entered in Genbank were resolved by re-sequencing the ITS-1 gene of NC-Liverpool. Discrepancies

remain with the NC-Ger isolate based on our sequencing, but this may have arisen from a sequencing error since a good sequence read was only obtained in one direction. The NC-Nowra isolate (Miller *et al.*, 2002) however appears somewhat divergent in the ITS-1 gene, which seems surprising. Such a discrepancy may be an error in the published sequence, but if not the phylogenetic position of NC-Nowra might have to be re-assessed. DNA from the NC-Nowra isolate was not available for analysis in this study. The ITS-1 sequencing results for *H. heydorni* were identical to the consensus *N. caninum* ITS-1 sequence as shown by its position in Figure 6.10. In addition, the intron regions of actin and tubulin also showed that *H. heydorni* clustered very closely amongst *N. caninum* isolates. These results are extremely interesting given the current controversy (discussed in section 1.8) over the phylogenetic placing of this organism and if this isolate proves to be representative of *H. heydorni* then it would suggest that *N. caninum* and *H. heydorni* are too similar to warrant separate genus and species status.

Chapter 7: General Discussion

The outbreak of neosporosis in the Astonwich dairy herd, Cheshire, provided a unique opportunity to study the seroepidemiology of *N. caninum* in a pedigree closed herd. Key results from this case study were the cattle age prevalence data, the dam-daughter pair analysis and the family tree data. All confirm the crucial role of vertical transmission in *N. caninum* infection. However, the question remains as to the route of entry of *N. caninum* into the herd in the first instance. The data are consistent with a point source infection with oocysts, most likely from an infected dog, but for this there is only circumstantial evidence.

Although most transmission appeared to be vertical, there is some suggestion that horizontal transmission may have continued in the herd to a small extent, but this appeared to be very low.

The confirmation of the importance of vertical transmission in this and other studies, raises the question as to whether *N. caninum* could be controlled, or even eliminated, by selective breeding within a closed herd or, by screening for *N. caninum* if replacement stock are bought-in. At first this might appear a straightforward solution for managing the disease, but there are some difficulties with this approach. If a closed herd is heavily infected, this strategy may severely restrict options for breeding. In addition, screening by antibody is not always guaranteed to find all infected animals due to fluctuations in antibody titres. Moreover, it would be very important to ensure that no horizontal transmission occurred subsequently on the farm. Dogs must therefore be managed and bovine placentas should be removed immediately to prevent

ingestion by other cows or dogs. One possible means of retaining family lines of high genetic merit, but which have become infected, is to use embryo transfer whereby an embryo of the infected cow is placed in a cow of lower genetic merit but is free of neosporosis. This approach has been adopted occasionally for cows within the Astonwich herd but it is an expensive option.

No study has yet addressed in a quantitative manner the impact of *N. caninum* infection on the fertility of cattle. Although it may appear obvious that neosporosis reduces fertility in cattle since it causes abortion, the relationship between herd serostatus and overall herd fertility has not been measured before. Fertility in cattle is an incredibly complex and multifactorial issue. Nevertheless, when all other factors are controlled for, as in this closed herd, there was a measurable and statistically significant increase in the number of AI services per successful pregnancy in cattle seropositive for *N. caninum* compared with seronegative cattle. This finding strongly justifies future work. If translated to a wider context, these data would have important implications for cattle management. However, the results from this case study need to be verified in a much larger survey. The economic impact of neosporosis is still not known, but is a very complex question with factors such as reduction in bovine fertility, yet to be quantified. An accurate assessment of the economic impact of neosporosis is essential for assigning future research priorities in animal health and also in attracting future funding to develop methods of control. A large cross-sectional study specifically to investigate the effect of *N. caninum* infection on fertility in cattle will be a considerable challenge; farm-to-farm variation will be large and multiple factors that affect fertility will need to be subtracted from the analysis.

For example, major variables affecting the success of AI include accurate heat detection and competent AI technique. Both of these factors can vary hugely between farms regardless of the infection status of the herd.

Chapter 3 described a cross-sectional study in two regions of Africa, Ghana in West Africa and Tanzania in East Africa. This work identified a region of West Africa in which *N. caninum* appears to be completely absent in cattle. This is in contrast to the closely-related parasite *T. gondii*, which has been found to have a truly world-wide distribution with remarkably similar seroprevalences in domestic animals regardless of geographic location or farming system.

Why has *T. gondii* been such a successful parasite and become so ubiquitous in comparison with *N. caninum*? The main transmission route for the spread of *T. gondii* to domestic animals such as sheep and goats, is thought to be via oocyst shedding by the cat. However, *N. caninum* seems to rely principally on vertical transmission in cattle. Perhaps the retention of efficient oocyst shedding in *T. gondii* has ensured its high overall prevalence and more cosmopolitan distribution compared to *N. caninum*. The apparent absence of *N. caninum* in West Africa raises the question as to whether the distribution of *N. caninum* reflects an association with intensive western-style farming systems and cattle breeds. Perhaps one of the most interesting questions is, did *N. caninum* evolve in this context? i.e. did the evolution of efficient vertical transmission go hand-in-hand with intensification of cattle farming? Perhaps *N. caninum* has been able to evolve through intensive farming systems by exploiting dairy cattle which are genetically selected specifically for high milk yields and may have reduced

tolerance to infection. Answers to these questions might be obtained from phylogenetic analysis to estimate the time-scale of evolution of *N. caninum*. The acquisition of exotic isolates of *N. caninum* might be necessary to gain an accurate measure of this. Unfortunately, none were obtained from West or East Africa, but collection of a larger number and wider range of isolates would be essential to study further the evolution of *N. caninum*. Rates of mutational change in the DNA of the three main lineages of *T. gondii* suggest that they may have diverged around 10,000 years ago, coincident with the time of agricultural expansion and domestication of the cat as a companion animal (Su *et al.*, 2003). A similar analysis could be undertaken with *N. caninum* by analysing SNPs within introns and antigen coding DNA regions and by comparison with rates for neutral mutations in closely related Apicomplexa such as *T. gondii* and *Plasmodium*. From this analysis the time to the most recent ancestor could be estimated for *N. caninum*.

RAPD and AFLP analysis showed that the genetic diversity of *N. caninum* appears to be even lower than that of *T. gondii*, although these analyses were limited to laboratory isolates. The apparently low genetic diversity in *N. caninum* is consistent with a highly clonal population structure, similar to that in *T. gondii*. It is important to note however, that this study has not formally demonstrated a clonal population structure for *N. caninum*, only that genetic diversity is low, which is usually consistent with a clonal structure. Clonality would have to be tested by a multi-locus genotyping approach to demonstrate linkage disequilibrium between populations (Mallon *et al.*, 2003). Unfortunately it proved difficult to extract markers from AFLP analysis that could be used for

such studies, but the markers developed in Chapter 6 could be used for future population genetic analysis.

A clonal population structure indicates little or no opportunity for sexual recombination. If indeed *N. caninum* is shown to have a clonal population structure, this may at first seem to indicate that the dog plays little role in transmission since it acts as a definitive host in which the sexual cycle takes place. However, for recombination to occur the dog must be infected simultaneously with two different genotypes, otherwise selfing occurs which will lead to genetically identical or 'clonal' progeny. Thus the dog may contribute significantly to the spread of disease (as does the cat in *T. gondii*), whilst still maintaining a clonal population structure. In contrast, the demonstration of a recombinant population for *N. caninum* would implicate the dog in transmission since sexual recombination can take place only in the dog.

Clonal population structures are also consistent with vertical transmission. Su *et al.* (2003) argued that clonal structures in *T. gondii* occurred after rapid expansion of successful recombinant genotypes following the evolution of oral infectivity of tissue cysts. However, vertical transmission also bypasses the definitive host and thus the opportunity for sexual recombination could also lead to a clonal population of low genetic diversity in *N. caninum*.

To begin to understand the population genetic structure of *N. caninum* and the relative importance of different transmission routes, a major aim of this study was to identify polymorphic molecular markers. Genetic diversity between

laboratory isolates was found to be relatively low. One concern is that this could reflect a genetic bottleneck caused by the processes of selection in tissue culture. As mentioned previously, the true diversity of *N. caninum* can only be determined directly from field samples and for this sequence-specific PCR typing methods were developed. Interestingly, even though only a small number of clinical samples were available, analysis of these clinical field samples revealed a marked increase in the number of SNPs found compared with the relative homogeneity of laboratory isolates. This suggests that until a wider range of clinical field samples are analysed it would be premature to draw any conclusions as to the true genetic diversity of this parasite.

RAPD and AFLP analyses were useful in measuring genetic diversity in laboratory isolates of *N. caninum*, but also confirmed previously reported genetic relationships in *T. gondii*. *T. gondii* has a population genetic structure that divides into three types: two clonal lineages and one recombinant lineage (Howe and Sibley, 1995). The population genetic structures of *T. gondii* may still be very skewed by the analysis of mainly laboratory stocks, some of which have been in culture for decades. This would specifically under-represent recombinant genotypes. As with *N. caninum*, there are still only a relatively small number of clinical samples available for *T. gondii*.

If *N. caninum* is a relatively recently evolved coccidian, it would be expected that the total genetic diversity observed, even in terms of SNPs and microsatellites, would be low. Regions of DNA most likely to be polymorphic include non-coding introns and it was in the introns of the actin and tubulin genes

that SNPs were observed in this study. What then is the next stage in molecular epidemiological studies? Clearly a larger number of clinical samples are required to be analysed to give a clearer picture of overall diversity. From this study, introns have been shown as useful polymorphic regions, so it would be worth sequencing introns in a wider range of genes. A proposal for the sequencing of the *N. caninum* genome is currently under consideration. The success of this application would have a significant impact on the approach to molecular epidemiological studies. It would allow the rapid identification of new markers, especially as intron sequences and microsatellite markers would be readily detectable. Microsatellites have been shown to be much more prevalent in the intron sequences of *Eimeria* for example (Al Ivens, pers. comm.). Knowledge of the *Neospora* genome would give huge potential for better understanding the biology of the protozoan through proteomics and comparative genomics, especially with the *Toxoplasma* (7 x coverage), *Eimeria* (8 x coverage) and *Cryptosporidium* (7 x coverage) genome sequencing projects almost complete. This approach will help address questions such as the basis of host specificity and virulence factors associated with *N. caninum*.

The development of sequence-specific PCR will be essential for widening the scope of genetic analysis to clinical field samples. Only then will comprehensive population genetic analysis be possible. The difficulty of extracting DNA from infected tissues must be overcome as currently the failure rate for PCR from field samples is too high. For all these approaches to be successful, it will be necessary to resolve the problem of clinical sample extraction. Currently, the failure to amplify by PCR from some field samples is disappointing. Perhaps

better collection methods would limit autolysis and more sensitive extraction methods combined with a nested PCR approach could increase the sensitivity of detection and the success of genotyping.

The evolution of vertical transmission in *N. caninum* and presumed avoidance of frequent sexual recombination raises the historical argument of the role of sex in the success of a species. The genetic diversity of *T. gondii* (in which recombination may be more frequent than in *N. caninum*) appeared to be slightly greater than that of *Neospora*. A population of sexually reproducing organisms can, under some conditions, evolve faster than a similar number of asexual organisms (Fisher, 1930). Sexual reproduction can greatly increase the rate at which beneficial mutations, at separate loci, can be combined in a single individual. This may partly explain the greater success in terms of host range and geographical range seen in *T. gondii* compared with *N. caninum*. Asexual lineages have a higher extinction rate than sexual ones by looking at taxonomic distributions and asexual lineages do not last long enough to diversify into a genus or higher taxonomic level (Ridley, 1996).

Perhaps the evolution of an efficient form of vertical transmission in cattle may provide a niche for the success of *N. caninum* in the short-term. However, the avoidance of sexual recombination may eventually limit its genetic diversity such that evolutionary extinction might become inevitable.

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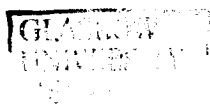
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Animals are such agreeable friends – they ask no questions, they pass no criticisms.

George Eliot