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A study of neosporosis in cattle and sheep in Ireland

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A Study of Neosporosis in Cattle and Sheep in Ireland

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This thesis is dedicated to my wife and family

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Summary

During the past decade, the intracellular protozoan parasite *Neospora caninum* has been identified as a major cause of reproductive disease in cattle throughout the world. Abortion occurring during the middle of gestation is the primary clinical sign of infection in cattle. These abortions and associated infertility lead to significant economic loss in the cattle industries of countries where the disease has been identified.

In this thesis, a major objective was to establish the prevalence of the disease, particularly in cattle, and in sheep in Ireland, and to identify the factors of epidemiological importance in cattle, which may be useful in designing any future eradication or control programmes at a later date. It was also decided to examine the pathogenesis of the disease in both species to determine the influence of infection at different stages of gestation on the outcome of the pregnancy following experimental infection. Finally, the role of vaccination in the prevention of abortion in cattle and sheep following experimental infection during pregnancy was also investigated.

The results of studies described in this thesis, confirm that neosporosis is very prevalent in the Irish cattle herd. It has also been shown to exist in the Irish sheep flock, but its importance has not yet been fully evaluated. The stage of gestation during which the dam becomes infected has been shown, at least in sheep, to determine the outcome of pregnancy. Finally, vaccination, in sheep, did not provide protection against abortion following experimental challenge of the dams during pregnancy. In the absence of abortion in the cattle trials it was not possible to comment on the efficacy of the vaccine in the bovine.

Table of Contents

Chapter 1. General Introduction .

Introduction 1

Chapter 2. The Seroprevalence of *N. caninum* Associated Abortions in the Irish Cattle Herd .

Summary 13

Introduction 14

Materials and Methods 16

Results 20

Discussion 23

Chapter 3. The Seroprevalence of *Neospora caninum* in the Irish Cattle Herd .

Summary 27

Introduction 28

Materials and Methods 30

Results 32

Discussion 33

Chapter 4. A Seroepidemiological Study of *Neospora caninum* in the Irish Cattle Herd .

Summary 35

Introduction 36

Materials and Methods 38

Results 41

Discussion 52

Chapter 5. A Study on the Seroprevalence of *Neospora caninum* in Sheep in Ireland .

Summary 56
Introduction 57
Materials and Methods 59
Results 60
Discussion 61

Chapter 6. A Study to Evaluate the Pathogenicity of *N. caninum* tachyzoites passaged in the Laboratory using the Murine Model .

Summary 62
Introduction 63
Materials and Methods 65
Results 72
Discussion 75

Chapter 7. A Study of the Pathogenesis of Experimental Infection in Pregnant Cattle with *Neospora caninum* .

Summary 77
Introduction 78
Materials and Methods 81
Results 89
Discussion 93

Chapter 8. A Study of the Pathogenesis of Experimental Infection in Pregnant Sheep with *Neospora caninum* .

Summary 95
Introduction 96
Materials and Methods 98
Results 109
Discussion 124

Chapter 9. The Antibody Response and Outcome of Pregnancy in Cattle Vaccinated against Neosporosis when Subsequently Challenged by Experimental Infection with *N. caninum*.

Summary 126
Introduction 127
Materials and Methods 129
Results 131
Discussion 133

Chapter 10. The Antibody Response, Pathogenesis of the Disease and Outcome of Pregnancy in Sheep Vaccinated against Neosporosis during Pregnancy when Subsequently Challenged by Experimental Infection with *N. caninum*.

Summary 137
Introduction 138
Materials and Methods 140
Results 143
Discussion 165

Chapter 11. General Discussion.

General Discussion 171

References 181

Appendices

Appendix A Chapter 10
Appendix B Chapter 8
Appendix C Chapter 10
Appendix D Chapter 9
Appendix E Chapter 6

Chapter 1

General Introduction

General Introduction

The apicomplexan protozoan parasite *Neospora caninum* is an important cause of abortion and infertility in cattle resulting in significant economic loss (Dubey and Lindsay, 1996, Trees et al., 1999, Anderson et al., 2000, Hemphill and Gottstein, 2000).

The parasite was first identified in Norway in 1984 as a previously unknown cyst-forming sporozoon causing encephalomyelitis and myositis in dogs (Bjerkås et al., 1984). It was later classified as a new genus within the protozoan apicomplexan phylum called *Neospora*, type species *Neospora caninum*, alongside other members of the family *Sarcocystidae* which includes *Toxoplasma*, *Sarcocystis* and *Hammondia* in 1988 (Dubey et al., 1988(b)). In 1989, *N. caninum* was identified in an aborted bovine foetus (Thilsted and Dubey, 1989). Two years later in 1991, it was recognised as a major cause of abortion in cattle in the United States (Anderson et al., 1991, Barr et al., 1991 (b)).

During the following five years, between 1991 and 1996, *N. caninum* was associated with bovine abortions in many regions of the world including Europe, Australia, Africa and North America (Dubey and Lindsay, 1996). More recently, it has been reported in parts of Latin America including Argentina (Venturini et al., 1999), Brazil, (Locatelli-Dittrich et al., 2001), Costa Rica, (Romero et al., 2002), and Mexico (García-Vázquez et al., 2002). Prior to 1996, Japan had been the only country in the Far East to report the presence of *N. caninum* in the bovine population. Since then, *N. caninum* has been identified in cattle in Thailand (Suteeraparp et al., 1999), and in Taiwan (Ooi et al., 2000). More recently, there have been reports in Europe from countries where the disease had been previously unreported, these included Portugal

(Thompson et al., 2001) and Hungary (Bacsadi et al., 2001). Neosporosis has been reported in a live 10 day calf in Ireland (Collery, 1995). It has also been associated with abortions in cattle in Northern Ireland (, McNamee and Jeffrey, 1994, McNamee et al., 1996). It appears more likely that the disease has a truly worldwide distribution, as more countries report the presence of the disease.

Antigenically, *Neospora caninum* has been shown to be closely related to *T. gondii* (Howe and Sibley, 1999), which is a confirmed zoonotic pathogen. There is no conclusive evidence that *N. caninum* can cause infection in humans (Graham et al., 1999, Petersen et al., 1999, Tranas et al., 1999,). The parasites recovered from dogs and cattle have been shown to be indistinguishable (Holmdahl and Mattsson, 1996). Recently, the dog has been described as the definitive host of *N. caninum* (McAllister et al., 1998). This finding was confirmed by Lindsay et al., in 1999.

While cattle are highly susceptible to natural infection leading to abortion and infertility, *N. caninum* is also pathogenic for dogs under natural conditions where it causes neuromuscular disorders, however, it is not normally associated with canine abortions (Dubey and Lindsay, 1996). There are some reports in the literature of natural infections with *N. caninum* in other species, however, the number of cases reported to date is very small. A case of congenital neosporosis has been reported in a lamb (Dubey and Lindsay., 1990). More recently, a case of natural infection in an ewe and her twin lambs was reported in Japan (Kobayashi et al., 2001). Antibodies to *N. caninum* have been found in camels in Egypt (Hilali et al., 1998) and in the sera of non-domestic felids in Southern Africa (Cheadle et al., 1999), indicating exposure of these species to the antigen. Neosporosis was first identified in an aborted equine foetus in 1988 (Dubey and Porterfield, 1990). *Neospora* organisms were found in the

brain and spinal cord of a horse with neurological signs, in California, U.S.A., where the parasite was isolated following inoculation of tissue culture with neural tissue of the horse (Marsh et al., 1996). These isolates were further differentiated, primarily on molecular differences, into a new species called *Neospora hughesi* (Marsh et al, 1998).

The life cycle of *N. caninum* involves a two host cycle, namely, an intermediate host and a definitive host. Cattle are by far the most common species that act as intermediate hosts. Natural infection has also been shown to occur infrequently in dogs, sheep, goats, horses and deer, for this reason they can all act as intermediate hosts though not all species have a similar host susceptibility. Under experimental conditions, it is possible to infect mice, rats, dogs, foxes, goats, cats, sheep, coyotes, pigs, gerbils, rabbits and cattle (Dubey and Lindsay, 1996, Dubey, 1999).

There are two distinct stages in the life cycle of *N. caninum*, the sexual and the asexual stages. Tachyzoites and tissue cysts comprise the asexual stages and are found in the intermediate hosts, where both are intracellular. Tissue cysts are round to oval in shape, up to 107 μm long and are found mainly in the central nervous system. The cyst wall may be up to 4 μm in thickness and the enclosed bradyzoites are 7 x 2 μm in size. The bradyzoites divide and multiply very slowly and represent latent infection. On the other hand, the tachyzoites which divide into two zoites by endogoney are the rapidly dividing stage of the parasite. They are ovoid, lunate or globular in shape and measure 3 to 7 μm long and 1 to 5 μm wide depending on stage of division (Dubey, 1999). Tachyzoites can penetrate the host cells by active invasion and can become intracellular within five minutes of contact (Hemphill et al., 1996).

After ingestion of the tissue cyst by the definitive host, the sexual stages of the *N. caninum* life cycle continues with the development of an ovum in the host's

intestine and the subsequent shedding of oocysts in the faeces. Having been passed in the faeces, the oocysts can sporulate within 24 hours. At this stage, each oocyst contains two sporocysts each with four sporozoites. The number of oocysts found in the faeces in dogs is invariable low and shedding of oocysts only continues for a few weeks (Dubey, 1999).

The transmission routes of *N. caninum* infection have been studied most extensively in cattle, where two routes have been described, namely vertical and horizontal. The term vertical means prenatal or congenital transmission and is used to describe the spread of infection from cow to calf during pregnancy. The term horizontal or post-natal transmission is used to described the infection in animals after birth.

Vertical transmission of *N. caninum,* through generations of cattle, appears to be the major route of transmission of the disease (Parė et al., 1997, Wouda et al., 1998(b), Thurmond and Hietala, 1999). This important route has been shown to be responsible for maintaining infection in a herd (French et al., 1999). There is no conclusive evidence that horizontal transmission plays a significant role in the spread of *N. caninum* in bovine herds. Under experimental conditions, calves were successfully infected with *N .caninum* by feeding them oocysts derived from a dog (De Marez et al., 1999). In a recent study, where calves were fed placental tissues from *N. caninum* infected cows, no transmission of infection took place. However, when calves were fed *N. caninum* tachyzoites orally in colostrum there was a serological response, but it was not possible to confirm by histology or PCR that infection had actually occurred. The conclusion was that the oral route is not an

important natural route of transmission of *N. caninum* between cattle (Davison et al., 2001).

Nevertheless, modelling studies appear to indicate that without an element of horizontal transmission *N. caninum* would disappear over time from cattle herds (French et al., 1999). Epidemiological investigations suggest a link between the presence of farm dogs and the occurrence of *N. caninum* associated abortions in cattle (Paré et al., 1998, Bartels et al., 1999, de Souza et al., 2002, Dijkstra et al., 2002(a)(b)). Dogs have been shown to shed oocysts at very low levels after eating mice experimentally infected with *N. caninum* (McAllister et al., 1998), and calves sero-converted after oral infection with oocysts (De Marez et al., 1999). Recently, *N. caninum* was isolated from the faeces of a naturally infected dog for the first time, showing that natural infection can take place in this species (Basso et al., 2001). Nevertheless, it is not fully understood how transmission takes place between dogs and cattle under natural conditions (Dubey, 1999).

In adult cows, abortion may be the only clinical sign of infection with *N. caninum*. Abortions, when they occur within herds, may be clustered, sporadic or epidemic and can occur at any stage of gestation between three months and term. Foetuses may also die in utero and be reabsorbed, mummified, autolysed or stillborn. The calves from an infected cow may be born alive but infected, and appear to be clinically normal. Some of these calves may sometimes be born underweight and showing neuromuscular symptoms manifested by the inability to stand upright (Dubey, 1999).

Following natural or experimental infection with *N. caninum*, cattle normally respond immunologically with the production of specific antibody and cell-mediated responses which involve proliferation of cells and cytokine production particularly of interferon gamma (IFNγ) (Lunden et al., 1998, De Marez et al., 1999, Williams et al., 2000, Guy et al., 2001, Andrianarivo et al., 2001,). Typically, these two types of responses are cross-regulatory and tend to down-regulate each other but not to the exclusion of either type of response. In most situations, the two mechanisms interact in a complex regulatory pathway (Allen and Maizels, 1997).

There is evidence that some *N. caninum* infected animals can develop a degree of protective immunity against abortion or congenital transmission.). Recently in cattle, it has been demonstrated that when cows are experimentally inoculated with *N. caninum* tachyzoites prior to mating, they developed sufficient immunity to protect against vertical transmission to the foetus following experimental challenge at the mid-trimester of pregnancy (Innes et al., 2001). In that study, there was a rapid onset of cell-mediated immune responses immediately following challenge which was accompanied by a significant specific antibody response.

The diagnosis of *N. caninum* infection in living animals is usually made by serology. There are a number of serological tests available which include the indirect fluorescent antibody test, the direct agglutination test and enzyme linked immunosorbent assays (Björkman and Uggla, 1999, Atkinson et al., 2000).The presence of specific antibodies is indicative only of exposure to *N. caninum* and does not necessarily mean that the animal is actually infected. The antibody levels in cattle may fluctuate particularly during pregnancy (Dannat, 1997). For a definitive diagnosis of *N. caninum* it is necessary to examine the foetus. Microscopic lesions may be found

in a variety of foetal tissues such as the brain, spinal cord, heart, lung and placenta (Barr et al., 1991, Lindsay et al., 1996). Foetal blood serum and other body fluids may be examined by serology, but a positive result is only indicative of neosporosis. The most characteristic lesion, which is found most commonly in the brain, consists of focal encephalitis, necrosis and non suppurative inflammation. There are no pathognomonic gross lesions of neosporosis (Dubey, 1999).

Immunohistochemistry (IHC) has been reported to give very good results when compared to other methods, however, there are other reports which suggest that the sensitivity of even the most sensitive IHC test to detect *N. caninum* in tissues is low (Dubey, 1999). A number of different polymerase chain reaction (PCR) methods have used been used to detect *N. caninum* DNA in bovine tissues with varying degrees of success (Lally et al., 1996 , Ellis, 1998, and Gottstein et al., 1998). However, more recently, PCR using the pNC-5 gene of *N. caninum* has been shown to have a much higher sensitivity in detecting *N. caninum* in tissues compared to IHC (Baszler et al., 1999).

While there are very few cases of naturally occurring infection in sheep reported in the literature, nevertheless, pregnant sheep are very susceptible to experimental infection (McAllister et al., 1996(a) , Buxton et al., 1997)). The lesions found in the foetal brains and placentae of experimentally infected ewes are very similar histologically (Buxton et al., 1998) to those reported in cattle (Dubey and Lindsay, 1996).

Although *N. caninum* has been identified in cattle in most parts of the world, mainly by serology, there is little information on the economic consequences. The parasite is recognised as an important cause of bovine abortion in many countries, it is

also associated with infertility, neonatal mortality, increased culling, reduced milk production and reduced value of breeding stock. Although many of these economic considerations apply mainly to the dairy sector, some are equally applicable to beef herds. To date, there is little information on neosporosis in beef herds (Trees et al., 1999). However, in spite of the absence of conclusive data regarding economic losses to the cattle industry, anywhere in the world, there is evidence that neosporosis costs Californian cattle producers approximately 35 million US dollars due to abortions alone (Dubey, 1999). In addition, milk yield of *N. caninum* infected, but non-aborting cows, is reduced by over 4% (Thurmond and Hietala, 1997(b)). Recently, in the United States it has been shown that the decrease in milk production alone can represent an annual loss of up to 128 $ per cow (Hernandez et al., 2001). It is clear that the figures quoted in relation to the true economic cost of neosporosis in cattle are only estimates based on incomplete data. In Ireland, there is an urgent need to determine the true prevalence of the disease on a countrywide basis and to investigate the epidemiological factors which influence the spread of the disease in a truly scientific manner so that the real economic importance of neosporosis in cattle can be accurately quantified.

Control and eradication of the disease is difficult in the absence of effective therapeutic agents and/or a reliable vaccine to prevent *Neospora* induced abortion or vertical spread in cattle (Dubey, 1999). The role of the dog in the transmission of infection to cattle has still not been fully quantified. Various measures to prevent close contact, between dogs shedding oocyts and cattle, may help in reducing the spread of disease within herds (Dijkstra et al., 2002,(a) (b)).

Outline of the Thesis

The global importance of neosporosis in cattle, as a cause of significant economic loss, is well recognised. However, even within countries, including Ireland, where the disease has been reported, information on the prevalence is often limited to individual herds, or at best to localised regions. To date, there are no reports in the literature, where comprehensive studies have been carried out to establish the prevalence of the disease in the national cattle herd of any country.

In this thesis, a major objective was to determine the seroprevalence of *N. caninum* in the Irish cattle population on a countrywide basis, and to evaluate the influence of various factors which may have a bearing on the epidemiology of the disease in the national herd. The factors evaluated during the course of the project included herd size, category of bovine animal, herd enterprise type, regional influences and season. This part of the project involved the setting up of three separate serological based studies which were carried out over a two year period from 1999 to 2001. In the initial study, 5,785 post abortion blood samples were collected from all bovine abortions reported to the Irish Department of Agriculture and Food , these were tested for the presence of specific antibodies to *N. caninum* (chapter 2). In the second study, a random sample comprising 2,253 bovine sera were collected countrywide from the Irish cattle population and were likewise screened for the presence of antibodies to neosporosis (chapter 3). In the final study, 871 bovine herds involving 21,351 sera were randomly selected from the national herd and tested for the presence of specific antibodies to the disease (chapter 4).

The protozoan parasite, *Toxoplasma gondii,* which is closely related to *Neospora caninum,* is a major cause of abortion in sheep in most countries of the

world including Ireland. Following experimental infection with *N. caninum,* sheep have been shown to be highly susceptible to infection, although there are very few reports in the literature of naturally occurring infection in sheep. In this project, a study was carried out, during which blood samples from 1,040 adult sheep were screened for the presence of antibodies to *N. caninum.* The sheep originated from 52 randomly selected flocks, distributed throughout the country (chapter 5).

The pathogenesis of neosporosis in cattle and sheep is not fully understood. It has been shown under experimental conditions that, in cattle or sheep infected early in pregnancy, the foetus generally dies in utero and is aborted, or it may also be mummified, autolysed or reabsorbed. Those animals infected at a more advanced stage of pregnancy, may be born alive but infected and appear clinically normal. Those animals that survive carry the infection on to the next generation mainly by the vertical transmission route. It appears that the stage of gestation at which infection takes place is critical in influencing the outcome of the pregnancy. In this study, it was decided to investigate the mechanisms involved by experimentally inoculating 22 cattle and a similar number of sheep at various stages during early to mid-pregnancy with NC-1 tachyzoites. The infected dams and control animals were slaughtered one month after challenge. The transmission of infection from dam to foetus across the placenta was studied by serology, histology, immunohistochemistry and PCR methods (chapters 7 and 8).

Control of the disease is difficult as the life cycle and exact mode of transmission in cases of natural infection is not fully understood. The dog is to date, the only species that has been shown to act as a definitive host. Dogs only pass oocysts for a limited period and then only in small numbers. Modelling studies have

indicated that without horizontal transmission of infection, neosporosis in cattle would die out, yet there is little evidence of horizontal infection playing a significant role in the spread of the disease. Current control strategies generally concentrate on culling animals and replacing them with heifers bred or purchased from non-infected herds. Measures to prevent the cross-contamination of pasture and feeding stuffs, with oocysts from infected dogs, are also important in controlling the spread of the disease but are not always practical at farm level.

In many other diseases of cattle and sheep, vaccination as a control measure has been shown to be very effective. With regards to neosporosis, there is one commercial vaccine available for use in cattle, however it is only currently licenced for use in cattle in the United States. There is no convincing evidence that it is effective in either preventing abortion or the spread of the disease within cattle herds.

In the final part of this project, the efficacy of vaccination of cattle and sheep during pregnancy as a means of preventing or reducing abortions was evaluated. These studies involved the experimental inoculation, with NC-1 tachyzoites, of 30 cattle and 29 sheep which had been vaccinated during early pregnancy with this commercial vaccine. The efficacy of vaccination, as a means of controlling the disease in these species, was determined by the outcome of pregnancy (chapters 9 and 10).

During the course of the different studies that constituted this thesis, various diagnostic methods including several serologically based tests, histology, immunohistochemistry and PCR methods were compared and contrasted in order to evaluate their effectiveness in diagnosing the presence of the disease in cattle and sheep.

Chapter 2

The seroprevalence of *N. caninum* associated abortions in the Irish Cattle Herd

Summary

Post abortion blood serum samples, sent over a period of one year to the Central Brucella Laboratory under the National Brucellosis Eradication Programme, were screened by ELISA for the presence of specific antibody to *Neospora caninum*. Positive results were confirmed by IFAT.

Statistical analysis of the results showed that animals seropositive for the disease were 2.5 times more likely to abort than seronegative animals. It was also shown that 9.0% of all abortions in Ireland can be attributed to neosporosis.

Introduction

The protozoan parasite *Neospora caninum* is now recognised as a major cause of abortion in cattle in many countries worldwide (Dubey and Lindsay, 1996, Anderson et al., 2000). It has been associated with up to 17% of aborted bovine foetuses in the Netherlands (Wouda et al., 1997). In England and Wales, it was estimated by serology that up to 12.5% of abortions in dairy herds were due to neosporosis (Davison et al., 1999(a)). Cattle which are serologically positive to *N. caninum* have an increased risk of abortion (Paré et al., 1997, Thurmond and Hietala, 1997(a), Davison et al., 1999(b)). It has been shown that seropositive cows have a two to four fold higher risk of abortion than seronegative cows (Sager et al., 2001, Pfeiffer et al., 2002).

Abortion can occur at any stage of pregnancy, but most abortions occur at four to six months of gestation. In cows and heifers, abortions may occur throughout the year but it is not clear if seasonality is an important factor. Cows may abort sporadically, or in groups, within a herd or the abortions may persist in the herd for a number of years (Anderson et al., 2000). Repeat abortions in the same animal can be due to *N. caninum* (Dannet et al., 1995).

In neosporosis of cattle, two types of abortions have been described in herds (Thurmond and Hietala, 1999(a)). In the endemic type, abortion rates within herds may exceed 5% per annum and may persist for years. In the epidemic type, the effect is more dramatic with abortion rates of up to 30% of pregnant animals within the herd aborting due to *N. caninum* infection, over a period of several months (Thilsted and Dubey, 1989). In some herds, both patterns of abortion may co-exist apparently, with

one or other becoming the predominant pattern over a period of time (Anderson et al., 2000).

There is no conclusive evidence available on the economic losses attributable to neosporosis in cattle. In addition to direct losses incurred due to abortion and stillbirths, there are also indirect costs which include possible loss of milk yield, infertility and culling of infected animals (Trees et al., 1999). Although abortion caused by *N. caninum* appears to be widespread in the world, there are no reliable estimates of the prevalence of the disease in the Irish National Herd. This information is fundamental in determining the true economic effect of the disease and evaluating future control or eradication programmes. For these reasons, this seroprevalence study was undertaken at both regional and national level. It was decided to carry out the study over a period of one year in order to look at the seasonal influence, if any, on the pattern of abortions.

It is well recognised that the prevalence of antibodies to *N. caninum* in the sera of cows, which had recently aborted, does not necessarily mean that the abortion was caused by the parasite, it only indicates that exposure to the organism has occurred. The results of this study and those described in chapter 3 were combined to estimate the true level of *Neospora*-associated abortion in the Irish cattle population and the number of abortions in seropositive cattle which were actually due to *N. caninum* infection.

Materials and Methods

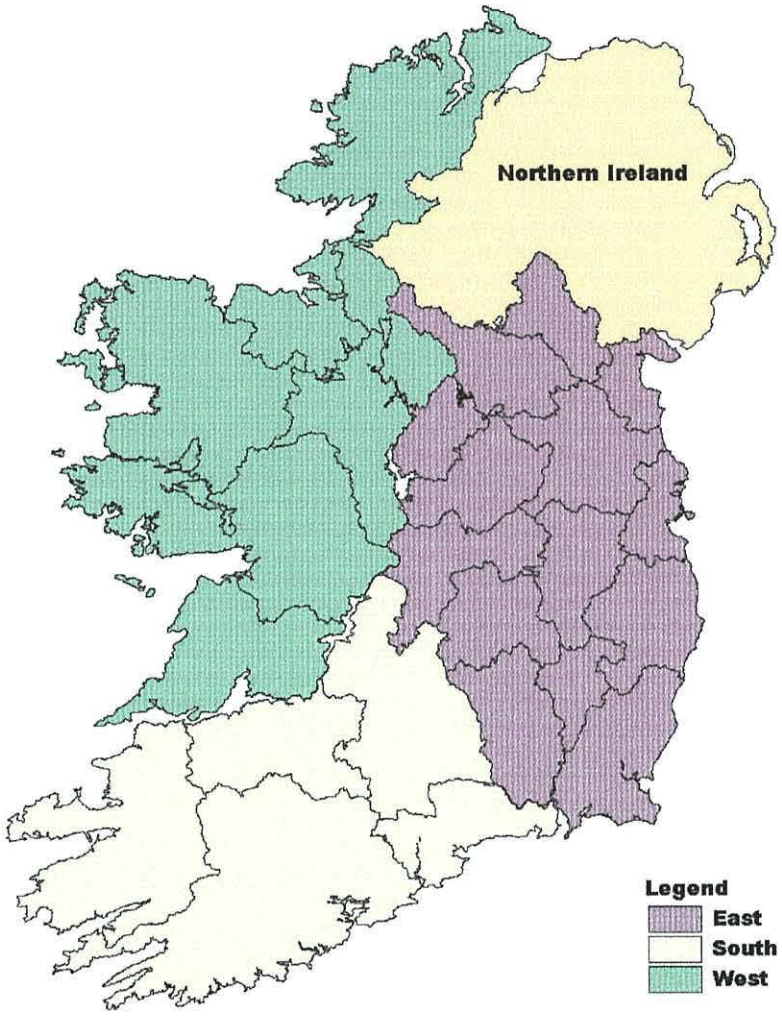
Survey Design

This survey was carried out during the 12 month period, 1st September, 1999 to 31st August, 2000. During that period, there were 108,989 individual herds containing 3,545,800 female animals over 12 months of age in the Irish National Herd (Department of Agriculture and Food ,1999 – 2000).

It is a requirement under Irish legislation, following an abortion in a cattle herd that a blood sample must be taken from the aborted animal as soon as possible after the event. This normally occurs within a period of one to fourteen days, following the abortion and it is usually taken by a veterinary practitioner under the direct supervision of the Department of Agriculture. The sample is then sent to the Central Brucella Laboratory in Cork for testing under the Irish Brucellosis Eradication Programme. Aliquots of serum from these samples were tested in the Central Veterinary Research Laboratory (CVRL) in Dublin for the presence of antibodies to *N. caninum*.

For the purposes of this study, the country was divided into three regions, the East comprising 14 counties, the West containing seven counties and the South with five counties, based on natural geographical boundaries and farming enterprises. The regions are clearly shown in figure 1 with the river Shannon dividing the East from West. The South is separated from the West by the Shannon and its southern border is formed by the Atlantic ocean. The East region lies between the Irish sea and the river Shannon, and is bordered by the southern region to the South and Northern Ireland to the North.

Figure 1. Map of Ireland showing the three regions



Serology

During the course of this study all the post abortion sera received in the Central Brucella Laboratory, were screened in the C.V.R.L., Dublin, for antibodies to *N. caninum* using a commercially available Enzyme linked immunosorbent assay (ELISA) test kit (Herd Check* *Neospora caninum* Antibody Test Kit, IDEXX, Westbrook, Main, USA). The test provides for the samples to be tested in duplicate;

however, in this case the samples were tested singly, otherwise the manufacturer's instructions regarding the test procedure and the interpretation of the results were followed exactly.

The results of the ELISA test were calculated as the serum/positive (S/P) ratio {(optical density (OD) test sample – OD negative control)/(OD positive control – OD negative control)}. A cut off S/P ratio of <0.5 was considered negative, an S/P ratio of 0.5 to 0.999 was a low positive, in which case, the test was repeated in duplicate. An S/P ratio ≥ 1.0 was considered to be a high positive and was repeated singly. All ELISA positive sera were subjected to further testing, using a commercially available immunofluorescent antibody test (IFAT), according to the manufacturers specifications (VMRD, Inc., PO Box 502, Pullman, WA 99163, USA). The IFAT at a dilution of $^{1}/_{100}$ was considered as the confirmatory test.

Statistical Analysis

The percentage of cows that had recently aborted and which were seropositive for antibodies to *N. caninum* was determined on a Regional and on a National basis and expressed as the percentage seropositive with the appropriate 95% confidence interval shown.

A Chi-square test was used to investigate the association between regional differences and the proportion of positive animals ($P < 0.005$). The effect of seasonality on the seroprevalence of antibodies to *N. caninum* in cows that had recently aborted was estimated on a monthly basis. Chi-square analysis was also used to determine the association between the month of the year and the proportion of seropositive cows ($P < 0.005$).

The true prevalence (TP) at animal level was expressed as a function of sensitivity / specificity of the test and the observed prevalence of the disease, sometimes referred to as the apparent prevalence (AP (Toma et al., 1999), using the formula :

$$TP = AP + (specificity - 1)/(sensitivity + specificity - 1)$$

The sensitivity and specificity quoted by the manufacturer, in respect of the indirect ELISA was 98.6% and 98.9% respectively with a sample to positive control max ratio = 0.5 using the test as described in this study (i.e. single ELISA) (Lawrence et al., 1996). When IFAT was included as the confirmatory test, the specificity rose to 99.5% following validation which involved a study of the immune response in 40 cattle. These were experimentally infected with NC-1 tachyzoites during pregnancy and screened by ELISA (in duplicate). The positive samples were confirmed by IFAT (Chapter 9). The test results of this survey were evaluated and showed a test sensitivity of 96% and a specificity of 99.5% when the results were interpreted as described above.

The number of actual abortions caused by *N. caninum* in cows, which were seropositive for the disease, was calculated using the attributable fraction statistical test (Martin et al., 1987). Likewise, the total number of abortions in the cattle population caused by *Neospora* were also calculated using the same statistical method.

Results

The total number of reported abortions in female animals over one year old at regional and country level during the period of this survey, are shown in table 1. There was a significant association using chi-square analysis between region and the proportion of positive results ($P < 0.05$). Most abortions occurred in the South, and significantly, the highest seroprevalence of *N. caninum* was also found in the same region. In the West and East, the seroprevalence was lower than that reported in the South. Overall, 15.3% of all cows aborting during the year were seropositive for antibodies to *N. caninum*, the details are illustrated in Figure 2.

Figure 2. Distribution of post abortion seropositive cattle in the country.

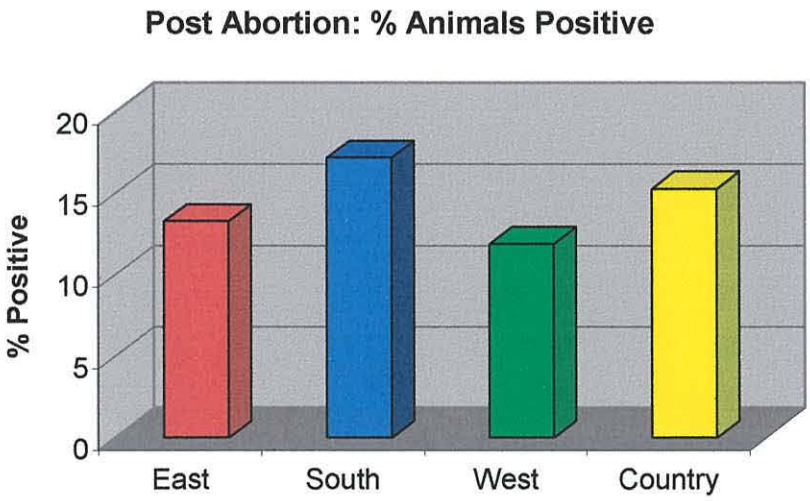


Table 1. Summary of seropositive *N. caninum*-associated abortions.

Number of Animals	East	South	West	Total
Aborted animals	2,042	3,146	597	5,785
Seropositive animals	271	541	71	883
% Positive	13.3%	17.2%	11.9%	15.3%
Confidence Limits	11.8 – 14.8	15.9 – 18.5	9.3 – 14.5	14.4 – 16.2
True Prevalence	13.9%	18.0%	12.5%	15.5%

The percentage of abortions in cows with seropositive titres of specific antibodies to *N. caninum* were compared to the month of year during which the abortion occurred in Figure 3 and Table 2. There was a significant association between the month of test and the proportion of seropositive animals using the chi-square test ($P<0.05$). In the case of the *N. caninum* associated abortions, there were two peaks, the first and by far the highest occurred during the period September to November reaching a maximum during October. The percentage of cows which aborted and were seropositive for *N. caninum* rapidly declined thereafter, and remained below the annual mean until the following May when a second, although smaller peak, occurred.

Figure 3. Number of *Neospora* associated abortions per month.

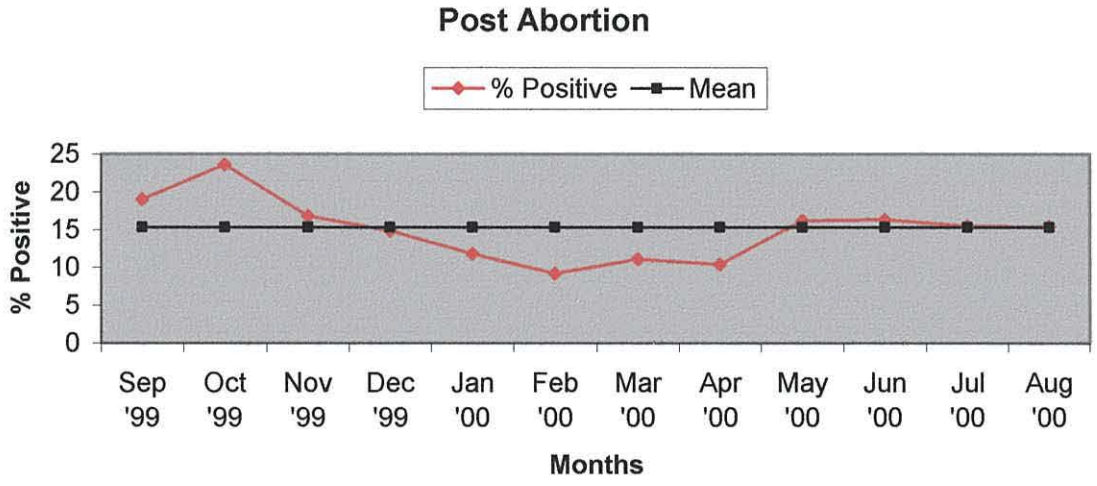


Table 2: Percentage of post abortion samples seropositive by month.

Number	Sep 99	Oct 99	Nov 99	Dec 99	Jan 00	Feb 00	Mar 00	Apr 00	May 00	Jun 00	Jul 00	Aug 00
Samples	421	745	763	913	1600	249	217	115	74	166	226	296
Positive	80	176	128	135	188	23	24	12	12	27	35	43
%Positive	19.0	23.6	16.8	14.8	11.8	9.2	11.1	10.4	16.2	16.3	15.5	14.5

The results of this study, were combined with those in chapter 3 and evaluated using the attributable fraction statistical test (Martin et al., 1987). The odds ratio was determined based on the calculation (883 x 2099)/(4902 x 154). The data used in both cases is show in table 3.

Table 3: Summary of numbers of seropositive cattle in the post abortion and animal prevalence studies.

Abortion Status	Serostatus for Antibodies to N. caninum		
	Positive	Negative	Total
Positive (chapter 2)	883	4902	5785
Negative (chapter 3)	154	2099	2253
Total	1037	7001	8038

The results of both studies can be summarised as follows:

- 59.3% of abortions in cattle that were seropositive for *N. caninum* in Ireland can be attributed to *N. caninum*.
- 9.0% of all abortions in the Irish cattle population can also be attributed to this disease.
- Cattle in Ireland that are seropositive for *N. caninum*, are 2.5 times more likely to abort than those that are seronegative based on an odds ratio of 2.5.

Discussion

The results of this survey showed that specific antibodies to *N. caninum* were detected in the blood of 15.3% of cows that aborted over a 12 month period in Ireland.

It is well recognised that the presence of specific antibodies to *N. caninum* in the sera of cows that recently aborted only indicates that exposure to the parasite had taken place. In some cases, even though exposure may have taken place, antibodies may not be detectable due to variations in the stages of seroconversion or to fluctuating titres which may have decreased below the cut off level some time after the abortion (Wouda et al., 1998(a), Schares et al., 1999). In other cases, even though exposure and infection has taken place, the subsequent abortion may not be due to *N. caninum* but caused by another abortifacient. It has been shown that many infected cows do not abort, but go on to give birth to a clinically normal, but infected calf (Paré et al., 1997, Thurmond and Hietala, 1997(a)).

Following abortions in cows, there has been shown to be a good correlation between foetal histology and the serology of the dam. Cows that abort *Neospora* infected foetuses mostly have high antibody levels (Otter et al., 1997(a)). High antibody titres are usually found in post abortion sera and during the second half of pregnancy (Schares et al., 1999, Stenlund et al., 1999). In the present study, the majority (75%) of the positive ELISA O.D. readings were in the high positive range (OD > 1.0) which subsequently tested positive by IFAT. These findings confirm that high levels of specific *N. caninum* antibody are normally present in post abortion blood sera, following *Neospora* associated abortions.

Infectivity studies in different hosts have shown that the *N. caninum* IFAT shows very little cross-reactivity with other coccidian parasites (Dubey and

Lindsay,1996). This is particularly important in relation to *Toxoplasma gondii*, consequently, the IFAT is often used as a reference test for *N. caninum* antibodies with which other tests are compared (Bjorkman et al., 1999, Anderson et al., 2000). Even though *T. gondii* is not considered a serious pathogen in cattle, there is the possibility that, in bovines, the parasite could interact with *N. caninum* or cross-react with the *Neospora* ELISA test. Furthermore, since this project also involved large scale experimental *N. caninum* infectivity study in sheep, where *T. gondii* is often a problem, it was decided to standardise the serological testing procedure in cattle and sheep by choosing IFAT as the confirmatory test throughout this project.

Furthermore, it has been recognised that within a given cattle population the sero-status of some individual animals will change over a period of time. In a recent Dutch study, 3.3% of animals changed from being seronegative, at the first sampling, to seropositive at later sampling, on the other hand 4.4% changed from being seropositive at the first sampling to seronegative at later sampling (Dijkstra et al., 2003).

It is difficult to compare the results of this survey, which was carried out at National Herd level, with surveys in other countries because of the lack of comparable data. To date, most seroprevalence studies have been carried out at herd or at regional level, where the sample size has been much smaller and the results may have been influenced by local factors and may therefore not be representative of the prevalence of the disease at the National level. In England and Wales, it has been estimated that 12.5% of abortions in dairy cattle may be caused by neosporosis. The seroprevalence of *N. caninum* in an aborting group of cattle at 18.0% was significantly higher when compared to 4.0% in the control group (Davison et al., 1999(a)). These results

compare closely with the findings in this study where 15.3% of aborting cows had specific antibodies to *N. caninum* in their sera when samples were collected within two weeks after the abortion having occurred. The true prevalence (T.P.) of the disease was estimated to be 15.5% in post abortion sera of cows in Ireland.

The relationship between the season and *Neospora* associated abortions has not been fully investigated. In Ireland, the majority of reported abortions due to all causes occur during the period November to January, reaching maximum level in the month of January after which the number of reported cases declines rapidly to relatively low levels for the remainder of the year.

However, it is worth noting that in the case of *Neospora* associated abortions, the numbers peak in October, some three months earlier than the period when the number of abortions, due to other causes, reach a maximum. In addition, in the case of *Neospora* associated abortions, there is a second but much smaller peak in the May to July period which does not seem to occur in abortions due to other causes.

In Ireland, the majority of cows and heifers are put in calf between May and June each year. These animals calve the following February to April to coincide with the onset of the grazing season. During the period September to November, the majority of the cows would be at the four to six month stage of gestation. It is during this period that most abortions associated with *Neospora* occur. Abortions due to other abortiofacients such as salmonellosis, leptospirosis or brucellosis, are much more likely to occur in the latter third of pregnancy which would coincide with the December to January peak. These findings indicate that there is a close association between the season of the year and the percentage of *Neospora* associated abortions,

which in turn is linked to the calving pattern and farm management practices rather than to other factors associated with the season.

It is well recognised that the presence of antibodies to *N. caninum* in the sera of cattle which had recently aborted is not conclusive evidence that the abortion was caused by this protozoan parasite. It only means that exposure to the organism had occurred which was followed by a humoral response (Dubey and Lindsay, 1996).

However, when the results of this study were combined with those described in chapter 3, it was possible to conclude following statistical analysis of the results that;

- Cattle which were seropositive for *N. caninum* in Ireland were 2.5 times more likely to abort than those that were seronegative.
- Almost 60% of abortions in cattle that were seropositive for N.caninum were due to infection with neosporosis.
- Within the national cattle population, that 9.0% of all abortions were attributable to *N. caninum.*

The economic significance of these findings is of major importance to all sectors involved in the cattle industry in Ireland. However, these conclusions are based on the results of one years study, it maybe necessary to repeat this investigation over a period of years to fully validate these findings.

Chapter 3

The Seroprevalence of *Neospora caninum* in the Irish Cattle Herd.

Summary

A study to determine the seroprevalence of *Neospora caninum* in the Irish cattle herd was carried out during the period September, 1999 to January, 2000. During the course of this project, a total of 2,253 blood samples were collected from female cattle over one year of age using a systematic random sample method. The sera were screened by ELISA for the presence of specific antibodies to *N. caninum*. Positive sera were confirmed by IFAT.

Introduction

The protozoan *Neospora caninum,* is now recognised as a major cause of abortion and infertility in cattle in many parts of the world including the USA., (Anderson et al., 1995), Netherlands, (Wouda, 1999), Australia and New Zealand, (Reichel, 2000), and the United Kingdom, (Davison et al., 1999(a)). The protozoan parasite has also been associated with sporadic disease in other livestock species including sheep (Dubey et al., 1990), goats (Barr et al., 1992) and horses (Marsh et al., 1996).

Surveys in California (Anderson et al., 1991), The Netherlands, (Wouda et al., 1998(b)), and New Zealand, (Thornton et al., 1991), indicated that approximately 20% of all aborted bovine foetuses were diagnosed with *N. caninum* infection. However, in herds in which abortion storms had occurred, the proportion of *N. caninum* infection in aborted foetuses was as high as 44% (Anderson et al., 1995). More recently, in a survey of 50 Dutch herds experiencing epidemic abortions, *N. caninum* infection was confirmed in 77% of the aborted foetuses (Wouda et al., 1999).

A number of limited studies have been carried out to estimate the seroprevalence of the disease in cattle herds. In Northern Spain, 83.2% of dairy herds and 55% of beef herds were seropositive for neosporosis. At animal level, 36.8% of the positive animals were in dairy herds and 17.9% in beef herds (Quintanilla – Gozalo et al., 1999). The estimated seroprevalence in dairy cattle in England and Wales is 6% (Davison et al., 1999(a)). A similar seroprevalence (6.75% to 8.5%) was identified in dairy cattle in New Zealand (Reichel, 1998). In Canada, a survey found a seroprevalence of 30% in beef cattle (Waldner et al., 1998). A study in Belgium reported that the seroprevalence in beef cows at 9.7% was higher than the 3.0%

prevalence found in dairy cows (De Meerschman et al., 2000). Within individual herds, the seroprevalence is variable; studies in the US have shown that *N. caninum* infection in dairy herds can vary from two to 98% (Anderson et al., 2000). It has been shown that seropositive cows have a two to four fold higher risk of abortion than seronegative cows (, Pfeiffer et al., 2002, Sager et al., 2001).

The economic follow-on effects of this situation, at farm and at national level, require that, before any eradication or control programme could be considered, the prevalence of the disease at national level must be first established, prior to putting any control measures in place. This study was undertaken with the objective of establishing the prevalence of neosporosis in cattle in Ireland. The ensuing information could form the basis of a comprehensive study of the prevalence of the disease in the National herd. The study would also investigate the influence of various factors including season, herd size, herd enterprise type and region on the level of the disease countrywide. For convenience of sample collection, rapid laboratory turn around time and cost considerations, a commercially available indirect ELISA was chosen as the preferred screening test for this study. A commercially available IFAT (VMRD, Washington, USA) was used as the confirmatory test; both tests were used as previously described in Chapter 2.

Materials and Methods

Survey Design

In the absence of reliable information, as to the prevalence of neosporosis in the Irish cattle herd, it was deemed necessary to carry out a seroprevalence study in two parts. In the first part, this study was carried out at animal level to establish the approximate seroprevalence of the disease nationwide. Having established an approximate seroprevalence at animal level, this data would then be used to carry out a second more comprehensive investigation to establish the level of the disease at herd, regional, and national level (Chapter 4). In addition, the effects of herd size, farm enterprise type, cow/heifer ratios and season on the seroprevalence of the disease, would also be investigated.

The blood samples were collected mainly by veterinary practitioners as part of the National Brucellosis Eradication Programme using sterile vacutainers and individual disposable 18g needles (Langan Bach Services, Ireland). The samples were sent using the postal services in insulated containers to the National Brucellosis Laboratory in Cork, where aliquots were collected, identified, coded and stored at –20°C before being sent to the Central Veterinary Research Laboratory (CVRL) in Dublin for testing for antibodies to *Neospora caninum*.

The number of cattle that would have to be sampled countrywide to establish the seroprevalence of *Neospora caninum* at animal leval in the national herd was determined using Epi_info Ver.6.0 statistical package (Dean et al., 1990). A worse case situation of 6.0% animal prevalence was assumed, with confidence limits set at 95% and a desired accuracy of 1% (ie. between the range of 5% to 7%) , it was calculated that at least 2165 cattle would have to be tested .

To ensure that sampling was spread evenly across the country, the sample size was predetermined on a county by county basis in direct proportion to the number of herds in each county. The routine blood samples, taken under the Brucellosis Eradication Programme, upon arrival each day in the National Brucellosis Laboratory are placed in racks each holding 96 samples, the racks are then grouped on a county by county basis and coded in order of the receipt in the laboratory. In this survey, systematic sampling was used to select samples at equal intervals for testing. In this case, intervals of 96 were chosen for logistical reasons.

The method involved the collection of the blood samples in the number one position in every rack containing the daily intake of samples from each county, thus minimising any element of periodicity in the sampling which could affect the accuracy of the sampling method (Thrusfield, 1995). Sampling continued until the required number of samples for each county was reached. All post abortion blood samples, received in the Cork Laboratory during this period were excluded from this exercise. For reporting purposes, the country was divided into three regions, East, West and South, similar to that already described in chapter 2.

Serology

All the blood serum samples were screened for antibodies to *N. caninum* in the CVRL, using a commercially available ELISA test (Herd Check* IDEXX) and a commercially available IFAT (VMRD), as previously described in chapter 2. The test results were also evaluated and interpreted, as previously described in Chapter 2.

Results

Nationwide, 6.8% of female cattle were found to be seropositive for antibodies to *N. caninum*. The highest percentage of seropositive animals was found in the east (9.1%) and the lowest in the west (5.6%) (Figure 1). Statistically, there was evidence of a significant association between the region and proportion of positive animals using a chi-square test ($P < 0.05\%$). The results are also expressed as the true prevalence (TP) of the disease (Table 1).

Figure 1. The distribution of seropositive cattle in Ireland

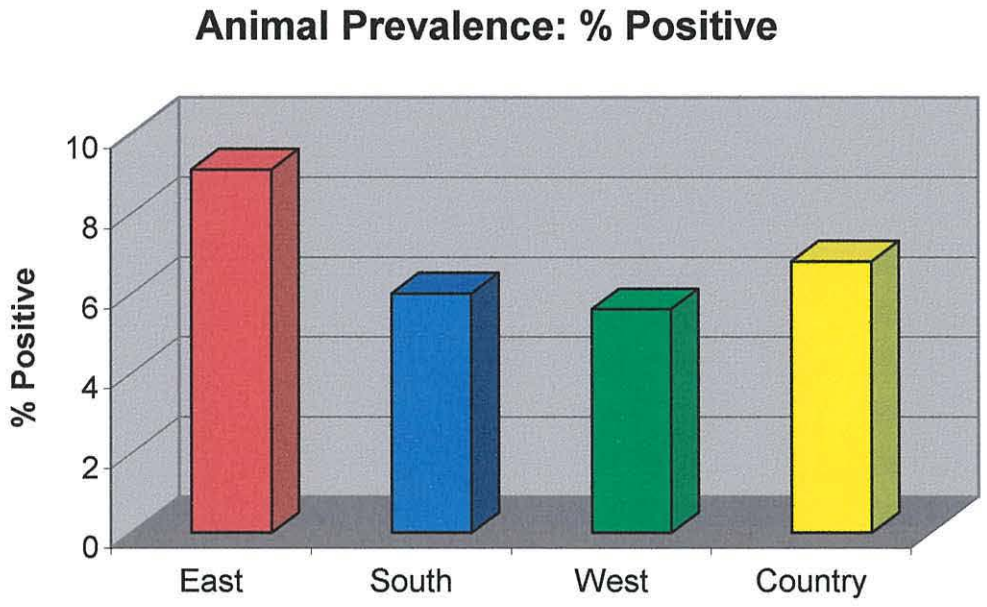


Table 1. The true prevalence (TP) of neosporosis in seropositive cattle in Ireland.

Animals Tested	East	South	West	Total
Number	717	683	853	2,253
Number Positive	65	41	48	154
% Positive	9.1	6.0	5.6	6.8
Confidence Limit	7.0 – 11.2	4.2 – 7.8	4.1 – 7.1	5.8 – 7.9
True Prevalence	9.6%	6%	5.8%	7.1%

Discussion

The results of this survey showed that the true seroprevalence (TP) to *N.caninum* the Irish cattle herd was 7.1%. This figure is similar to that found in a recent survey of neosporosis in dairy herds in England and Wales where 6.0% of cows were shown to be seropositive (Davison et al., 1999(a)). However, these results are not directly comparable, as in the latter study, dairy herds only were investigated, whereas in this study, there was no distinction between livestock production systems (enterprise type) sampled. In this survey the targeted population was the Irish Cattle Herd, whereas in the England/Wales study, the collection of samples was not implemented on a randomised countrywide basis.

It is recognised that testing of blood sera for antibodies to *N.caninum* has limitations in the diagnosis of infection, nevertheless, it is useful in identifying exposure to the disease (Anderson et al., 2000). A positive serological result does not necessarily confirm the presence of infection, it only shows that exposure to *N. caninum* has occurred and that the animal's humoral immune system has responded accordingly. Confirmation of the disease requires the availability of tissue from an aborted foetus or a congenitally infected calf for examination by histology and immunohistochemistry and/or PCR methods. Under field conditions, especially where a large scale survey is being carried out, such tissues are not always readily available, hence the reliance on serology.

In this study, it was decided, on economic and logistical grounds, to screen the serum samples with a single indirect ELISA and to test any positive results by means of a repeat ELISA. If the initial screening ELISA had a low OD reading (0.5 – 0.999), the sample was repeated in duplicate. Samples with high OD readings $\geq$ 1.00 were

repeated singly. Confirmation of positive status was carried out by means of IFAT as previously described in Chapter 2. This procedure has been shown to have a sensitivity of 96% and a specificity of 99.5% when used in a trial where negative cattle were inoculated with live *N. caninum* tachyzoites and the serological response monitored using a combination of ELISA and IFAT tests (Chapter 9).

In Ireland, four main types of livestock production systems (enterprise types) exist, namely, beef herds which are entirely devoted to beef production, dairy herds which are devoted to milk production, suckler herds which are entirely involved in producing calves for beef herds or replacement female stock for suckler herds themselves, and finally mixed herds which are a combination, to varying degrees of the other three systems.

The percentage of seropositive cattle in the eastern region (9.1%) was higher than in the southern (6.0%) or western regions (5.6%). The reasons for these regional differences are not clear, however, they are probably related to herd sizes and to the different types of livestock production systems which predominate in the regions themselves. The true prevalence (TP) of the disease was calculated from the formula shown in chapter 2. The sensitivity and specificity of the test methods used in the study must be included in the calculation of the true prevalence (TP) of the disease, however, this increases the accuracy of the result. In this study, using the true prevalence there was a slight increase in the seroprevalence of the disease at animal level from 6.8% to 7.1% at county level, the results were more variable at regional level. Nevertheless, the results of this study show that significant levels of antibodies to *N.caninum* are present in the Irish cattle population indicating that significant exposure to the parasite occurs in cattle in Ireland.

Chapter 4

A seroepidemiological study of *Neospora caninum* infection in the Irish Cattle Herd

Summary

The purpose of this study was to establish the level of neosporosis in the Irish Cattle Herd and to study factors which may influence the seroprevalence of the disease in the country.

A total of 21,351 female cattle aged one year or more from 871 cattle herds were screened for the presence of antibodies to *N. caninum* by ELISA during the period 2000 to 2001. The herds were randomly selected from the National Cattle Herd which contained 128,872 individual herds at that time.

The results of this study were evaluated using a logistic regression statistical model. It was shown that the total herd size and the herd enterprise type were the main significant risk factors in determining whether a herd was seropositive for neosporosis or otherwise.

Introduction

The protozoan parasite *Neospora caninum* was first identified and described in dogs (Bjërkås et al., 1984, Dubey et al., 1988(a)). It is now recognised as a major cause of abortion and infertility in cattle in many parts of the world (Jardine and Last, 1993, Duivenvoorden and Lusis, 1995, Dubey and Lindsay, 1996, McNamee et al., 1996, Moen et al., 1998). Surveys in California (Anderson et al., 1991, Barr et al., 1999, ,The Netherlands (Wouda et al., 1998 (b)) and New Zealand (Thornton et al., 1991) have shown that up to 20% of all bovine abortions are caused by *N. caninum*. It has been reported that in dairy herds with a history of *Neospora* endemic abortions, the proportion of *Neospora* infection in aborted foetuses is as high as 44% (Anderson et al., 1995).

While *Neospora* associated abortions and congenital infections have been reported in both beef and dairy herds, most of those reports relate to dairy herds. It is possible that a sample bias exists in favour of dairy herds, because the farm management systems in place on such farms may tend to lead to reports of abortions more frequently than on beef farms, where abortions may not always be observed. It has also been suggested that the close contact which exists in a dairy environment may be more conducive to the spread of the disease (Anderson et al., 2000). In a survey of beef and dairy cattle in Northern Spain, the seroprevalence in dairy cattle was shown to be significantly higher (35.9%) when compared to beef cattle (17.9%) (Quintanilla-Gozalo et al., 1999). A survey of beef cattle in Canada found a seroprevalence of 30% (Waldner et al., 1998). In one investigation carried out in New Zealand to establish the national prevalence of *Neospora* infection in dairy cattle, 400 sera from a historical serum bank were tested for antibodies to the disease. The results showed that the

seroprevalence in cows with no history of abortion varied between 6.75% and 8.5% (Reichel, 1998). Similar seroprevalence levels (6%) were reported in dairy cattle in England and Wales (Davison et al., 1999(a)). In Belgium, the seroprevalence in dairy cows (3.0%) was found to be significantly lower than that found in beef cows (9.7%) (De Meerschman et al., 2000).

There are several serological tests currently available to detect the presence of specific antibody to *N. caninum*, these include a number of different enzyme linked immunosorbent assay tests (ELISA) and the Direct Agglutination (DA) test (Bjorkman and Uggla, 1999, Anderson et al., 2000). Normally these tests are validated against post abortion blood sera from cattle with confirmed *N. caninum* aborted foetuses or with the immunofluorescent antibody test (IFAT) (Anderson et al., 2000). The accuracy of serological tests for random samples is normally lower than for post abortion sera due to fluctuations in the antibody titres, these variations have also been shown to occur in chronically infected animals (Dannatt, 1997, Schares et al.,1999, Stenlund et al., 1999, Maley et al., 2001 (a)).

In this project, the prevalence of neosporosis in the National Cattle Herd was determined by means of serology. The study was designed to run for a period of 12 months to monitor the effects of season, if any, on the seroprevalence levels. The purpose of the study was also to evaluate the effects of herd enterprise types, herd size, region and animal categories on the seroprevalence levels. The effects of these parameters were studied at both regional and national level.

Materials and Methods

Study Design

This investigation was carried out during the period 1st September, 2000 and 31st August, 2001. It was designed so as to take any seasonal influences into account by spreading the sampling rate evenly over each of the twelve months of the year long survey. The estimated numbers of herds in each county were based on 1998 data which contained the most recent validated figures available (Collins and Hammond, 1998, Central Statistics Office, 1998). According to this data, there were 123,835 individual herds containing 3,657,000 female cattle > 12 months of age in the Irish Cattle Herd.

In the previous study (Chapter 3), antibodies to *N. caninum* were found in the sera of 7.1% of female cattle belonging to this age group, indicating that exposure to *N.caninum* was widespread in Irish cattle herds. Using Epi-info Ver. 6.0 statistical package (Dean et al., 1990), a worse case situation of 50% herd prevalence was assumed, with confidence limits set at 95% and a desired accuracy of 5.0% (i.e. between the range 45% to 55%), it was calculated that 383 herds would have to be tested countrywide to establish the national herd prevalence. A herd was considered positive when at least one animal was seropositive.

However, to ensure that there was an even distribution of sampling throughout the country and to account for seasonal factors, samples were taken monthly and in direct proportion to the total number of herds containing female cattle aged one year or more, within each of the 26 counties of Ireland. These requirements resulted in the total herd sample size being increased from 383 to 876 herds or 73 herds per month.

Samples and Data Collection

The blood samples were collected mainly by veterinary practitioners when carrying out herd tests under the national brucellosis eradication programme. Under this scheme, all herds with female bovine animals > 12 months of age must be blood tested annually. The blood samples were sent in insulated boxes to the Brucellosis Laboratory, using the postal service. The boxes, upon arrival in the laboratory, were sorted by county and opened, the blood sample tubes were then placed in special racks. These racks were part of a dedicated system used to assemble blood samples from each herd. A separate racking system was used for each county. The data collected at the time of sampling at farm level included the unique herd number, name and address of herd owner, the age and category of the animal (cow, heifer, maiden heifer), ear tag number; each blood sample was identified by a sequential coding system.

Random Selection of Samples within the Brucellosis Laboratory

In the laboratory, systematic selection of counties for sampling was achieved by selecting, in alphabetical order, three different counties each day from the list of the 26 counties in the country. The selection of counties was repeated using the same system, after the initial 26 counties were sampled, but omitting counties when the required number of county samples for the month had been reached. Each day, the blood samples from the herd occupying position one in the racking system for each of the selected counties were collected. The methods described above ensued that bias did not occur in selecting the herds. Sampling was spread evenly throughout the period of the study. Checks for bias in relation to the numbers of samples collected per herd were carried out at the end of the study, using Microsoft Visual Basic for applications (VBA) Ver. 2000 software programme. The results showed that the mean

herd sample size of the herds sampled was 24.2, this figure compared closely to a mean herd sample size of 23.4 which is likely to have been the outcome were a computerised random number generator based system used to select the samples.

In view of the large scale testing programme envisaged in this study, the logistical considerations and the overall costs involved, it was decided to use stratified sampling within the herds selected for testing. This sampling protocol required, that for herds with between 1 – 29 eligible female cattle (i.e. > 1 year old), all were sampled. Herds with between 30 – 99, the first 30 samples in the racks were collected, for herds with between 100 – 200 cattle, the first 50 samples were used and for those over 200 animals, the first 55 samples were removed for testing.

On the basis of the formula $n = [1 - (1\text{-}CL)^{1/D}][N - (D\text{-}1)/2]$, (Martin et al., 1987) where n was the required sample size, CL the confidence interval, D the supposed number of infected cattle (7.1% from chapter 2) and N was the herd size, the following levels of confidence were obtained using the sampling protocol described in this study, the details are shown in Table 1. The samples were stored at –20°C until tested in the Central Veterinary Research Laboratory, Dublin

Table 1. Summary of confidence levels of detecting at least one positive animal at a seroprevalence level of 7.1%.

Herd Size	Number of Herds		Number of Samples	Confidence Levels
	Herds	Animals		
1-29	368	4480	All samples	100%
30-60	305	13432	30	95%
61-99	119	9187	30	92.8%
100-200	71	9216	50	98.5%
201-400	8	1881	55	98.5%

Results

Prevalence of *N. caninum* seropositive herds at regional and country (national) level

In this study, blood sera from 21,351 adult female cattle in 871 herds were tested for antibodies to *N. caninum,* 49.8% of the herds were seropositive for the disease. The highest level of infection as demonstrated by the presence of specific antibodies was found in the South (53.7%) and the lowest in the West (44.1%). There was a significant association between the regions and the proportion of positive herds using chi-square test analysis ($P < 0.05$), see Table 2 and Figure 1 for details.

Figure 1. Percentage of herds seropositive for antibodies to *N. caninum* in the country and at regional level.

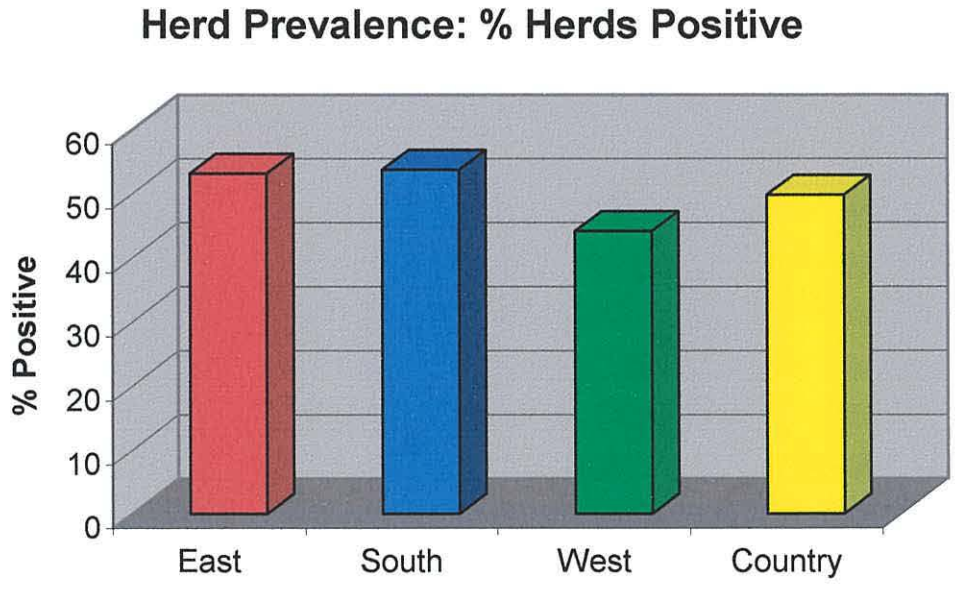


Table 2: Sample size and number of herds positive at country and regional levels.

Herds	East	South	West	Country
No. Tested	307	231	333	871
No. Positive	163	124	147	434
% Positive	53.1	53.7	44.1	49.8

Prevalence of *N. caninum* seropositive herds according to enterprise type at country (national) level.

In this study, the seroprevalence of neosporosis in four categories of cattle enterprise types (production systems) was examined. The classifications used were based on the various types of subsidies being claimed by the herd owner under the EU Common Agriculture Policy (CAP). This information is held on the Department of Agriculture and Food's (DAF) database and is validated by regular on-farm checks carried out by DAF staff in accordance with EU legislation.

The results showed that the highest seroprevalence level (68.5%) was found in suckler herds, the lowest level (47.6%) was reported in mixed herd enterprises. Statistically, there is evidence of a significant association between herd enterprise and the proportion of positive herds when tested using a chi-square test ($P < 0.05$). See Table 3 and Figure 2 for details.

Figure 2. Positive herds by enterprise type at country (national) level .

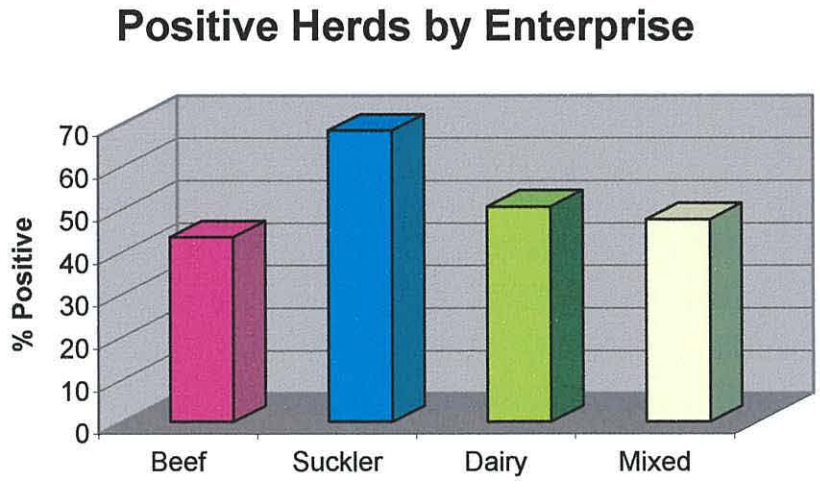


Table 3: Numbers of herds sampled and number seropositive.

Herd – Enterprise	Beef	Suckler	Dairy	Mixed	TOTAL
Herds Tested	53	54	344	420	871
Herds Positive	23	37	174	200	434
% Positive	43.4	68.5	50.6	47.6	

Prevalence of *N. caninum* seropositive herds at regional level according to enterprise type .

It has been shown that there is a significant association between herd enterprise type and seroprevalence of *N. caninum* in herds at national level (Table 3, Fig. 2). However, when examined at regional level, no association was observed between regions using chi-square test analysis ($P > 0.05$). The seroprevalence of *N. caninum* in different herd enterprises at regional level is illustrated in figure 3, and the details with the appropriate odds ratio and P. values are shown in Tables 4 and 5.

Figure 3. The seroprevalence of *N. caninum* in different herd enterprise types at regional level .

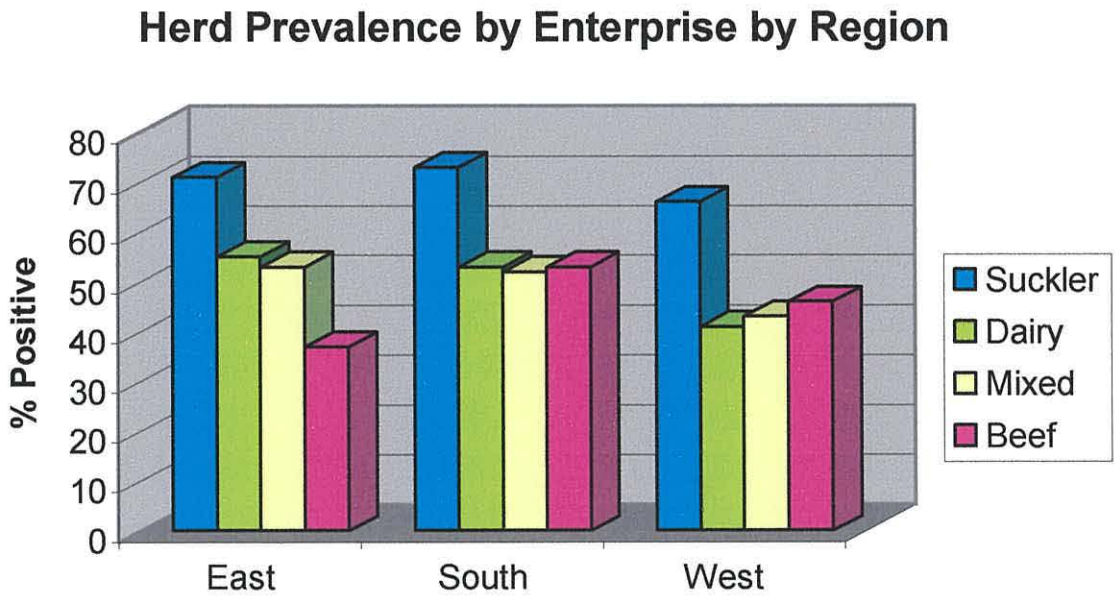


Table 4: The number of herds sampled and the number seropositive at regional level.

Region	Enterprise Type	Herds Sampled		
		No. Pos	Total	% Pos
East	Beef	10	27	37
	Dairy	64	117	55
	Mixed	79	149	53
	Suckler	10	14	71
South	Beef	8	15	53
	Dairy	76	143	53
	Mixed	32	62	52
	Suckler	8	11	73
West	Beef	5	11	45
	Dairy	34	84	40
	Mixed	89	209	43
	Suckler	19	29	66

Table 5: Odds Ratio for different Herd Enterprise types.

Effect	Odds Ratio	Lower	Upper	P. Value
Beef V. Suckler	0.304	0.136	0.677	0.004
Dairy V. Suckler	0.412	0.221	0.768	0.005
Mixed V. Suckler	0.408	0.222	0.750	0.004
Beef V. Mixed	0.745	0.416	1.337	0.324
Dairy V. Mixed	1.011	0.748	1.336	0.944
Beef V. Dairy	0.738	0.410	1.327	0.309

Prevalence of seropositive animals according to animal category at regional and country (national) level

The results of this study show that the level of neosporosis in female cattle over one year old in the country was highest in cows (74%) and lowest in maiden heifers (10%) when expressed as a percentage of seropositive animals. However, the level in the regions showed similar variations for the different categories of animals (see Table 6 and Figure 4 for details). There was a significant association between the region and the animal category for positive herds ($P < 0.05$). Relevant data necessary to evaluate negative herds in a similar way was not available.

Figure 4. Percentage prevalence of *N. caninum* in various female animal categories in the country and at regional level.

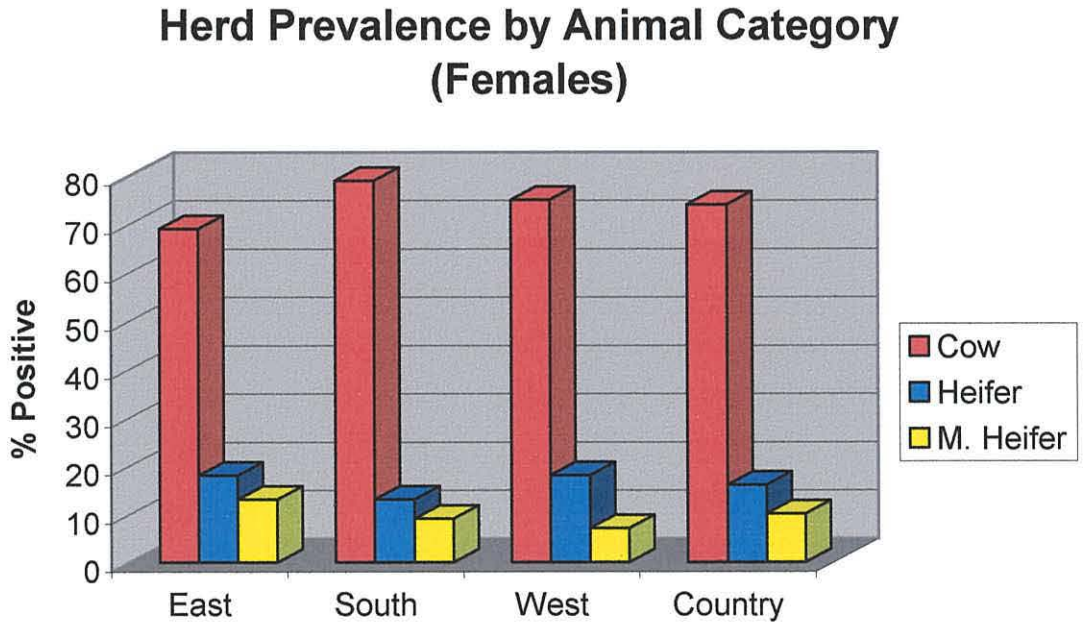


Table 6: Percentage of seropositive cows, heifers and maiden heifers in positive herds.

Region	M.Heifer	Heifer	Cow
East	13%	18%	69%
South	9%	13%	79%
West	7%	18%	75%
Country	10%	16%	74%

The effect of herd size and composition on the prevalence of *N. caninum* in seropositive herds

The herd composition and mean herd size data were obtained from the annual TB herd test results. If there had been more than one full herd test carried out during the survey period, the figures were averaged accordingly. The positive herds contained an average of 104 cattle of all categories , on the other hand, herds that were negative, were smaller, and contained an average of 75 cattle.

In this study, the average herd size and the breakdown of the animal categories (cows, heifers, bullocks, bulls and calves) within each of the 871 herds sampled were analysed to see if there was an association between the different categories of animals and the sero-status of these herds for neosporosis. Herd size played a key role in determining whether a herd was seropositive or not. The larger the herd, the more likely it was to contain at least one positive animal thereby classifying the herd as seropositive. On the other hand, the smaller the herd the less likely the chance of finding at least one positive, therefore the herd was more likely to be deemed seronegative.

However, while the overall mean herd size (all categories of cattle) was a factor likely to influence a herd being classified as being seropositive or not, the average number of females > 12 months of age within the herd was also an important consideration (see Table 7 and Figure 5 for details). Positive herds contained significantly more cows ($P < .0001$) heifers ($P < 0.0001$) and calves ($P < 0.0002$) than negative herds. The details are shown in Table 7 and 8, and the results are illustrated in figure 5.

Figure 5. The mean number of animals in the different categories in positive and negative herds.

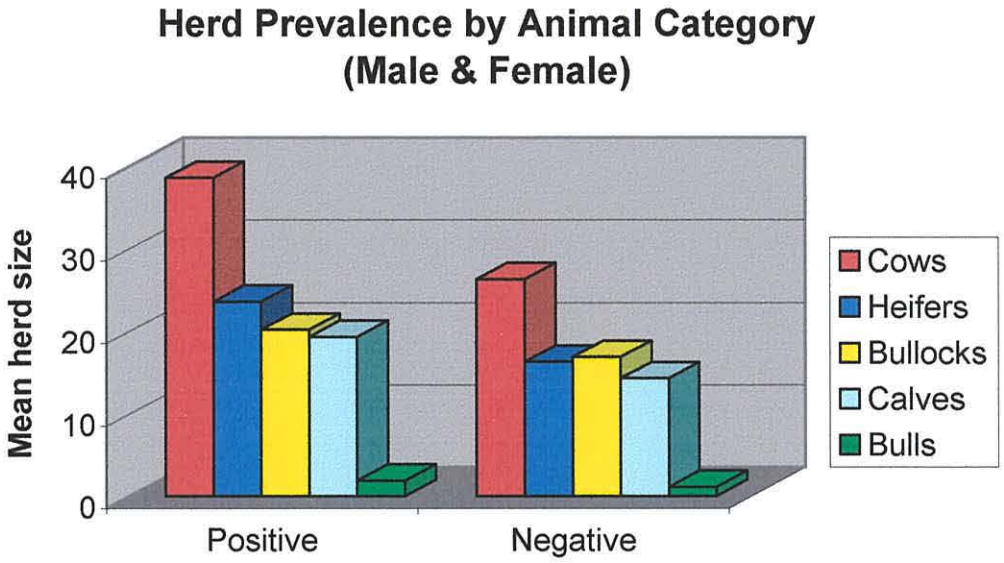


Table 7: The mean herd size and total number of animals in different categories in negative and positive herds.

Animal Category	Negative Herds		Positive Herds	
	Total	Mean	Total	Mean
Sum of Cows	11,483	26.3	16,764	38.6
Sum of Heifers	7,106	16.3	10,229	23.6
Sum of Calves	6,244	14.3	8,381	19.3
Sum of Bullocks	7,377	16.9	8,751	20.2
Sum of Bulls	496	1.1	818	1.9
Total	32,706	74.8	44,943	103.6

Table 8: Comparison of the mean herd size of male and female animal categories in negative and positive herds.

Animal Type	Average Herd Size		P. Value of t-test
	Neg. Herds	Pos. Herds	
Bulls	1	2	0.050
Bullocks	17	20	0.099
Calves	14	19	0.0002
Heifers	16	23	0.0001
Cows	26	39	0.0001

Prevalence of seropositive herds grouped according to month of the year

The mean proportion of seropositive herds reached a maximum of 59% during the month of October before falling back to a low of 44% by the following January. A second but smaller peak occurred during the period February to April when the levels rose to 52% before falling back to around 45%. The two peaks and troughs are very similar to those reported in the post abortion study (Chapter 2). However, in this instance, while there was a trend showing evidence of an association between the month of test and the proportion of positive herds, it was not significant ($P > 0.05$). The details are shown in Table 9 and illustrated in Figure 6.

Figure 6. The percentage of positive herds identified by month.

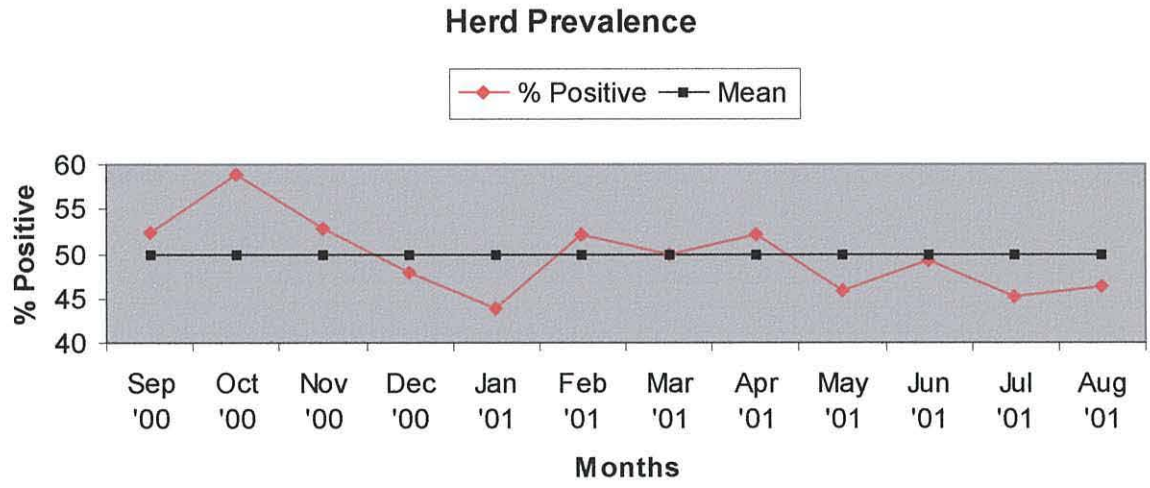


Table 9: The number of herds tested and the percentage positive per month.

Herd Prevalence : Positive by Month													
	2000								2001				
	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Total
No Herds Tested	65	85	74	71	66	73	70	73	72	71	71	80	871
No Pos	34	50	39	34	29	38	35	38	33	35	32	37	434
% Pos	52	59	53	48	44	52	50	52	46	49	45	46	50 (Mean)

Statistical Analysis of the Results

A logistic regression model was developed to determine the probability of a herd testing positive for *N. caninum*. The risk factors also known as confounders, used in the model included total herd size, (all bovine animals), herd enterprise type and region of the country. The herd size was initially tested as a continuous variable and then as a categorical variable.

Nested models were compared using the likelihood ratio tests, the risk factors were dropped from the model if their deletion involved a non-significant change in deviance ($p > 0.05$). The goodness of fit of the model was assessed by means of the Hosmer-Lemeshow statistic (Hosmer and Lemeshow, 1989). The effect of any large outliers was also examined.

In the final model, total herd size and herd enterprise were identified as the two significant risk factors involved in determining whether a herd tested positive for neosporosis or otherwise. While the herd size was originally considered as a continuous variable and shown to be a significant risk factor, it's overall fit of the final model was poor. However, when herd size was treated as a categorical variable, it's fit of the final model was good as shown by the Hosmer-Lemeshow statistic with a P-value of 0.343, which indicated no lack of fit in the model.

The odds ratio in the final model showed that the likelihood of a herd being seropositive for neosporosis increased with herd size up to 40 bovine animals. Increases in herd size above this figure did not increase the likelihood of a herd testing positive for *N. caninum*. The details are shown in Table 10 and illustrated in figure 7.

Table 10: Summary of data used in the Hosmer-Lemeshow statistic to compare the effects of herd size using herd size 1-3 as the base line (not shown).

Herd Size	Odds ratio	P- Value	95% Confidence Interval	
4-8	4.32	0.005	1.56	11.99
9-16	5.40	0.001	1.94	14.81
17-24	11.28	0.000	4.07	31.3
25-34	17.15	0.000	6.28	46.84
35-43	22.88	0.000	8.21	63.75
44-50	20.15	0.000	7.35	55.24
51-63	15.38	0.000	5.59	42.35
64-93	19.12	0.000	6.95	52.54
94-200	21.38	0.000	7.70	59.38

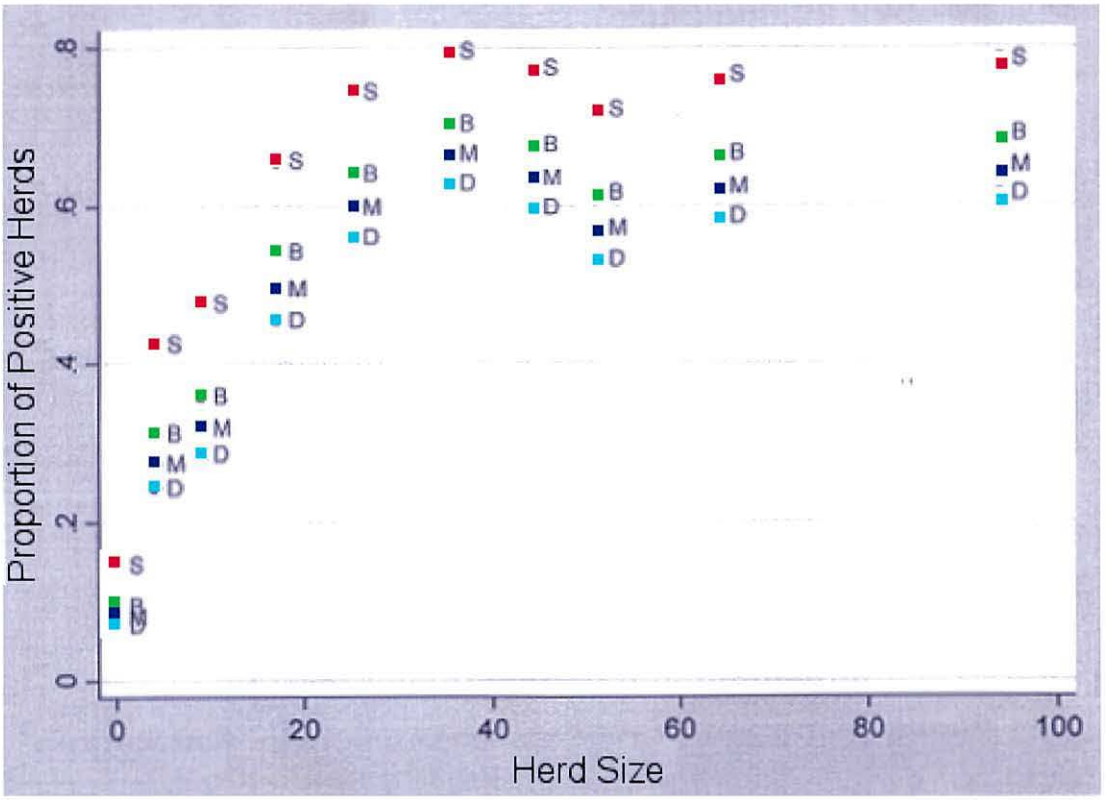
Suckler herds followed by beef and mixed herds were shown to have the highest risk of testing positive for the disease. Dairy herds had the lowest risk. These results are shown in Table 11 and illustrated in figure 7.

Finally, the effect of outliers amongst the covariate patterns were examined. The largest outlier was for mixed herd enterprise type with a total herd size of 51 cattle. When this covariate was dropped, the fit of the model improved but the coefficients of the model remained the same, indicating that the outlier was not influential in the final model and should be included in the results.

Table 11: Summary of data used in the Hosmer-Lemeshow statistic to compare the different herd enterprise types against suckler herds (not shown).

Herd Enterprise Type	Odds ratio	P- Value	95% Confidence Interval	
Beef	0.61	0.265	0.26	1.45
Dairy	0.44	0.014	0.23	0.84
Mixed	0.51	0.041	0.27	0.97

Figure 7: Summary of the effect of herd size on the proportion of herds seropositive for *N. caninum* categorised by herd enterprise type. The graph shows that very small herds have major increases in risk of being infected as the herd size increases up to 40 animals. However, with herd sizes above this level there is no corresponding increase in risk. Herd Enterprise type : S= Suckler, B= Beef, M = Mixed, D = Dairy.



Discussion

In any serological study, the sensitivity and specificity of the test methods employed are critical in determining the final result. In general, the sensitivity of a test is greater in detecting the presence of disease in a herd than at individual animal level. Conversely, the specificity of a test is always lower at herd level than in the individual animal (Toma et al., 1999).

For these reasons and including cost and laboratory capacity limitations, it was decided to maximise specificity at the expense of slightly reduced sensitivity by screening with a single ELISA (IDEXX), and confirming the positive samples by IFAT (VMRD). It was accepted that the use of a single ELISA, when compared to carrying out the test in duplicate, would reduce the sensitivity of the test by about 2.6% (i.e. from 98.6 to 96.0%). This 2.6% reduction in sensitivity was determined when cattle were experimentally inoculated with NC-1 tachyzoites and the immune response was measured by ELISA carried out both singly and in duplicate. However, the specificity of the test method was improved from a claimed 98.9% to 99.5% when the ELISA positives were subsequently confirmed by IFAT (Chapter 9). This two-stage test methodology allowed twice the number of samples to be screened for the same cost as the ELISA in duplicate method, with only minimal loss in sensitivity, while at the same time improving the specificity which is critical to the interpretation of test results applied at herd level (Toma et al., 1999).

The primary objective of this study was to establish the seroprevalence to *N. caninum* in the Irish cattle herd at country and at regional levels. It was also decided to evaluate the effect of other parameters on the seroprevalence of the disease; these included seasonal effects, herd size, category of animal, regional effects and enterprise

types. There was considerable evidence from both the post abortion and animal studies carried out during the previous 12 months that *N. caninum* is widely prevalent in Ireland and therefore the cause of significant loss to the economy. If national control measures were to be put in place, they would ultimately have to be implemented on a herd basis to ensure success. Before implementing any control measures, it is essential to establish the likely herd prevalence of the disease within the country.

The results of this study, which is the first investigation into the seroprevalence to *N.caninum* in cattle at National Herd level carried out in any country, showed that 49.8% of the herds which make up the Irish National Herd were seropositive for the disease. However, a few reports of similar studies at regional level exist. In Northern Spain, 83.2% of dairy herds and 55.1% of beef herds investigated have been shown to be seropositive for *N. caninum* (Quintanilla et al., 1999). This figure is significantly higher than that reported for any herd in the three regions in Ireland, where all herd enterprise types were tested. In another random epidemiological survey carried out in dairy herds in the department of Orne, France, 64% of the herds were seropositive (Ould-Amrouche et al.(1999). It is not clear how other herd enterprise types, if included in the survey, would have affected the final outcome in either Spain or France.

In a recent report of a survey of cattle production systems carried out over a six year period in Ireland, it was found that mixed herd enterprises are concentrated in the East, dairy herds predominate in the South while suckler herds are found mainly in the West. In the case of beef herds, while the majority are located in the East, a very

sizeable number composed mainly of small herds are found in the West (Fallon and Hammond, 1999, Unpublished data).

In this project, the highest seroprevalence of neosporosis nationally was found in suckler herd enterprises (68.5%) closely followed by dairying (50.6%), the levels in beef (43.4%) and mixed herds (47.6%) were lower. The seroprevalence of the disease was consistently higher in suckler herds compared to other herd enterprises in all three regions, however there was no significant association between the other herd enterprises and regions. It is difficult to ascertain why the seroprevalence of neosporosis is highest in suckler herds, but it is probably linked to the type of loose herd management which often exists in these herds when compared to tightly run dairy herds.

It has become apparent in this study that there is a close relationship between the month of the year and the herd prevalence levels. Comparison with other data is difficult, as similar studies do not seem to have been carried out. The season itself is unlikely to be directly involved in the higher prevalence which occurs at certain times of the year. In Ireland at least, it can be associated with calving patterns as already discussed in the earlier post abortion study (chapter 2). Assuming that calving patterns in other countries are not evenly spread throughout the year for various reasons, the timing of any seroprevalence study is critical in determining the significance of the result obtained. The abortion or herd seroprevalence surveys that have been reported on to date may have been carried out at a time which may unintentionally coincide with a natural peak or trough in the seroprevalence of the disease in that animal population thereby leading to unreliable conclusions.

In conclusion, the results of this study show that:

- Suckler herds in Ireland are more likely to be seropositive to *N. caninum* than other enterprise types.
- There is an increased risk of a herd being seropositive for *N. caninum* as the herd size increases up to a maximum of 40 animals ,there is no association between increasing herd size and risk in herds with more than 40 cattle.
- Season of the year is not a significant factor in determining the sero-status of a herd.
- Region was shown to have no influence on the sero-status of cattle herds in Ireland.

Chapter 5

A study on the seroprevalence of *N. caninum* in sheep in Ireland

Summary

Fifty two sheep flocks were randomly selected from the national sheep flock for screening for antibodies to *N. caninum.* Blood samples were taken from all ewes up to a maximum of twenty per flock.

A total of 1040 sera were screened by a monoclonal ELISA. Six seropositive samples were sent to the Moredun Institute in Scotland, where they were confirmed positive for antibodies to *N. caninum* by IFAT. However, 5/6 were also seropositive by IFAT for *T. gondii.* These results indicate that although *N. caninum* is not a major cause of disease in sheep, there is evidence that it does exist and that further studies are necessary to establish more precisely the seroprevalence to this parasite in the national sheep flock.

Introduction

In 1984, an unidentified protozoan parasite was reported as the cause of encephalitis and myositis in dogs in Norway (Bjerkas et al., 1984). The parasite resembled *Toxoplasma gondii* in morphology but it did not react serologically with antibodies to *T. gondii*. Similar clinical findings were reported in dogs in the USA in 1988 (Dubey, et al., 1988 (a)). Subsequently the parasite was successfully isolated and grown in cell culture, following which it was identified as a new genus *Neospora* type species *Neospora caninum* (Dubey, et al., 1988(b)). Serological and immunohistological tests were developed using specific antibodies which were raised in rabbits following inoculation with *N. caninum*, these tests were used to distinguish *Neospora* from *Toxoplasma* (Lindsay and Dubey, 1989(a)). More recent work has confirmed that these two protozoan parasites are two separate organisms, although they are genetically related and have a similar structure and life cycle, nevertheless, different intermediate and definitive hosts are involved (Dubey and Lindsay, 1996, Dubey, 1999, Lindsay et al., 1999,).

Neosporosis is a common cause of abortion and infertility in cattle, particularly dairy cattle (Gottstein et al., 1998, Schares et al., 1998), Thurmond and Hietala, 1999). *Neospora* associated abortions have been reported throughout the world (Dubey and Lindsay, 1996). Naturally occurring abortion due to *Neospora caninum* has also been reported in goats and horses, but not in sheep (Dubey and Porterfield, 1990, Barr et al., 1992, Dubey et al., 1992,). *Neospora caninum* has been found in the brain of a congenitally infected one week old lamb, which was born alive and ataxic, but died after one week (Dubey and Lindsay, 1990). More recently, *N. caninum* DNA was identified by PCR in the brain of a clinically normal pregnant ewe thought to have

been naturally infected (Koyama et al., 2001). Tissue cysts very similar histologically to those associated with neosporosis were found in the brains of this ewe's twin foetuses surgically removed at 119 days of gestation (Kobayashi et al., 2001).

In a study of 281 aborted ovine foetuses in England and Wales, in which the tissues and sera were examined by serology, histology and immunocytochemistry, no evidence of *Neospora* infection was found (Otter et al., 1997(b)). More recently, in a larger study in the same region of the United Kingdom, the sera of 660 aborted sheep were tested using a direct ELISA and an IFAT test. Only three (0.45%) of the ovine sera were seropositive to both tests (Helmick et al., 2002). It is possible that with such a low prevalence that these three sera were false positives as the proportion of false positives increases where such low prevalence rates are found (Thrusfield, 1995). These findings suggest that while the possibility of naturally occurring *N. caninum* infection may exist in British sheep, its prevalence is extremely low.

The purpose of this study was to determine the presence of *N. caninum* in the Irish sheep population, by means of serology. If present, it was important to establish the seroprevalence with a view to determining the association of infection in the sheep population, if any, with the presence of the disease in the Irish Cattle herd.

Materials and Methods

Study Design

During 1999, one thousand four hundred and forty eight flocks in Ireland were randomly selected from the National Sheep Flock for testing under European Community legislation to confirm national freedom from Maedi Visna, Contagious Agalactia and *Brucella ovis*. The numbers of flocks selected in each county was in direct proportion to the number of flocks present. Blood samples were collected from all female sheep over six months of age up to a maximum of twenty samples per flock. Samples were sent to the Central Veterinary Research Laboratory (CVRL) and stored at –20°C prior to testing under these programmes.

In this study, a sub set of two flocks per county were randomly selected from the sera of 1448 flocks which were stored in the CVRL. A total of 1044 sheep in 52 flocks were tested for antibodies to *N. caninum* in this investigation.

Serology

The samples were tested using a commercial indirect monoclonal ELISA test according to the manufacturers instructions (Chekit®, Bommeli Diagnostics, Bern, Switzerland). Wash steps were carried out using a LKF (Austria) automatic plate washer. A plate shaker was used to ensure adequate mixing of reagents. Plates were read with an LKF (Austria) automated plate reader. The optical density (OD) was read using an ELISA reader (LKF, Austria) at a wavelength of 405 nm (reference wavelength 492 nm). The OD of the duplicate samples was the average of the two readings. The interpretation of the results was in accordance with the manufacturers protocols, OD < 0.400 = negative, OD between 0.400 and 0.500 = inconclusive, OD > 0.500 = positive. The optimum stopping time was five to eight minutes.

Results

A total of six samples out of a total of 1044 tested were seropositive by ELISA for *N. caninum*. The seropositive samples originated in three flocks (two per flock) from counties Cork, Waterford and Wicklow, all of which were located in the southern part of the country. The six samples sent to the Moredun Research Institute, Edinburgh, were seropositive for antibodies to *N. caninum* by IFAT, five out of the six were also seropositive for antibodies to *T. gondii* by IFAT. The results are shown in Table 1.

Table 1: A comparison of the ELISA results in seropositive sheep for antibodies to *N. caninum* with IFAT results for both *N. caninum* and *T. gondii.*

Sample Number	ELISA	* IFAT (*N. caninum*)	* IFAT (*T. gondii*)
1	0.595	1:1024	1:512
2	0.595	1:1024	1:512
3	1.346	1:256	1:64
4	0.657	1:512	1:256
5	0.686	1:128	1:512
6	0.763	1:256	1:128
Positive cut off readings	≥ 0.500	$\geq$ 1:128	> 1:64

* Moredun Research Institute (Samples were not titrated beyond 1:1024)

Discussion

In this study, 6/1044 (0.57%) random sheep samples were seropositive for *N. caninum*. This finding suggests that the prevalence of neosporosis in sheep in Ireland is very low. Similar findings were reported in England and Wales where 0.45% of ewes which had aborted were seropositive (Helmick et al., 2002).

In this survey, 5/6 ewes that were seropositive for *N. caninum* were also positive for *T. gondii* suggesting that cross reactivity may be involved. However, it is now recognised from other studies in sheep that this is unlikely (Buxton et al., 1997, Helmick et al., 2002,). This is supported by infection studies in dogs and cattle, where very little if any cross reactivity between *N. caninum* and other coccidian parasites has been shown to exist using the IFAT test (Trees et al., 1994, Williams et al., 1997). In another part of this thesis, experimental infection of sheep with *N. caninum,* which were already seropositive to *T. gondii,* showed no evidence of significant subsequent cross-reactivity (Chapter 10).

In conclusion, the evidence from this limited survey suggests that *N. caninum* is not a major source of infection in sheep in Ireland and that the seroprevalence in sheep is similar to that already reported in the UK. Nevertheless, the finding that 10% of sheep sampled in 3 out of 52 flocks were seropositive for *N. caninum* cannot be ignored. It would be an interesting project to follow up on the prevalence of neosporosis in the three flocks and to investigate the abortion history (if any). The interaction with concurrent toxoplasmosis and a serological analysis of post abortion sheep blood sera should also be included in any future study.

Chapter 6

A study to evaluate the pathogenicity of NC-1 tachyzoites of *N. caninum* passaged in the laboratory using the murine model

Summary

Inbred BALB/c mice were inoculated with NC-1 tachyzoites previously passaged through Vero cells in the Central Veterinary Research Laboratory, Dublin, to evaluate their pathogenicity prior to their use in experimental cattle and sheep.

BALB/c mice inoculated with large doses of NC-1 tachyzoites (3.0×10^6) were euthanised at 80 days post inoculation. They showed no evidence of infection, when their tissues were examined by histology and immunohistochemistry. It was necessary to immunocompromise the mice by including methylpredniselone in the inoculum to produce the classical symptoms and histopathological lesions of the disease in this strain of mice.

The failure to infect inbred BALB/c mice following experimental inoculation with NC-1 tachyzoites passaged in this laboratory, without having to immunocompromise them, suggests that this isolate may have had a reduced virulence for BALB/c mice compared to other NC-1 isolates.

Introduction

The protozoan parasite, *Neospora caninum,* is a major cause of abortion resulting in significant economic loss in cattle worldwide (Dubey and Lindsay, 1996 , Trees et al., 1999, Anderson et al., 2000, Hemphill and Gottstein, 2000,). The parasite was first recognised as a cause of neuromuscular disease in dogs in Norway in 1984 (Bjerkas et al., 1984). It was described as a new genus *Neospora*, type species *N. caninum* in 1988 (Dubey et al., 1988 (b)). It was recognised later as a major cause of abortion in cattle (Anderson et al., 1991, Barr et al., 1991(b)).

In addition to cattle and dogs, *N. caninum* has been associated with sporadic disease in other livestock species including sheep (Dubey et al., 1990), goats (Barr et al., 1992), and horses (Marsh et al., 1996, Hamir et al., 1998). A wide range of other animals have been infected under experimental conditions; these include cats, foxes, coyotes, monkeys, pigs, laboratory mice and gerbils (Dubey, 1999). Recently, dogs have been shown to be a definitive host (Lindsay et al., 1999).

While *N. caninum* has been shown to be infective for mice, the development of the clinical disease depends on the strain, route of administration and the dose of tachyzoites given. The strain of the mouse is also a very important factor in determining whether that animal becomes infected and goes on to develop clinical disease. Outbred Swiss Webster adult mice do not develop clinical disease, but *N. caninum* can encyst in them (Dubey et al., 1988 (b)). It was possible, using corticosteroids and varying doses of tachyzoites, to set up clinical disease in this strain of mouse (Lindsay and Dubey, 1989(b), Lindsay and Dubey, 1990). The NC-1 isolate was more virulent than the NC-2 or NC-3 isolates.

Immunodeficient nude mice can develop severe clinical symptoms of neosporosis after inoculation with NC-1 isolate tachyzoites (Yamage et al., 1996). Outbred BALB/c mice are relatively resistant to infection with *N. caninum*, however depending on the isolate and dose, inbred BALB/c mice can develop both clinical and sub-clinical symptoms. The NC-1 isolate is more virulent than the NC-3. Characteristically in BALB/c mice, *N. caninum* tissue cysts are rarely found in histological examination of brain tissue (Lindsay et al., 1995).

The purpose of this study was to evaluate the pathogenicity of the NC-1 isolate, kindly provided by the Department of Veterinary Parasitology, University of Liege, Belgium, using the murine model prior to its use, in the experimental infection studies of cattle and sheep in this thesis.

Materials and Methods

Experimental Design

Forty inbred adult BALB/c mice were purchased from the Bio Resources Unit, Trinity College, Dublin, (TCD). The mice were kept in plastic cages which were maintained in the dedicated laboratory animal facilities of this unit in TCD.

The mice were divided into two experimental groups, each consisting of 20 animals which were administered various infective doses of NC-1 tachyzoites subcutaneously in phosphate buffered saline (PBS), with and without the addition of Methylprednisolone Acetate (*MPA),(Pharmacia & Upjohn), and appropriate control groups. The details of the two experimental groups are shown in Tables 1 and 2.

Table 1: Mice inoculated with NC-1 tachyzoites and MPA with appropriate control groups, the survivors were euthanised at 25 days post inoculation (dpi.).

Experiment 1		
Group	Number of Mice	Inoculum
Infected Mice	15	2.4×10^5 NC-1 tachyzoites with 4.0 mg MPA in 0.2 ml PBS
Negative Control	4	0.2 ml PBS only
*MPA Control	1	4.0 mg *MPA in 0.2 ml PBS

Table 2: Mice infected with NC-1 tachyzoites and euthanised at 80 dpi.

Experiment 2		
Group	Number of Mice	Inoculum
Infected Mice	7	3.0×10^6 NC-1 tachyzoites in 0.5 ml PBS
Infected Mice	7	1.5×10^6 NC-1 tachyzoites in 0.5 ml PBS
Vero Control	4	6.0×10^5 Vero cells in 0.5 ml PBS
Negative Control	2	0.5 ml PBS

Tissue Culture Techniques

Preparation of Stock Culture of Vero Cell Lines

Vero cell lines (African green monkey kidney cells) were obtained from the European Collection of Cell Cultures (ECACC) in the United Kingdom. The cell lines provided were part of the World Health Organisations (WHO) seed culture stock passage 134. Prior to use, the cells were resuscitated from the frozen state in accordance with the supplier's instructions.

Briefly: Resuscitated vero cells were grown in 25 cm^2 vented tissue culture flasks containing minimal essential medium with 10% foetal calf serum (MEM-10), flasks were incubated at 37°C with 4.0% CO_2. Harvesting of the cell line commenced when the monolayer was 80 – 90% confluent. The supernatant MEM-10 media was decanted and 0.5 ml of trypsin added to detach the monolayer from the bottom of the flask, taking care that overexposure to trypsin did not occur. Once detachment of the monolayer was completed, the trypsin was neutralised by the addition of 10 ml of RPMI-1640 media containing 10% horse serum. The contents of the flask were split 1:3, additional vented flasks were inoculated and incubated as before. The build up of a stock of Vero cells proceeded by harvesting of the cells as described, except that subsequent detachments were carried out using sterile Costar Cell Scrapers (Corning Incorporated, Corning NY 14831 USA) in a laminar flow cabinet.

Following each passage, 80 – 90% of the yield was pipetted into 1 ml cryovials containing 20% foetal calf serum and 10% dimethyl sulfoxide (Sigma) which acted as a cryoprotectant. The cryovials were placed in polystyrene boxes and surrounded by cotton wool to minimise cold shock when frozen at –70°C for 24 hours prior to being stored in liquid nitrogen at –180°C.

Preparation of Stock Cultures of NC-1 Tachyzoites

Neospora caninum tachyzoites (NC-1) growing on Vero cell monolayer in 25 cm^2 flasks in RPMI-1640 medium were kindly provided by the Department of Veterinary Parasitology, University of Liege, Belgium. These were inoculated onto 24 hour old stationary Vero cell monolayers in RPMI-1640 medium with antibiotic supplements in 75 cm^2 ventilated flasks and incubated at 37°C with 4.0% CO_2. The detailed composition of the RPMI-1640 media used is given in Appendix E. Flasks were examined daily using an inverted Olympus Microscope with a x 40 objective. A Vero cell monolayer culture in a 25 cm^2 flask was included as a control. When the Vero cell monolayer showed 80 – 90% lysis (cytopathogenic effect), it was scraped into the supernatant as before. Further passages were carried out, by inoculating 75 sq cm^2 flasks with 1.0 ml of supernatant from the previous passage, 20% of the yield of each passage was used to inoculate additional flasks, the remainder was stored at –180°C.

Prior to storage, the supernatant was centrifuged at 1250 G for 20 minutes at 18°C. The supernatant liquid and debris were discarded and the NC-1 pellet was resuspended in 2 ml of storage medium containing 90% horse serum (decomplemented – GIBCO) and 10% sterile dimethyl sulfoxide (Sigma). The suspension was aliquoted into cryovials (max 1.25 ml per vial) and stored at –180°C using the method previously described for Vero cells.

Preparation of challenge doses of NC-1 tachyzoites

A standardised procedure was developed to prepare the challenge doses of NC-1 tachyzoites. This procedure involved the withdrawal of a cryovial containing 1.00 – 1.25 ml of NC-1 tachyzoites from stocks stored at –180°C and defrosting as

previously described. The contents of the vial were inoculated onto a 24 hour old Vero cell monolayer in a 75 cm^2 vented flask. After 48 hours, the cell sheet was scraped using a costar scraper and $^1/_{10}$ of the volume (1.8 ml) was inoculated into 2 x 75 cm^2 vented flasks containing 24 hour old Vero cell monolayers. These flasks were again scraped into the supernatant 48 hours later and $^1/_{10}$ of the total volume (3.6 ml) was inoculated on each of two 150 cm^2 vented flasks containing 24 hour old Vero cell monolayers as previously described. The cell monolayers were incubated at 37°C in 4.0% CO_2 and were inspected daily using an Olympus Iverted Microscope with a x 40 objective to ensure that lysis of the monolayer did not exceed 80 – 90% before the next passage was carried out. Depending on the required numbers of NC-1 tachyzoites this process was continued using 150 cm^2 flasks until the required concentrations of tachyzoites were achieved. Challenge inoculua were obtained from NC-1 tachyzoites obtained from passages 10 to 15 and grown on Vero cells from passages 145 to 155.

Harvesting of NC-1 tachyzoites was carried out by scraping the Vero cell monolayer which was 80 – 90% lysed into the supernatant media as previously described. The contents of the flasks were poured into sterile universal screw cap containers which were centrifuged at 1250 reciprocal centrifugal force (RCF) for 20 minutes at 18°C. The supernatant was discarded and the pellet in each universal container was re-suspended in 2.0 ml of sterile phosphate buffer saline PH 7.4 (PBS). This suspension was passed through 23 gauge needles using disposable syringes to disrupt the Vero cell walls and facilitate the release of any tachyzoites still contained in them. The contents of the universals were then bulked and mixed gently to form the concentrated inoculum.

Counting of the parasites was carried out using an improved Neubauer Haemacytometer (Weber, England) and a 0.1% trypan blue vital stain. The count procedure was repeated by an experienced colleague and the results averaged. Only full sized, lunar shaped tachyzoites, which did not stain with trypan blue, were included in the count. The desired concentrations of the inoculum doses were calculated and the concentrated suspension diluted with PBS accordingly. All animal inoculations were made within 1.5 hours of the harvesting of the Vero cell cultures.

Histology

Following euthanasia of the mice, using CO_2, specimens of brain, lungs, liver, heart and kidneys were collected at necropsy and placed in 10% buffered formol saline for seven days to allow fixation to occur.

Specimens were subsequently cassetted and processed in paraffin wax blocks from which sections 4-um in thickness were cut on a microtome (Micron HM 340E). These sections were stained by the standard Ehrlich's haematoxylin and eosin method (H & E). Routine histopathogical examination of these sections was carried out using a Nikon E-600 brightlight microscope.

Immunohistochemistry (IHC)

Immunostaining was carried out using the immunoproxidase technique with Avidin-Biotin Complex ABC on tissue sections, which had been serially sectioned from the same wax blocks, from which the H & E sections had been previously cut. This method has been shown to increase the sensitivity of the test (Haines and Clark, 1991(b).

The tissue sections were cut at 4-5 μm in thickness and were mounted on positively charged ChemMate^{TM} 75 μm capillary gap microscope slides (Dako A/S,

BioTek Solutions, USA). Prior to immunostaining, the sections were de-waxed in 100% xylene for 5 minutes x 2 and the tissues were then gradually rehydrated in decreasing concentrations of ethanol for two minutes each (100%, 95% and 70% and finally 100% water) using a robotic autostainer (Sakura DRS 601). The immunochemical detection of most antigens in fixed paraffin embedded tissues is improved when the tissue sections are subjected to proteolytic enzyme digestion (Haines & Chelack, 1991(a)). In this study, proteolytic enzyme digestion was carried out prior to immunostaining by allowing the tissue sections to stand in pre-warmed 0.4% pepsin (Sigma) at 37°C for 10 minutes. Slides were then washed twice in distilled H_2O before being treated with 3.0% H_2O_2 (BDH) in 100% methanol which acted as a blocking agent for endogenous peroxidases. Slides were washed twice in distilled H_2O followed by buffered PBS PH 7.4 twice for 5 minutes, after which they were blocked with 2% heat inactivated normal horse serum (Vector Laboratories Cat. S-2000) for 30 minutes. Finally slides were washed in PBS twice for 5 minutes.

Immunostaining was performed using a Dako Techmate 500 robotic immunostainer with a pre-programmed immunostaining programme MSIPI, optimised to contain the following protocols:

(a) Primary antibody = 60.00 mins.

(b) Secondary antibody = 30.00 mins.

(c) HRP (Avidin-Biotin) = 30.00 mins.

(d) Chromogen DAB = 3.00 mins.

(e) Haematoxylin = 1.00 min.

The dilution of the primary antibody goat anti-*Neospora caninum* (VMRD Inc., Pullman, Washington, USA) was optimised at $^1/_{2500}$ with PBS. Biotinylated anti-goat

IgG prepared in a horse (Vector Laboratories Cat. No. BA-9500 UK) was used as the secondary antibody. It was mixed with the Avidin-Biotin Reagent ABC (Vectastain Universal Elite ® ABC kit PK 6200 USA) in accordance with the manufacturers instructions. The wash buffers, chromogen and the DAB/HRP substrate were provided by Dako Chem Mate^{TM}.

Upon completion of the immunostaining procedure, the slides were "blued" using a 10% dilution of Scotts tap water solution (Surgipath UK) for two minutes. Slides were rehydrated in ethanol (70%, 95% and 100%) and xylene for two minutes using the robotic autostainer as previously described. Coverslips were put on the slides using an automated system (Tissue Tek, Bayer, Germany).

The controls consisted of a positive tissue control and negative reagent controls, which were run with each batch of slides. The positive control was a section of bovine foetal heart positive by IHC and PCR for *N. caninum* (kindly provided by Dr. Meerschman, Department of Parasitology, University of Liege). The negative reagent controls included the substition of the primary antibody with normal goat serum (Vector laboratories S-1000), omitting the secondary antibody and replacing it with normal horse serum (Vector Laboratories S-2000), or both, and using DAB only.

Results

The mice in experiment 1, which were inoculated subcutaneously with 2.4 x 10^5 NC-1 tachyzoites together with 4.0 mg of Methylprednisolone (MPA) (Depo-Medrone, Pharmacia S Upjohn) became thin and emaciated from around 14 days post inoculation (dpi). These symptoms became progressively worse, six died on day 24. The remaining 14 mice were euthanised on day 25 using CO_2. All of the mice in the control groups appeared clinically normal.

Typical lesions associated with *N. caninum* were found in the brains of 5/9 mice from the infected group following histopathological examination of sections stained by H & E. The lesions in the cerebrum included focal encephalitis with localised gliosis and perivasular cuffing, some of the blood vessels were engorged with red blood cells. In sections of mid brain and cerebellum, there was evidence of focal malacia with localised meningitis but the degree varied between animals. In some cases, small numbers of gitter cells were present. Areas of vascular proliferation were associated with the edges of areas of necrosis which in some cases showed some vacuole formation within the brain. However, there was no evidence of cyst formation in any of the mice brains examined.

When these brain sections, cut serially from the same paraffin wax blocks were examined by IHC, 7/8 were positive for *N. caninum* antigen. Individual and sometimes small groups of tachyzoites were present in areas where a non-suppuritive meningoencephalitis was present. There were also small pieces of antigen floating free in the necrotic area or within the cytoplasm of the phagocytic cells. However, the amount varied greatly from lesion to lesion. In areas of the brain, where there was extensive vacuolation, small pieces of particulate antigen were occasionally scattered

diffusely throughout the lesion. A typical lesion stained by IHC is shown in Figures 1 to 3. The brains of the control mice were normal, when sections stained by H & E and IHC were examined microscopically.

However, in experiment 2 where infected mice received a high dose 3.0 x 10^6 NC-1 tachyzoites and a medium dose of 1.5 x 10^6, without the addition of MPA, no significant clinical symptoms appeared and all the animals survived until euthanised at 84 days post inoculation. Specimens of brain, heart, lung, kidney and liver taken at post mortem examination were fixed in 10% buffered formol saline for 14 days. These sections when stained by H&E and examined microscopically were normal.

Figure 1. Characteristic focus of necrosis (N) accompanied by vaculation in the brain of a mouse inoculated with NC-1 tachyzoites. An area of inflammatory cells (➔) adjoins a blood vessel with perivascular cuffing (←).
ABC Immunoperoxidase stain. X 100.

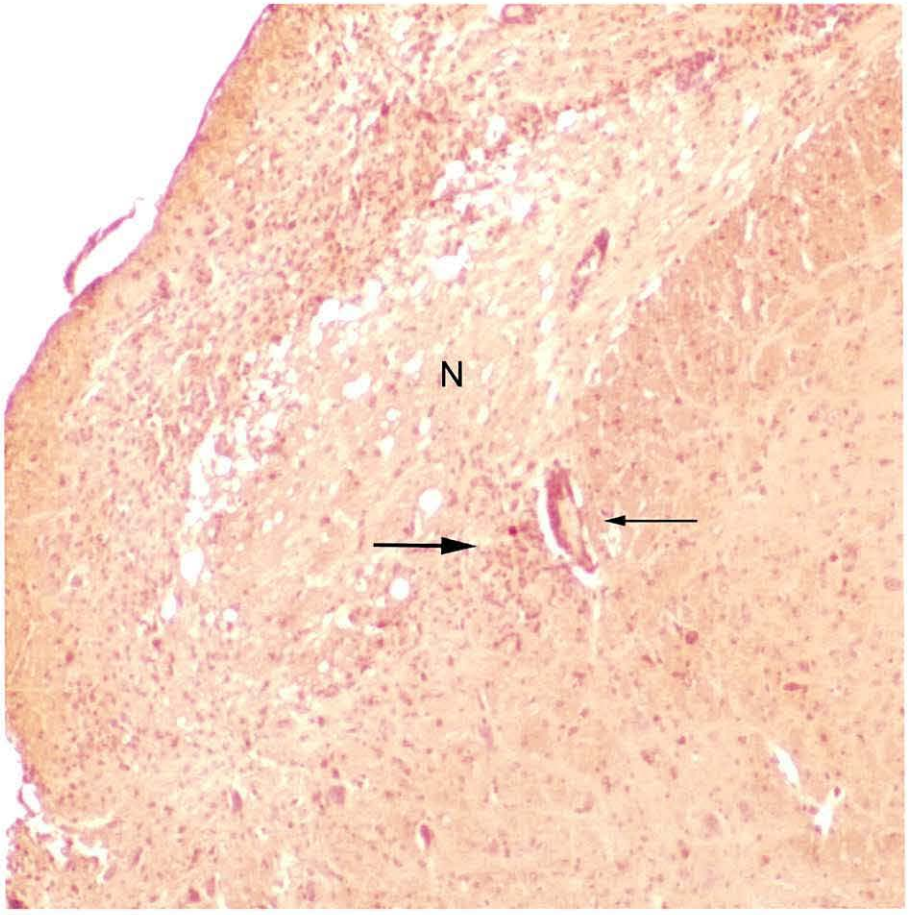


Figure 2. Higher magnification of figure 1 showing focal microgliosis (→) and unilateral perivascular cuffing by mononuclear cells (←). A single group of *N. caninum tachyzoites* is present near the center of the area of microgliosis.
ABC Immunoperoxidase stain. X 200.

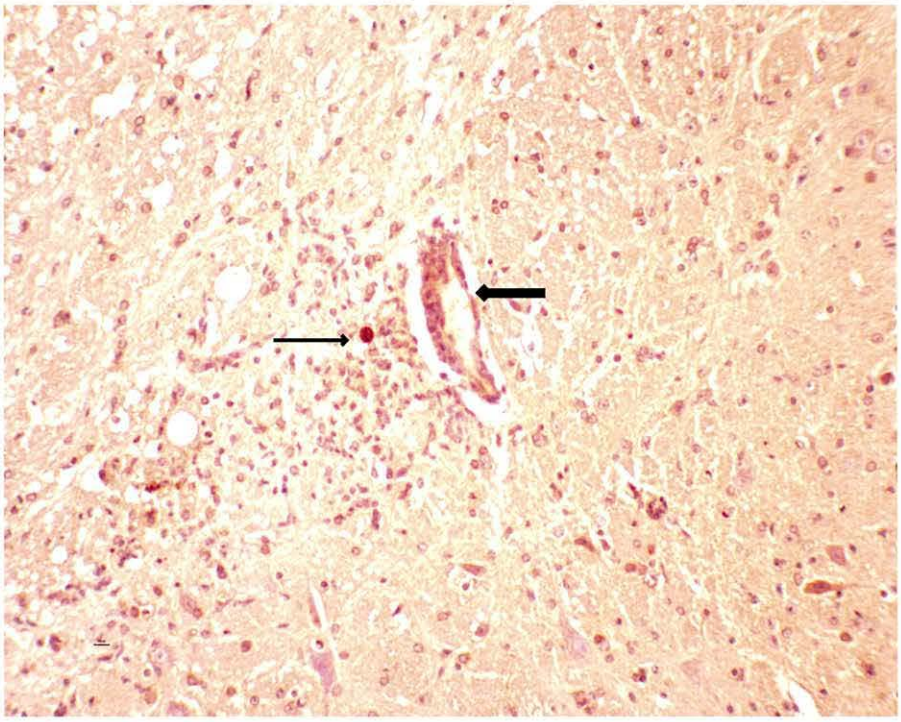
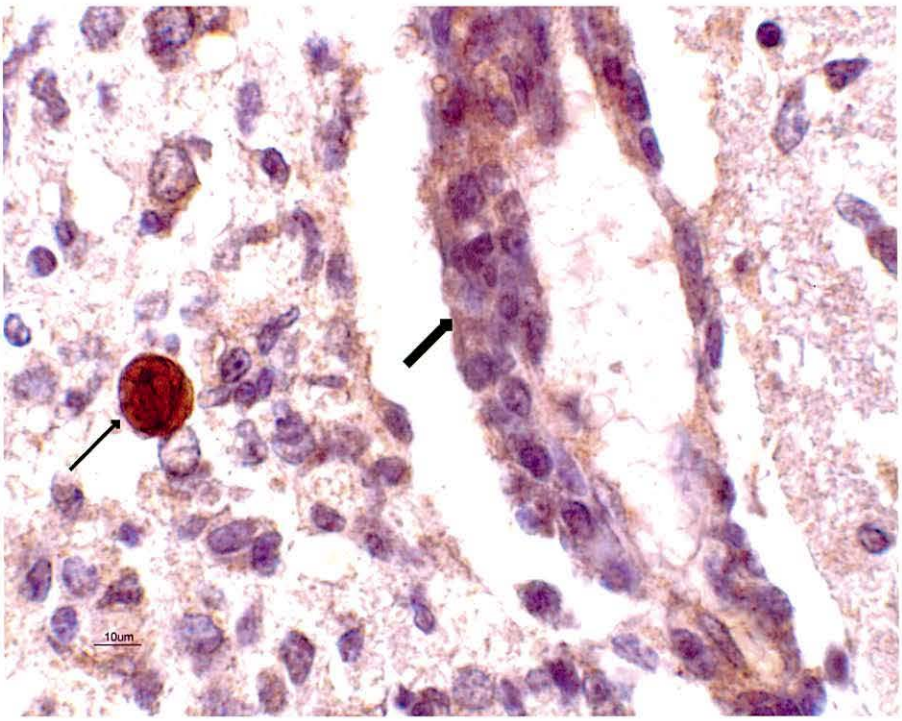


Figure 3. Higher magnification of figure 2 showing a cluster of clearly defined NC-1 tachyzoites (↗). The wall of the blood shows unilateral perivascular cuffing (↗)adjacent to the area of microgliosis.
ABC Immunoperoxidase stain. X 1000.



Discussion

Most mouse strains are highly resistant to infection with *Neospora caninum*, however, some mouse strains have been shown to be susceptible to infection caused by the parasite. The murine model has been shown to be useful in the study of the pathogenesis of the disease in laboratory animals. The development of clinical disease in mice depends on the strain of the mouse, the strain and dose of the *N. caninum* and the treatment given to the mouse (Dubey and Lindsay, 1996). Inbred BALB/c mice have been shown to develop clinical symptoms after inoculation with the parasite (Lindsay et al., 1995). Typical symptoms appear after two to three weeks and include incoordination, wasting and progressive weakness.

This study was carried out in order to evaluate the virulence of the NC-1 strain kindly provided by the Faculty of Veterinary Medicine, University of Liege, using the tissue culture methods in the Central Veterinary Research Laboratory (CVRL), to grow sufficient numbers of tachyzoites required to carry out the challenge and vaccination experiments in this project which involved relatively large numbers of cattle and sheep.

The results of this study have shown that the NC-1 isolate successfully propagated by Vero cell culture in this laboratory even when administered in large doses was not as virulent in BALB/c mice as had been previously reported. Only when the mice were immunocompromised, by the addition of methylprednisolone (MPA), did very obvious clinical symptoms develop. The brains of these mice following histopathology examination showed characteristic lesions of *N. caninum* infection which were subsequently confirmed by immunohistochemistry.

More recently, Lunden et al, (2002), have reported that inbred BALB/c mice were only relatively susceptible to infection with the NC-1 isolate of *N. caninum* when it was administered alone without ISCOMS. In that study, the clinical symptoms which appeared in 3/9 of the mice which received low doses of the live parasite (2.4×10^4) were mild and transitory. In those mice that received larger doses of NC-1 tachyzoites (2.4×10^6), 9/10 showed some clinical symptoms. Mild inflammatory changes were found in the brains of some of these mice following histopathological examination, however, in the low dose group, no significant histopathological changes were reported. Antigen was not detected, by immunohistochemistry, in the brains of any mice immunised with the live parasite.

The findings by Lunden et al., (2002) support the results of this study in which response of inbred BALB/c mice to experimental challenge with NC-1 isolates was shown to be variable; especially when mice had to be immunocompromised with MPA before infection could be established.

Following the results of this study, the size of the experimental challenge doses which were used later in this project to infect cattle and sheep (chapters 7 – 10) were maximised to the limit of the laboratory resources with regard to culturing the large numbers of NC-1 tachyzoites which were required on any specific day in these experiments.

Chapter 7

A study of the Pathogenesis of Experimental Infection of Pregnant Cattle with *Neospora caninum*

Summary

Twenty two cows, in three groups, were experimentally inoculated at different stages of gestation with 8.8 x 10^7 NC-1 tachyzoites. In addition, three pregnant cows were inoculated with 2.0 x 10^6 Vero cells to act as a negative control.

The cows were killed 30 days later, and samples of placenta, foetal brain, liver, lungs, heart and kidney were collected for examination by histology, immunohistochemistry (IHC) and PCR. Blood serum samples were collected immediately from the dams prior to the study, and at necropsy, from both dam and foetus. These samples were tested by ELISA and IFAT for antibodies to *N. caninum.*

The results of this study showed that while seroconversion occurred in the dams, following inoculation with tachyzoites, there was no evidence that infection had been transmitted to any of their foetuses. Foetal sera were tested by ELISA and IFAT with negative results. Foetal tissues examined by histology, immunohistochemistry and PCR were also negative for *N. caninum*. These findings demonstrated that the virulence of the NC-1 tachyzoites used in this study, was not sufficient to set up infection in the dam which was capable of transmission to the foetus.

Introduction

Neosporosis is a major cause of disease in cattle throughout the world (Dubey and Lindsay, 1996, Dubey, 1999, Anderson et al., 2000). It is characterised by abortion which may be endemic or epidemic particularly in intensive dairy herds (Anderson et al., 1991, Gottstein et al., 1998, Thurmond and Hietala, 1999). It is also a significant cause of abortion in intensive beef herds (Waldner et al., 1998, Quintanilla et al., 1999).

There are no clinical signs in cows that abort due to *Neospora* infection. The aborted foetuses are usually autolysed with no gross lesions and the placentae are not retained. The majority of the abortions occur between 4-6 months of gestation (Anderson et al., 2000). It has been shown in sheep, following experimental infection with *N. caninum,* that it may take up to four weeks for the foetus to die and for the dam to recognise this and abort the foetus (McAllister et al., 1996 (a)). In other species, where foetal death due to other causes has occured , the abortion may take weeks or even months to occur .(Kennedy and Miller., 1993).

It appears that the stage of pregnancy, at which the foetus becomes exposed to infection, may determine the outcome of the pregnancy. In normal circumstances, the majority of infected cattle will have been congenitally infected at birth (Davison et al., 1999(b)). In determining the outcome of a pregnancy in a *Neospora* infected dam, it appears that two factors are involved, the first is the age of the foetus at the time of challenge (Gonzalez et al., 1999), and second, the time of recrudesence of a persistent maternal infection (Buxton et al., 1998). In a minority of cases, the infection of a naïve dam may be due to ingestion of sporulated oocysts (De Marez et al., 1999). The size of the challenge dose and the strain of the *N. caninum* isolate involved must also be important considerations in the development of the parasitaemia in the dam which ultimately result in a challenge infection to the developing foetus via the placenta (Innes et al., 2002).

In cattle, pregnancy lasts approximately 280 days. During the first third of pregnancy, the foetal immune system is immunologically incompetent (Nettleton and Entrican, 1995). Sometime between 100 to 150 days, the foetus is beginning to be able to mount an immune response, after which time it becomes progressively more immunologically competent at recognising and responding to various pathogens (Osburn, 1986). As a result, the foetus is extremely vulnerable to *N. caninum* infection during the first three months of pregnancy and is unlikely to survive. Between the three and six months, the foetus is capable of mounting a rudimentary immune response which might still not be sufficient to allow it to survive. It is during this period that most of the abortions due to neosporosis occur in cattle. However, by the third trimester, the immune response of the foetus has progressively improved leading to the survival of the foetus.

In ruminants (e.g. cattle, sheep, goats and deer), the immunology of pregnancy is mediated through a materno-foetal interface consisting of up to 100 points of contact called placentomes. Each placentome comprises a foetal placental cotyledon which intimately interdigitates with a maternal caruncle projecting from the inner surface of the uterus. Transfer of nutrients and oxygen from mother to foetus takes place within this structure across the interface between the maternal caruncular septa and the foetal placental villi (Buxton et al., 2002).

When *N. caninum* invades the cells of the bovine uterus, following a maternal parasitaemia, it multiplies and causes an inflammatory reaction and destruction of both the maternal and foetal tissue at the materno-foetal interface. From here, the damage extends out into the chorioallantois (foetal placental membranes) between the cotyledons both in naturally occurring (Otter et al., 1995) and experimentally induced lesions (Barr et al., 1994). Concurrent with the inflammatory process taking place at the materno-foetal tissue interface

the parasite enters the foetal circulation by means of which it invades the developing foetal tissues, showing a predilection for brain tissue. The age of the foetus, which in turn largely determines its immunocompentancy to combat the disease plays an important role in the degree to which the disease develops in the foetus. In general, the younger the foetus the greater the destructive damage caused by the parasite in the brain leading to foetal death (Ogino et al., 1992). In older foetuses, which are immunologically more competent, the ability of the parasite to cause extensive CNS lesions appears to be more limited and as a result the foetus survives (Otter et al., 1995, Barr et al., 1994).

The purpose of this project was to study the pathogenesis of neosporosis in cows, experimentally infected with Nc-1 tachyzoites during the 4^{th}, 5^{th} and 6^{th} month of pregnancy, to determine if there is an association between the stage of placental development and the invasion of the foetus itself by tachyzoites. It is also during this critical middle trimester of pregnancy that the immune system of the foetus is developing a capability to launch a rudimentary immune response which may be sufficient to allow it to survive. It is likely that the two events, that is the degree of invasion of foetal tissues and the development of the foetal immune system are mutually interdependent.

Materials and Methods

Herd

This study was carried out in the period March, 2000 to April, 2001, during which a suckler herd was established on the Central Veterinary Laboratory's farm at Abbotstown, Dublin. It was operated as a separate herd enterprise with dedicated pasture and housing facilities. The herd consisted of 99 female cattle aged two years or more. Thirty nine of these cattle were indigenous, having been born on the farm, while the remainder had been purchased in various parts of the country during the two preceding years. The majority of the animals were friesian breeds (64/99), with charolais, limousin and hereford accounting for the remainder.

The herd was blood tested for brucellosis during March, 2000 as part of the National Brucellosis Eradication programme, all animals were negative. Blood sera samples were collected in March, 2000 from all 99 cattle and tested for antibodies to *Brucella abortus* and *Salmonella dublin* (O and H antigens), all animals were negative to the tests. However, when tested for antibodies to *Leptospira hardjo*, 50% were seropositive indicating previous exposure. It was known that vaccine had been used in the past in some or all of the herds of origin. However, it was not possible to determine the vaccinal status of the seropositive animals. The herd was also tested for evidence of exposure to *N. caninum,* using both the enzyme linked immunosorbent assey (Elisa, IDEXX) and immunofluorescent antibody test (IFAT, VMRD), according to both manufacturers instructions. Six cattle were seropositive and these were immediately culled from the herd.

The animals in the herd were mated under natural conditions, using stock bulls, between the period April to July, 2000. To ensure that accurate service dates were observed the bulls were fitted with roller ball chin straps (Shoof, N.Z.) and likewise the cows with

Kramer pads (Steamboat Springs, USA), the colour of the marking fluid in each device was changed at 21 day intervals. The animals were inspected daily and the service dates recorded. The animals were examined in August and again 56 days later in October by ultrasound using the presence of foetal heart beat as an indicator of foetal viability. Foetal size was also assessed as an indication of foetal age.

Cattle

Twenty five pregnant cattle were randomly selected from the above herd and divided into three groups on the basis of the number of days pregnant. Group I contained nine cows which were 120 days ± 10 days pregnant. Within this group, eight animals were inoculated intramuscularly with 8.8×10^7 tachyzoites of the NC-1 isolate of *N. caninum* in 10 ml of phosphate-buffered saline (PBS) pH 7.4. The tachyzoites had been passaged in Vero cells as previously described in chapter 6. The ninth animal in this group was inoculated with 2.0×10^6 Vero cells (a similar number to that contained in the inoculum of tachyzoites) to act as a negative control.

Group II contained eight cows which were 150 days ± 10 days pregnant. Seven of these were inoculated with the same dose of *N. caninum* tachyzoites as above while the eighth received Vero cells as in Group I. Likewise Group III contained eight cows which were 180 days ± 10 days pregnant. Seven of these were administered tachyzoites, as in Group I, the eighth animal was inoculated with Vero cells, as before, to act as a negative control.

Collection of Tissue Samples

The adult cattle were stunned using a captive bolt and killed by exsanguation at 30 days post-inoculation. The foetuses were examined macroscopically at necropsy; sex, weight and crown-rump measurements were recorded. Samples of foetal tissue, including brain, heart, liver, lung, kidney and cotyledons with associated placenta, were collected for

examination by the polymerase chain reaction (PCR). Disposable, forceps, scalpels and gloves were used in such a manner as to avoid cross-contamination between foetuses, samples were stored at –20°C. Specimen samples of the same tissues were also collected and placed in 10% buffered formol saline for histology and immunohistochemistry. Blood serum samples were collected and stored initially at –20°C for 72 hours before final storage at –80°C.

Serology

Foetal and maternal blood serum samples were tested for the presence of antibodies to *N. caninum* using an indirect ELISA test (IDEXX, Maine, USA) and an IFAT (VMRD) according to the manufacturers instructions. The IFAT was considered as the confirmatory test with a dilution of $^{1}/_{200}$ for maternal serum and 1/80 for foetal serum, all IFAT positive sera were titred out to their end point titre.

Polymerase Chain Reaction (PCR)

DNA Extraction from Tissue

Samples of brain, liver, lung, kidney, smooth muscle and cotyledon from each foetus in this study were examined by PCR to detect the presence of NC-1 genomic DNA. Approximately 200 mg of sample material, which was removed from each specimen using a disposable forceps, scalpel and petri dish, was finely diced before being placed in a screw top microcentrifuge tube. This procedure was carried out in a laminar flow class II safety cabinet (Nuoire, UK) to minimise cross-contamination.

The extraction of the genomic DNA from the tissues was performed using Roche High Pure Template preparation kit (Roche Diagnostics GMBH, Mannheim, Germany) in accordance with the manufacturers instructions, manual version 2, 2000 with certain

modifications. These involved mainly using larger tissue sample weights and increased volumes of kit reagents. The technique was optimised as outlined below.

Briefly: The tissue sample (200 mg) was placed in a 1.5 ml microcentrifuge tube and was incubated at 55°C for 20 hours following the addition of 300 µl of tissue lysis buffer and 60 µl of Proteinase K to lyse the tissue the cell walls and release the cellular DNA. During this process any nucleases present were also inactivated. The released cellular DNA was selectively bound to the special glass fibers, prepacked in the High Pure Purification filter tubes by the addition of 300 µl of binding buffer and 150 µl of isopropanol (Sigma). The bound DNA was purified in a series of rapid "wash and spin" steps during which 500 µl of wash buffer was added to the filter tube followed by centrifugation at 9,750 RCF (MSE Hawk 15/05 refrigerated microcentrifuge) for two minutes. This step was repeated three times to force the elution of the wash buffer through the glass fibers in the filter tube, thereby purifying the attached DNA. A prewarmed elution buffer (200 µl) at 70°C was added, which freed the DNA bound to the glass fibers in the filter tube, and allowed it to pass into a fresh collection tube as eluted DNA which could be immediately analysed or stored at –20°C.

Quantification of DNA

Both the purity and the concentration of the extracted cellular DNA present in a sample was determined by measuring their absorbance at 260 nm and 280 nm using a UV spectrophotometer (Campspec M320). The reading at 260 nm allowed the calculation of the concentration of DNA in the sample. An optical density (OD) of 0.1 at 260 nm corresponded to approximately 100 ng/µl of DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provided an indication of sample purity. Pure preparations of DNA had OD_{260}/OD_{280} values of approximately 1.8.

Amplification of extracted DNA

The extracted DNA was amplified using the polymerase chain reaction (PCR). This technique required the identification of the sequence on either side of the target region of the gene thereby allowing a region between two defined points on a template molecule to be amplified (Harris and Jones, 1997).

Briefly: The technique involved the denaturation of a double stranded template of DNA by heat treatment resulting in the double stranded helix being separated into two single strands. The single stranded DNA was annealed with two shorter primer sequences (each typically circa 20 bases in length) that are complementary to sites on opposite strands on either side of the target region at a lower temperature. A thermostable DNA polymerase facilitated the extension of the primers from 3' – OH end. The entire cycle could then be repeated by denaturing the preparation and starting again.

It has been shown when using the Np21 (19 bases) and Np6 (19 bases) primers which target the pNc5 region of the gene, as the desired sequence for DNA amplification (Kaufmann et al., 1996), that a single specific fragment of DNA could be amplified in the presence of NC-1 *Neospora* tachyzoite derived DNA. This was equivalent to a single parasite in 2 mg of brain tissue (Yamage et al., 1996). In this study, an improved version of these primers called the Np21 plus (23 bases) and Np6 plus (24 bases), which also target the pNc5 region of the gene, was used in the PCR reaction. These primers have been shown to allow improved sensitivity and specificity of routine PCR methods to detect the presence of amplified DNA (Muller et al., 1996). The sequences used in these primers were:-

Np21 plus : 5'- C C C A G T G C G T C C A A T C C T G T A A C- 3'
Np6 Plus : 5' – C T C G C C A G T C A A C C T A C G T C T T C T – 3'

Similarly, two primers N91F (22 bases) and N91R (24 bases) which target the GLUCOSE TRANSPORTER TYPE 4 (Gtt4) region of the bovine gene were used for each tissue sample as a "housekeeping gene", to ensure that the initial DNA extraction procedure had been successful and that the DNA obtained was of sufficiently good quality. The sequences used in these primers were:-

N91F : 5' – A G A G C C A G T T C T C T C C A G T T G C – 3'
N91R: 5' – C A C A G T C A C T G C T A A C C A C A A C A C – 3'

All the primers were supplied by Genossys (Genosys Biotechnology, UK).

The Taq polymerase (jump start Taq) buffers and other reagents were supplied by Sigma (UK), unless otherwise stated. All PCR reaction mixes in this project were optimised, the details are shown in Table 1.

Table 1. Standard PCR mix.

Reagent	Concentration	Volume
Taq Polymerase	2.5 units/µl	0.5 µl
Primers	5.0 pMol/µl	5.0 µl
PCR Buffer x 10 without $MgCl_2$		5.0 µl
$MgCl_2$	25 mMol	2.0 µl
dNTP's	2.5 mMol	4.0 µl
Purified H_2O		28.5 µl
		Total 45.0 µl

Finally, 5.0 µl of the template (normally containing between 250 – 1000 ng of DNA depending on the concentration in the tissue source) was added to give a final volume of 50 µl in 0.5 ml thin walled lidded PCR tubes (AB gene, Surrey, UK). Two drops of mineral oil were added to the reaction mix, prior to the addition of the template DNA, to prevent evaporation of the PCR reaction mix during the heating phase of the thermocycling process. Separate laminar flow biosafety cabinets in different rooms were used to perform the DNA isolation, pipetting of reaction mixes, addition of template DNA and preparation of mixes for gel running. In addition, thermal amplification and gel running were also performed in separate rooms to minimise the possibility of cross-contamination.

PCR amplification was carried out in a thermocycler (Perkin-Elmer Thermal Cycler 480) using an initial denaturation step, during which the reaction sample mix was incubated at 95°C for 12 minutes. This was followed by 39 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C for 75 seconds and primer extension at 72°C for 60 seconds. A final extension step of 72°C for 10 minutes after which the amplified product was stored at 4°C until removed from the thermocycler. All PCR amplifications included positive and negative reaction mixes, in addition to the "housekeeping" control template DNA, already described. The positive control consisted of DNA, extracted from pure washed culture of NC-1 isolates of *N. caninum* grown in Vero cells, which was optimised to contain 100 fg/µl. This concentration of DNA was found to give a clear single band at around 337 bp position using the reaction mix already described. The negative control contained 5.0 µl of purified water in place of the DNA template in addition to 45 µl of reaction mix.

Agar Gel Electrophoresis

Briefly: Amplified products were analysed by electrophoresis through a 1.5% analytical grade agarose gel (Promega Corporation, Madison, USA) made up using 1 x TAE electrophoresis buffer (40 mM Tris-Hcl, 1.0 mM EDTA, 0.11% v/v glacial acetic acid, pH 8.0). The mixture was dissolved by heating in a microwave oven taking care to avoid boiling. The mixture was then cooled to 55°C before ethidium bromide was added to give a final concentration of 0.5 ug/ml. The agarose solution was mixed thoroughly and poured into a clean perspex template and combs were inserted to form sample wells. Depending on the number of samples to be run, midi gels (20 wells) or maxi gels (72 wells of which only 48 were ever used for various operational reasons) were used. Once the gel was set, the combs were removed and the gel placed in the electrophoresis tank and covered with 1 x TAE electrophoresis buffer.

Each well in the gel was loaded with 21 µl of a sample mix containing 10 µl of the amplified DNA sample, 3.5 µl of loading buffer (x6) and 7.5 µl of purified water. Appropriate molecular weight size markers were used to determine the DNA fragment size. For most runs 100 bp or 50 bpDNA ladders (Invitrogen Life Technologies, USA) were used. Depending on the size of the gel and the number of samples involved, one to three positive controls and a negative control were used . A seperate "housekeeping control" for each tissue DNA sample was always included. In order to achieve adequate separation and resolution, midi gels were run for 1.5 hours at 80 volts and maxi gels for 1.75 hours at 150 volts. Checks for adequate separation of the bands were made during each run by means of a UV transluminator. At the end of each electrophoretic run, the images were recorded using a Polaroid camera.

Results

Macroscopic necropsy examination of the uterus, placental membranes and foetuses at post mortem showed no evidence of pathological changes. All tissues and foetuses appeared normal indicating that the foetuses were alive prior to the death of their dams.

Histopathology

Samples of foetal brain, heart, lung, liver and kidney were sectioned and stained by Ehrlich's routine H & E method. All tissues appeared normal with no evidence of any pathological changes. In the case of the placentomes which are composed of maternal and foetal tissue as already described, the interface where the maternal septa and the foetal villi come into direct contact was normal indicating that infection by NC-1 tachyzoites had not occurred.

Serology

Blood serum samples taken from all the cows in the experimental group including the controls immediately prior to inoculation were negative when tested by ELISA (IDEXX) and IFAT (VMRD) in accordance with the manufacturers instruction literature. However, when re-sampled at 30 days post inoculation (dpi.), all animals except the controls, had responded by producing high levels of *Neospora caninum* specific antibodies. The IFAT tested samples were titred out to one dilution past their end point (Table 2).

In the control group 4, one cow was inoculated at 120 days, a second at 150 days and a third at 180 days of gestation (dg) with Vero cells to act as negative controls. Foetal blood samples were examined using the same ELISA and IFAT test kits. A cut off titre of $^{1}/_{80}$ for the IFAT was used in the case of foetal sera. All the samples were seronegative to both ELISA and IFAT.

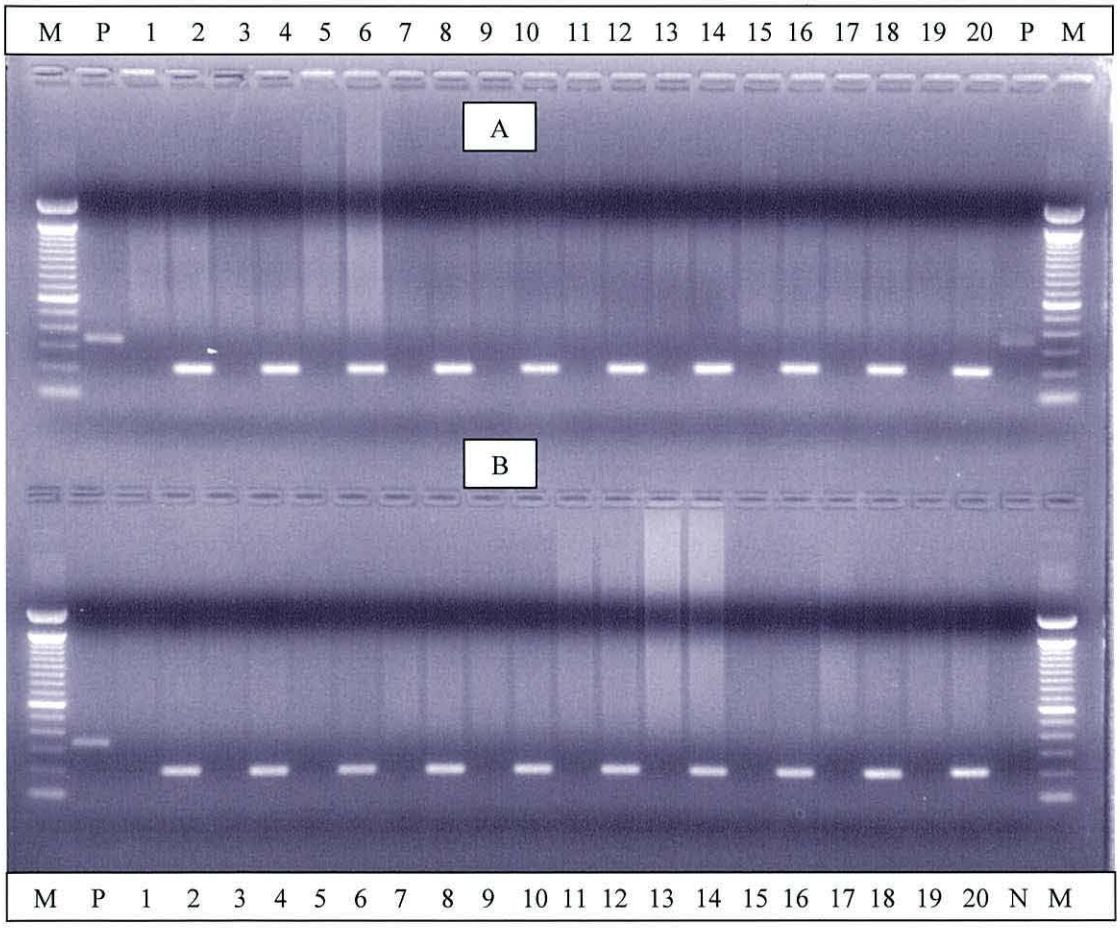
Table 2: Antibody titres (IFAT) to *N. caninum* in cows inoculated intramuscularly at 120, 150 and 180 days of gestation with NC-1 tachyzoites.

Titre (Reciprocal)	Number of cows with Titre 30 days post inoculation. (dpi)			
	Group 1 120 days	Group 2 150 days	Group 3 180 days	Group 4 Vero cells
<200	0	0	0	3
400	0	0	0	0
800	0	0	0	0
1,600	1	0	0	0
3,200	1	2	0	0
6,400	2	2	3	0
12,800	4	3	4	0

Polymerase Chain Reaction

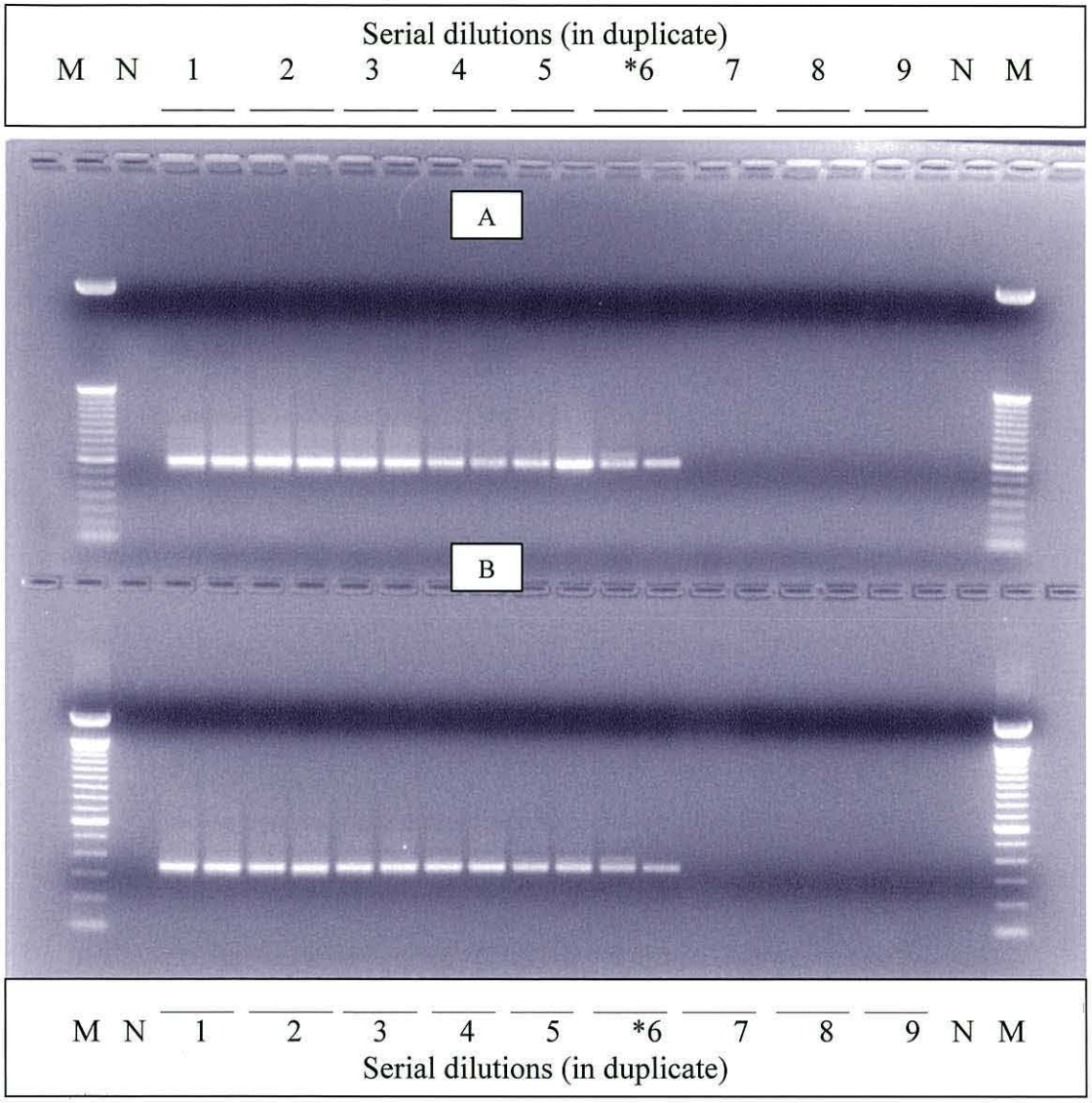
Samples of DNA were extracted from foetal brain, heart, kidney, liver and lung and tested for the presence of *N. caninum* specific DNA, as previously described, with negative results. Similarly, *N. caninum* specific DNA was not identified in the material/foetal tissue of the placentomes, (Figure 1). Titration of DNA extracted from NC-1 tachyzoites to determine the limit of detection for this PCR method are shown in Figure 2.

Figure 1. Detection of *Neospora caninum* by PCR on a 1.5% agarose gel stained with ethidium bromide.



Lanes
M: = 100 bp DNA size ladders, 4.0 µg/lane.
P: = Positive NC-1 DNA control. 337 bp.
N: = Negative purified H_2O control.
1-19: = (Odd numbers, Comb A + B) DNA extracted from brain tissue of bovine foetuses. Extracted bovine foetal brain DNA.
2-20: = (Even numbers, Comb A + B) "House keeping" DNA control. 200 bp.

Figure 2. Titration of DNA extracted from NC-1 tachyzoites to determine the limit of detection of the PCR method used in this study. Test was carried out in duplicate (i.e. comb A and B contain the same samples in duplicate).



Lane M: = Comb A: 50 bp DNA size ladder. 4.0 µg/µl.

= Comb B: 100 bp DNA size ladder. 4.0 µg/µl.

Lane N: = Negative control, purified H_2O and PCR reaction mix.

* The limit of detection was determined by making 1:10 serial dilutions of the "NC-1 DNA standard" which was extracted from NC-1 tachyzoites and standardised to contain 10 ng of DNA/µl

1 = "DNA standard" diluted to 10 ng/µl. 2 = "DNA standard" diluted to 1 ng/µl.

3 = "DNA standard" diluted to 100 pg/µl. 4 = "DNA standard" diluted to 10 pg/µl.

5 = "DNA standard" diluted to 1 pg/µl.

* 6 = "DNA standard" diluted to 100 fg/µl. = End Point Titre.

Discussion

The results of this experiment show that when cattle were inoculated intramuscularly with 8.8 x 10^7 tachyzoites of the NC-1 isolate of *N. caninum* during the middle trimester of pregnancy, they mounted a strong humoral response which indicated that the cows had been successfully challenged. Similar humoral responses accompanied by foetal death and/or abortion were reported in cattle challenged by intravenous inoculation with 1.0 x 10^7 *N. caninum* tachyzoites (NC-Liverpool) administered intravenously at 10 and 30 weeks of gestation (Williams et al., 2000).

In the present study, seroconversion did not take place in any of the foetuses indicating that transplacental infection had not occurred. If this had taken place, it would have been reasonable to assume that the foetuses in the 150 to 180 day stage of gestation would have been immunologically competent and would have responded with detectable levels of *N. caninum* specific antibody in their blood serum. The absence of histopathological changes and the failure to detect *Neospora* DNA in either the foetal tissues or more significantly in the maternal/foetal tissue of the placentome further indicated that infection was not established in the dams or their foetuses in this study.

Several studies have indicated that spread of the disease from dam to foetus occurs mainly by vertical transmission in utero (Wouda, 2000). Nevertheless, this apparently did not happen in this experiment, in spite of a strong challenge by inoculation of the dams with large numbers of tachyzoites. The resulting high antibody titre in the dams is indicative of a challenge by viable tachyzoites. Experiments carried out in sheep, using the same passages and proportionate doses at similar stages of pregnancy, resulted in extensive foetalopathy. Furthermore on two occasions, cattle and sheep were inoculated on the same day from the same batch of tachyzoites. These findings would suggest that the virulence of the

tachyzoites, at least for sheep, was not a problem. However, it is recognised that sheep are very susceptible to experimental challenge with *Neospora* (Dubey & Lindsay, 1990, McAllister et al., 1996(a), Buxton et al., 1997). It has been shown that in cattle challenged by the intravenous route with low doses (1×10^7) of NC-Liv isolate of *N. caninum* at 70 days of gestation, 6/7 foetuses died. However, in cattle inoculated with the same dose by the same route, at 210 days of gestation, 7/7 survived (Williams et al., 2000). More recently, in cattle infected subcutanously at 140 and 210 days of gestation with the same dose of NC-1 isolate, all the foetuses, except one out of the 140 day group, survived (Maley et al., 2001, (b)). In both experiments, the calves that survived were antibody positive at birth, prior to ingestion of colostrum, confirming that infection had occurred in the dams which was transmitted across the placenta to their foetuses.

While molecular diagnostic techniques are normally highly sensitive and specific, PCR amplification of parasitic DNA does not work well when foetuses are autolysed (Anderson et al.,2000) or when the parasite is present in small amounts as is often the case with *Neospora* (Dubey et al ., 1998 In this experiment, the failure to confirm infection in either the placentome of the dam or in brain or other foetal tissues by PCR or histology, is unlikely to have any relationship with the inoculation route. It has been shown in other studies (Williams et al., 2000, Maley et al., 2001,(b)) that successful transmission was achieved using much smaller doses of either NC-1 or NC-Liv isolate, by the intravenous or subcutaneous routes. The response using the intramuscular route should be intermediate between the other two routes. It is likely, that in this study, the virulence of the NC-1 isolate of *N. caninum,* passaged in this laboratory, has a reduced virulence for adult pregnant cattle).

Chapter 8

A study of the Pathogenesis of Experimental Infection in Pregnant Sheep with *Neospora caninum*

Summary

Twenty four pregnant sheep were divided into three groups according to their stage of gestation. Each group contained eight ewes, seven of which were challenged by an experimental inoculum of 10^7 NC-1 tachyzoites, the eighth animal was inoculated with 10^6 Vero cells to act as a negative control. Group I were challenged at 53 ± 8 days of gestation (dg.), Group II at 70 ± 8 dg. and Group III at 87 ± 8 dg.

The ewes were slaughtered at 28 days post inoculation and blood serum samples were tested by ELISA, IFAT and Direct Agglutination for antibodies to *T. gondii* and *N. caninum.* Tissue samples collected at necropsy, were examined by histology, immunohistochemistry (IHC) and PCR for the presence of *N. caninum.* During this study, one ewe aborted, DNA extracted from the foetal brain and from the NC-1 tachyzoites used to infect the dam were cloned, and the sequences of the cloned products were compared; there was a 98.3% similarity between the resulting sequences.

The stage of gestation at which the dam was experimentally inoculated with NC-1 tachyzoites was shown to influence the outcome of the pregnancy. Foetal death was more likely to occur during the early stages rather than the latter stages of gestation following experimental inoculation.

Introduction

The apicomplexan parasite *Neospora caninum* was first described in dogs as a cause of encephalomyelitis and myositis in Norway in 1984 (Bjerkas et al.,1984). It was subsequently isolated and named in 1988 (Dubey et al., 1988(a), Dubey et al., 1988(b)). Neosporosis has since been shown to be a major cause of abortion and neonatal mortality in cattle throughout the world (Dubey and Lindsay, 1996, Dubey, 1999, Anderson et al., 2000).

The parasite has been shown to be associated with disease in other livestock species including sheep (Dubey and Lindsay, 1990), goats (Barr et al., 1992) and horses (Marsh et al., 1996). The life cycle of *N. caninum* is not fully understood but the dog has been shown to be a definitive host (Lindsay et al., 1999). The only known route of transmission from dam to offspring is transplacental (Dubey and Lindsay, 1996). *Neospora caninum* has a very similar life cycle to *Toxoplasma gondii,* which is another apicomplexan parasite; however, the dog has been shown to be a definitive host for the former and the cat for the latter. Although the two parasites are also structurally, genetically and antigenically related (McAllister et al., 1996 (b) , Beckers et al., 1997, Lindsay et al., 1998, Speer et al., 1999 , and Fazaeli et al., 2000,), they cause biologically different diseases. *Toxoplasma gondii* is associated with abortion in sheep and goats, while cattle are clinically unaffected. In pregnant sheep and goats, *T. gondii* can establish a placentitis with consequent foetal death and re-absorption, abortion or stillbirth. Infection is worldwide and follows ingestion of contaminated food or water with sporulated oocysts. In the case of neosporosis, natural infection of sheep and goats is uncommon (Dubey et al., 1996, Kobayashi et al., 2001, Koyama et al., 2001). In sheep and goats, toxoplasmosis only causes disease following primary

infection, after which a strong immunity develops, which normally protects subsequent pregnancies against the disease (Buxton et al., 2002).

Nevertheless, sheep under experimental conditions are highly susceptible to infection with *N. caninum*. It has been shown that, when pregnant ewes are inoculated with tachyzoites, during pregnancy, the clinical symptoms and pathological changes in the placenta and foetus are very similar to those observed in cattle infected with neosporosis (Buxton et al., 1998). These clinical and histopathological changes in sheep are also very similar to those observed in ovine toxoplasmosis (Buxton et al., 1982, Buxton and Finlayson, 1986,).

In view of the worldwide distribution of neosporosis in cattle, and of toxoplasmosis in sheep, it is reasonable to assume that both species of animals have been exposed to infection by one or other parasite from time to time. The life cycles of the two protozoan parasites are very similar, except that two different definitive hosts have been identified, i.e. dog and cat, nevertheless, contamination of food and water supplies by sporulated oocysts of both parasites has been suggested as being involved in the spread of the neosporosis in cattle and toxoplasmosis in sheep.

The objective of this project was to study the pathogenesis of *N.caninum* in pregnant sheep, which were experimentally inoculated with NC-1 tachyzoites at different stages of gestation during early to mid-pregnancy. It was also proposed to investigate the effects of experimental challenge with NC-1 tachyzoites in sheep, which had been previously exposed to *T. gondii* infection.

Materials and Methods

Sheep Flock

This study was carried out during the period November, 2000 to April, 2001. The experimental flock assembled for this study consisted of 75 cross bred grey faced ewes. These were maintained as a single unit for the period of the experiment with dedicated pasture and housing on the Central Veterinary Research Laboratory farm at Abbotstown, Dublin. Oestrus was synchronised using chronogest sponges (Intervet Irl, Dublin) in accordance with the manufacturers instructions. The sponges were removed after 14 days and the ewes were mated using rams with harnesses and colour coded markers. The colours were changed at 14 day intervals to identify repeat services. The sheep were observed daily and the matings were recorded.

In this project, twenty four adult pregnant ewes were randomly selected and divided into three groups on the basis of the number of days pregnant (Table 1). The infected groups of sheep were inoculated intramuscularly with 1×10^7 NC-1 tachyzoites, while the control groups received 1.0×10^6 Vero cells administered by the same route and prepared according to the method described in Chapter 6 (The experimental group details are shown in Table 1).

Table 1. Experimental infected and control groups.

Challenge Groups	Day of Gestation	Infected Groups Number of Sheep	Control Group 4 Number of Sheep
1	53 ± 8	7	1
2	70 ± 8	7	1
3	87 ± 8	7	1

Collection of Tissue Samples

The ewes were slaughtered 30 days post inoculation using a captive bolt followed by bleeding. The foetuses were examined macroscopically at necroposy. The sex, weight and crown-rump measurements of the foetuses were recorded. Samples of foetal brain, heart, liver, lung, kidney and at least two placentomes were collected for histopathology, immunohistochemistry (IHC) and PCR as previously described for cattle in Chapter 7.

Serology

Blood samples were collected from the 24 sheep in the challenge and control groups immediately prior to inoculation and again 28 days later at the time of slaughter. Blood samples were also collected from the foetuses at time of necropsy. The serum was separated by centrifugation at 3500 reciprocal centrifugal force (RCF) for five minutes (Megafuge 1.0, Herareus Sepatech, Germany) and stored at –20°C.

The serum samples were screened for the presence of specific antibodies to *Toxoplasma gondii* and *Neospora caninum*. In the case of *T. gondii,* screening was carried out using a commercially available indirect monoclonal ELISA test (Chekit-toxotest, Bommeli Diagnostics, Bern, Switzerland). A similar commercially available monoclonal ELISA kit (Chekit-*Neospora*, Bommeli Diagnostics, Bern, Switzerland) was used to detect antibodies to *N. caninum*. Both tests were performed in accordance with the manufacturers instructions. A second non-ELISA based test was used to confirm the presence and titre of specific antibodies to both diseases. In the case of *T. gondii,* a commercially available direct agglutination test (bio-Merieux, Lyon, France) was used in accordance with the manufacturers instructions.

However, in the case of *N. caninum*, a similar commercial kit was not available. It was therefore necessary to develop and validate an alternative test method. The immunofluorescent antibody test (IFAT) was considered to be the most reliable alternative.

Briefly: Serum from sheep under test was diluted and added to wells on a slide coated with whole cell NC-1 tachyzoites and incubated at 37°C. Anti-sheep gamma globulin prepared in donkeys and labelled with fluorescein isothiocyanate (FITC), was added to wells which bound to the ovine antibody attached to the tachyzoites coating the surfaces of the wells. The list of reagents used, are shown in Table 1.

Table 1: Reagents used in the ovine immunofluorescent antibody test (IFAT) .

(1) *Neospora caninum* FA Substrate slides (VMRD Inc. USA).

(2) Anti-sheep IgG labelled with FITC prepared in a donkey (Sigma code F7634).

(3) Moredun standard ovine positive control sera, IFAT titre $^{1}/_{8192}$ (Moredun Research Institute, Edinburgh, U.K.).

(4) Moredun standard ovine negative control sera, IFAT titre $< {}^{1}/_{64}$ (Moredun Research Institute, Edinburgh, U.K.).

(5) FA conjugate diluting buffer: containing 1.0% bovine serum albumin, PH 7.2, and 0.09% sodium azide (VMRD Inc. USA).

(6) Serum diluting buffer: 1.0% BSA, pH 7.2 and 0.09% sodium azide (VMRD Inc. USA).

(7) Wash buffer: Cat. No. 210-90-RB (VMRD Inc. USA).

The test was carried out using the methodology already described for the commercially available IFAT kit (VMRD Inc., Pullman, USA)for use with bovine sera with certain modifications. These included the replacement of the bovine positive and negative control sera with ovine positive and negative controls. The anti-bovine FITC

labelled conjugate was replaced with anti-sheep FITC labelled conjugate prepared in a donkey. The optimum dilution of the latter conjugate was determined by using increasing serial dilutions of the conjugate until a dilution was reached where the negative and positive controls were observed and the end point titre of $^{1}/_{8192}$ (Moredun control) was reached. A dilution of $^{1}/_{800}$ of the anti-sheep FITC conjugate with FA conjugate diluting buffer was found to give the best results. All optimisation trials were carried out in duplicate.

The blood samples (105) from the negative control sheep, which had been tested on two or more occasions with the monoclonal ELISA test, were used to calculate the "cut off" titre for this IFAT test. Titres $< {}^{1}/_{256}$ were considered negative using the geometric mean titre (GMT) of the negative control bloods plus two standard deviations. This cut off titre compared favourably with the $< {}^{1}/_{128}$ cut off used in the Moredun Research Institute's IFAT test, which incorporates the same controls but different reagents and methodology.

Foetal sera were also examined for the presence of IgM antibodies using the same IFAT test, except that the secondary FITC antibody was replaced by a FITC labelled anti-sheep IgM conjugate prepared in rabbits (Kirkegaard & Perry Laboratories Inc., MD, USA). Sera with titres $\geq {}^{1}/_{80}$ were deemed positive.

Histology

At necropsy, specimens of brain, liver, lung, heart, kidney and at least two cotyledons were collected from each foetus and associated placenta and placed in buffered formol saline for 14 days to allow fixation to occur. Sections were cut at 4 um in thickness, stained by the standard Ehrilchs H & E method and examined by bright light microscopy as previously described in chapter 6.

Immunohistochemistry

Immunostaining was carried out on tissues, which had been serially cut from the same paraffin wax blocks as the H & E stained sections to enhance sensitivity of the test. Two immunostaining methods were evaluated, the immunoperoxidase Avidin Biotin ABC method (Vectastain Universal Elite ABC Kit) previously described in chapter 6 and the Dako EnVision ^{TM} peroxidase system (Dako Corporation, California, USA). The latter system is based on a HRP labelled polymer, which is conjugated with the secondary antibody. The labelled polymer does not contain avidin or biotin, consequently non-specific staining from endogenous avidin/biotin activity is eliminated or significantly reduced. The results obtained using this technique, were superior to those achieved with the Avidin Biotin Complex ABC immunoperoxidase method.

Using the EnVision ^{TM} peroxidase system, section cutting, mounting, paraffin dewaxing and dehydration steps in ethanol were similar to those described for the Vectastain Elite Universal ABC method already described in chapter 6. The enzyme digestion stage, using pepsin and the quenching of endogenous peroxidase activity using H_2O_2, were also similar. However, in the blocking step, for non-specific binding, Dako protein serum-free blocking solution (Dako Ltd., UK, Code No. X0909), was used instead of horse serum.

Immunostaining was performed using a Dako Techmate 500 robotic immunostainer. The various programme incubation and wash times were optimised as follows:-

(1) *Primary antibody: 38 minutes (Polyclonal rabbit antiserum to BPA-1 isolate *N. caninum*, dilution $^{1}/_{400}$).

(2) Secondary Antibody: 35 minutes (Dako EnVision ^{TM} labelled polymer)

(3) Chromogen DAB: 3 minutes x 3 (Diaminobenzidine tetrahydrochloride, Dako Ltd).

(4) Haematoxylin: 1.0 minute.

* The primary antibody was a polyclonal antibody, raised in rabbits, following inoculation with the BPA-I isolate of *N. caninum,* was kindly provided by Dr. B. Barr, University of Davis, California, USA.

Sections were "blued" by soaking in Scotts solution for two minutes (Surgipath UK) and rehydrated using an automated stainer (DRS-601 Sakura). Cover slips were applied to the slides using an automated system (Tissue Tek, Bayer, Germany). All the buffer reagents used were supplied by Dako Ltd. Positive and negative controls were included with each batch of slides processed. The positive tissue controls included a section of foetal bovine heart, which was positive for *N. caninum* by IHC and PCR (kindly provided by Dr. Merschmann,Faculty of Veterinary Medicine, University of Liege, Belgium), and a section of ovine brain which was positive by serology, histology and PCR. The negative tissue control was a section of ovine brain (Pi-4) which was negative by serology, histology and PCR for *N. caninum*. The negative reagent controls included substitution of the primary antibody with normal rabbit serum (Vectorstain S-500) and omitting the secondary antibody and replacing it with normal rabbit serum as above.

Polymerase Chain Reaction

A PCR test was used to detect the presence of *N. caninum* DNA in samples of foetal brain, heart, liver, lung, kidney and cotyledons. The technique was identical to that previously described in chapter 7 except that different primers were used to detect

a suitable "housekeeping" gene. In this study, SPF and SPR primers which target the ovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used as the "housekeeping" control. These primers were each 20 bp long and the sequences give rise to a PCR product of 818 bp in length. The primers used were:-

SPF: 5' – C C C A T G G C A A G T T C C A C G G C –3'
SPR: 5' – C A C C C T G T T G C T G T A G C C G A – 3'

Cloning

Extracted tissue DNA from the brain of an aborted foetus which was positive by serology, histology, immunohistochemistry and PCR, as well as DNA extracted from *N. caninum* tachyzoites was used to prepare fresh PCR product as previously described. The final extension step of 72°C for 10 mins was extended to 20 mins to facilitate additional 3' poly A overhangs which are necessary for TA cloning. Electrophoresis was carried out on a 1.5% agarose gel to confirm the presence of NC-1 specific DNA.

Cloning in this study was carried out using the commercially available Topo TA Cloning® Kit (Invitrogen Life Technologies, California, USA) according to the manufacturers instructions with minor modifications.

Briefly: A Topo Cloning reaction mix containing 4 µl of fresh PCR Product, 1.0 µl of salt solution and 1.0 µl of Topo® Vector was prepared and mixed in a 0.5 ml PCR micro tube. Care was taken to add the Topo Vector first, followed by the salt solution, finally the PCR product was added taking care not to mix the contents by flipping the tube or using a pipette. The tubes were incubated at room temperature (22-23°C) for 20 mins. The cloning reaction mix was then placed on ice before proceeding to the next step called the transformation stage.

Transformation in this experiment was carried out using One Shot® chemically competent *E. coli* cells. In this method, 2.0 µl of the Topo® Cloning reaction was added into a vial of One Shot® Top10 chemically competent *E. coli* and mixed GENTLY, followed by incubation on ice for 30 minutes and heat shock for exactly 30 seconds at 42°C without shaking. Immediately, the tubes were returned to ice. The next step involved the addition of 250µl of SOC enrichment medium which had been pre-heated to room temperature, the contents of the vial were then incubated in a shaking waterbath at 37°C for 1 hour. Pre-warmed petri dishes (plates) containing LB agar alone (Sigma), LB agar and 50 µl Kanamycin, LB agar and 100 µl Kanamycin and LB agar plates with 50 µl of ampicillin were inoculated with 50 to 100 µl transformation mix containing the SOC enrichment medium. It was found that when using smaller volumes (10 – 50 µl), as recommended in the manufacturers instructions, that the colonies failed to grow following overnight incubation at 37.5°C. However, when 100 ul of transformation mix was used ,small numbers of colonies were present following overnight incubation at 37.5 degrees centigrade,thus indicating low transformation efficiency.

The plates were examined after 24 hours and ten colonies showing good growth characteristics were selected from each DNA sample growing on the LB agar plates containing 50µl of Kanamycin. The selected colonies were stabbed onto fresh plates divided into sections using the end point of a sterile loop (one side only).

Analysis of the Positive Clones

This was achieved using two methods:-

(a) In the first method, PCR was used to directly analyse the positive transformants.

Briefly: This was achieved by preparing a PCR reaction mix as before but using a final volume of 20 µl of the mix instead of 45 µl. Ten colonies were picked and resuspended individually in 20 µl of the reaction mix. PCR amplification was carried out using a hot lid thermocycler (DNA Engine: PTC 200 Petier Thermal Cycler). In the initial denaturation step, the reaction sample mix was incubated at 94°C for 10 minutes. This was followed by 25 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds and primer extension at 72°C for 10 minutes after which the amplified product was stored at +4°C until removed from the thermocycler. Positive and negative controls were included. Agarose gel electrophoresis was carried out on the PCR product and the presence of the 337 bp band confirmed the presence of NC-1 *N. caninum* DNA.

(b) In the second method, the plasmid DNA from the positive clones was extracted and isolated, the NC-1 DNA insert was then removed using restriction analysis (EcoRi digest).

Briefly: The first step in this process was achieved using a commercially available Qia Prep^{R} Spin Miniprep Kit (Qiagen, California, USA) in accordance with the manufacturer's instructions. This procedure involved a series of repeated wash/spin steps using different kit buffers to purify the plasmid DNA. It is possible using this method to purify up to 20 µg of high copy plasmid DNA from overnight cultures of *E. coli* in LB Broth/Kanamycin medium. The final step involved the elution of the

plasmid DNA from the filter pad in the Qia prep column into a collection tube using an elution buffer.

Restriction Enzyme Analysis

A restriction enzyme EcoR1 (Biolabs Inc. New England, USA) was used to cleave the *N. caninum* DNA insert in the plasmid. A reaction mix containing 1.0 µl of enzyme buffer (1 x NE buffer EcoR1 supplied with the kit), 1.0 µl of EcoR1 restriction enzyme and 8.0 µl of plasmid DNA extract was made up in a PCR tube. Each plasmid sample was tested x 10. The mixtures were incubated at 37°C for 2.0 hours before storage at +4°C overnight.

Amplification was carried out using 20 µl of the standard PCR reaction mix and the 25 cycle programme described above. Agarose gel electrophoresis with appropriate positive and negative controls were also carried out as previously described. The size markers used were individual 1 Kbp and 100 bp DNA ladders (Invitrogen Inc, USA).

Nucleic Acid Sequencing

To sequence DNA, an ABI Prism® Big Dye Terminator cycle sequencing ready reaction kit (AB applied biosystems) was used in accordance with the manufacturer's instructions. Sequencing of the plasmid insert DNA products which were cleaved with the EcoR1 digest restriction enzyme involved three stages:-

(a) Amplification of the plasmid insert DNA by PCR.

(b) Purification of the amplified products.

(c) Sample electrophoresis using an ABI Prism 310 Genetic Analyser.

Amplification was carried out on a PCR reaction mix consisting of 8.0 µl of Terminator Ready Reaction mix, 1.0 µl of Template (EcoR1 digest double stranded DNA), 3.2 µl of T3 Primer, deionised H_2O to 20 µl. The PCR was carried out in thermocycler (Gene amp 2700) during which the initial denaturation step was carried out at 96°C using a rapid thermal ramp of 1°C/Sec. The cycle procedure itself consisted of 25 cycles during which denaturation occurred at 96°C for 15 secs, primer annealing at 50°C for 5 secs and extension using a rapid thermal ramp (1°C/Sec) to 60°C. Cycling concluded with a final extension step at 60°C for 4 mins followed by a rapid thermal ramp (1°C/Sec) to +4°C where it was stored until ready to purify. Purification of the PCR product was carried out using the ethanol/sodium acetate method.

Briefly: The following were added to capped 1.5 ml micro centrifuge tubes,

(a) 3.0 µl – 3 M sodium acetate Ph 4.6, (b) 62.5 µL – 95% ethanol,

(c) 14.5 µl – deionised H_2O, (d) 20.0 µl – PCR reaction mix, total 100.00 µl. (separate tube for each reaction). After a series of washes/spins by centrifugation, making sure to remove all the ethanol, the final pellet was allowed to dry at room temperature.

Sample electrophoresis was carried out using an ABI Prism 310 genetic analyser. The purified pellet of template DNA was resuspended in 20 µl of template suppression reagent (TSR). The sample was vortexed and spun, heated to 95°C for 2 mins before being chilled on ice, this process of vortex and spinning was repeated twice returning to ice each time. The samples were loaded on the ABI genetic analyser and sequenced according to the manufacturers users manual (P/N 903565).

Results

Maternal: Serology

The sera of the 21 ewes in the experimental groups, challenged with 1 x 10^7 *N. caninum* tachyzoites and the three ewes in the control group were screened for the presence of specific antibodies to *N. caninum* and *T. gondii* at day 0 post inoculation (dpi) and 28 dpi by means of ELISA and IFAT in the case of *N. caninum,* by Elisa and the direct agglutination (DA) test for *T. gondii.* The IFAT and DA tests were regarded as the confirmatory tests for each disease as appropriate.

All 24 ewes were seronegative by ELISA and IFAT for antibodies to *N. caninum* at 0 dpi, however, by 28 dpi all the ewes in the experimental groups were seropositive by IFAT, while those in the control groups remained negative. The ELISA test only identified one seropositive animal out of seven in each experimental group at day 28 dpi.. When the sera of the 24 ewes were screened by ELISA for antibodies to *T. gondii,* 11/21 in the experimental group and 2/3 in the control groups were seropositive at 0 dpi, the same ewes remained positive by 28 dpi. The results were confirmed using DA test, the findings are shown in Table 2.

Table 2. Summary of seropositive ewes for antibodies to *N. caninum* and *T. gondii* by ELISA or IFAT/DA test.

	Number of ewes giving positive results							
	N. caninum				*T. gondii*			
Group	ELISA		IFAT		ELISA		DA test	
	0 dpi	28 dpi	0 dpi	28 dpi	0 dpi	28 dpi	0 dpi	28 dpi
I	0	1	0	7	4	4	4	4
II	0	1	0	7	3	3	3	3
III	0	1	0	7	4	4	4	4
IV	0	0	0	0	2	2	2	2

When the antibody titres to *N. caninum* were measured in the sera of the 24 ewes in this study using IFAT, seroconversion was shown to occur between 0 dpi and 28 dpi in the experimental groups I to III. Prior to this study eleven experimental and two control ewes were seropositive for antibodies to *Toxoplasma gondii* (titre > $^1/_{40}$). Following challenge with *N. caninum*, the *T. gondii* antibody titre increased (not significantly) in the animals in groups II and III and IV which were already seropositive at 0 dpi. The antibody titres were expressed as the geometric mean titre (GMT). Tests were considered positive at a titre of $^1/_{256}$ for IFAT and $^1/_{40}$ for the DA test. The results are shown in Table 3.

Table 3. Endpoint titres expressed as the geometric mean titre (GMT) of *N. caninum* and *T. gondii* IgG antibodies in sheep infected with NC-1 tachyzoites during pregnancy showing 95% confidence intervals (CI's)

Group	*N. caninum*		*T. gondii*	
	[Log_2 x 10^3] (95% of CI's)		[Log_2 x 10^3] (95% of CI's)	
	0 dpi	28 dpi	0 dpi	28 dpi
I	0	1.7 (0.6 – 4.7)	1.7 (0.4 – 7.9)	1.7 (0.3 – 10.5)
II	0	1.4 (0.2 – 9.2)	1.4 (0.1 – 15.2)	3.4 (0.6 – 45.1)
III	0	0.6 (0.1 – 4.1)	1.4 (0.1 – 15.2)	2.1 (0.1 – 24.5)
IV	0	0.0	1.0 (0.0 – 47.8)	1.4 (0.1 – 25.9)

In this study, the monoclonal ELISA test identified specific antibody in 3/21 ewes at 28 dpi with NC-1 tachyzoites, however, IFAT identified all 21/21. (Table 2). Nevertheless, when the monoclonal ELISA test was used to detect specific antibody to *T. gondii*, it identified all 13/24 ewes that were seropositive for *T. gondii* at 0 dpi and at 28 dpi. However, these sheep had been infected with *T. gondii* prior to being included in this study, consequently high levels of circulating antibody were already present in their blood serum (Table 4).

Table 4. Initial and endpoint ELISA mean OD's at 0 dpi and 28 dpi for antibodies to *N. caninum* and *T. gondii* following challenge with *Neospora caninum* tachyzoites.

Group	* *N. caninum* [Mean OD]		** *T. gondii* [Mean OD]	
	0 dpi	28 dpi	0 dpi	28 dpi
I	0.06	0.37	0.50	0.82
II	0.03	0.47	0.40	0.57
III	0.04	0.29	0.64	0.63
IV	0.05	0.03	0.83	0.53

* Positive *Neospora* test interpretation = ≥ 30% OD, expressed as = ≥ 0.30.
** Positive *Toxoplasma* test interpretation = ≥ 50% OD, expressed as = ≥ 0.50.

Foetal Serology

The ELISA test failed to detect antibodies to *N. caninum* or *T. gondii* in any of the 26 foetal sera. However, when the foetal sera were examined by IFAT for the presence of IgG and IgM antibodies to *N. caninum*, both classes of antibody were found in 3/26 foetal sera whose dams had been experimentally infected during pregnancy. Low levels of IgG antibody were also found in the serum of a fourth foetus, however, there was no evidence of IgM present, these results are shown in Table 5.

Table 5. Endpoint titres (IFAT) of IgG and IgM in foetuses seropositive for *N. caninum*

Group	IgG		IgM	
	Number +/-	*Titre	Number +/-	*Titre
I	0/7	-	0/7	-
II	1/7	1280	1/7	2560
III	3/9	320	2/9	2560
		80		-
		2560		20,480
IV	0/3	-	0/3	-

* Titre expressed as a reciprocal.
** Number +/- = positive/negative.

Necropsy

Following inoculation of 21 pregnant ewes with 1 x 10^7 *N. caninum* during early to mid pregnancy, 17 out of 28 foetuses were shown to have died when their dams were examined by ultrasonography shortly before necropsy at 28 dpi. There was no evidence of foetal death prior to necropsy in the control group. Most foetal deaths occurred in dams which had been inoculated during the early stages of pregnancy (see Table 6 for details).

Table 6: Number of foetuses that were alive or died in utero prior to necropsy following experimental inoculation of the dam with NC-1 tachyzoites at different stages of gestation.

Group	Stage of Gestation (Inoculated)	Foetuses		
		Dead	Alive	Total
1	53 days ± 8 days	8	0	8
2	70 days ± 8 days	7	4	11
3	87 days ± 8 days	2	9	11
4	Control ± 8 days	0	3	3

In all three groups, in which foetal death was recorded, the most consistent gross finding was the presence of extensive oedema in the foetal body cavities. The abdominal cavities of most dead foetuses, depending on the degree of autolysis, contained significant volumes of blood stained fluid. The livers were enlarged and congested, subcutaneous oedema was also present in most cases. The placentae from foetuses which had died recently in utero were usually intact and provided good specimen material for histopathology, immunohistochemistry and PCR. In other cases where extensive autolysis had occurred, samples were deemed unsuitable for examination by any of these methods.

Histological and Immunohistochemistry Findings

Characteristic lesions of neosporosis were most frequently observed in the foetal brain and placenta, lesions were rarely observed in the myocardium. Tissue sections were initially examined using slides stained by Erhlick's routine H and E method. Confirmation of the presence of the parasite was made by immunostaining, the results are summarised in Table 8.

Brain: In the brain, encephalitis was characterised by multifocal microgliosis and leucomalacia. Cellular infiltration by mononuclear cells, probably of the monocyte and lymphoid series, frequently surrounded the areas of necrotic foci. Dystrophic mineralisation, in the centre of the necrotic area, was not a feature of the lesion. In specimens where the meninges were still attached, encephalitis was usually accompanied by small localised areas of meningitis with varying degrees of mononuclear cell infiltration. Perivascular cuffing with mononuclear cells was frequently observed in areas bordering areas where gliosis had occurred. A feature of this cuffing was that it often tended to be concentrated to one side of the blood vessel wall. Lesions were most commonly found in the cerebrum and mid-brain, protozoal cysts were not observed in any sections of brain examined.

Immunostaining with Envision^{TM} polymer and BPA-I antiserum revealed the presence of dark brown staining tachyzoites, cut in various planes, in the areas where focal gliosis and necrosis was most pronounced. Individual or small clumps of tachyzoites were frequently located near the periphery of the area of necrosis. Numerous small pieces of particulate antigen, which were probably tachyzoite debris resulting from the cellular response, were usually found amongst the much less numerous intact tachyzoites in the areas of gliosis (Figures. 1 and 2).

Figure 1 A characteristic focus of necrosis (↙) recovered surrounded by diffuse areas of gliosis (G) in the cerebrum of a foetus recovered at 28 dpi from a dam inoculated with NC-1 tachyzoites at 70 dg. A discrete group of tachyzoites are shown arrow (→). En Vision™ Immunoperoxidase stain. X 100.

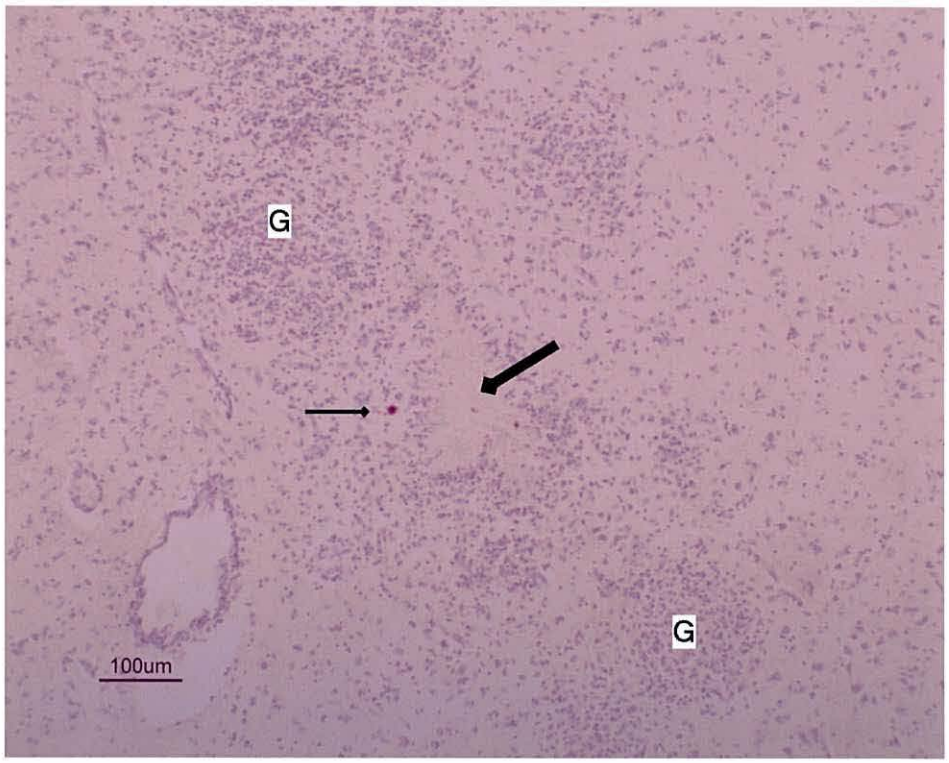
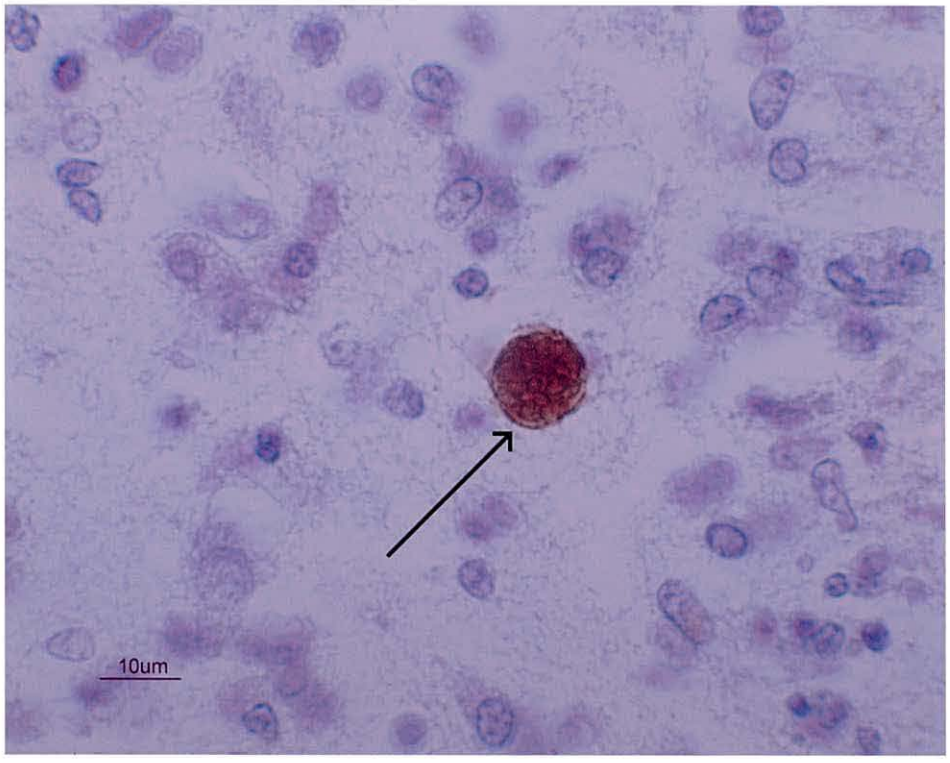


Figure 2 Higher magnification of the group of tachyzoites shown in figure 1. Tachyzoites are clearly visible(↗) . En Vision™ Immunoperoxidase stain. X 1000.



Placenta: The histopathological changes in the placenta were often extensive and primarily involved the placentomes, which consist of foetal placental cotyledonary villi, interdigitating intimately with maternal caruncular septae. The characteristic lesion consisted of focal necrosis of the surface endothelial and other cells of the maternal carunclar septae. The adjoining trophoblast cells of the foetal villi were largely intact, indicating that the early stages of transplacental infection of the foetus was taking place. The degree of necrosis varied between different foetuses and between different areas of the same placentomes. The lesion was sometimes quite extensive, leading to necrosis of the underlying connective tissue and even the walls of the smaller blood vessels, causing localised areas of haemorrhage. Immunostaining confirmed the presence of positively staining antigen which usually consisted of brown staining particulate material similar to that observed in brain. Tachyzoites occurring either singly or in small clumps, were also present in these lesions. The amount of positively labelled *N. caninum* tachyzoite-like material varied greatly in different placentomes. Mineralisation was not observed in any of the lesions (Fig. 3, 4 and 5).

Other tissues: In one case, a mild non-suppurative mononuclear infiltration with oedema, was observed within the epicardium. These lesions were confirmed by immunostaining but the amount of positively labelled antigen observed was very small and involved single tachyzoites with small amounts of particulate material. No significant lesions could be confirmed by immunostaining in the other foetal tissues examined.

All foetal placental, brain, heart and other tissues from the control group appeared normal when examined by H & E and immunostaining. Due to extensive autolysis in some foetal tissues at the time of necropsy, not all samples were deemed

suitable for histopathology or immunohistochemistry. The results are summarised in Table 7.

Table 7. Summary of lesions found in 33 foetuses obtained from ewes slaughtered 28 days after inoculation with NC-1 tachyzoites during pregnancy. *Number of samples positive/total number of samples (-/-). Day of gestation (dg).

Tissue	Group 1 49 dg 9 Foeti	Group 2 70 dg 11 Foeti	Group 3 87 dg 9 Foeti	Positive Samples Groups 1-3	Total Feotal Samples Groups 1-3	Group 4 Control 3 Foeti
Brain	*4/7	5/9	5/7	14	23	0/3
Placenta	3/9	5/11	3/6	11	26	0/3
Heart	0/5	1/10	0/7	1	22	0/3
Lung	0/2	1/10	0/6	1	18	0/3
Liver	0/3	0/11	0/7	0	21	0/3
Kidney		0/3	0/4	0	7	0/1

Figure 3 Extensive area of inflammation indicated by arrow (→) primarily involving the maternal caruncular septa (MC) of the placentomes obtained from a ewe at 98 dg which had been inoculated with tachyzoites at 70 dg. Foetal placental villi (FV). H + E. X 40.

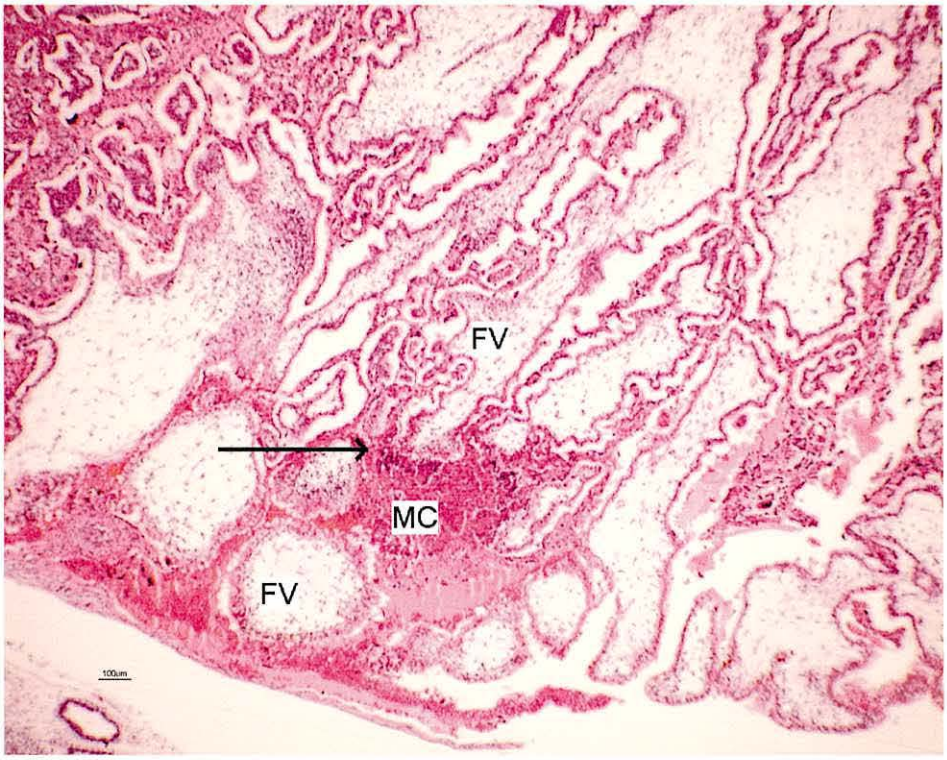


Figure 4 Higher magnification of figure 3 showing large numbers of mononuclear inflammatory cells (↗) in the area of the maternal caruncular septa (MC) close to the placental foetal villi (FV). H + E. X 1000.

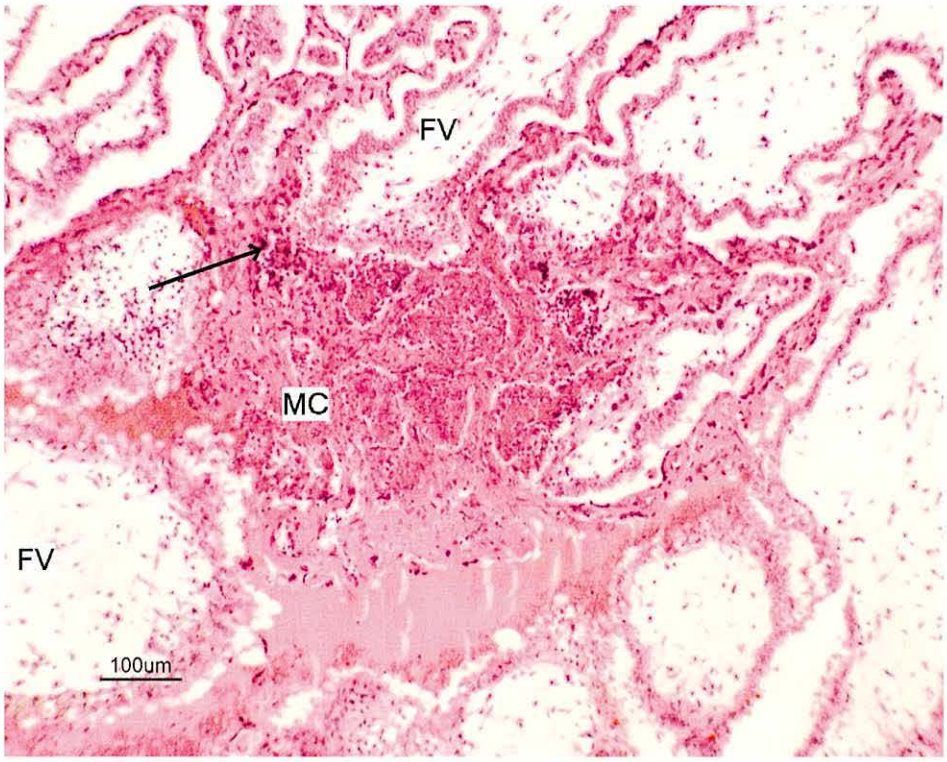


Figure 5 Higher magnification of figure 4 showing large numbers of inflammatory cells with dark staining material which are the remains of broken down tachyzoite antigen (↗). Trophoblast cells of foetal villus (FV) are largely intact except at tip of villus (↙). H + E. X 200.

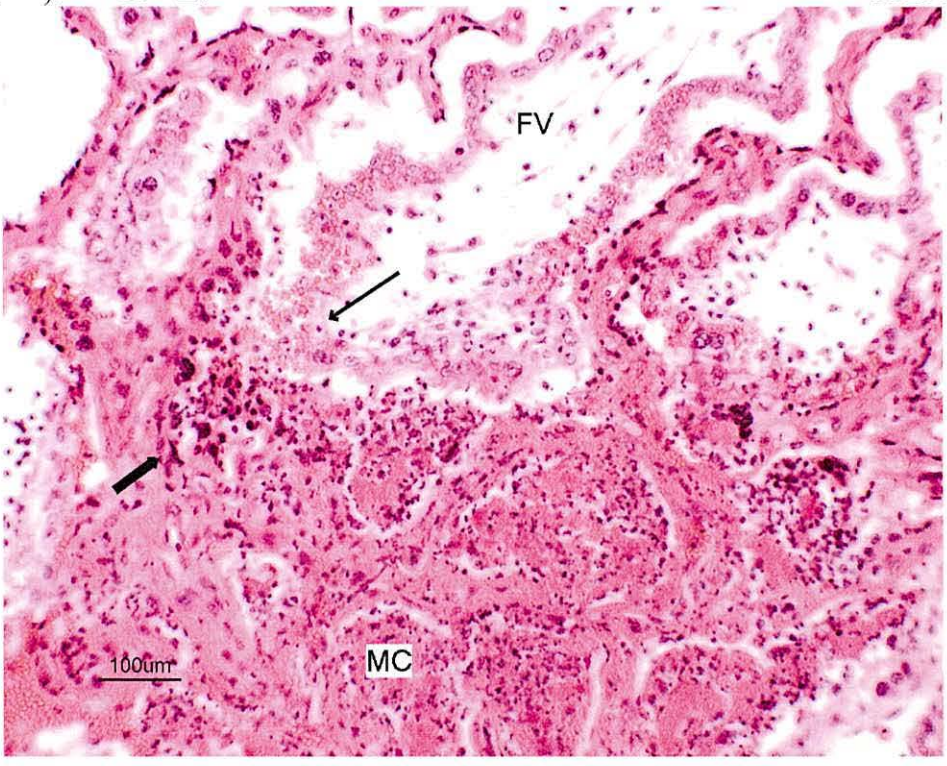


Figure 6 A section of a placentome removed from a ewe at 98 dg. which was infected at 70 dg with tachyzoites. The lesion consisting of inflammatory cells and areas of necrosis is largely confined to the maternal caruncular septa (→ MC). FV= Foetal Villus. En Vision™ Immunoperoxidate stain . X 40.

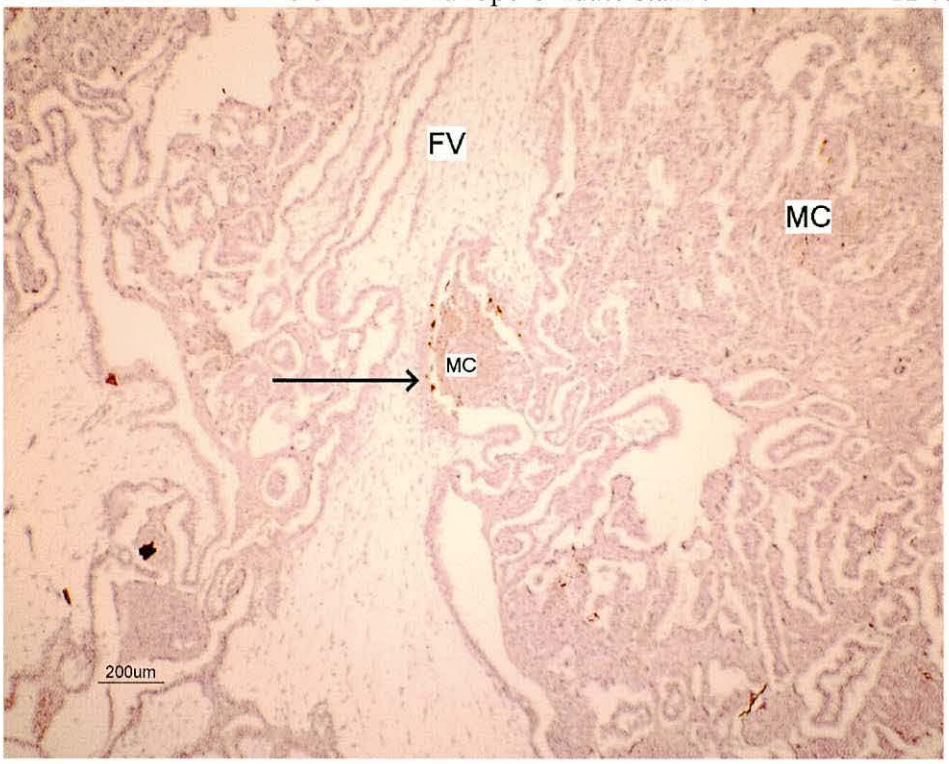


Figure 7 Higher magnification of figure 6 showing discrete brown stain foci which represent immunohistochemical demonstration of *N. caninum* tachyzoites (→). Extensive necrosis of the maternal caruncular septal cells is present (←). FV= Foetal villus. MC= Maternal caruncule. En Vision™ immunoperoxidase stain. X 100.

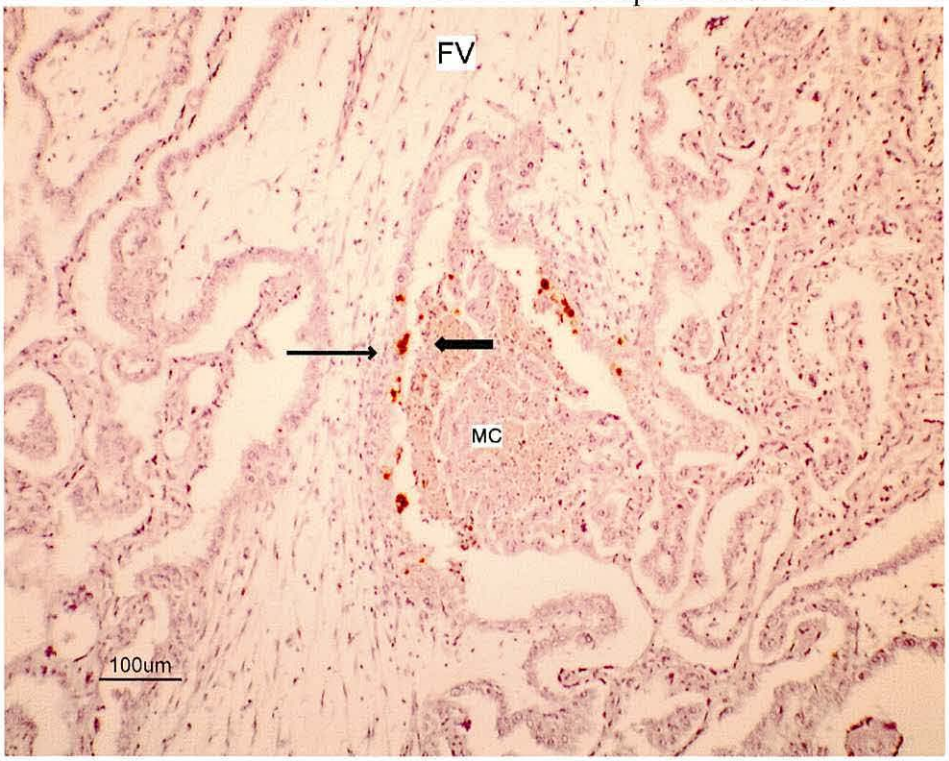


Figure 8 Higher magnification of figure 7, showing early invasion of the of the trophoblast cells lining the outer surface of the foetal placental villi by intact tachyzoites (→). FV= Foetal placental villus. MC= Maternal caruncular septa. En Vision™ immunoperoxidase stain. X 200.

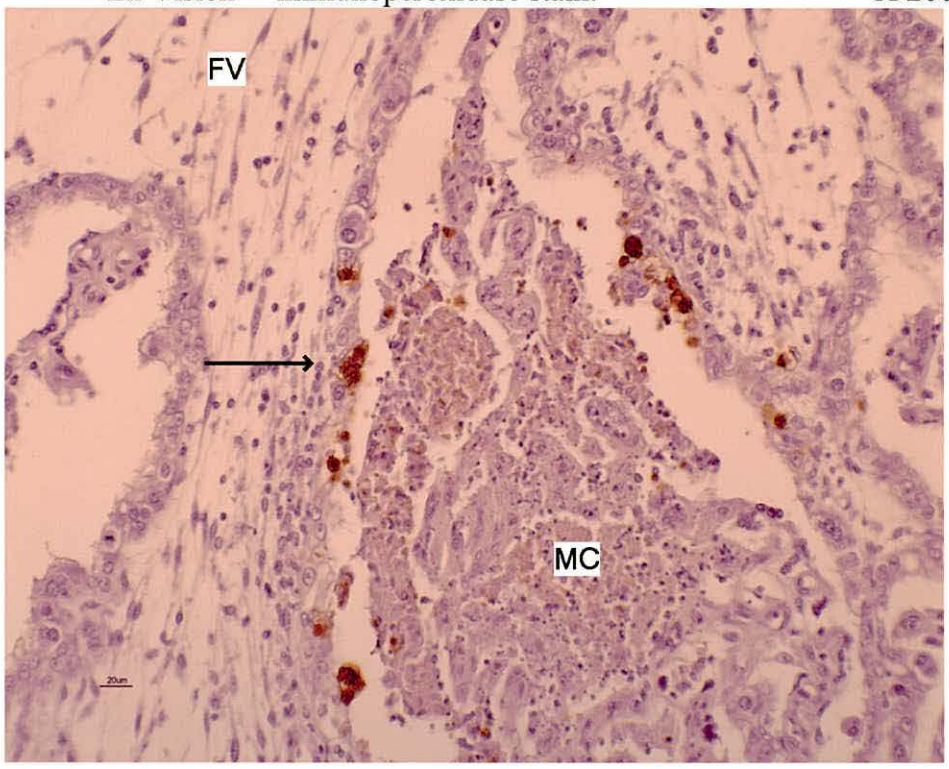


Figure 9 Higher magnification of figure 8 showing two clusters of tachyzoites within the trophoblast cells (↘) of the placental foetal villi. FV= Foetal placental villi. MC= Maternal caruncule septa. En Vision™ immunoperoxidase stain X 400.

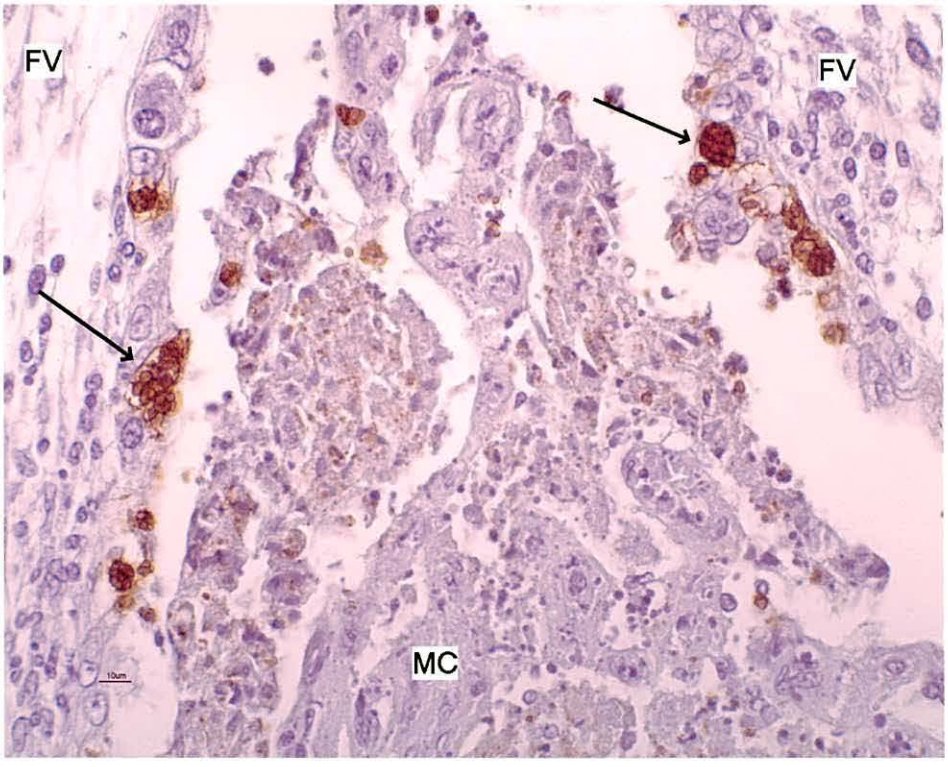
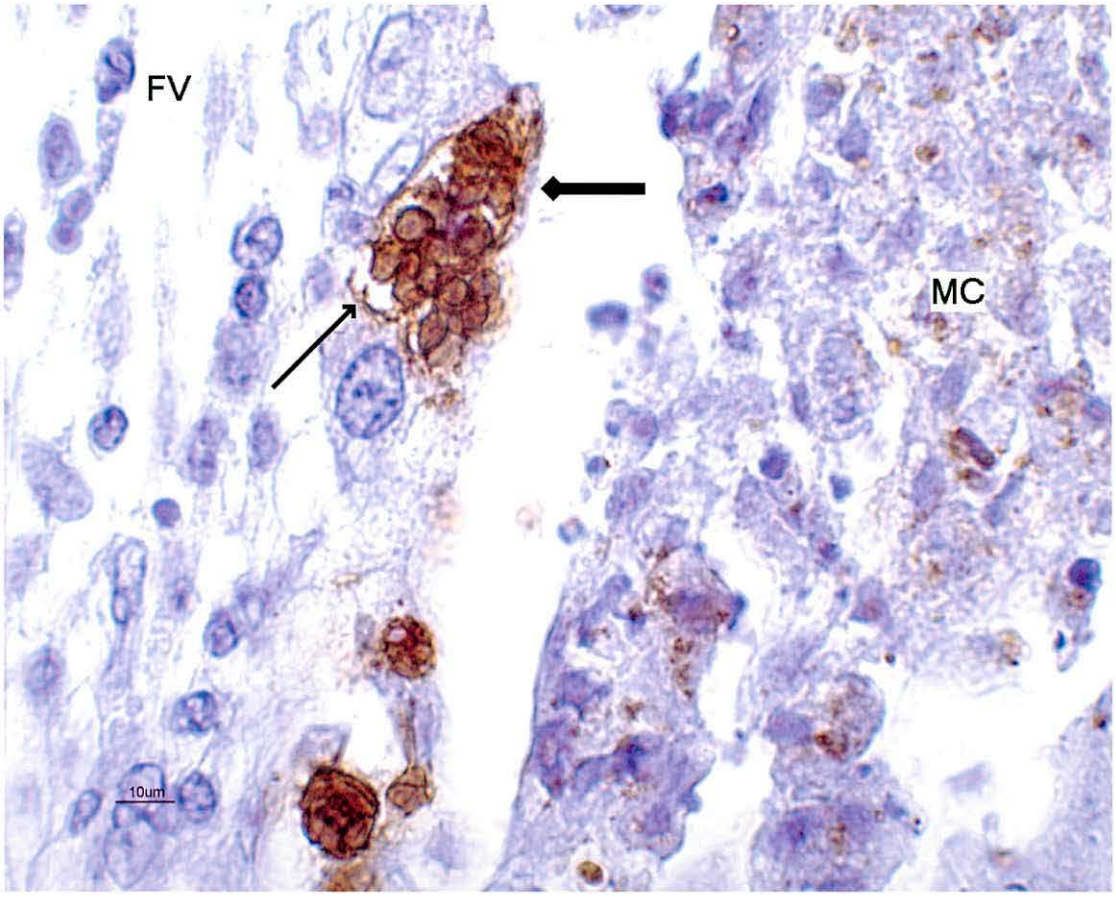


Figure 10 Higher magnification of figure 9 showing large numbers of intact tachyzoites within the trophoblast cell (←) wall of a foetal placental villus (FV). The parasitophorous vacuole surrounding the tachyzoites is clearly visible (↗). Areas of extensive necrosis of the cells in the maternal caruncular septa (MC) are also present. En Vision™ immunoperoxidase stain. X 1000.



Polymerase Chain Reaction

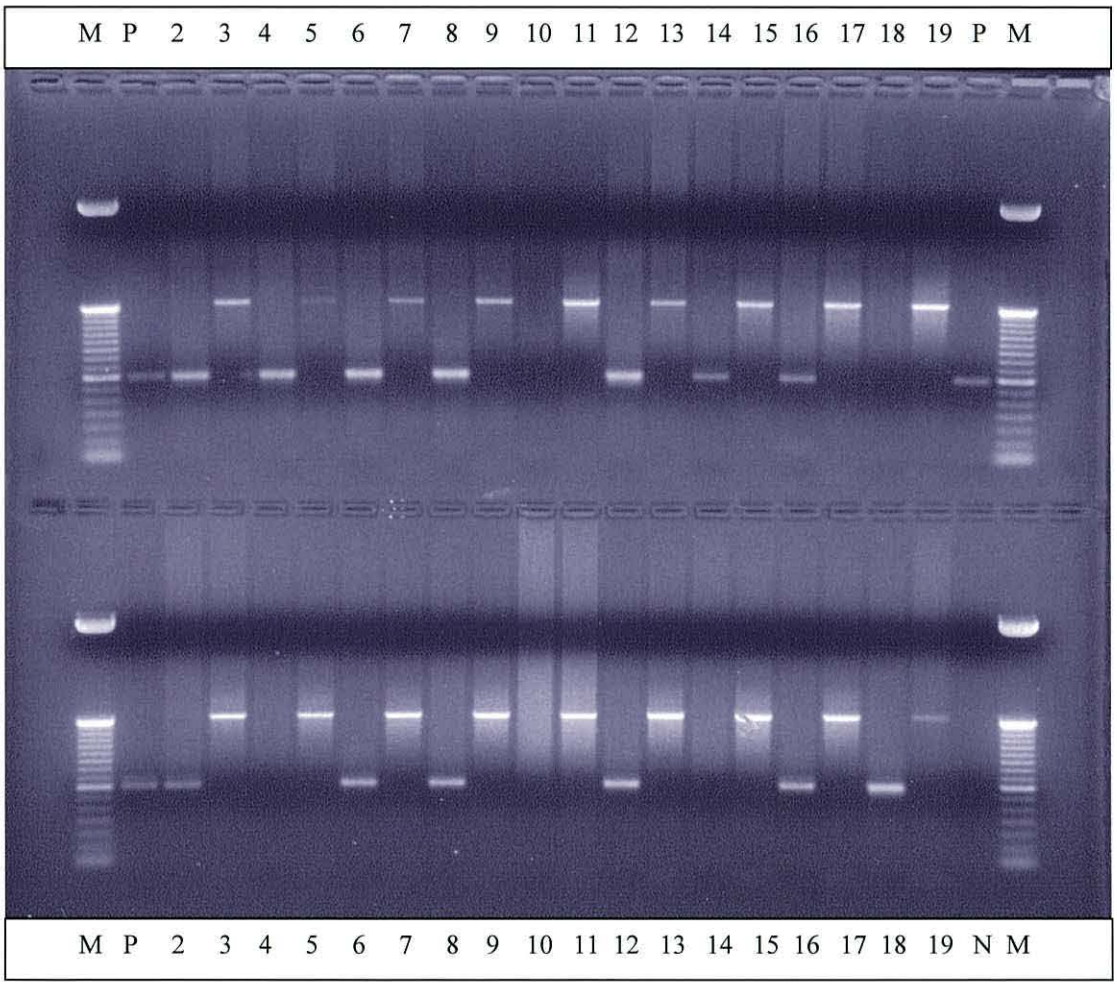
The PCR results in Table 8 are not directly comparable to those achieved using histology and immunohistochemistry, because sometimes, suitable sufficient tissue was not available.

The combined histological/immunohistochemical and PCR results for brain and placenta were almost identical, with both test methods identifying the same positive tissues. However, in the case of the other tissues, the PCR had a much higher detection rate. A typical agarose gel showing the detection of *Neospora caninum* by PCR of samples extracted from the brain and other tissues of foetuses obtained from ewes following experimental infection with NC-1 tachyzoites in this study is shown in Figure 11.

Table 8. Comparison of Histological/Immunohistochemistry and PCR results following examination of foetal tissues obtained from ewes inoculated during early pregnancy with NC-1 tachyzoites. *Number of positives/number of samples tested (-/-).

Tissue	PCR				Total PCR Excluding Control	Histology & IHC Positive
	Group 1 49 dg	Group 2 70 dg	Group 3 87 dg	Group 4 Control		
Brain	*5/8	3/7	5/9	0/3	13/24	14/23
Placenta	5/5	5/8	2/9	0/3	12/22	11/26
Heart	4/8	3/8	3/9	0/3	10/25	3/22
Liver	4/6	4/7	2/9	0/3	10/22	1/18
Lung	5/6	3/6	3/9	0/3	11/21	0/21
Kidney	N/T	4/6	4/9	0/3	8/15	0/7

Figure 11 Detection of *Neospora caninum* DNA in various ovine foetal tissues by PCR on a 1.5% agarose gel stained with ethidium bromide.



Lanes

M	50 bp DNA size ladder	
P	Positive control (3) *N. caninum* DNA	
N	Negative control (1) Purified H_2O and PCR reaction mix	
2 → 18	Even numbered lanes extracted DNA from ovine foetal tissues including liver, lung, placenta and kidney.	337 bp
3 → 19	Odd numbered lanes house keeping control DNA.	818 bp

Cloning and Sequencing

A Sample of brain DNA recovered from a foetus which had been aborted at 98 days of gestation (dg) whose dam had been inoculated with tachyzoites at 70 dg was cloned in *E. coli*. Similarly, a sample of DNA extracted from the stock culture of *N. caninum* tachyzoites used to infect the dam was similarly cloned. Cleavage of the plasmid inserts was achieved by means of EcoRI restriction enzyme digestion. The cleaved plasmid inserts were sequenced in triplicate in one direction using the T3 primers previously described. The resulting sequences were aligned using the European Bioinformatics Institute Software programme and a consensus sequence determined for each of the two samples. Comparison of the sequences, which were 344 bp long showed 98.3% similarity with no gaps (fig. 12).

Figure 12. Nucleotide alignment of *Neospora caninum* (NC-1) used to infect the dam and the PCR amplification product recovered from the brain of the foetus. Dots indicate nucleotides identical in both sequences.

NC-1	1	CTCGCCAGTC	AACCTACGTC	TTCTGCCTCT	TCCCTCGTCC
612-4		.....	.....	.....C.....	.....
NC-1	41	GCTTGCTCCC	TATGCATAAT	CTCCCCCGTC	ATCAGTGCCG
612-4		.....	.....	.....A.....	.....
NC-1	81	CCGGTGTTGC	CTCAACACAG	AACACTGAAC	TCTGGATAAG
612-4		.....	.....	.....	.....
NC-1	121	TATCATTGAC	ACACTGTCCA	CACCCTGACG	CAGGCTGATT
612-4		.....	.....	.....	.....C....G
NC-1	161	TCAACGTGAC	GAATGACTAA	CCACAAACCA	CGTATCCCAC
612-4		.....	.....	.....	.....
NC-1	201	CTCTCACCGC	TACCAACTCC	CTCGGTTCAC	CCGTTCACAC
612-4		.....	.....	.....	.....
NC-1	241	ACTATAGCCA	CAAACAAAAA	AGGAGCCTTG	CTGCCGCAGG
612-4		.....	.....	.....	.....
NC-1	281	CTGCGCCCAA	CAACGACACG	TCCGCATCCG	CACAGCAACA
612-4		.....	.....	.....	.....
NC-1	321	CGTTACAGGA	TTGGACGCAC	TGGG	
612-4		.....	.....	.....	

Discussion

Experimental neosporosis in sheep is clinically and histopathologically similar to that reported in cattle (McAllister et al., 1996(a)). Pregnant and non-pregnant sheep, inoculated with *N. caninum* tachyzoites under experimental conditions have been shown to provide a good model for the study of bovine neosporosis (Buxton et al., 1997 , Jolley et al., 1999,). The protozoan parasite, *T. gondii* has a worldwide distribution and is recognised as a major pathogen for pregnant ewes (Buxton, 1998). In Ireland, it has been reported that up to 55.6% of aborted ewes were seropositive for the disease (O'Brien and Geraghty, 1990). It has been suggested that there is some serological cross reactivity in animals exposed to *T. gondii* or *N. caninum* antigens. It has been shown that pregnant ewes developed low antibody titres to *T. gondii* following exposure to *N. caninum* using the IFAT (McAllister et al., 1996(a)).

In this study, pregnant ewes, whose pregnancies had been confirmed using ultrasound imaging, were randomly assigned into four groups of ewes without prior confirmation of their serological status for antibodies to *N. caninum* or *T. gondii*. During the course of the project, blood serum samples taken immediately prior to the experiment from all 24 ewes, were found to be seronegative for antibodies to *N. caninum*. However, 13/24 were seropositive for *Toxoplasma gondii,* the remainder were seronegative. Following inoculation of the experimental groups with 1 x 10^7 *N. caninum* tachyzoites, antibody titres to *T. gondii* slightly increased in 5/13 of those ewes already seropositive for this parasite, in one ewe a slight decrease occurred. The sero-status of all eleven ewes that were seronegative for antibodies to *T. gondii* remained unchanged throughout the period of the study.

The results of this study show that a slight serological cross-reactivity occurred when sheep, which were already seropositive for *T. gondii* were inoculated with *N. caninum* antigen.

The outcome of pregnancy was determined by the stage of pregnancy at which the ewes were infected with *N. caninum*. In ewes in Group 1, all 8/8 foeti had died at 28 dpi following challenge at 49 ± 8 dg. However, 7/11 died in Group II when the dams were challenged at 70 ± 8 dpi. In contrast only 2/9 were dead in Group III at the time of necropsy of ewes which had been inoculated at 87 ± 8 dg. The lesions in the placenta and foetal brain in all three experimental groups were very similar to those previously reported by Buxton et al.(1997), where sheep were infected at 90 dg and slaughtered at 25 dpi. The results in this study showed that in ewes that were infected at 49 dpi, foetal death occurred in all cases before necropsy at 28 dpi. In some cases, extensive autolysis had taken place confirming early foetal death. With increasing days of gestation, the number of foetal deaths occurring by 28 dpi had decreased significantly resulting in excellent tissue for analysis.

In this study, *N. caninum* was shown to be responsible for the lesions observed using PCR and IHC. However, when DNA from the brain of the only foetus which was aborted in this study was extracted, cloned and sequenced, four base pair changes were identified when the sequence was compared with that of the DNA extracted from the NC-1 tachyzoites which were used to infect the dam. It has been suggested that the differences in the sequence were due to the primers being directed towards the non-conserved areas of the gene rather than a new strain of *N.caninum* being identified in the brain of this aborted foetus (Muller, personal communication). Another possible explaination is that multiple copies of the same gene exist in *N.caninum* .

Chapter 9

The antibody response and outcome of pregnancy in cattle vaccinated against neosporosis when subsequently challenged by experimental infection with *N. caninum*

Summary

In this study, 35 cows were experimentally challenged with NC-1 tachyzoites and/or vaccinated against neosporosis during pregnancy using a commercially available vaccine at 115 ± 10 days of gestation (dg). Five pregnant cows were inoculated with Vero cells and served as negative controls.

All forty cows were seronegative for antibodies to *N. caninum* prior to the experiment. Following challenge and/or vaccination, the cows all seroconverted. However, the level of antibody response varied between groups depending on the treatment given. The negative control animals remained seronegative throughout the period of the study. The cows gave birth to 40 full term calves, however, while 37 were clinically normal two were stillborn and a third died twelve hours after birth. At necropsy, there was no evidence following histopathological and PCR examination of brain and placental tissues that *N. caninum* was associated with the deaths of these calves.

In the absence of abortions, it was not possible to draw conclusions as to the efficacy of vaccination in preventing foetal death in cows experimentally inoculated with *N. caninum* tachyzoites during pregnancy.

Introduction

The intracellular *Neospora caninum* protozoan parasite is closely related to *Toxoplasma gondii* (Dubey et al., 1988(a)). It has been shown to be a major cause of abortion in cattle throughout the world (Dubey and Lindsey, 1996 , Dubey, 1999). In cows, infected with *N. caninum* abortion is the only clinical symptom observed which may occur at any stage of gestation from three months to term (Dubey and Lindsay, 1996). However, the majority of abortions occur during the 4^{th} to 6^{th} month of pregnancy (Anderson et al., 2000). The foetus may also die in utero, be reabsorbed, mummified, autolysed, stillborn, born alive but diseased or born clinically normal but congenitally infected (Dubey and Lindsay, 1996).

Following either natural or experimental challenge with *N. caninum* tachyzoites or oocysts, the immune system has been shown to respond with the production of specific antibodies, cytokines, and associated cells of the cellular immune system (De Marez et al., 1999, Guy et al., 2001, Williams et al., 2000, Andrianarivo et al., 2001). It is not clear how effective the humoral response may be in providing protective immunity against intracellular protozoan parasites. Protection against such organisms has been shown to most likely involve a cell-mediated immune response, similar to that involved with host resistance to another protozoan parasite, *T. gondii*, which is itself, closely related to *N. caninum* (Gazinelli et al., 1993). It has been suggested that the humoral response may be involved in controlling the spread of *N. caninum* by neutralising infection of host tissue cells at the extracellular stages of the parasite; however, the exact mechanisms involved are not clear (Innes et al., 2002).

The stage of gestation, during which infection occurs, has been shown to determine the outcome of pregnancy in cattle and sheep. Foetuses of cattle and sheep

infected in the early stages of pregnancy generally die in utero or are aborted whereas those infected late in gestation often survive (, Barr et al., 1994, Buxton et al., 1998, Williams et al., 2000). Recently, it has been shown in cattle that protective immunity against vertical transmission, which is the major route of infection, could be induced by experimental infection with *N. caninum* tachyzoites before pregnancy (Innes et al., 2001). These findings suggest that vaccination using live virulent tachyzoites may provide a protective immunity in cattle and possibly other species.

Currently, there is no effective method for the control of neosporosis. Measures adopted at farm management level include those aimed at reducing the contamination of feed and water supplies, these may not be economical or indeed practical.

The purpose of this study was to evaluate the effectiveness of vaccination of pregnant cows, using a commercially available vaccine against experimental challenge following inoculation with *N. caninum* tachyzoites during pregnancy. This vaccine was the first commercially available vaccine on the market in 2000. It was produced by the Bayer Corporation (Kansas, USA) and was provisionally licenced for use in cattle. The vaccine consisted of dead *N. caninum* tachyzoites combined with an adjuvant. The antibody response and the outcome of pregnancy were the parameters used to evaluate the efficacy of this vaccine as a method of reducing the number of abortions in cattle due to infection with *N. caninum.*

Materials and Methods

Cattle

This study was carried out during the period March, 2000 to April, 2001. The composition and management of the herd was as previously described in Chapter 7. Forty cows at 90 ± 10 days of gestation (d.g.) were randomly selected from this herd and were divided into four experimental groups (Table 1).

Table 1: **Experimental Design**

Group	Number of Cows	Treatment
I	15	Vaccine & 2.2 x 10^7 *N. caninum* tachyzoites
II	5	Vaccine only
III	15	2.2 x 10^7 *N. caninum* tachyzoites
IV	5	2 x 10^6 Vero cells

Group 1 was vaccinated in accordance with the instructions provided by the manufacturer (Bayer Corp. KS, USA). The cows received the first dose of 5 ml subcutaneously at 90 ± 10 days of gestation (d.g.). A second dose was administered 21 days later. After a further 14 days (125 ± 10 d.g.) the cows were inoculated intramuscularly with 2.2 x 10^7 NC-1 tachyzoites of *N.caninum* in 10 ml of phosphate buffered saline (PBS). Blood samples for serology were taken immediately prior to each inoculation and thereafter at 28 day intervals until calving.

Group 2 served as the vaccine control group and received 5 ml subcutaneously of vaccine on two occasions as described above, however, they were not challenged by an experimental inoculum of NC-1 tachyzoites . Group 3 served as the challenge group and were inoculated with 2.2 x 10 NC-1 tachyzoites at 125± 10 d.g. as previously described. Group 4 served as the negative control group and were inoculated with 2.0 x 10^6 Vero cells in 10 ml sterile PBS intramuscularly, this dose

was equivalent to that contained in the inoculum of NC-1 tachyzoites administered to the cows in the two challenge groups (I and III).

Serology

Blood samples were tested for the presence of antibodies to *N. caninum* using a commercially available ELISA kit (herd check* *Neospora caninum* antibody test kit, IDEXX, Westbrook, Main, USA) and a commercially available IFAT test kit (VMRD, Inc., P.O. Box 502, Pullman, WA, 99163, USA,). Both kits were used in accordance with the manufacturers instructions.

Histology

Routine histology involving the cutting of 4μm sections followed by staining with the standard Ehrlich's haemotoxylin and eosin method was used to examine the tissues from any cases of neonatal mortality which occurred during the study.

Tissue Culture

Experimental inoculations contained *Neospora caninum* (NC-1) tachyzoites grown in Vero cell tissue cultures and harvested according to the method described in chapter 6.

PCR

The PCR method described in chapter 7 was used to examine tissues from any cases of neonatal mortalities which occurred during this study.

Results

Outcome of Pregnancy

All cows calved normally, two full time calves were stillborn and a third died twelve hours after birth. Following necropsy, histopathological examination, serology and PCR of brain and placental tissues, there was no evidence that *N. caninum* was associated with the deaths of these calves.

Serology

The levels of specific antibody to *N. caninum* in the blood serum of cows, which had been vaccinated and/or challenged with experimental inoculations of NC-1 tachyzoites during pregnancy were measured by the immunofluorescent antibody test (IFAT) and by enzyme linked immunosorbent assay (ELISA). Serum antibody titres $\geq {}^{1}/_{200}$ (IFAT) or those with an optical density (OD) ≥ 0.500 (ELISA) were considered positive.

The cattle in this study were all seronegative by both test methods prior to the experiment. The negative control group IV remained seronegative throughout the period of the trial. Seroconversion occurred in those cattle which had been vaccinated and subsequently challenged with an experimental inoculation of NC-1 tachyzoites (Group I) shortly after the initial dose of vaccine. Antibody levels increased rapidly after the challenge dose at 125 dg, before decreasing as calving approached.

A similar, but lower level antibody response occurred in those cattle which had been challenged at 125 dg with NC-1 tachyzoites without prior vaccination (Group III). However, the cattle in Group II which had been inoculated with vaccine only

showed a low level transient antibody response. The results are shown in tabular form in appendix D and are illustrated in Fig. 1 and Fig. 2.

Figure 1: Summary of antibody titres (IFAT) to *N. caninum* in cattle following vaccination and challenge with NC-1 tachyzoites (Group 1), vaccine only (Group II), challenge without prior vaccination (Group III). The negative control (Group IV) was seronegative and is not shown.

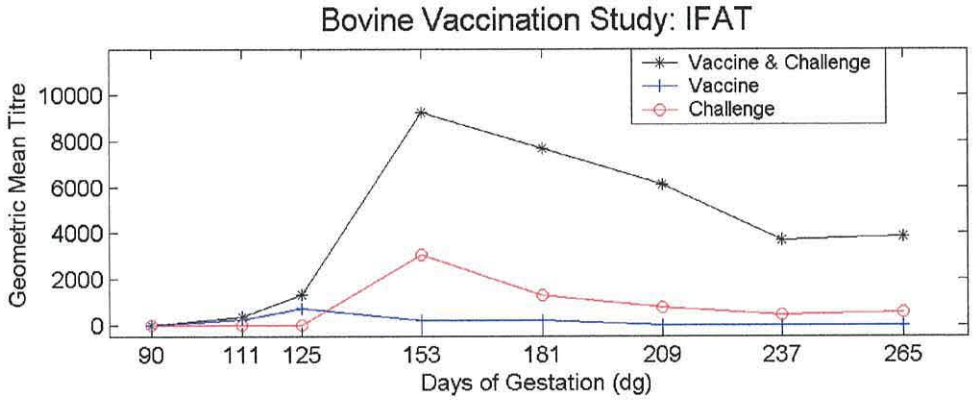
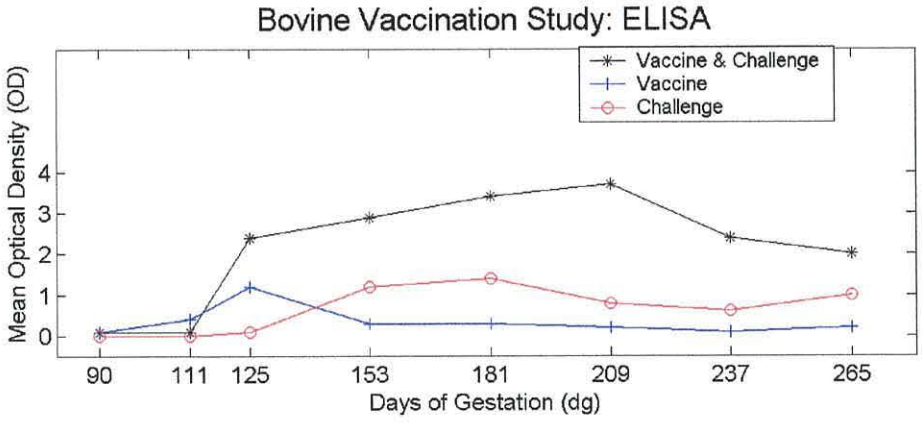


Figure 2: Summary of antibody titres (ELISA) to *N. caninum* in cattle following vaccination and challenge with NC-1 tachyzoites (Group 1), vaccine only (Group II), challenge without prior vaccination (Group III). The negative control (Group IV) was seronegative and is not shown.



Discussion

Currently, the control of neosporosis in cattle is limited to adopting measures at farm level aimed at preventing or reducing contamination of cattle feed and water by dogs and culling of infected cows. However, these control methods are often not practical or economically possible.

Following natural or experimental infection with *N. caninum*, cattle normally respond immunologically with the production of specific antibody and cell-mediated responses which involve proliferation of cells and cytokine production particularly of interferon gamma (IFN-γ) (Lunden et al., 1998, De Marez et al., 1999, Williams et al., 2000, Guy et al., 2001, Andrianarivo et al., 2001,). Typically, these two types of responses are cross-regulatory and tend to down-regulate each other but not to the exclusion of either type of response. In most situations, the two mechanisms interact in a complex regulatory pathway (Allen and Maizels, 1997).

There is evidence that some *N. caninum* infected animals can develop a degree of protective immunity against abortion or congenital transmission. Most of the experimental work has been carried out in mice, where it has been shown that vertical transmission was completely prevented when BALB/c mice were inoculated with *N. caninum* tachyzoites (Liddel et al., 1999). Recently in cattle, it has been demonstrated that when cows are experimentally inoculated with *N. caninum* tachyzoites prior to mating, they developed sufficient immunity to protect against vertical transmission to the foetus following experimental challenge at the mid-trimester of pregnancy (Innes et al., 2001). In that study, there was a rapid onset of cell-mediated immune responses immediately following challenge which was accompanied by a significant specific antibody response. In a separate study, when pregnant cattle were challenged by

inoculation with *N. caninum* tachyzoites at various stages of gestation, there was a very significant rise in specific IgG_2 antibody levels (Williams et al., 2000). It is not clear how effective antibodies are in controlling the spread of infection due to neosporosis, as in general, immunity to intracellular parasites is mainly cell-mediated (Lunden et al., 2002, Ritter et al., 2002). It has been postulated that they may have a role in limiting the spread of the disease by neutralising the infection of cells by the extracellular stages of this parasite ([Innes et al., 2002). While their exact role in the control of neosporosis is unclear, their presence in the sera of animals infected with *N. caninum* provides an useful method of tracking the development of the disease at various stages of infection in host animals.

In this project, the efficacy of a commercial vaccine to prevent abortion in pregnant cows which had been vaccinated during early pregnancy and subsequently challenged by an experimental inoculation of *N. caninum* tachyzoites was evaluated. The effectiveness of vaccination was assessed by measuring the antibody response and evidence of foetal death or abortion. Previous work has shown that experimental inoculation of cows with NC-1 tachyzoites during pregnancy resulted in foetal death followed by mummification, re-absorption or abortion (Barr et al., 1994, Dubey and Lindsay, 1996 , Andrianario et al., 2000). While the aim of the present study was to determine whether cattle vaccinated during pregnancy with *N. caninum* would develop sufficient immunity to protect against experimental challenge in mid-pregnancy, in the absence of abortion, particularly in Group III which were challenged but not vaccinated, it was not possible to evaluate the effectiveness of vaccination in preventing foetal death.

Nevertheless, during the course of the study, several interesting observations occurred in relation to the challenge trials and to the serological response. In this study, cattle were inoculated with 2.2 x 10^7 NC-I tachyzoites by the intramuscular ($^I/_M$) route. This dose was large when compared to more recent work where foetal death and abortion occurred in cattle inoculated during mid-pregnancy with much smaller doses given subcutaneously (Maley et al., 2001(b)), the latter route could be deemed less invasive to the animals immune system than the $^I/_M$ route.

It is possible that the NC-I tachyzoites used in this study may have a reduced virulence for cattle. When inbred BALB/c mice, which are relatively susceptible to experimental infection with *N. caninum* (Dubey and Lindsay, 1996) were inoculated with the NC-1 tachyzoites, there was no evidence of the disease in brain tissues subsequently examined by histology and immunohistochemistry. This loss in virulence may have been associated with repeated passages in tissue culture in this or other laboratories or due to the low numbers of viable NC-I being present in the inoculum using the culture methods described in this laboratory. However, the latter is unlikely since infection was readily achieved in sheep which were inoculated with the same isolate and cell culture techniques. On several occasions, cattle and sheep were inoculated on the same day with challenge doses of *N. caninum* inoculum prepared from the same cell culture. The conclusion that reduced virulence was involved is further reinforced by the finding that the disease could only be induced in inbred BALB/c mice which were immunocompromised using methylprednisolone acetate.

The strong specific antibody response indicated by the high geometric mean titre (IFAT) and the OD's (ELISA) indicate that this was due to stimulation by live tachyzoites rather than dead antigen. In group II, in which the cattle were inoculated

with vaccine containing dead tachyzoites of *N. caninum,* the antibody titre reached was only 1/700 at 125 ± 10 d.g., after which it rapidly declined until no antibody could be detected four weeks later.

These findings suggest that in order to stimulate maximum antibody levels, the initial immune response should be followed by an amnestic response using live parasite. Inoculation with a single dose of live antigen, while much more effective than repeated doses of dead antigen did not produce the same level of antibody response when compared to that where an amnestic response was involved. It has been shown that when cattle were immunised with a Polygen^{TM} adjuvanted killed *Neospora caninum* tachyzoite preparation, they developed very high levels of specific antibodies in their sera which were accentuated by an amnestic response following tachyzoite challenge (Andrianarivo et al., 2000).

Recently, a new commercial vaccine NeoGuard^{TM} which contains Havlogen-adjuvanted killed *N. caninum* tachyzoites has been approved by the US Department of Agriculture (US DA) for use in cattle. In trials, it has been reported as being effective in reducing abortions in healthy pregnant heifers when challenged with *N. caninum*, however, using immunohistochemistry, it has been shown to be unable to prevent foetal or placental infection in vaccinated heifers (Purtle et al., 2001). In conclusion, the goal of producing an effective vaccine which is capable of preventing abortion in subsequently challenged animals has not yet been achieved.

Chapter 10

The antibody response, pathogenesis of the disease and outcome of pregnancy in sheep vaccinated against neosporosis during pregnancy when subsequently challenged by experimental infection with *N. caninum*

Summary

Forty eight sheep, including controls, were used as the model species to evaluate vaccination as a means of preventing abortion and thereby controlling the spread of neosporosis in a susceptible animal population. The vaccine was produced by the Bayer Corporation, KA., and was provisionally licensed for use in cattle in the USA.

The efficacy of vaccination was evaluated against the number of abortions which occurred in vaccinated sheep which were subsequently challenged by experimental inoculation with *N. caninum* tachyzoites during pregnancy. DNA recovered from brains of aborted foetuses were cloned and the sequences compared with that of the NC-1 isolate used to infect the dams during pregnancy.

The results of this study showed that vaccination of sheep using this vaccine did not prevent abortion following experimental challenge during pregnancy.

Introduction

During the last decade, neosporosis has emerged as a major disease of cattle, causing foetal death and abortion resulting in serious economic loss throughout the world (Dubey and Lindsay, 1996, Dubey, 1999, and Anderson et al., 2000,). The disease is caused by an intercellular protozoan parasite which is closely related genetically, structurally and antigenically to *Toxoplasma gondii* (McAllister, 1996(b), Carreno et al., 1998, Speer et al., 1999 , and Fazaeli et al., 2000,). The parasite was isolated and named *Neospora caninum* as the type species of the genus (Dubey et al., 1988, (a)(b)).

Under natural conditions, the parasite mainly infects cattle and dogs. There have been isolated reports of natural infection occurring in sheep, horses, goats and deer, however, experimentally, a wide range of domestic and laboratory animals, including sheep, can be infected (Dubey and Lindsay, 1996).

It has been shown that experimental infection of pregnant sheep with *N. caninum* tachyzoites causes lesions in foetal tissues and placentae which resemble those of bovine neosporosis, thereby suggesting that pregnant sheep provide a good model for the study of bovine neosporosis (Buxton et al., 1997). It has been shown that the stage of gestation at which cattle and sheep become infected largely determines the outcome of pregnancy. When animals of both species were infected early in gestation, foetal death occurred, however, in those that were infected towards the end of pregnancy, the foetuses survived (, McAllister et al., 1996 (a), Williams et al., 2000) These findings suggest that the development of the immune system of the foetus may be involved in determining its survival. It has been shown in cattle, that the foetal immune system begins to develop around the 4^{th} to 5^{th} month of pregnancy,

after which it rapidly develops until it is able to mount a significant immune response to various pathogens, as term approaches (Osburn, 1986, Nettleton and Entrican, 1995,). Most of the work to date on evaluating the role of the immune system, in preventing transmission of *N. caninum,* has been carried out in mice (Liddell et al., 1999, Long and Bazler, 2000, and Nishikawa et al., 2001). Recently, vaccination has been used in cattle in an effort to control both vertical transmission of the disease and abortion in animals subsequently challenged during pregnancy. It has been shown that cattle immunised prior to pregnancy did not transmit the disease vertically following challenge during pregnancy (Innes et al., 2001). However, when cattle were immunised with a commercial vaccine during the early stages of gestation and subsequently challenged, it did not afford total protection against abortion (Andrianarivo et al., 2000).

In sheep, where the lesions of the disease following experimental challenge closely resemble those in cattle (Buxton et al., 1997), but the recrudescence of maternal infection is rare under experimental conditions (Buxton et al., 2001). It could be postulated that the immune system of sheep may be able to control the disease better than cattle where recrudescence of the disease is common (Dubey and Lindsay, 1996, Dubey, 1999).

In this project, sheep were chosen for the reasons already described above as a suitable model in which to evaluate the effectiveness of vaccination against neosporosis in preventing foetal death and abortion in pregnant ewes subsequently challenged in mid-pregnancy with experimental infection.

Materials and Methods

Sheep Flock

This study was carried out during the period November, 2000 to April, 2001. The composition and maintenance of the flock was previously described in chapter 8.

Sheep

Forty eight ewes at 35 days of gestation (dg) were randomly selected, and assigned to five experimental groups (Table 1). The ewes were seronegative for antibodies to *N. caninum* immediately prior to the commencement of the experiment, however, when tested for antibodies to *Toxoplasma gondii*, 70% (31/48) were seropositive. Pregnancy was confirmed by ultrasound scanning at regular intervals during gestation. Blood samples were taken from ewes in all five groups at 28 day intervals, until lambing.

Table 1. Experimental Design

Group	No. of Ewes	* No. Positive for *T. gondii*	Experimental Group	Treatment
I	14	8/14	Vaccine & Challange	Vaccine and 1 x 10^7 NC-1 tachyzoites
II	5	4/5	Vaccine Control	Vaccine only
III	15	11/15	Callange	1x10^7 NC-1 tachyzoites
IV	8	4/8	Negative Control	1x10^6 Vero cells
V	6	4/6	Negative Control	5 ml sterile H_2O

* (-/-) Number of ewes positive for antibodies to *T. gondii*/Number of ewes in group.

Group I was vaccinated with a commercial vaccine, pro rata, in accordance with the manufacturers instructions for cattle (Bayer Corp., KS, USA). The ewes received the first dose of 5 ml vaccine subcutaneously at 35 days of gestation (dg). A booster dose was administered 21 days later (56 dg). After a further 14 days (70 dg), the ewes were inoculated intramuscularly with an experimental inoculum containing 1

x 10^7 *N. caninum* tachyzoites in 5 ml of sterile phosphate buffered saline (PBS) as outlined in Table 1. The sheep, in Group II, served as a vaccine control and received 5 ml of vaccine only, according to the schedule described for Group 1, while those in Group III, were inoculated with 1.0 x 10^7 *N. caninum* tachyzoites intramuscularly at 70 dg. The sheep, in Group IV, acted as the negative control and were inoculated at 70 dg with 1.0 x 10^7 uninfected Vero cells in 5 ml of sterile PBS administered by the intramuscular route. This dose was equivalent to that used to prepare the inoculum administered to the infected animals. Group V received 5 ml sterile H_2O at 70 d.g. and also served as a negative control.

Serology

Blood serum samples from the forty eight experimental ewes were examined for the presence of specific antibody to *N. caninum* by IFAT and an indirect ELISA and for antibodies to *T. gondii* by DA test and indirect ELISA tests, as previously described in chapter 8. Aborted foetal serum was examined for IgM by IFAT and for IgG by ELISA and IFAT.

Histology and Immunohistochemistry

At necropsy, specimens of brain, liver, lung, heart, kidney and at least two cotyledons were collected from each aborted foetus and associated placenta. However, in some cases, the placenta was not available or was so heavily contaminated or autolysed that it was deemed unsuitable for examination, other foetal tissues were also sometimes unsuitable and were not processed. Histology and immunohistochemistry were carried out using the methods described in chapter 8.

PCR

Foetal tissue samples including brain, liver, lung, heart, kidney and placenta were collected for analysis by PCR in a manner designed to avoid cross contamination. However, not all samples were deemed suitable for the same reasons as above and therefore were not processed. PCR was carried out as previously described in chapter 8.

Cloning and Sequencing

Extracted tissue DNA from the brains of seven aborted foetuses, which were positive for *N. caninum* by histology, immunohistochemistry and serology, were cloned using chemically competent *E. coli* cells as previously described in chapter 8. A restriction enzyme, EcoRI, was used to cleave the *N. caninum* DNA insert in the plasmid. The plasmid insert was subsequently amplified by PCR and identified by Agarose gel electrophorosis. These seven cloned *N. caninum* DNA inserts were sequenced using an ABI Prism 310 genetic analyser as previously described in chapter 8.

Results

Outcome of Pregnancy

Confirmation of pregnancy and foetal survival, as indicated by the presence of a heartbeat, was carried out at regular intervals during the latter part of pregnancy by ultrasanography. All 48 sheep successfully went in lamb, however, only 39 lambs were born alive, the remaining 35 were either autolysed,mummified,stillborn or aborted (Table 2).

Table 2. The outcome of pregnancy in sheep inoculated with *N. caninum* tachyzoites and/or Vero cells at 70 days of gestation (dg)

Group	Number of Sheep	Number of Foetuses				
		Born Alive	Autolysed Or Mummified	Died Recently	Stillborn	Total
I	14	2	11	10	2	25
II	5	7	-	-	-	7
III	15	12	9	1	2	24
IV	8	8	-	-	-	8
V	6	10	-	-	-	10

Sheep in Groups11, IV and V (negative and vaccine control groups) gave birth to clinically normal lambs. The mean size (29.6 cm) and weight (1.44 kg) of the non-viable foetuses in Group I was smaller than that in Group III (34.5 cms and 2.0kg), indicating that foetal development had ceased and foetal death had occurred at an earlier stage of gestation in Group I compared to Group III. The absence of a foetal heart beat during ultrasound scanning of the dams of these foetuses during early pregnancy confirmed these findings. All the foetal deaths occurred during the period 110 to 150 days of gestation.

Group 1: Vaccinated and Challange Sheep

There was a progressive increase in the specific antibody levels for *N. caninum* following vaccination which was augmented by an amnestic response following challange at 70 dg. The responses were compared with the antibody levels for *T. gondii,* which did not vary significantly during this period. The changes are illustrated in Figures 1 and 2. The antibody levels were expressed as the mean geometric titre using IFAT and DA tests and as the mean % positivity using ELISA. The results are shown in Appendix C – Table 1.

Figure 1. Mean antibody titres for *N. caninum* (IFAT) and *T. gondii* (DA) in sheep in Group I

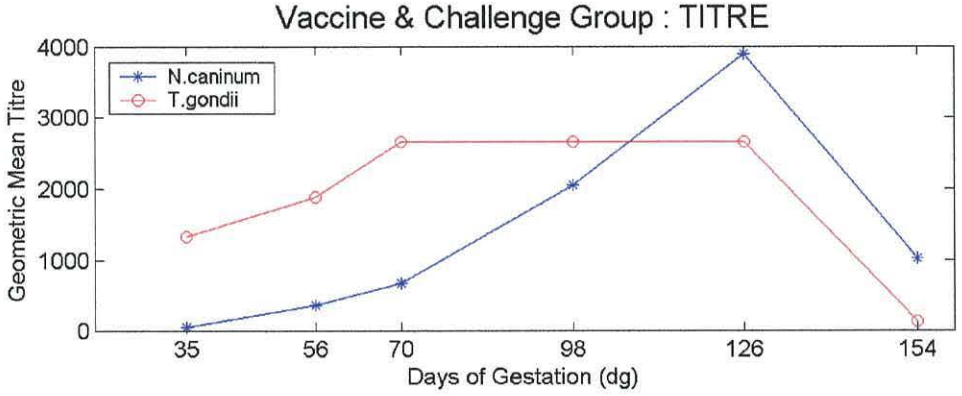
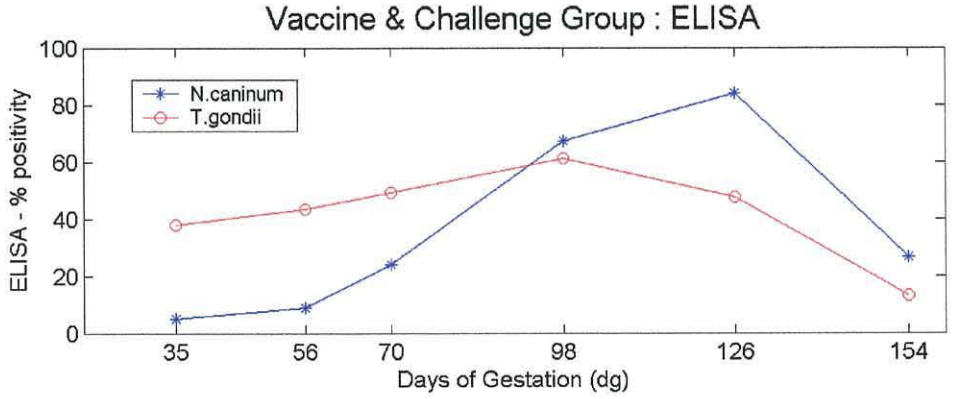


Figure 2. Mean % positivity (ELISA) for antibodies to *N. caninum* and *T. gondii* in sheep in Group I



There was a significant increase in *N. caninum* antibody levels measured by IFAT and ELISA in sheep experimentally challanged with NC-1 tachyzoites at 70 dg. The increase was greater in sheep which were seronegative for antibodies to *T. gondii* when compared to those that were seropositive. The antibody responses measured by ELISA, IFAT and DA tests are compared and contrasted with the mean titre of *T. gondii,* the results are illustrated in Figures 3 and 4.

Figure 3. Mean titre (IFAT) for antibodies to *N. caninum* in *T. gondii* seropositive and seronegative sheep in Group 1. The titre (DA) of T.gondii antibodies is also shown.

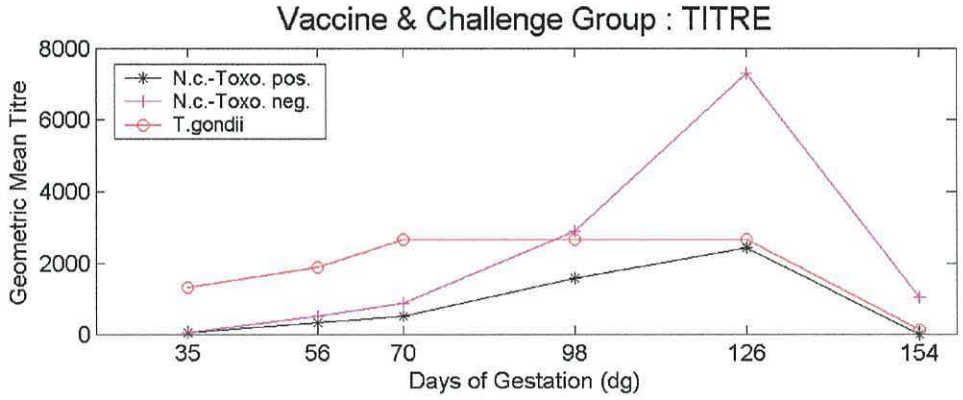
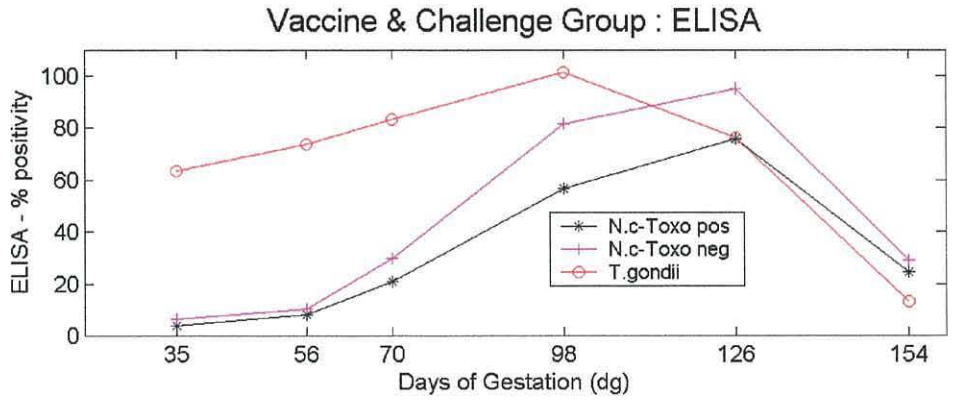


Figure 4. Mean % positivity (ELISA) for antibodies to *N. caninum* and *T. gondii* in Group 1



Group II: Vaccination Control Sheep

In this group the levels of specific antibody to *N. caninum* measured by IFAT and ELISA increased slightly following vaccination before rapidly falling to very low levels at lambing. The levels of antibody to *T. gondii* remained relatively constant during the period of the study. The antibody titres throughout this period are illustrated in Figures 5 and 6. These results are tabulated in Appendix C (Table 2)

Figure 5. Mean antibody titres for *N. caninum* (IFAT) and *T. gondii* (DA) in sheep in Group II

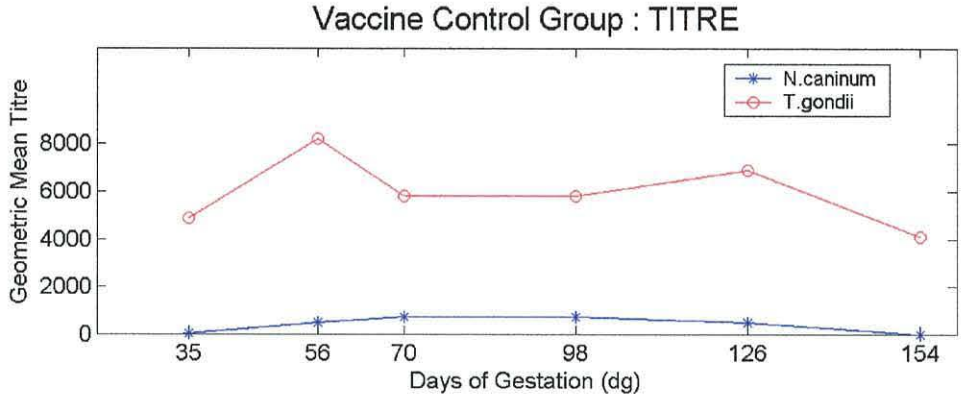
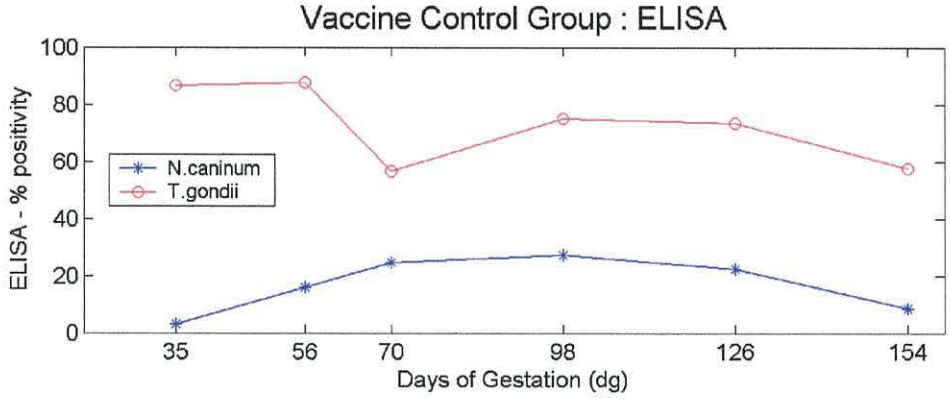


Figure 6. Mean % positivity (ELISA) for antibodies to *N. caninum* and *T. gondii* in sheep in Group II



The antibody profile of sheep which were seropositive for antibodies to *T. gondii* did not change significantly following inoculation with the *N. caninum* vaccine alone. The results are shown in figures 7 and 8.

Figure 7. Mean titre (IFAT) for antibodies to *N. caninum* in *T. gondii* seropositive and seronegative sheep in Group II. The titre (DA) of *T. gondii* antibodies is also shown

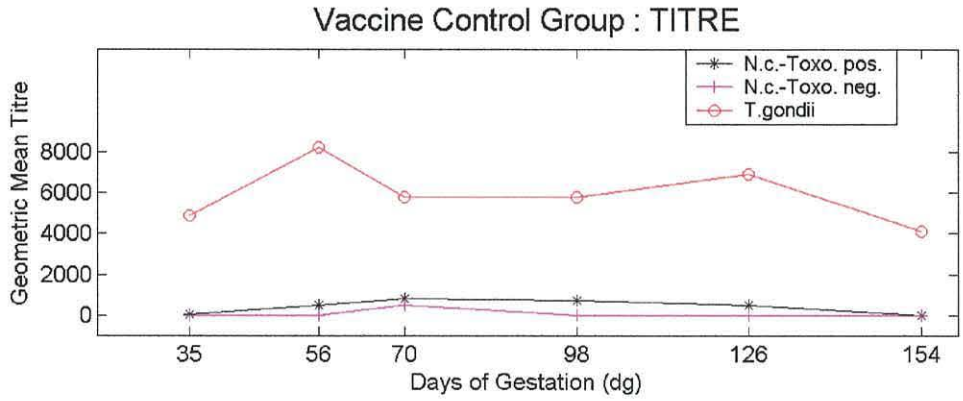
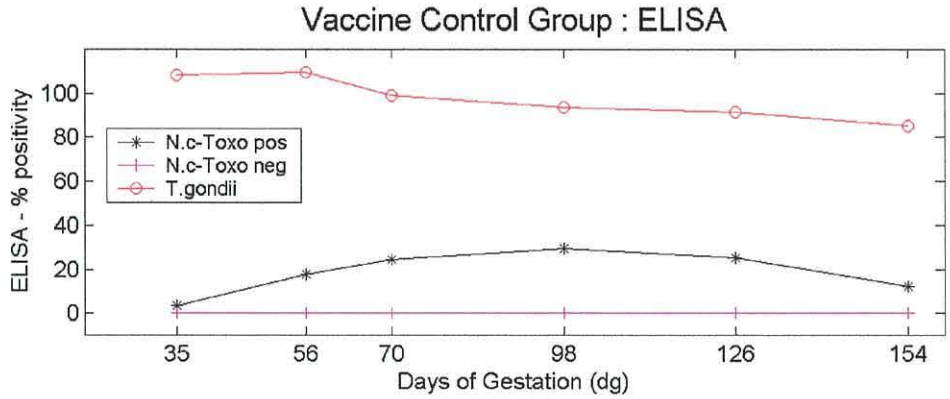


Figure 8. Mean % positivity (ELISA) for antibodies to *N. caninum* in *T. gondii* seronegative and seropositive sheep in Group II, the % positivity for *T. gondii* is also shown



Group III: Challange Sheep :

Specific antibody production, in response to experimental challenge with NC-1 tachyzoites, increased during the four weeks following challenge, after which it levelled off and remained constant until parturition. The details are illustrated in Figures 9 and 10. The antibody levels were expressed as the geometric mean titre using IFAT and DA tests and as the % positivity for the ELISA, the results are shown in Appendix C – Table 3.

Figure 9. Mean antibody titres for *N. caninum* (IFAT) and *T. gondii* (DA) in sheep in Group III

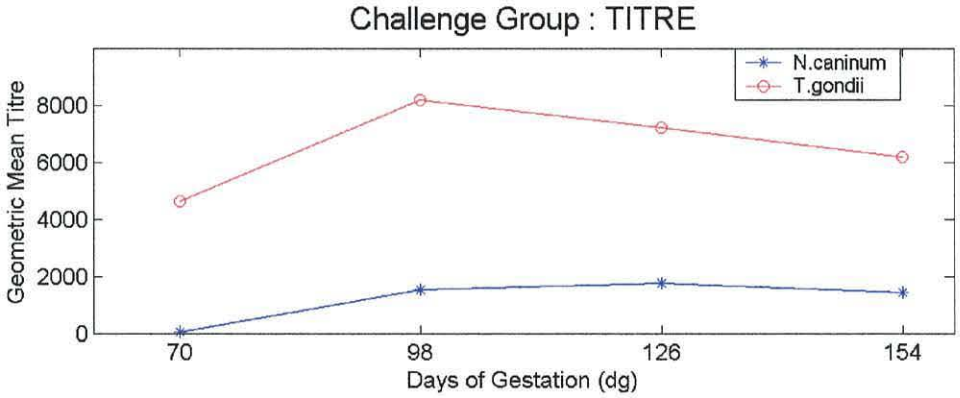
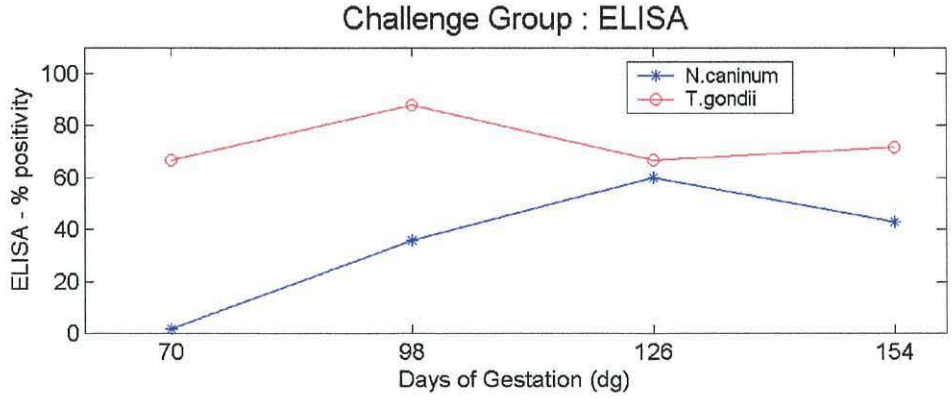


Figure 10. Mean % positivity (ELISA) for antibodies to *N. caninum* and *T. gondii* in sheep in Group III



There was no significant difference between the antibody titre to *N. caninum* in those sheep which were seropositive for *T. gondii* and those which were seronegative following infection with *N. caninum*. Between the period, 98 dg and parturition, a slight decrease in antibody titres for both protozoan parasites took place. These changes are illustrated in Figures 11 and 12 and are shown in Appendix C – Table 3.

Figure 11. Mean titre (IFAT) for antibodies to *N. caninum* in *T. gondii* seropositive and seronegative sheep in Group III. The antibody titre (DA) to *T. gondii* is also shown

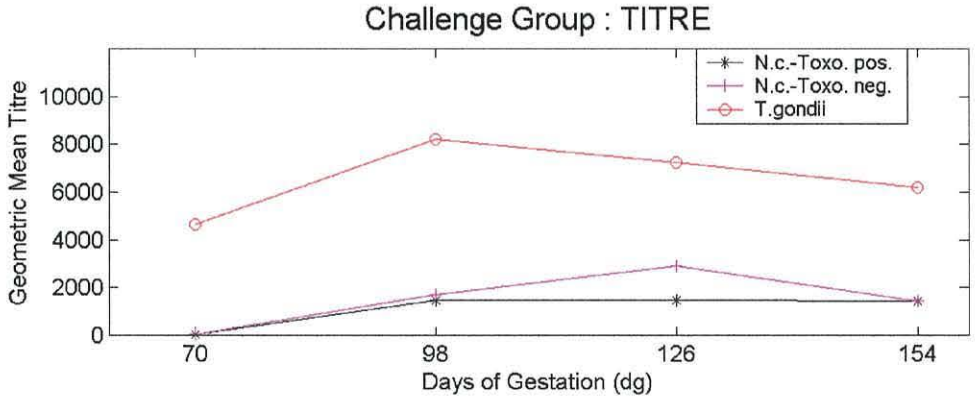
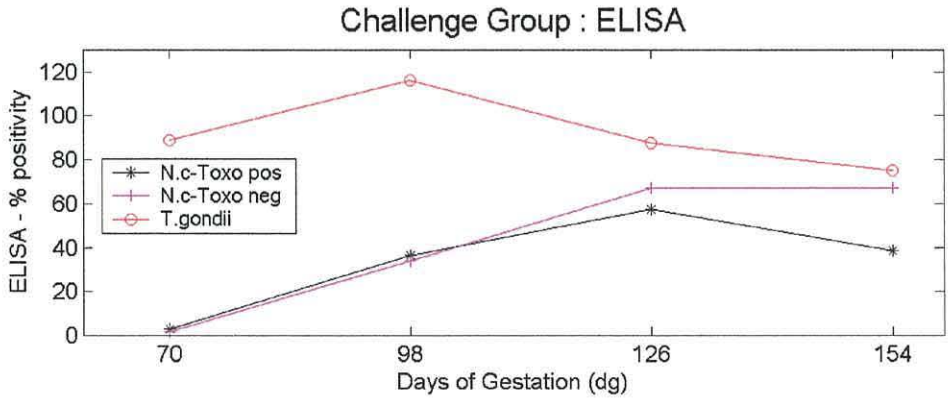


Figure 12. Mean % positivity (ELISA) for antibodies to *N. caninum* in *T. gondii* seropositive and seronegative sheep in Group III. The % positivity for *T.gondi* is also shown.



Group IV and Group V: Vero Cell and Negative Control Sheep

The sheep in these groups remained seronegative for specific antibodies to *N. caninum* throughout the period of this study. Sheep that were seronegative for *T. gondii* at the start of the study remained negative while those that were seropositive so remained positive throughout the period of the experiment, the details are shown in figures 13 and 14 for Group IV. The results were expressed as the geometric mean titre using the IFAT and DA test and as the % positivity using the ELISA. The results for Groups IV and V are tabulated in Appendix C, Table 4 and 5. Group V results are almost identical to those in Group IV and therefore are not shown in graphical form.

Figure 13. Mean antibody titres for *N. caninum* (IFAT) and *T. gondii* (DA) in sheep in Group IV.

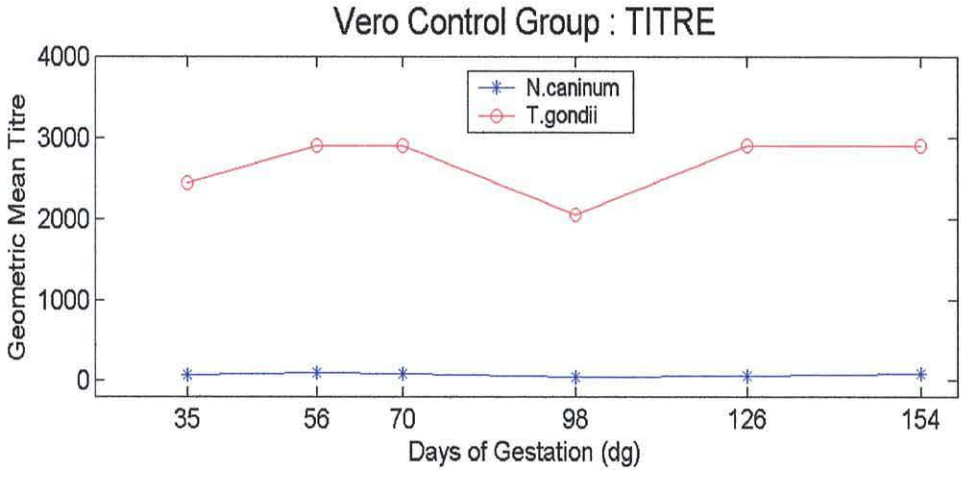
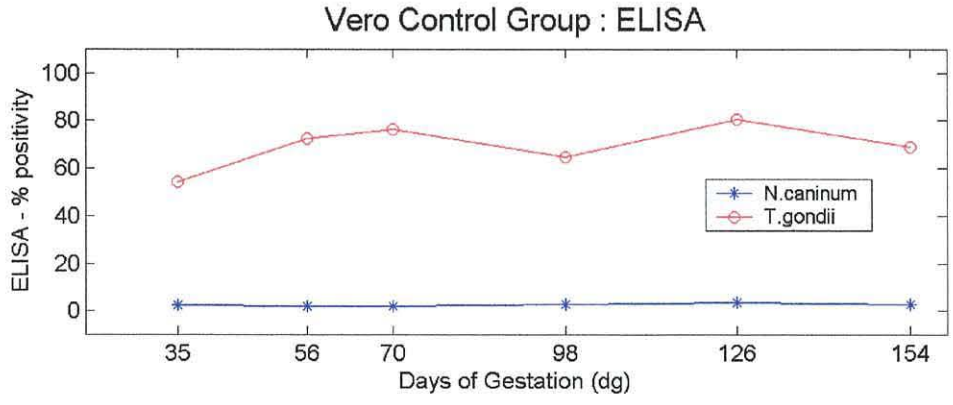


Figure 14. Mean % positivity (ELISA) for antibodies to *N. caninum* in *T. gondii* in sheep in Group IV.



Antibodies to *N. caninum* were not detected in the sera of any of these sheep in this group by either IFAT or ELISA. The level of antibodies to *T. gondii* in those sheep which were seropositive immediately prior to the study did not alter significantly during the period of this project.

Figure 15. Mean titre (IFAT) for antibodies to *N. caninum* in *T. gondii* seropositive and seronegative sheep in Group IV. The mean antibody titre for the seropositive *T. gondii* sheep is also shown

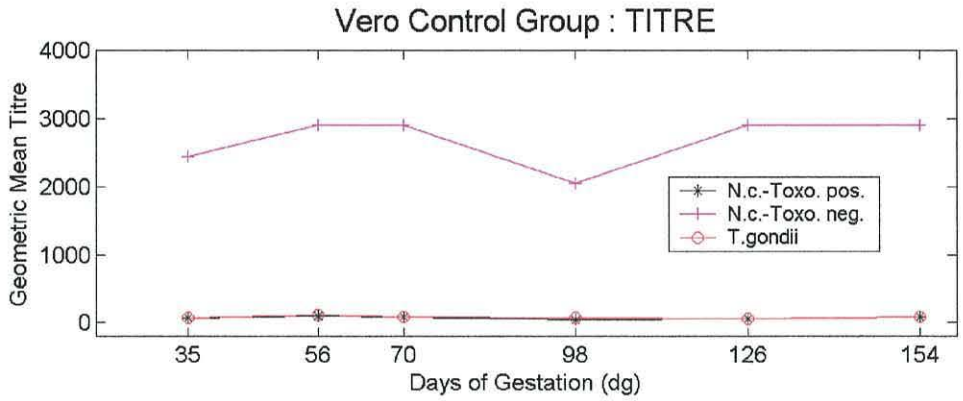
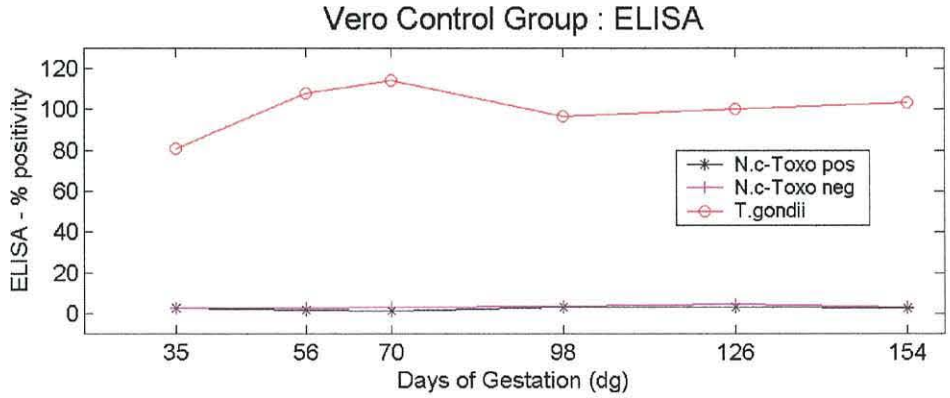


Figure 16. Mean % positivity (ELISA) for antibodies to *N. caninum* in *T. gondii* seropositive and seronegative sheep in Group IV. The % positivity for *T. gondii* is also shown.



Foetal Serology

Foetal serum samples from *N. caninum* challenged groups were not always available at necropsy for testing due to extensive foetal mummification or autolysis. The available samples were seronegative for antibodies to *T. gondii* when screened at a $^{1}/_{80}$ dilution by ELISA and DA test.

When the samples were screened for antibodies to *N. caninum* by ELISA at $^{1}/_{80}$ dilution, all were negative, however, when tested by IFAT at $^{1}/_{80}$, the majority were positive for 1gG and/or 1gM. The results are presented in Table 7.

Table 7. Mean titre (IFAT) (95% confidence level) of 1gG and 1gM antibody to *N. caninum* in aborted ovine foetal serum from sheep in Groups I and III experimentally infected with NC-1 tachyzoites

Group	Number of Foetuses	Geometric Mean Titre [Log $_{10}$] (95% Confidence Interval)			
		1gG	Number Positive	1gM	Number Positive
I	25	1350 (369 – 4935)	13/16	211 (100 – 444)	5/16
III	12	905 (183 – 4488)	10/11	254 (62 – 1033)	6/11

Histology and Immunohistochemistry Findings

Foetal Brains: In the foetal brain, localised meningitis with mononuclear cell infiltration was present in six out of twenty five brains examined, the lesions were largely confined to the cerebrum. The characteristic lesion of multifocal gliosis accompanied by mononuclear cellular infiltration was found in eight out of twenty five brains examined. Perivascular cuffing with mononuclear cells was frequently observed in the normal brain tissue bordering areas of gliosis. Lesions were also found in the midbrain. Protozoan cysts were not observed, however, mineralisation of one necrotic area was observed in a brain, but it was not confirmed as being due to *N. caninum* by immunohistochemistry. Following histology, immunostaining was used to confirm the presence of neosporosis in positive brains. *N. caninum* tachyzoites could be easily identified, sometimes appearing singly or in small clumps. Pieces of antigen were frequently present which probably represented the breakdown of the parasite by the hosts cellular immune response. Typical lesions are shown in figures 17- 19.

Placenta: In this study, samples from the placenta of four ewes which had aborted were deemed suitable for histology and immunohistochemistry. Lesions typical of neosporosis, were present in all four samples, these included focal necrosis of the foetal villous tissue and adjacent maternal caruncular septae. In some sections, a single area of necrosis was present which was quite large, in others, a number of small areas were involved.

The characteristic lesion consisted of focal necrosis of the surface endothelial cells of the maternal septae and adjoining trophoblast cells of the foetal villi. The inflammatory response was generally mild and often only involved increased

vascularity of the capillaries in the foetal villi and maternal septae adjoining the area of necrosis, these lesions were confirmed by immunostaining. Particulate antigen, which appeared as brown staining granules scattered in the inflammatory area surrounding the necrotic area, was the most common finding observed. Typical lesions stained by H +E and immunoperoxidase are shown in figures 20-25.

Other Foetal Tissues: Mild myocarditis, with non-suppurative mononuclear cell infiltration and oedema were found in two foetuses by H + E. However, only one was confirmed positive by immunostaining. Other tissues examined included liver, lung and kidney. However, due to varying degrees of autolysis, no definitive lesions were observed. Immunostaining of these tissues, particularly the liver and kidney, was inconclusive due to the presence of non-specific background staining.

Figure 17 Characteristic focus of necrosis (↙) surrounded by a mild diffuse microgliosis in the brain of an ovine foetus aborted at 120 dg. The dam was inoculated with tachyzoites at 70 dg. A blood vessel with mild perivascular cuffing is also shown (↓). En Vision™ Immunoperoxidase stain. X 100.

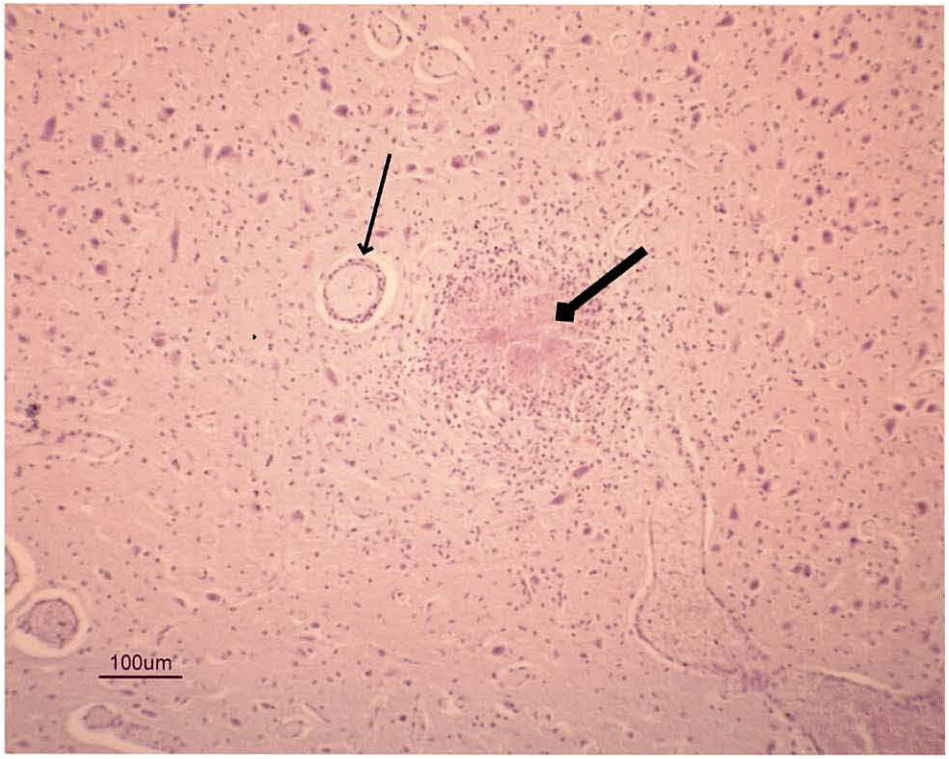


Figure 18 Higher magnification of figure 17 showing the area of necrosis surrounded by small numbers of brown staining antigen representing immunochemically stained tachyzoites, some of which are circled. En Vision™ Immunoperoxidase stain. X 200.

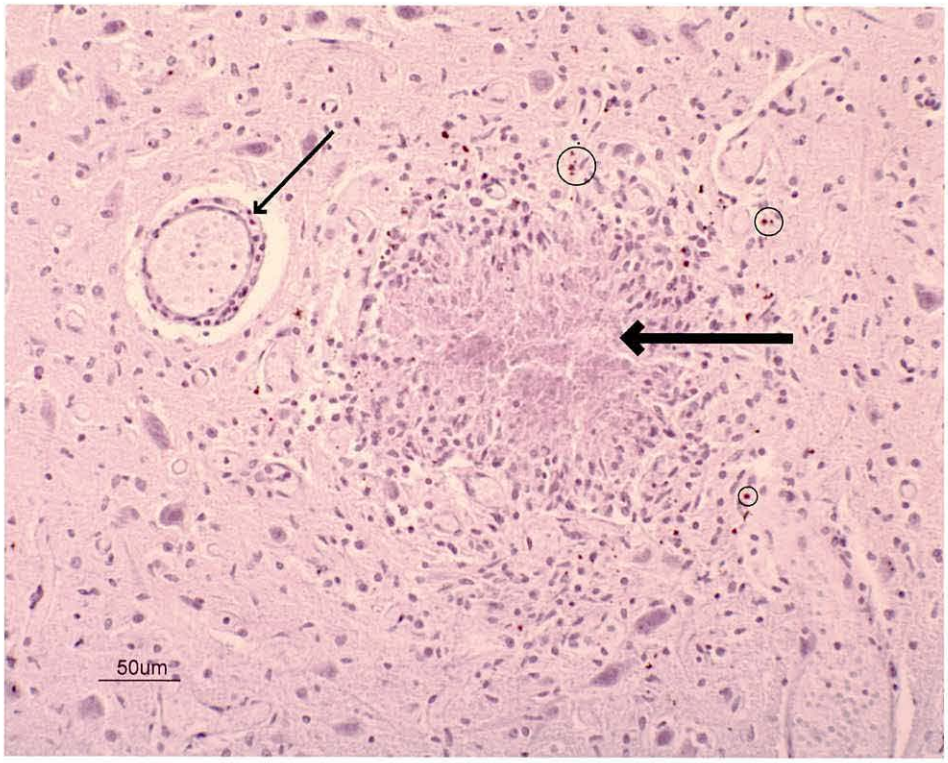


Figure 19 Higher magnification of figure 18. Some intact tachyzoites as well as particulate antigen (circled) are visible surrounding the area of necrosis.

En Vision ™ Immunoperoxidase stain X 400.

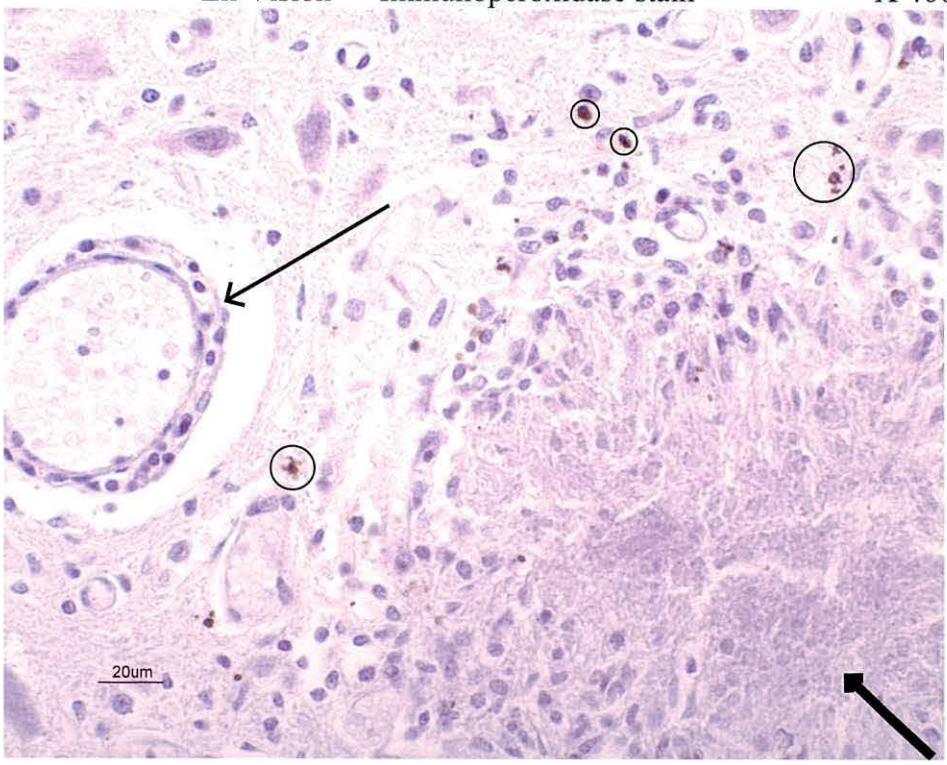


Figure 20 A placentome from a ewe which had aborted at 130 dg. Following inoculation with tachyzoites at 70 dg. Extensive areas of inflammation (→)are visible which involve both foetal placental villus (FV) and maternal caruncular septa (MC) tissues. H + E. X 100.

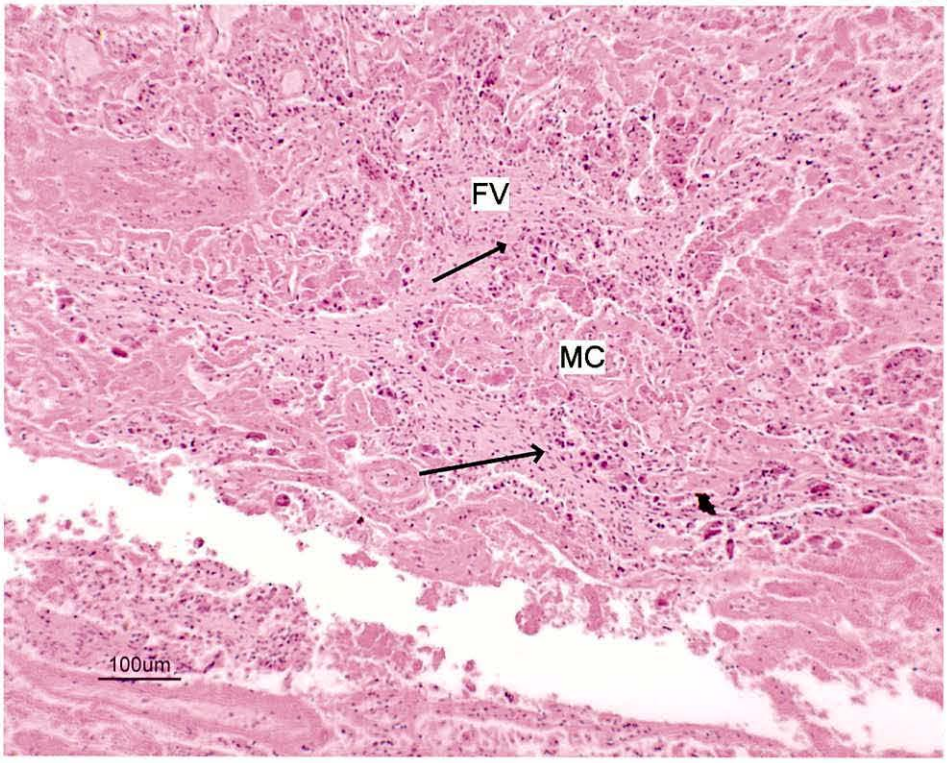


Figure 21 A higher magnification of figure 20 showing extensive necrosis of the cells of the maternal caruncular septa (MC) with an extensive inflammatory response (→) in the adjoining foetal placental villi (FV). H + E. X 200.

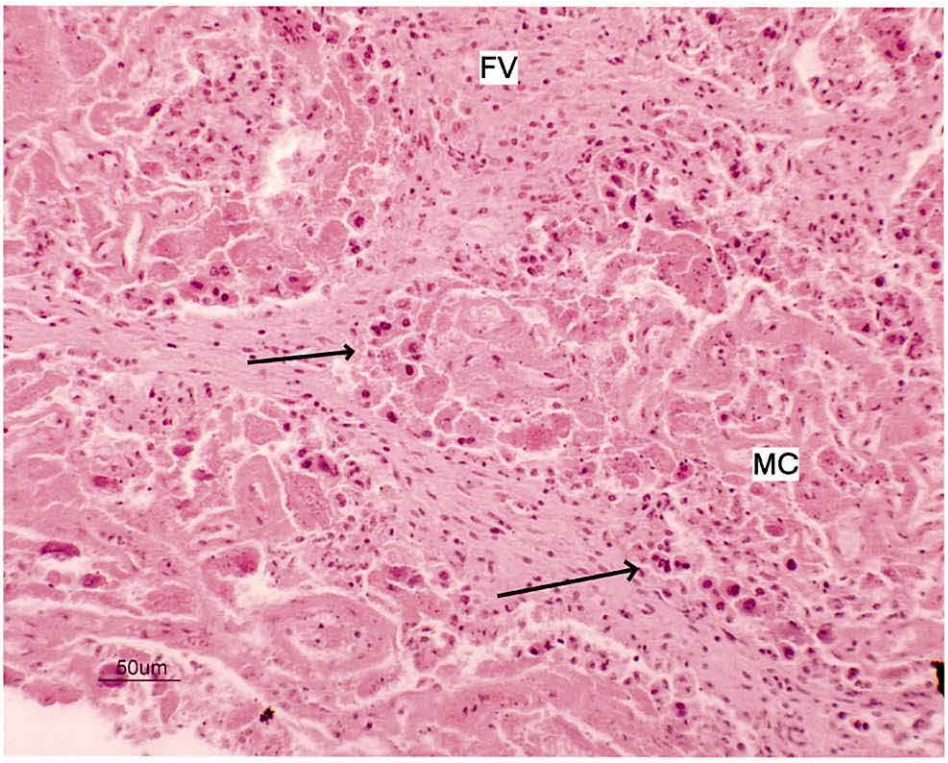


Figure 22 The same ovine placentome tissue stained by H + E in figures 20 and 21 are immunohistochemically stained in this section. The brown staining tachyzoite antigen is clearly visible (↗) at the junction of the foetal placental villi (FV) and maternal caruncular septa (MC). En Vision™ Immunoperoxidase stain. X 40.

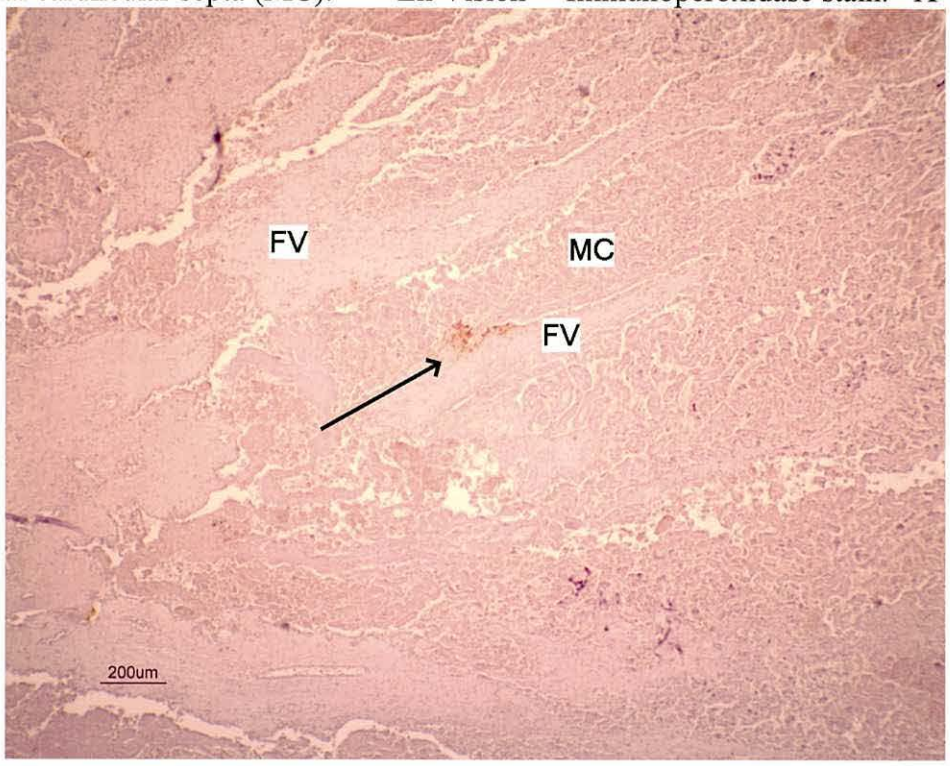


Figure 23 Higher magnification of figure 22 showing the brown staining tachyzoite antigen located mainly in the tissue of the foetal placental villus (FV). The adjoining maternal caruncular septal tissue shows evidence of extensive necrosis (↗).
En Vision™ Immunoperoxidase X 100.

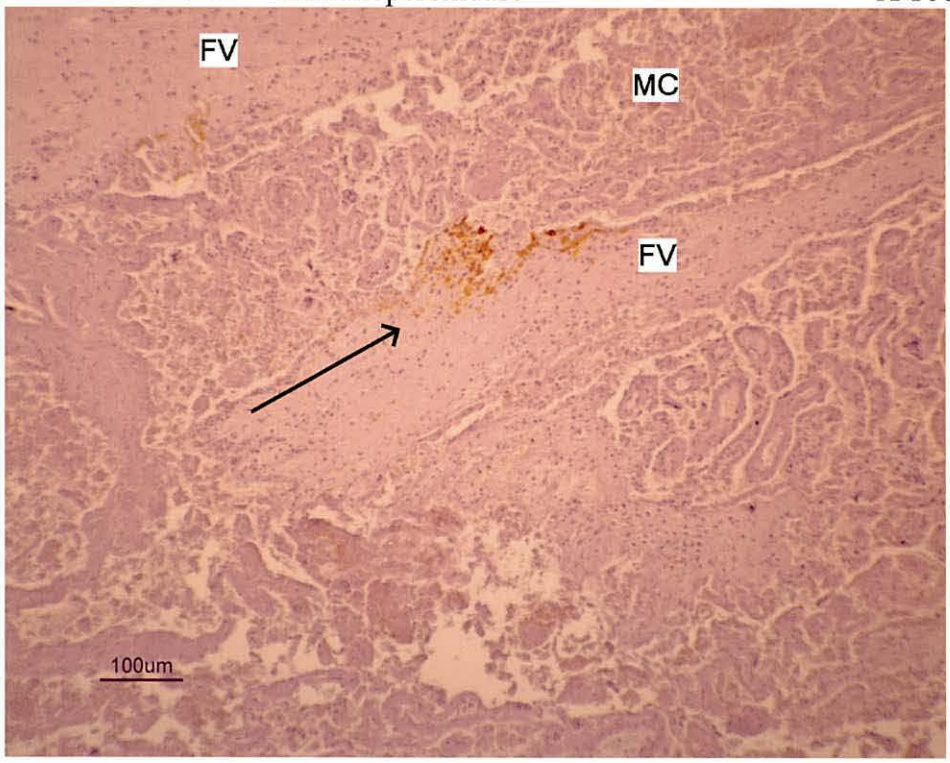


Figure 24 Higher magnification of figure 23 clearly demonstrating that infection in the form of immunohistochemically stained tachyzoite antigen (↙) had crossed from the adjoining maternal caruncular septa (MC) to infect the foetal placental villus (FV). En Vision™ Immunoperoxidase stain. X 200.

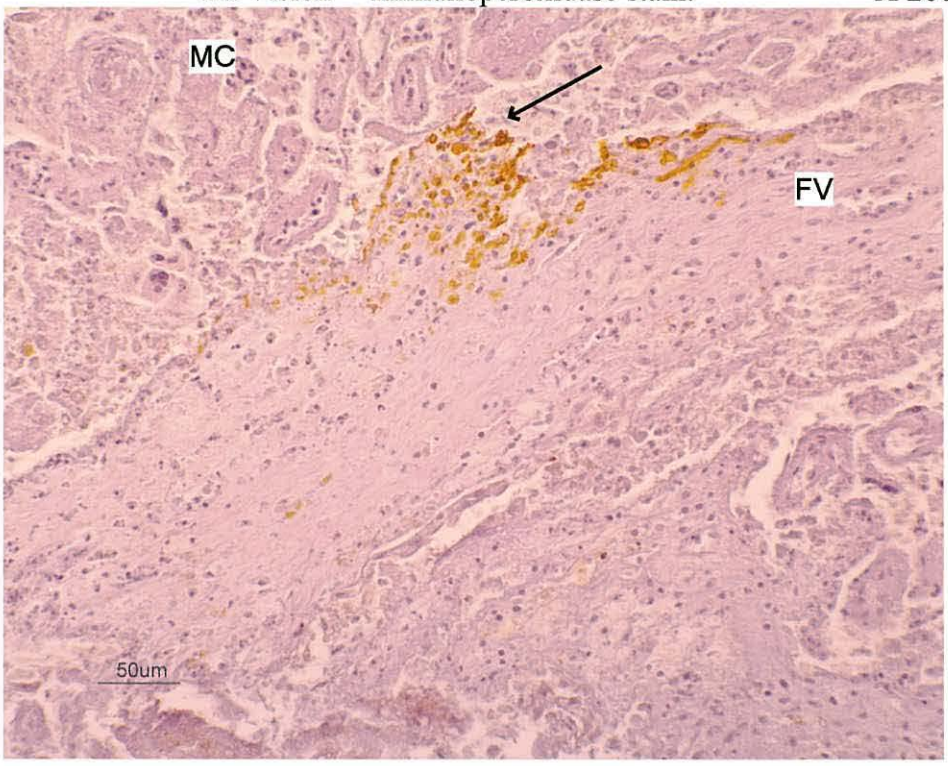
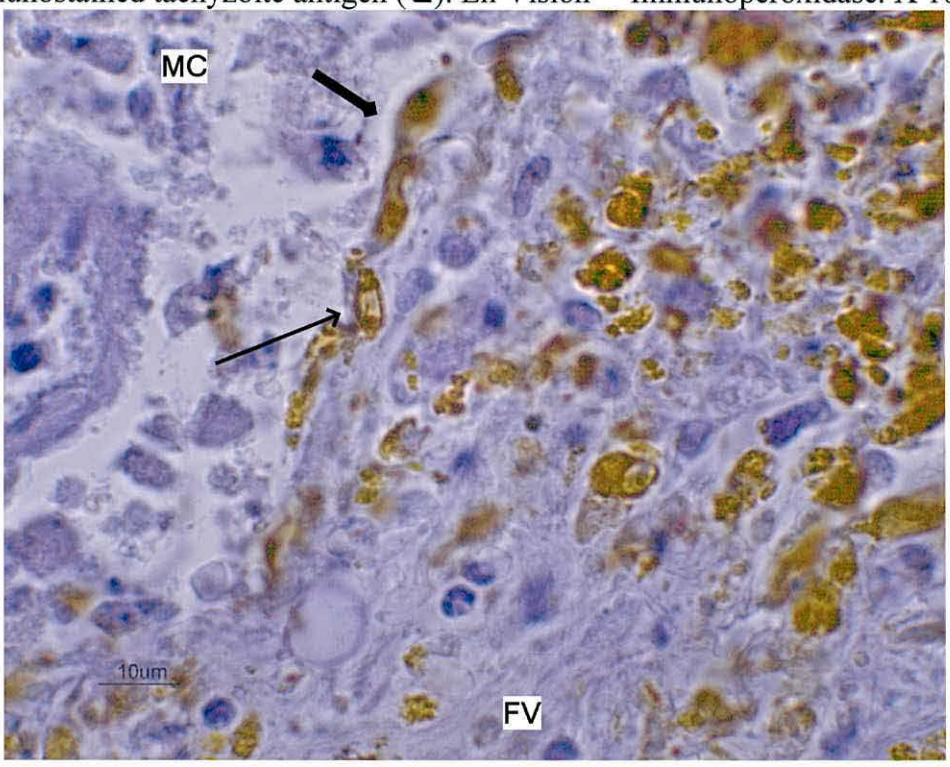


Figure 25 Higher magnification of Figure 24 showing an intact tachyzoite (↗)having passed into the trophoblast cell layer of the foetal placental villus (FV) from the maternal caruncular septa (MC). Both tissues especially the FV contain large amounts of immunostained tachyzoite antigen (↘). En Vision™ Immunoperoxidase. X 1000.



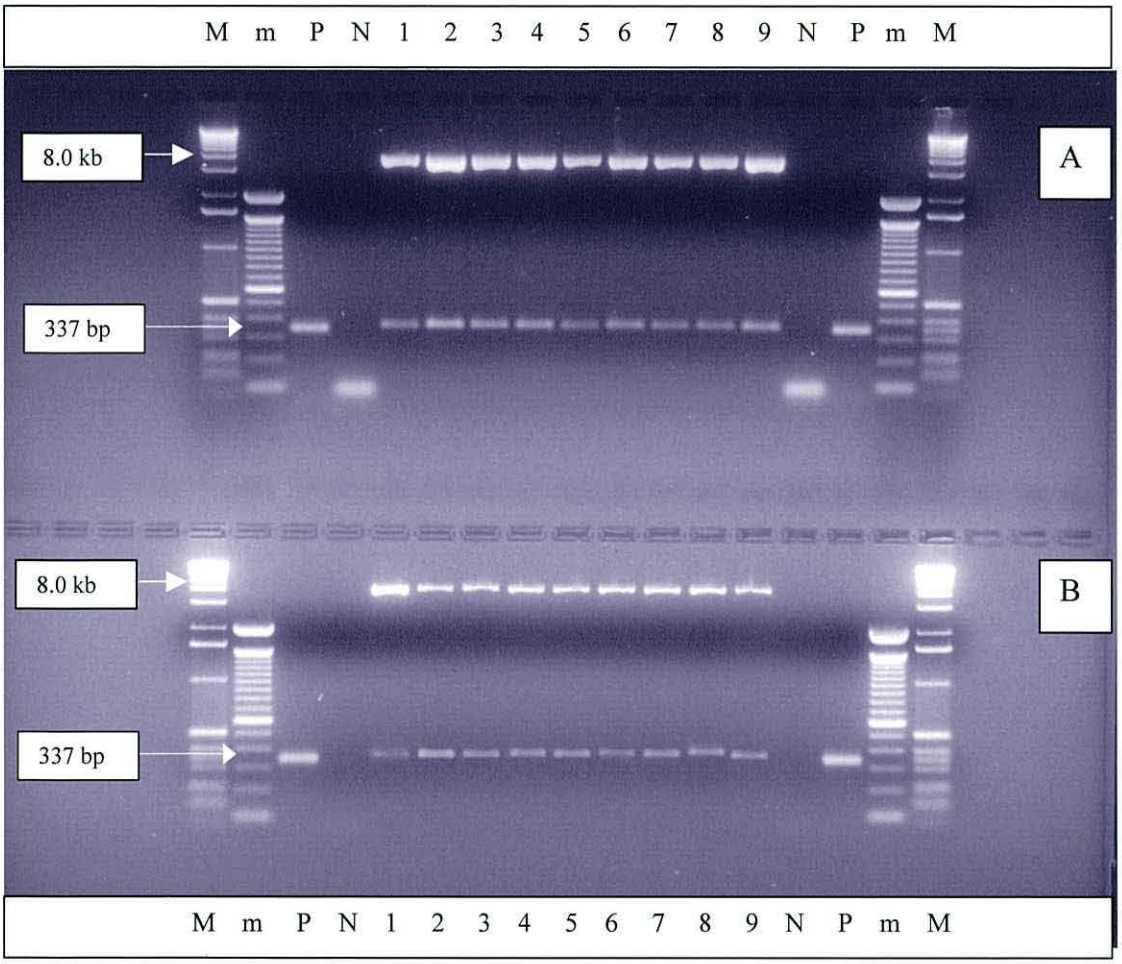
Polymerase Chain Reaction

The PCR results were influenced by the degree of autolysis of the tissue samples prior to extraction. In severely autolysed tissue samples, it was sometimes necessary to repeat the extraction and amplification stages in order to obtain sufficient good quality DNA for gel electrophoresis. The presence of *N. caninum* was confirmed in 27 out of the 35 aborted, mummified or stillborn foetuses examined. The tissues examined included brain, heart, lung, kidney and placenta, however not all these tissues were always available for analysis from the same foetus. The results of the PCR analysis of these tissues is shown in Table 7.

Table 7. Summary of the results following PCR analysis of various foetal tissues from aborted foetuses in experimental Groups I and III. *Number positive/number tested (-/-).

Group	Number of Samples Positive/Tested by IHC						
	Brain	Heart	Liver	Kidney	Lung	Placenta	No. Pos. Foetuses
1	* 14/22	6/9	4/20	1/14	8/17	2/2	17/22
3	6/12	5/12	5/12	1/8	2/4	2/2	10/12
Total	20/34	11/31	9/32	2/22	10/21	4/2	27/34

Figure 26. Detection of *Neospora caninum* and *E. coli* plasmid DNA by PCR following cloning and cleavage using EcoRI restriction enzymes in a 1.5% agarose gel stained by ethidium bromide.



The wells numbered 1 → 9 in combs A and B each contained DNA extracted by EcoRI digest from 9 cloned colonies grown on LB agar.

Lanes

M 1 kb DNA size ladder

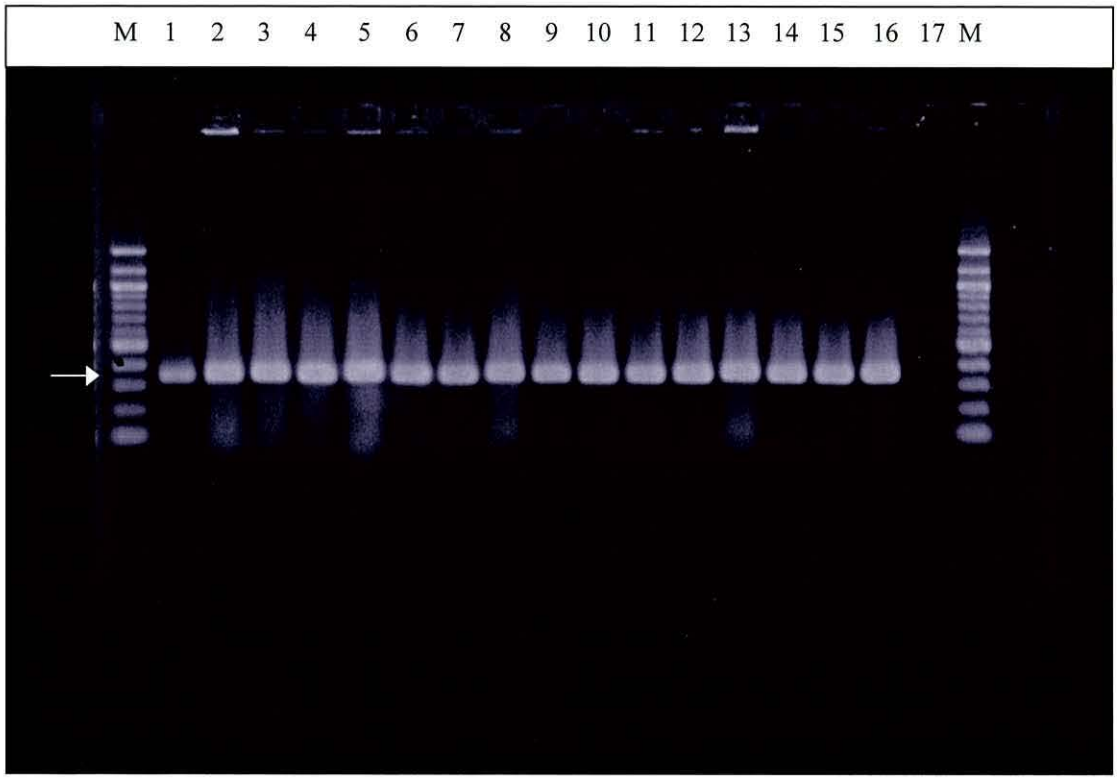
m 100 bp

P Positive control: DNA extracted from NC-1 tachyzoites.

N Negative control: Purified H_2O containing PCR reaction mix.

1 → 9 DNA extracted from plasmids in nine different *E. coli* colonies.

Figure 27. Detection of *Neospora caninum* by PCR from cloned colonies of *E. coli* containing plasmids with *N. caninum* inserts, grown on LB agar plates using 1.5% agarose gel stained with ethidium bromide.



Lanes

M	100 bp DNA size marker.
1	Positive control: DNA extracted from *N. caninum* tachyzoites.
17	Negative control: Purified H_2O and PCR reaction mix.
2 → 16	DNA extracted from cloned colonies of *E. coli* with *N. caninum* inserts in their plasmids.

Cloning and Sequencing

DNA was extracted from the brains of seven aborted foetuses whose dams had been inoculated with *N. caninum* tachyzoites at 70 days of gestation. The DNA was cloned and the sequences compared with that of the DNA extracted from *N. caninum* tachyzoites used to inoculate the dams as described in chapter 8. The results are illustrated in figure 28 and shown in Table 8. The sequence of the NC-1 isolate used to infect the dams of the aborted foetuses was compared to the corresponding reference strains of NC-1 in the GeneBank (accession numbers X-84288) [95.9% identity in the 334 bp compared] and AF 190701 [100% identity in the 261 bp compared]).

Figure 28. Nucleotide alignment of *Neospora caninum* (NC-1) used to infect the dam and the PCR amplification products (601.3 to 609.6) recovered from the brain of the aborted foetuses. Dots indicate nucleotides identical in both sequences, ***X*** indicates gaps.

NC-1	1	CTCGCCAGTC	AACCTACGTC	TTCTGCCTCT	TCCCTCGTCC
601-3		....	....	..........C.....	....
602-7		....	....	..........C.....	....
604-3		....	....	..........C.....	....
605-8		....	....	....	....
606-2		....	....	..........C.....	....
608-8		....	....	....	....
609-6		....	....	..........C.....	....
NC-1	41	GCTTGCTCCC	TATGCATAAT	CTCCCCCGTC	ATCAGTGCCG
601-3		....	....	.....A.....	....
602-7		....	....	....	.....T.....
604-3		....	....	....	.....C.....
605-8		....	....	....	....
606-2		....	....	....	.....C.....
608-8		....	....	....	....
609-6		....	....	....	.....C.....
NC-1	81	CCGGTGTTGC	CTCAACACAG	AACACTGAAC	TCTGGATAAG
601-3		....	....	....	....
602-7		....	....	.....A.....	....
604-3		....	....	....	.....G.....
605-8		....	....	....	....
606-2		....	....	....	.....G.....
608-8		.....C.....	....	G.....	....
609-6		....	....	....	.....G.....

NC-1	121	TATCATTGAC	ACACTGTCCA	CACCCTGACG	CAGGCTGATT
601-3		.....	.....	.....	.....G
602-7		.....	.....T..C.....	...T.....	.....
604-3		.....	.....T	.....	.....
605-8		.....	.....	.....	.....
606-2		.....T.....G...	.....	.....	.....
608-8		.....	.....	.....	.....
609-6		.....	.....T	.....	.....
NC-1	161	TCAACGTGAC	GAATGACTAA	CCACAAACCA	CGTATCCCAC
601-3		.....	.....	.....	.....
602-7		.....	.....	.....	.....
604-3		.....	.....	.....	.....
605-8		.....	.....	.....	.....
606-2		.....	.....	.....	.....
608-8		.....	.....	.....	.....
609-6		.....	.....	.....	.....
NC-1	201	CTCTCACCGC	TACCAACTCC	CTCGGTTCAC	CCGTTCACAC
601-3		.....	.....	.....	.....
602-7		.....	.....A.....	.....	.....
604-3		.....	.....	.....	.....
605-8		.....	.....	.....	.....
606-2		...C.....	.....	.....	.....
608-8		.....	.....	.....	.....
609-6		.....	.....	.....	.....
NC-1	241	ACTATAGCCA	CAAACAAAAA	AGGAGCCTTG	CTGCCGCAGG
601-3		.....	.....	.....	.....
602-7		.....	.....	.....	.....
604-3		.....	.....	.....	.....
605-8		.....	.....	.....	.....
606-2		.....	....C.....	.....	.....
608-8		.....	.....	...AA.....	.....
609-6		.....	.....	.....	.....
NC-1	281	CTGCGCCCAA	CAACGACACG	TCCGCATCCG	CACAGCAACA
601-3		.....	.....	.....	.....
602-7		.....	.....	.....	.....
604-3		.....	.....	....A.....XXX	XXX.....
605-8		.....	.....	.....	.....
606-2		.....	.....	....A.....XXX	XXX.....
608-8		.....	.....	.....	.....A.....
609-6		.....	.....	....A.....XXX	XXX.....
NC-1	321	CGTTACAGGA	TTGGACGCAC	TGGG	
601-3		.....	.....	.....	
602-7		.....	.....	.....	
604-3		.....	.....	.....	
605-8		.....	.....	.....	
606-2		.....	.....	.....	
608-8		.....	.....	.....	
609-6		.....	.....	.....	

Table 8: Showing details of comparison of sequences obtained following sequencing of DNA obtained from brains of aborted ovine foetuses whose dams were inoculated with tachyzoites at 70 days of gestation.

Sample Identification	Sequence Length	Identity	Similarity %	Gap Number	Gaps %
601-3	344	340/344	98.6	0/344	
602.7	344	336/344	97.4	0/344	
604.3	344	334/344	96.8	6/344	1.7
605.8	344	344/344	99.7	0/344	
606.2	344	328/344	95.1	6/344	1.7
608.8	344	336/344	97.4	0/344	
609.6	344	331/344	95.9	6/344	1.7
* 612.4	344	399/344	98.3	0/344	

* Chapter 8

Discussion

Naturally occurring neosporosis in sheep is rare, it has only been reported twice, once in a weak lamb (Dubey and Lindsay, 1990) and once in a live ewe and her twin lambs (Kobayashi et al., 2001). A survey of aborted lambs in the UK did not reveal evidence of neosporosis (Otter et al., 1997(b)). A more recent serological survey of ewes that had aborted in the United Kingdom suggested that abortion due to neosporosis is rare (Helmick et al., 2002).

In many countries in the world including Ireland and the United Kingdom, cattle and sheep often share the same pasture and water supply. While neosporosis in cattle is a worldwide problem it is difficult to understand why the disease does seem to cause natural infection in sheep. Susceptibility does not appear to be an issue since it has been shown that pregnant sheep are very prone to experimental infection at least with NC-1 tachyzoites of *N. caninum* (McAllister et al., 1996(a), Buxton et al., 1997). Natural infection normally occurs by ingestion of infected sporulated oocysts, it is possible that sheep are less susceptible to oral infection than cattle. Nevertheless, whatever the cause of the low prevalence of the disease in sheep, the clinical outcome of experimental infection in pregnant ewes and the associated lesions in the aborted foetuses and placenta closely resemble those of neosporosis in cattle (Buxton et al., 1997).

For these reasons, sheep were chosen as the model to evaluate the effectiveness of vaccination during pregnancy in preventing abortion in a commercially assembled flock maintained under field conditions and subsequently challenged by experimental inoculation with *N. caninum* tachyzoites. However, *N. caninum* is closely related antigenically (Beckers et al., 1997) and morphologically (Speer et al., 1999) to

Toxoplasma gondii which has a worldwide distribution and is a major cause of abortion in sheep. In Ireland, an extensive serological survey over a five year period of ewes which had come from flocks with abortion problems showed that 55.6% of ewes were seropositive for the disease (O'Brien and Geraghty, 1990). In this situation it was judged imperative that any field trials involving neosporosis in sheep should include adequate controls for toxoplasmosis. To avoid any element of bias in the selection of sheep, both within and between groups, and to reflect the natural *Toxoplasma* disease status of the sheep population, blood samples which were collected prior to and during the study, were not tested for antibodies to *T. gondii* until the end of the experiment.

It was anticipated that each experimental group should contain approximately 33% sheep seronegative and 66% seropositive for antibodies to *T. gondii*. The actual outcome for the two challenge groups I and III was 43/57 and 27/73 percent *T. gondii* seronegative/*T. gondii* seropositive. In the control groups II and IV, the ratio was 20/80 and 33/66 percent respectively. In situations such as this, it was important to identify the immune response in sheep following inoculation with *N. caninum* tachyzoites and to differentiate it from acute and chronic *T. gondii* infection. This was achieved using monoclonal ELISA tests for both *T. gondii* and *N. caninum* and confirming the results by IFAT for *N. caninum* and DA test for *T. gondii*.

There are conflicting reports in the literature on the sensitivity and specificity of various serological tests for *N. caninum* particularly when ELISAs were compared to IFAT (Schares et al., 1998, Björkman and Uggla, 1999). In studies in cattle, there was good agreement between ELISA and IFAT in differentiating between negative and positive sera, but there was a poor correlation in titre and absorbance values (Björkman

and Uggla, 1999). In contrast, in this study, this was true only during the initial phase of seroconversion where the IFAT consistently identified the presence of specific antibody before the ELISA test. However, once seroconversion had peaked there was a reasonably good relationship between the two test methods. In the case of toxoplasmosis, the serological results obtained using DAT also compared favourably with the monoclonal ELISA test method.

Vaccination of the pregnant ewes did not protect them against experimental challenge with *N. caninum* tachyzoites. It is interesting to note that a significantly higher percentage of sheep in Group I aborted (86%) compared to Group III (53%) while no abortions occurred in the Control Groups II, IV and V . It was also significant that the abortions which occurred in Group I, that is the vaccine group, occurred much sooner after challenge than those in Group III as indicated by the mean weight and size of the aborted foetuses. The reason for this is not clear but it may be associated with the large doses of vaccine which these sheep received (same dose rate as for cattle) prior to challenge. These doses in conjunction with a large challenge dose may in some way have overwhelmed the immune system of the sheep in Group I.

It is now recognised that while the immune response to intracellular protozoan parasites is largely cell-mediated, there is normally a concurrent humoral response. Typically, these two types of responses are cross-regulating and tend to down grade each other but not to their mutual exclusion, the exact mechanisms involved are extremely complex (Allen and Maizels, 1997). In these circumstances, it is possible that the initial antibody response to the large dose of vaccine followed by the challenge with a relatively large dose of *N. caninum* tachyzoites in a very susceptible host species partially suppressed the cell-mediated response resulting in the rapid spread of

infection within the affected animal which resulted in early foetal death. Experimental inoculation of virulent *N. caninum* prior to pregnancy might confer immunity.

Meanwhile, the specific antibody levels to *T. gondii* in all groups of sheep measured by ELISA and DA test remained relatively constant throughout the period of the study showing that recent or new infection with toxoplasmosis, resulting from field exposure during the study, was not involved. Previous studies have shown that sheep recently infected with toxoplasmosis show rising titres which persist at high levels over time (Buxton et al., 1991, 1993). Similarly, field or natural infection with neosporosis was not involved since all the control groups remained seronegative for neosporosis.

Both IgG and IgM antibodies to *N. caninum* were found in the sera of many of the aborted foetuses. However, the finding of antibody in the dam is only indicative of exposure, similarly in the foetus, it is indicative of intra uterine exposure but not necessarily of having been the cause of foetal death. Confirmation of the presence of the disease normally depends on the presence of characteristic lesions of focal encephalitis accompanied by necrosis and non-supporative inflammation. This is normally achieved by histology followed by immunohistochemistry (IHC), for a definitive diagnosis. The sensitivity of even the most sensitive IHC method to detect *N. caninum* in tissues is low (Dubey, 1999). This is partially due to low levels of *N. caninum* tachyzoites present in tissues in the first place, thus requiring the examination of large numbers of sections from the same specimen, to identify the organism.

Immunohistochemistry was found to give very poor results in this study when used to confirm the presence of neosporosis in aborted foetal tissue, thus confirming the above report. Foetal autolysis appeared to be largely responsible for this

considering that excellent results were obtained when IHC was used in fresh foetal tissue and placenta in chapter 6 of this project.

In cattle, histopathological lesions have been most commonly reported in foetal brain and heart, but rarely in other organs including the placenta (Dubey and Lindsay, 1996). However, in this study and in chapter 6, examination of the placenta for lesions gave the most frequent and consistent positive results. Characteristic lesions of neosporosis were sometimes difficult to find and identify in brain sections using routine H & E staining. Confirmation with IHC was not always possible. Lesions in other tissues including the heart were infrequent and also difficult to identify by histology and immunohistochemistry .

Several polymerase chain reaction PCR methods have been reported as being able to detect *N. caninum* in tissues (Kaufmann et al., 1996, Lally et al., 1996, Muller et al., 1996,Yamage et al., 1996, Ellis et al., 1998). Some of the PCR methods described in the literature use highly conserved genes like the 16s-like RNA genes which may result in cross reactions with unknown organisms. In this study, the primers described by Yamage et al., 1996 which are directed at the pNC_5 *Neospora* specific genomic DNA sequence, found outside the coding region in the variable region of the gene where the sequence is less conserved were used. The primers Np 21 plus/Np 6 plus used in this study proved very reliable, when used to detect the presence of *Neospora* specific DNA in aborted foetal tissues. However, in severely autolysed tissues it was often necessary to run the PCR reaction in triplicate in order to detect very low levels of good quality *Neospora* specific DNA that were present. PCR when compared to IHC was superior in identifying *N. caninum* in aborted ovine foetal tissues.

PCR methods have not yet been evaluated critically for the diagnosis of *N. caninum* induced abortion (Dubey, 1999). There is always the possibility that DNA of unknown origin will cross react with *N. caninum* specific DNA. In this study, DNA extracted from *N. caninum* tachyzoites grown in tissue culture and from the brains of seven aborted ovine foetuses was cloned into *E. coli* and subsequently sequenced.

The resulting sequences when compared to that of the DNA extracted from the original inoculum of *N. caninum* grown in tissue culture showed from 2 to 11 base pair changes in a total sequence of 344 bp. In three out of nine samples sequenced, the changes were almost identical suggesting that these were not random errors. It is possible that the changes were associated with the primers being directed towards non-conserved areas of the gene (Muller personal communication). However, the finding that a 261bp of DNA from the NC-1 strain to infect the dams showed a 100% similarity with one of the Gene Bank sequences(AF 109701) does not support this suggestion. It is possible that mutiple copies of the same gene exist in *N. caninum* which could help explain these findings. In any case, these results clearly indicate that the genetics of the parasite are not currently fully understood and that a further investigation of these findings could form the basis of a very interesting project at a future date.

The results of this study also show that while sheep are a good model in which to study the outcome of experimental challenge in vaccinated sheep, further work is needed to evaluate the efficacy of a live versus a dead vaccine and to ascertain the best time for such vaccinations. It is important in susceptible species, such as sheep, not to either over-vaccinate or over-challenge the immune system during pregnancy when they appear to be very susceptible to experimental infection.

Chapter 11

General Discussion

The study of neosporosis in cattle and sheep in Ireland described in this thesis, was divided into three parts, an epidemiological investigation based on three seroprevalence studies, a pathogenesis study, an evaluation of the available diagnostic methods and of vaccination as a means of controlling the disease.

Prior to this project, there was little information on the prevalence of the disease in cattle and none in sheep in Ireland. The existing information was limited to the sporadic finding in a number of Regional Veterinary Laboratories, of antibodies in blood samples from cattle that had recently aborted. Occasionally, characteristic lesions were observed in brains from aborted foetuses when examined by histology using routine Erhlicks H and E stained sections. However, in most cases, there were no follow up investigations carried out or notifications of the disease recorded.

Neosporosis has been shown to be a major cause of abortion and infertility in cattle in most countries in the world, it was reasonable to assume that a similar situation would exist in this country. While there is no conclusive information on the economic losses due to neosporosis in the cattle industry anywhere in the world, it has been estimated that in California the annual loss to the industry, has been put at $35 million per year (Dubey, 1999). In another report the losses incurred have been estimated to cost the Australian dairy industry and beef industry 85 and 25 million dollars respectively every year (Ellis, 1997). In Ireland, there are 7.5 million cattle, approximately 90% of the output from which is exported in the form of live cattle, beef or dairy products to many regions of the world thereby contributing significantly to the Irish economy in terms of foreign earnings. If there were a significant level of

infection with *N. caninum* in the Irish National herd, the effects on the cattle livestock industry and ultimately to the country would have serious economic consequences. The losses would be reflected in terms of reduced milk production, infertility, abortions, stillbirths, birth of weakly calves and extensive culling. In such a situation,it may be necessary to introduce legislation to put in place measures to control the disease before it became endemic in herds.

This project was the first concerted attempt to study the epidemiology of the neosporosis and to establish the seroprevalence of the disease in Ireland. It was decided to investigate the level of the disease in the national herd and to compare it at regional level. In Ireland, the country is geographically divided into three main regions in which different livestock enterprises predominate; this information would be vital in drawing up any future national control or eradication programme for this disease.

The results of these three seroprevalence studies, described in chapters 2, 3 and 4 showed that infection with *N. caninum* is a significant problem for the Irish cattle industry with consequent economic loss to the economy. Since these studies were the first to be carried out at National level in any country and encompass all main cattle livestock production systems, they are particularly useful in contributing to our understanding of the disease in the country with consequent universal significance. These results, which were also broken down into regional level, are particularly useful to those sectors including the regulatory authorities, farming organisations and the agri-food industry who are likely to become involved in trying to control the spread of the disease within the cattle population. Prior to this project, the seroprevalence of neosporosis in the country was not available to either the regulatory authorities,

farmers or veterinary practitioners, all of whom are currently involved in the constant improvement in the health standards of the national cattle herd. This is evidence by fact, that the Irish government currently spends in excess of €100 million annually on the eradication of bovine TB and Brucellosis alone. Knowledge of the prevalence of the disease and the other parameters described are essential perquisites in drawing up an effective control and an eventual eradication programme for this disease.

The influence of season on the prevalence of neosporosis has been often mentioned indirectly in the literature. Prior to this study, there appears to have been no comprehensive study carried out on this aspect of the disease. The available, scant information has been accumulated from the results of various herd studies from some diagnostic laboratories on investigations carried out on aborted foetuses. In this project, the association between season and the occurrence of the disease was investigated in the post abortion and herd prevalence studies. Both investigations were carried out at national herd level and each lasted for one full year covering the period September, 1999 to August, 2001 and involved sampling and testing 27,136 cattle for specific antibodies to *N.caninum*.. While the investigation at herd level showed trends, the post abortion study, which by its nature was more targeted, revealed a significant association between the months of the year and the numbers of reported abortions. The results of these studies show that herd management factors, and in particular the month of the year during which the majority of the cows in the Irish national herd were at the four to six month stage of gestation was the main factor involved. It is of course, at this stage of pregnancy when cattle are at their most vulnerable, as regards becoming infected with *Neospora*. This information should allow precautionary measures to be adopted at farm level at the appropriate time.

Sheep have been shown in this project, and in previous work, to be very susceptible to experimental challenge with the disease. However, there are few reports of natural infection having occurred in this species, in spite of the fact that cattle and sheep often graze the same pastures, though not always at the same time, at least in Ireland. Sheep often also share the same water supply and consume the feedstuffs which are equally liable to contamination by infected oocysts passed by dogs. While the transmission of the disease in cattle has been shown to be largely transplacental, with horizontal transfer not playing a very significant role, nevertheless it is difficult to understand why the disease is not self limiting under these circumstances. Horizontal transfer in cattle must be involved to a greater extent than has been reported to date, possibly involving other definitive and/or intermediate hosts. In sheep, the route of transmission of infection under natural conditions has not been elucidated, due to the low number of reported cases of natural infection, in these species. If vertical transmission is not as important as in cattle, it may explain the relative infrequency of the disease in sheep.

In this project, a study was carried out in flocks countrywide, 5.8% of sheep flocks were found to be seropositive for antibodies to *N. caninum*. Within these positive flocks, 10% of those animals sampled were seropositive indicating that the sheep had been exposed to *N. caninum*. However, five out of six of these *N. caninum* seropositive sheep also showed antibodies to *T. gondii*. In a previous study almost 66% of Irish sheep were positive for antibodies to *T. gondii*. While the two parasites are closely related phylogenetically and antigenically, there are a number of reports which failed to find any evidence of major cross-reactivity (Dubey et al.,1996, Wouda et al., 1998(a)). The results described in chapter 10 of this thesis confirm these reports. However, the susceptibility to challenge by *N. caninum* may be influenced by the

concurrent *T. gondii* infection. This association between the two parasites may be more involved with the extremely low levels of *Neospora* associated abortions in sheep in Ireland than the serological evidence from the study in this thesis would suggest. The serological relationship between sheep which were already seropositive for antibodies to *T. gondii* when subsequently challenged experimentally with *N. caninum,* was investigated in chapter 10. There were no significant changes in titres in either disease following challenge with *N. caninum* which could be related to serological cross-reactivity between the two protozoan parasites thus indicating that the two disease conditions proceed independently of each other. These findings indicate that neosporosis in sheep, at least in Ireland, maybe present in significant levels in some Irish sheep flocks. In the absence of this information, farmers, veterinary practitioners and veterinary diagnostic laboratories are currently not specifically looking for *N. caninum.* They are accepting the serological or histopathological confirmation of toxoplasmosis as being the sole cause of the abortion, while at the same time possible concurrent *N. caninum* infection goes unrecognised.

Routine diagnosis of neosporosis in cattle in Ireland is normally carried out by serology. Blood samples from the dam, and if available from the aborted foetus, are usually examined by ELISA or IFAT. ELISA is more commonly used as a screening test especially if large numbers of samples are involved because many of the steps in the test method can be automated. If the foetus is available, histology combined with immunohistochemistry is frequently used, with varying degrees of success, depending on the degree of tissue autolysis.

In this project, ELISA was found to give reliable results when compared to IFAT which is often regarded as the "gold standard" of serological tests. However, when antibody levels were low for any reason such as during the early stages of seroconversion, or when the titre is falling off some time after exposure having occurred, the IFAT test was consistently better in identifying the presence of specific antibody in these circumstances.

Histology used in conjunction with immunohistochemistry has been shown to be an effective method of identifying *N. caninum* tachyzoites in aborted foetal tissues (Anderson et al., 2000). In this study, good results were achieved when using fresh foetal tissue, for example, from those in the serial kill trials. However, in the case of aborted foetuses, especially if extensive autolysis was present, as it usually was, the results were much more variable. In such cases it was often not possible to identify specific lesions or to clearly differentiate specific staining from non-specific background staining. There are reports in the literature (Dubey and Lindsay, 1996) that brain and heart were the foetal tissues where most lesions were found and that lesions were found infrequently in the placenta. In this study, on the contrary, lesions were found in the placenta on the same frequency as in brain tissue. However, the lesions in the placenta were very obvious when examined by histology using routine H & E and they were also easily observed by IHC largely due to the size of the lesion and due to the amount of stained antigen (IHC) visible when compared to brain tissue. In this study, placental tissue, if available, was the tissue of choice for examination by IHC.

Various PCR methods have been reported to be limited in their use as diagnostic methods for neosporosis because of the presence of foetal autolysis, which

appears to limit the sensitivity of the test method. However, in this study, PCR using Np21 plus and Np6 plus primers was found to give very consistent and reliable results once sufficient DNA was extracted from the tissue samples. In some samples, which were severely autolysed, it was still possible using these primers to identify *N. caninum* in the sample tissue by running the reactions in triplicate. It was found that the reliability of the test improved when a "housekeeping" control PCR reaction was included in the test method for each sample tested. In the absence of a "housekeeping" control, a negative PCR result could have been due to the absence of the specific DNA or to the failure to successfully extract it from the tissue in the first place. It is possible that sufficient emphasis was not attached to including these controls during the evaluation of some other PCR methods in the past.

A number of different strains of *N. caninum* have been reported from different countries, these include NC-1, NC-2, NC-3, NC-Liv amongst others. It has been shown that there are differences in virulence amongst strains, NC-1 was more pathogenic for mice than either NC-2 or NC-3. However, there are no reports of a change in virulence of a particular isolate having occurred over a period of time. As previously stated, Dubey and Lindsay (1996), failed to find any evidence of reduced virulence for NC-1 passaged in tissue culture over an eight year period when inoculated into mice.

In this project, using large inoculations of NC-1 tachyzoites prepared in this laboratory administered by the intramuscular route, it was not possible to infect either cattle or inbred BALB/c mice. However, when sheep and immunocompromised BALB/c mice were administered challenge doses of the same passages of NC-1 tachyzoites prepared in the same way, severe clinical symptoms appeared in both

species. Furthermore, on two occasions, cattle and sheep were inoculated with aliquots of the same preparation of inoculum on the same day. The cattle showed no evidence of infection while most of the sheep subsequently aborted.

It is possible to postulate that species susceptibility was the main factor involved, however, in this study, cattle and inbred BALB/c mice were administered large doses by an invasive route (I/M) when compared to other reports in the literature where infection in these species was readily achieved, using similar doses of NC-1 tachyzoites and administration routes. The results of this project strongly indicate that changes in virulence must have been a factor involved in the failure to set up experimental infection in the cattle and in the non-immunocmprimised inbred BALB/c mice.

Various measures including the use of antimicrobial agents have been tested *in vitro*(Dubey and Lindsay, 1996, Lindsay et al., 1997, Kim et al., 2002) or *in vivo* in mice (Gottstein et al., 2001) . However, there is no known drug that can be used to clear N. caninum infection in cattle (Dubey and Lindsay,1996). In the literature, in so far as it is a practical method at farm level is concerned, vaccination appears to be the method of choice to control the disease in cattle, when compared to the alternatives already discussed. However, to-date only one vaccine, which is a dead product, is commercially available at least in the USA. However, while the results achieved in cattle were disappointing, nevertheless in the absence of abortion in any of the challenge groups, it is not possible to evaluate the efficacy of the vaccine for use in this species .However, in sheep where it was used as an experimental vaccine, the results certainly were not encouraging. In animals that had been vaccinated, there was an amnestic response when the challenge dose of NC-1 tachyzoites was administered,

high, but decreased antibody levels, persisted until parturition. The antibody titre following vaccination alone was low suggesting that the administration of dead vaccine on its own did not stimulate a sufficient antibody response that might play a role in the protection of the foetus. Normally, in intracellular protozoan parasitic diseases, the cell-mediated response is the main protective type of response involved in protecting the dam from these diseases. Nevertheless, the low level of antibody produced, suggests that inoculation, at least during pregnancy with this vaccine and followed so closely by challenge did not afford protection. It is possible that vaccination of cattle and sheep well in advance of pregnancy may have resulted in a different outcome. This is supported by the finding in a recent study where cattle, which were previously exposed to infection and subsequently challenged during later pregnancies, did not abort.

While the results of work in this thesis, carried out in cattle and sheep in Ireland have greatly contributed to the knowledge of the disease both in this country and generally, a lot of gaps remain in the understanding of the disease. In particular, now that it is known that the disease is very prevalent in the Irish cattle herd and possibly is a significant disease in the sheep flock, the development of control measures, which are practical and economically feasible, remain a very important goal. The use of vaccination as a control measure is probably the ultimate goal, but the development of an effective vaccine will undoubtedly involve gene deletion in some form or other. However, probably the most important goal to be achieved is to disseminate the results of this project to the larger audience of herd and flock owners, veterinary practitioners, regulatory authorities and scientists working in the various areas covered by this project.

In this project considerable progress has been made in furthering the understanding of neosporosis in cattle and sheep, nevertheless, many unanswered questions remain. For instance, having established the levels of the disease in the Irish cattle herd, there is still no information on the true economic significance of the disease on the national economy of the country. The results of the studies, described in chapters 2, 3 and 4, provide an ideal database for such a study to be carried out in the future. No comparable database at national level is currently available in any other country worldwide.

To date, the prevalence of naturally occurring neosporosis in sheep has been described as sporadic. The results described in chapter 5 of this thesis suggest that this is not accurate, and that the level of disease in sheep in Ireland may be significantly higher than that reported in other countries. Furthermore, there is evidence in chapters 8 and 10, that the disease could easily be misdiagnosed as toxoplasmosis, which is very prevalent in sheep in in Ireland (O'Brien and Geraghty,1990). It would be a very interesting project for the future, to carry out a countrywide seroepidemiological study in sheep to investigate any association between the two closely related protozoan parasites *Toxoplasma gondii* and *N. caninum* in causing abortion in the Irish sheep flock.

Finally, the failure to set up infection in cattle, following experimental inoculation with large doses of NC-1 tachyzoites, raises very interesting questions in relation to the virulence of the NC-1 isolate used in cattle in this thesis. Currently, work is in progress in this laboratory, to determine if the isolate used in the cattle trials described in chapters 7 and 9 could be used to produce a live vaccine for use in cattle.

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Appendix A

Final Date: 21/1/02

Sheep Flock 2 (CVRL) – Titres - Toxoplasma Elisa – Toxoplasma Direct Agglutination Titres (Logs) - Neospora Elisa – Neospora IFAT Titres (Logs)

Experimental Group **Vaccine Group [A]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Yellow	Pre Inoc 27/12/00	*	Booster 17/1/01		31/1/01		28/2/01		28/3/01		25/4/01		Pre Inoc 27/12/00	**	Booster 17/1/01	1/256	31/1/01	1/256	28/2/01	1/256	28/3/01	1/256	25/4/01	1/256
Y-2	**.231**	2	0.172	2	**.280**	2	**.247**	2	.174	2	**.214**	2	.112	1	.060	5	.110	4	**.537**	6	**.404**	5	.244	-
Y-3	**.658**	8	**.774**	7	**.990**	8	**1.439**	8	**1.054**	7			.004	1	.065	-	.289	4	**.539**	6	**.925**	8		
Y-4	**1.129**	8	**1.039**	8	**1.357**	8	**1.442**	8	**1.032**	8			.038	3	.104	5	.307	6	**.743**	7	**1.000**	9		
Y-6	**.726**	6	**1.185**	4	**1.119**	6	**1.089**	5	**.662**	5			.036	0	.060	-	.384	7	**.658**	7	**.504**	6		
Y-7	**.412**		**0.579**	8	**0.627**	7	**1.192**	8	**.644**	8			.052		.052	-	.068	-	**.432**	7	**.900**	9		
Y-11	.037	0	.004	0	.070	0	0.050	0	.020	0	.051	0	.024	2	.132	-	.393	4	**1.017**	9	**1.164**	10	.290	6
Y-12	**.840**	6	**1.019**	6	**0.871**	6	**.987**	6	**1.062**	7			.004	2	.045	4	.116	-	.348	6	**.659**	6		
Y-14	.022	0	.005	0	.036	0	.074	0	.007	0			.020	3	.142	5	**.418**	6	**.725**	6	**.593**	6		
Y-15	**0.771**	9	**0.862**	9	**1.088**	9	**1.136**	9	**1.033**	9			.068	2	.173	4	.233	-	**.725**	9	**.691**	7		
Y-16	0.043	0	.029	0	.036	0	.067	0	.107	0			.033	0	.010	-	.005	-	**.653**	7	**.963**	9		
Y-17	**0.315**	4	**0.270**	5	**.327**	5	**.584**	5	**.438**	5			.013	2	.092	4	.154	4	**.572**	5	**.998**	8		
Y-18	.106	0	0.147	0	.060	0	.096	0	.194	0			.069	2	.246	-	.336	6	**.862**	8	**1.110**	9		
Y-20	.031	0	0.012	0	0.014	0	.031	0	.088	0			.220	2	.010	-	.259	6	**.918**	8	**.951**	10		
Y-187	.023	0	0.010	0	0.043	0	.134	0	.164	0			.029	1	.071	-	.326	7	**.720**	7	**.934**	9		

Toxoplasma Interpretation:

< 20% OD = negative
20 → 30% OD = $^1/_C$
30 → 100% OD = weak positive
> 100% OD = positive

*** = Toxo D.A. Log Titre**

0 = < $^1/_{32}$ 3 = $^1/_{256}$ 6 = $^1/_{2048}$
1 = $^1/_{64}$ 4 = $^1/_{512}$ 7 = $^1/_{4196}$
2 = $^1/_{128}$ 5 = $^1/_{1024}$ 8 = $^1/_{8192}$ 9 = $^1/_{16,384}$

Neospora Interpretation:

< 40% OD = negative
40 → 50% OD = $^1/_C$
> 50% = positive

**** = Neospora IFAT – Log Titre**

1 = $^1/_{32}$ 4 = $^1/_{256}$ 7 = $^1/_{2048}$
2 = $^1/_{64}$ 5 = $^1/_{512}$ 8 = $^1/_{4096}$
3 = $^1/_{128}$ 6 = $^1/_{1024}$ 9 = $^1/_{8192}$ 10 = $^1/_{16,384}$

Appendix A (cont'd)

Sheep Flock (CVRL) - Titres

Experimental Group **Vaccine Control Group [B]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Yellow	Pre Inoc 27/12/00	*	Booster 17/1/01		31/1/01		28/2/01		28/3/01		25/4/01		Pre Inoc 27/12/00	**	Booster 17/1/01	1/256	31/1/01	1/256	28/2/01	1/256	28/3/01	1/256	25/4/01	1/256
Y-5	**0.583**	7	**.870**	8	**.732**	6	**.712**	8	**.790**	7	**.550**	7	.071	0	.266	-	.325	-	.386	-	.341	-	.139	-
Y-8	**1.597**	6	**1.133**	7	**.950**	7	**1.006**	6	**.989**	7	**1.152**	7	.049	0	.169	5	.221	6	.256	-	.218	-	.105	-
Y-10	**1.389**	9	**1.541**	9	**1.295**	10	**1.226**	9	**.988**	9			.008	2	.108	-	.158	6	.211	6	.118	-		
Y-13	0.008	0	.006	0	.003	0	.001	0	.012	0	.025	0	.026	0	.094	-	.280	5	.193	-	.129	-	.023	-
Red-16	**0.758**	7	**.840**	8	**.988**	7	**.804**	7	**.890**	8			.012	2	.173	-	.266	5	.331	5	.331	5		

Toxoplasma Interpretation:

< 20% OD = negative
20 → 30% OD = $^1/_C$
30 → 100% OD = weak positive
> 100% OD = positive

Neospora Interpretation:

< 40% OD = negative
40 → 50% OD = $^1/_C$
> 50% = positive

*** = Toxo D.A. Log Titre**

0 = < $^1/_{32}$ 3 = $^1/_{256}$ 6 = $^1/_{2048}$
1 = $^1/_{64}$ 4 = $^1/_{512}$ 7 = $^1/_{4196}$
2 = $^1/_{128}$ 5 = $^1/_{1024}$ 8 = $^1/_{8192}$ 9 = $^1/_{16,384}$

**** = Neospora IFAT – Log Titre**

1 = $^1/_{32}$ 4 = $^1/_{256}$ 7 = $^1/_{2048}$
2 = $^1/_{64}$ 5 = $^1/_{512}$ 8 = $^1/_{4096}$
3 = $^1/_{128}$ 6 = $^1/_{1024}$ 9 = $^1/_{8192}$ 10 = $^1/_{16,384}$

Appendix A (cont'd)

Sheep Flock (CVRL) - Titres

Experimental Group **Challenge Group [C]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Red	31/01/01	*	28/2/01		28/3/01		25/4/01						31/1/01	**	28/2/01	1/256	28/3/01		25/4/01	1/256				
R-1	0.104	0	0.132	0	.071	0							.032	1	.328	7	**.520**	9						
R-2	**1.477**	8	**1.478**	9	**1.556**	9	**1.347**	9					.003	2	.246	7	.208	4	.198	4				
R-3	**0.393**	7	**0.644**	8	**.439**	7							.023	2	**.416**	7	**.868**	9						
R-4	**0.922**	8	**1.143**	9	**.940**	9							.025	1	.293	6	**.638**	8						
R-5	**1.211**	9	**1.514**	9	**1.314**	9	**1.042**	9					.020	3	.389	7	**.452**	7	.294	7				
R-6	0.023	0	0.049	0	.060	0							.018	0	.267	7	**.735**	8						
R-7	**.886**	8	**1.481**	8	**1.061**	9	**1.138**	8					.035	2	**.574**	6	**.466**	6	.325	6				
R-8	**1.166**	6	**1.699**	7	**.966**	6							.032	2	.374	7	**.843**	8						
R-10	**0.676**	7	**1.284**	8	**.750**	8							.004	2	.257	6	.259	4						
R-11	0.445	7	**0.615**	7	**.464**	7	**.311**	7					.027	2	**.524**	6	**.745**	8	**.745**	9				
R-12	**1.080**	7	**1.186**	9	**.825**	9							.014	2	.263	6	**.507**	6						
R-13	**0.605**	4	**0.634**	5	**.443**	5	**.937**	5					.019	0	.383	7	.392	4	.359	-				
R-14	**0.905**	8	**1.107**	9	**.875**	8							.028	0	.296	7	**.946**	8						
R-15	0.048	0	**0.089**	0	.076	0	.097	0					.014	2	.357	7	**.449**	5	.275	5				
R-17	0.047	0	0.125	0	.154	0	.132	0					.007	2	**.401**	6	**.984**	8	**.798**	8				

Toxoplasma Interpretation:
< 20% OD = negative
20 → 30% OD = 1/C
30 → 100% OD = weak positive
> 100% OD = positive

Neospora Interpretation:
< 40% OD = negative
40 → 50% OD = 1/C
> 50% = positive

*** = Toxo D.A. Log Titre**

0 = < 1/32 3 = 1/256 6 = 1/2048
1 = 1/64 4 = 1/512 7 = 1/4196
2 = 1/128 5 = 1/ 1024 8 = 1/8192 9 = 1/16,384

**** = Neospora IFAT – Log Titre**

1 = 1/32 4 = 1/256 7 = 1/2048
2 = 1/64 5 = 1/512 8 = 1/ 4096
3 = 1/128 6 = 1/1024 9 = 1/8192 10 = 1/16,384

Appendix A (cont'd)

Sheep Flock (CVRL) - Titres

Experimental Group **Verro Control Group [K]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Pink	31/1/01	*	28/2/01		28/3/01		25/4/01						31/1/01	**	28/2/01		28/3/01		25/4/01					
Pi-1	.004	0	.003	0	.012	0							.022	2	.003	2	.007	2						
Pi-2	**.642**	6	**.789**	6	**.824**	7	**.814**	6					.004	1	.038	2	.034	3	.043	1				
Pi-3	.047	0	.013	0	.006	0							.062	2	.023	2	.019	2						
Pi-5	.007	0	.013	0	.005	0	.003	0					.067	2	.031	0	.036	1	.042	2				
Pi-7	**.603**	6	**.678**	6	**.594**	6							.035	3	.012	1	.028	3						
Pi-8	**.790**	4	**1.863**	7	**.882**	5							.023	0	.030	0	.021	2						
Pi-14	.012	0	.018	0	.005	0							.054	2	.020	0	.025	2						
Pi-13	**.560**	5	**.574**	5	**.541**	6							.002	2	.006	2	.007	1						

Toxoplasma Interpretation:
< 20% OD = negative
20 → 30% OD = $^1/_C$
30 → 100% OD = weak positive
> 100% OD = positive

Neospora Interpretation:
< 40% OD = negative
40 → 50% OD = $^1/_C$
> 50% = positive

*** = Toxo D.A. Log Titre**
0 = < $^1/_{32}$ 3 = $^1/_{256}$ 6 = $^1/_{2048}$
1 = $^1/_{64}$ 4 = $^1/_{512}$ 7 = $^1/_{4196}$
2 = $^1/_{128}$ 5 = $^1/_{1024}$ 8 = $^1/_{8192}$ 9 = $^1/_{16,384}$

**** = Neospora IFAT – Log Titre**
1 = $^1/_{32}$ 4 = $^1/_{256}$ 7 = $^1/_{2048}$
2 = $^1/_{64}$ 5 = $^1/_{512}$ 8 = $^1/_{4096}$
3 = $^1/_{128}$ 6 = $^1/_{1024}$ 9 = $^1/_{8192}$ 10 = $^1/_{16,384}$

Appendix A (cont'd)

Sheep Flock (CVRL) - Titres

Experimental Group **Non Vaccinated Control Group [D]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Green	27/12/00	*	17/1/01		31/1/01		28/2/01		28/3/01		25/4/01		27/12/00	**	17/1/01		31/1/01		28/2/01		28/3/01		25/4/01	
G-4	**.859**	7	**1.252**	7	**1.380**	7	**1.355**	7	**1.330**	8	**1.400**	8	.014	3	.001	3	.022	3	.039	3	.040	3		
G-5	**.297**	5	**.580**	5	**0.622**	5	**0.398**	4	**.453**	4	**.580**	5	.034	2	.015	2	.002	3	.015	3	.036			
G-6	**.847**	5	**.958**	6	**0.915**	6	**.897**	5	**.788**	6	**.658**	5	.047	2	.030	3	.009	2	.032	1	.021	1	.035	3
G-7	.010		.006	0	.011	0	.017	0	.000	0	.006	0	.026		.019	2	.041	0	.051	0	.047	2		
G-8	**1.225**	8	**1.522**	8	**1.651**	8	**1.210**	8	**1.440**	8	**1.485**	8	.009	2	.023	3	.023	2	.032		.035	2	.029	2
G-10	.009		.017	0	0.002	0	.012	0	.012	0	.007	0	.027		.033	3	.021	2	.018	2	.043	2		

Toxoplasma Interpretation:
< 20% OD = negative
20 → 30% OD = $^1/_C$
30 → 100% OD = weak positive
> 100% OD = positive

Neospora Interpretation:
< 40% OD = negative
40 → 50% OD = $^1/_C$
> 50% = positive

*** = Toxo D.A. Log Titre**
0 = < $^1/_{32}$ 3 = $^1/_{256}$ 6 = $^1/_{2048}$
1 = $^1/_{64}$ 4 = $^1/_{512}$ 7 = $^1/_{4196}$
2 = $^1/_{128}$ 5 = $^1/_{1024}$ 8 = $^1/_{8192}$ 9 = $^1/_{16,384}$

**** = Neospora IFAT – Log Titre**
1 = $^1/_{32}$ 4 = $^1/_{256}$ 7 = $^1/_{2048}$
2 = $^1/_{64}$ 5 = $^1/_{512}$ 8 = $^1/_{4096}$
3 = $^1/_{128}$ 6 = $^1/_{1024}$ 9 = $^1/_{8192}$ 10 = $^1/_{16,384}$

Appendix B

Final Date: 21/1/02
Page 1

Sheep Flock (CVRL) - Titres

Experimental Group **Slaughter Group @ 49 days (Infection) [F]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Pink	10/1/01	*	6/2/01		Foetal Sera								10/1/01	**	6/2/01	$^1/_{256}$	Foetal Sera							
					F-1		F-2										F-1	$^1/_{80}$	F-2	$^1/_{80}$				
Pi-4	**1.053**	8	**1.141**	8	.016	0					VC		.050	3	.007	-	.060	-						
Pi-15	**0.794**	7	**1.133**	8	N.I.L.								.015	3	.329	7	N.I.L.							
Pi-16	**0.837**	7	**1.393**	6	.014	0	.019	0					0.142	3	.392	7	.058	-	.027	-				
Pi-17	0.062	0	**0.240**	0	N.I.L.								.069	2	.248	6	N.I.L.							
Pi-18	0.059	0	**0.304**	0	.017	0	.033	0					.030	3	**.605**	8	.065	-	.063	-				
Pi-19	**0.943**	5	**1.305**	6	.023	0							.035	2	.439	7	.063	-						
Pi-20	**0.811**	7	**1.364**	7	.020	0							.015	2	.265	6	.054	-						
B-20	.023	0	.010	0	.039	0							.091	2	.341	6	.065	-						

Toxoplasma Interpretation:
< 20% OD = negative
20 → 30% OD = $^1/_C$
30 → 100% OD = weak positive
> 100% OD = positive

Neospora Interpretation:
< 40% OD = negative
40 → 50% OD = $^1/_C$
> 50% = positive

*** = Toxo D.A. Log Titre**

0 = < $^1/_{32}$ 3 = $^1/_{256}$ 6 = $^1/_{2048}$
1 = $^1/_{64}$ 4 = $^1/_{512}$ 7 = $^1/_{4196}$
2 = $^1/_{128}$ 5 = $^1/_{1024}$ 8 = $^1/_{8192}$ 9 = $^1/_{16,384}$

**** = Neospora IFAT – Log Titre**

1 = $^1/_{32}$ 4 = $^1/_{256}$ 7 = $^1/_{2048}$
2 = $^1/_{64}$ 5 = $^1/_{512}$ 8 = $^1/_{4096}$
3 = $^1/_{128}$ 6 = $^1/_{1024}$ 9 = $^1/_{8192}$ 10 = $^1/_{16,384}$

N.I.L. = Not in Lamb
VC = Vero Control

Appendix B (cont'd)

Sheep Flock (CVRL) - Titres

Experimental Group **Slaughter Group @ 70 days (Infect) [H]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Blue	31/1/01	*	12/2/01		28/2/01		Foetal Sera						31/1/01	**	12/2/01	1/256	28/2/01	1/256	Foetal Sera					
							F-1		F-2										F-1	1/80	F-2	1/80		
B-1	0.023	0	**.247**	0	.176	0							.047	2	.190		**.432**	7						
B-2	**0.628**	7	**.948**	7	**.843**	8	.031	0	.023	0			.027	2	.168		.440	5	.067	-	.068	-		
B-3	0.010	0	.005	0	.002	0	.031	0					.053	3	.290		.459	7	.065	1/1280				
B-4	**1.097**	7	**1.454**	10	**1.558**	9	.018	0					.027	3	.338		**.436**	5	.069	-				
B-5	**0.379**	4	**0.326**	4	**0.439**	5	.039	0			VC		.036	2	.061		.250	-	.069	-				
B-6	**0.966**	8	**1.066**	9	**1.125**	9	.031	0	.017	0			.011	1	.175		.366	6	.065	-		-		
B-7	.006	0	0.083	0	.062	0	.042	0	.015	0			.015	1	.208		**.699**	9	.057	-		-		
B-8	.062	0	**.251**	0	**.254**	0	N.I.L.						.011	1	.258		**.456**	6	N.I.L.					

Toxoplasma Interpretation:

< 20% OD = negative
20 → 30% OD = 1/C
30 → 100% OD = weak positive
> 100% OD = positive

*** = Toxo D.A. Log Titre**

0 = < 1/32 3 = 1/256 6 = 1/2048
1 = 1/64 4 = 1/512 7 = 1/4196
2 = 1/128 5 = 1/1024 8 = 1/8192 9 = 1/16,384

N.I.L. = Not in Lamb

VC = Vero Control

Neospora Interpretation:

< 40% OD = negative
40 → 50% OD = 1/C
> 50% = positive

**** = Neospora IFAT – Log Titre**

1 = 1/32 4 = 1/256 7 = 1/2048
2 = 1/64 5 = 1/512 8 = 1/4096
3 = 1/128 6 = 1/1024 9 = 1/8192 10 = 1/16,384

Appendix B (cont'd)

Final Date: 21/1/02
Page 3

Sheep Flock (CVRL) - Titres

Experimental Group **Slaughter Group @ 87 days (Infect) [H]**

Exper Tag	Mean O.D. Toxoplasma												Mean O.D. Neospora											
Orange	14/2/01	*	13/3/01		13/3/01								14/2/01	**	13/3/01	1/256	13/3/01				28/3/01		25/4/01	
					Foetus 1		Foetus 2										Foetus 1	1/80	Foetus 2	1/80				
O-1	0.145	0	**.645**	0	.002	0							.052	3	**.403**	4	.067	-						
O-2	0.012	0	.104	0	.007	0	.003	0					.047	0	.128	5	.065	-	.067	-				
O-3	0.034	0	.083	0	.002	0							.030	0	**.443**	4	.068	1/320						
O-4	**0.916**	5	**.923**	5	.007	0							.028	0	.246	4	.068	1/80						
O-5	**0.819**	5	**.689**	6	N.I.L.								.017	0	.178	7	NIL							
O-6	**1.115**	8	**1.056**	8	.007	0	.006	0					.053	0	.245	7	.068	-	.068	-				
O-7	**1.459**	8	**1.319**	9	.004	0							.059	0	.358	6	.069	1/2560						
Pi-10	0.051	0	.011	0	.004	0					VC		.027	2	.009	-	.058	-						

Toxoplasma Interpretation:
< 20% OD = negative
20 → 30% OD = 1/C
30 → 100% OD = weak positive

Neospora Interpretation:
< 40% OD = negative
40 → 50% OD = 1/C
> 50% = positive
100% OD = positive

*** = Toxo D.A. Log Titre**
0 = < 1/32, 1 = 1/64, 2 = 1/128, 3 = 1/256, 4 = 1/512, 5 = 1/1024, 6 = 1/2048, 7 = 1/4196, 8 = 1/8192, 9 = 1/16,384

**** = Neospora IFAT – Log Titre**
1 = 1/32, 2 = 1/64, 3 = 1/128, 4 = 1/256, 5 = 1/512, 6 = 1/1024, 7 = 1/2048, 8 = 1/4096, 9 = 1/8192, 10 = 1/16,384

N.I.L. = Not in Lamb
VC = Vero Control

Appendix C

Table 1: A comparison of the specific antibody response in sheep following vaccination and subsequent challenge with *N. caninum* tachyzoites at 70 days gestation.

Days of gestation	Antibodies to *N. caninum* in *T. gondii* Seronegative Sheep (6)		Antibodies to *N. caninum* in *T. gondii* Seropositive Sheep (8)		Antibodies to *N. caninum* in all sheep in Group I	
	* IFAT (Titre)	** ELISA % Positivity	* IFAT (Titre)	ELISA % Positivity	IFAT (Titre)	ELISA % Positivity
35	0.1 (0.3 – 1.6)	6.5	0.1 (0.2 – 1.5)	4.1	0.1 (0.0 – 1.6)	5.2
56	0.5 N/A	10.2	0.3 (1.6– 0.7)	8.1	0.4 (0.2 – 0.8)	9.0
70	0.9 (0.2 – 3.9)	28.9	1.0 (0.6 – 3.5)	20.8	0.7 (0.1 – 3.8)	24.3
98	2.9 (0.6 – 12.0)	81.5	1.5 (0.3 – 8.0)	57.0	2.1 (0.4 – 10.1)	67.4
126	7.3 (1.0 – 53.9)	95.2	2.2 (0.4 – 14.1)	76.0	3.9 (0.4 – 36.1)	84.2

* IFAT Titre expressed as $Log_2 \times 10^3$ (95% confidence interval)

** ELISA % Positivity expressed as a percentage of the test sample divided by positive control, positive result $\geq$ 50%.

Appendix C

Table 2: A comparison of the specific antibody response in sheep following vaccination alone without subsequent challenge at 70 days of gestation. Four of the sheep were seropositive for antibodies to *T. gondii* prior to the study indicating previous field exposure

Days of gestation	Antibodies to *N. caninum* in *T. gondii* Seronegative Sheep (1)		Antibodies to *N. caninum* in *T. gondii* Seropositive Sheep (4)		Antibodies to *N. caninum* in Group II Sheep (5)	
	* IFAT (Titre)	** ELISA % Positivity	IFAT (Titre)	ELISA % Positivity	IFAT Titre	ELISA % Positivity
35	0.0	0.0	0.1 (0.0 – 0.1)	3.5	0.1 (0.1 – 0.1)	3.3
56	0.0	0.0	0.4 (0.1 – 0.7)	17.9	0.5 (N/A)	16.2
70	0.5 N/A	0.0	0.5 (0.7 – 3.6)	24.3	0.7 (0.3 – 15.9)	20.0
98	0.0	0.0	1.5 (0.3 – 7.9)	29.6	0.7 (0.3 – 18.9)	27.6
126	0.0	0.0	2.2 (0.4 – 14.1)	25.2	0.5 (N/A)	15.8

* IFAT Titre expressed as $Log_2 \times 10^3$ (95% confidence interval)

** ELISA % Positivity expressed as a percentage of the test sample divided by positive control< positive result ≥ 50%

Appendix C

Table 3: A comparison of the specific antibody response in sheep following challenge with *N. caninum* tachyzoites at 70 days of gestation. In this Group 11 sheep were seropositive for antibodies to *T. gondii* during the period of the study

Days of gestation	Antibodies to *N. caninum* in *T. gondii* Seronegative Sheep (4)		Antibodies to *N. caninum* in *T. gondii* Seropositive Sheep (11)		Antibodies to *N. caninum* in Group III Sheep (15)	
	* IFAT (Titre)	** ELISA % Positivity	* IFAT (Titre)	** ELISA % Positivity	* IFAT (Titre)	** ELISA % Positivity
70	0.1 (.00 – 0.1)	1.8	0.1 (0.3 – 1.3)	2.1	0.1 (0.3 – 1.2)	2.0
98	1.7 (0.9 – 3.4)	33.8	1.5 (0.7 – 3.0)	36.5	1.6 (0.8 – 3.1)	35.8
126	2.9 (0.3 – 30.5)	67.2	1.7 (0.1 – 22.5)	57.5	1.8 (0.2 – 211.5)	60.1
154	1.4 (0.1 – 25.9)	67.2	1.5 (0.1 – 24.5)	38.2	1.5 (0.1 – 183.9)	42.8

* IFAT Titre expressed as $Log_2 \times 10^3$ (95% confidence interval)

** ELISA % Positivity expressed as a percentage of the test sample divided by positive control, positive result $\geq 50\%$

Appendix C

Table 4: A comparison of the specific antibody response to *N. caninum* in sheep following inoculation with Vero cells at 70 days of gestation. In this Group 4 these sheep were seropositive for antibodies to *T. gondii* indicating previous exposure to natural infection

Days of gestation	Antibodies to *N. caninum* in *T. gondii* Seronegative Sheep (2)		Antibodies to *N. caninum* in *T. gondii* Seropositive Sheep (4)		Antibodies to *N. caninum* in Group IV Sheep (6)	
	* IFAT (Titre)	ELISA % Positivity	* IFAT (Titre)	ELISA % Positivity	IFAT (Titre)	ELISA % Positivity
35	0.1 (0.1 – 0.1)	2.7	0.1 (0.0 – 0.1)	2.6	0.1 (0.0 – 0.1)	2.6
56	0.1 (0.0 – 0.2)	2.6	0.1 (0.1 – 0.2)	1.7	0.1 (0.1 – 0.2)	2.0
70	0.1 (N/A)	3.1	0.1 (0.0 – 0.2)	1.4	0.1 (0.0 – 0.2)	2.0
98	0.1 (N/A)	3.5	0.1 (0.0 – 0.3)	3.0	0.0 (0.0 – 0.7)	3.1
126	0.1 (0.1 – 0.1)	4.5	0.1 (0.0 – 0.2)	3.3	0.1 (0.0 – 0.1)	3.7

* IFAT Titre expressed as $Log_2 \times 10^2$ (95% confidence interval)

** ELISA % Positivity expressed as a percentage of the test sample divided by positive control, positive result $\geq$ 50%

Appendix D

The antibody levels in the cows following vaccination and/or challenge with an experimental inoculation of NC-1 tachyzoites at 125 days of gestation (Chapter 9).

Days of Gestation ± 10	Blood Serum Antibody Levels to *N. caninum*					
	Group I (Vaccine & Challenge)		Group II (Vaccine Only)		Group III (Challenge)	
	* IFAT Titre	* ELISA OD	IFAT Titre	ELISA OD	IFAT Titre	ELISA OD
90	0.0	0.130	0.0	0.119	-	-
111	0.3 (0.1 – 1.6)	0.057	0.2 (0.1 – 0.6)	0.352	-	-
125	1.3 (0.4 – 4.9)	2.360	0.7 (0.2 – 2.2)	1.238	0.0	0.118
153	9.3 (4.6 – 18.7)	2.940	0.2 (0.2 – 0.2)	0.272	30.1	1.195
181	7.7 (1.9 – (31.3)	3.400	0.2 (0.1 – 0.4)	0.250	12.8	1.347
209	6.1 (2.1 – 18.1)	3.712	0.0	0.200	7.6	0.807
237	3.7 (1.7 – 7.9)	2.394	0.0	0.127	4.2	0.607
265	3.9 (2.1 – 7.2)	1.968	0.0	0.159	5.7	1.034

* IFAT titre expressed as Geometric Mean Titre (G.M.T.) ($Log_2 \times 10^3$) [95% confidence interval]).

** ELISA OD = Mean Optical Density.

Summary of antibody levels to *N. caninum* in blood sera of pregnant cows following vaccination and/or challenge.

Appendix E

RPMI-1640 Medium

(5 Litres)

RPMI-1640 10 x Liquid 500 ml (GIBCO BRL cat. No. 22511-026)
Sodium Pyruvate 0.5 g (DBH 44094)
Sodium Bicarbonate 11 g (BDH 102475W)
Non-Essential Amino Acids (100x) 50 ml (GIBCO BRL cat. 11140-035)
Essential Amino Acids (50x) 100 ml (GIBCO BRL cat. 11130-036)
Vitamin Solution (100x) 50 ml (GIBCO BRL cat. 11120-037)
Horse Serum 500 ml (GIBCO BRL cat. 16050-098)
Penicillin G Final Conc. 100 μ/ml (10 ml)
Streptomycin SulphateFinal Conc. 100 μg/ml (of pen/strep soln.)
Polymixin B Final Conc. 20 μ/ml (1 ml of pre-made 10 ml soln.)
Nystatin Final Conc. 18.1 μ/ml (5 ml of pre made 10 ml soln.)
Sterile Distilled H_2O up to 50 Litres

ALL ANTIBIOTICS FROM SIGMA:

Penicillin G	P-3032
Streptomycin Sulphate	S-9137
Polymixin B	P-4932
Nystatin	N-4014