Review article

# *Neospora caninum* – how close are we to development of an efficacious vaccine that prevents abortion in cattle?

Michael P. Reichel[1], John T. Ellis*

*Department of Medical and Molecular Biosciences, University of Technology, Sydney, P.O. Box 123, Broadway, NSW 2007, Australia.*

*Corresponding author.

Tel.: +61-2-9514-4161; fax: 61-2-9514-8206. *E-mail address*: john.ellis@uts.edu.au

[1]Present address: School of Veterinary Science, Adelaide University, South Australia

**Abstract**

*Neospora caninum* is a protozoan parasite that causes abortion in cattle around the world. Although the clinical signs of disease in both dogs and cattle have now been recognised for over 20 years, treatment and control options are still limited, despite the availability of a commercial vaccine in some countries of the world. The case for an efficacious vaccine has not been convincingly waged by farmers, veterinarians and other members of the agricultural and rural communities. In recent times, however, economic modelling has been used to estimate the industry losses due to *Neospora*-associated abortion, providing, in turn, the business case for forms of control for this parasite, including the development of vaccines. In this review, we document progress in all areas of the vaccine development pipeline, including live, killed and recombinant forms and the animal models available for vaccine evaluation. In addition, we summarise the main outcomes on the economics of *Neospora* control and suggest that the current boom in the global dairy industry increases the specific need for a vaccine against *N. caninum*-associated abortion.

*Keywords*: *Neospora caninum*, Cattle, Abortions, Vaccine, Control, Economics

## 1. Introduction

*Neospora caninum*, a protozoan parasite closely related to *Toxoplasma gondii*, is recognized as a major cause of disease in dogs (Reichel et al., 2007) and, in particular, abortions in cattle around the world (Dubey et al., 2007). The parasite impacts seriously on the economic performance of the dairy and beef industries (Reichel and Ellis, 2006), with attributable losses measured in the millions of dollars. Early research efforts were focussed on the diagnosis of disease (Dubey and Schares, 2006) and the use of diagnostic tools to obtain a better understanding of the pathology and pathogenesis (Dubey et al., 2006). It is only more recently that controlling infection and/or abortions have come to the fore of research into neosporosis (Reichel and Ellis, 2002). Subsequent reviews on this topic include those by Brake (2002), Hemphill et al. (2006), Innes and Vermeulen (2006), Nishikawa et al. (2002) and Williams and Trees (2006).

Treatment options in dogs have recently been reviewed (Reichel et al., 2007). Essentially, there are a number of drugs available for the treatment of clinically affected dogs, which may result in the resolution of lesions. Treatment needs to commence promptly and be initiated as early as possible before clinical symptoms become irreversible.

To the primary producer, principally three control options appear available for dealing with *N. caninum* in cattle: a (as yet hypothetical) treatment with a parasiticide efficacious against *N. caninum*, a test-and-cull approach where infected animals are identified and eliminated from the herd (or breeding) and, lastly, vaccination.

Coccidiostatic drugs have been found to be efficacious against some stages of the parasite life cycle in vitro (Lindsay et al., 1994) and experimentally in vivo (Kritzner et

al., 2002) and appear to be reasonably efficacious (>90%). Questions, however, remain regarding the costs of such treatment and the likely meat and milk with-holding periods.

Test-and-cull approaches have been advocated (Larson et al., 2004) and successfully implemented in the field (Hall et al., 2005). *Neospora caninum*-infected cows were culled from the herd or not re-bred, and a reduction of the within-herd prevalence of infection immediately achieved, and preserved, as the rate of new infection (from post-natal infection) was very low. Economic analysis suggests, however, that this approach to controlling neosporosis is also very expensive (Reichel and Ellis, 2006) and unlikely to be economical, especially if a large part of the herd is infected.

Economic analysis does suggest that vaccination might be the most cost-effective approach to controlling neosporosis (Reichel and Ellis, 2006), and, largely, preferable to continuing to live with the disease (Reichel and Ellis, 2008). An inactivated vaccine against neosporosis has been available commercially for a number of years, but appears only partially (~50%) successful in preventing abortions in cattle (Romero et al., 2004). Live vaccination, utilising tachyzoites of an attenuated strain of *N. caninum* (NC-Nowra (Miller et al., 2002)), has demonstrated exceedingly high efficacy (measured as the ability to prevent abortions due to *N. caninum*) (Williams et al., 2007), but may suffer as a commercial product from limited shelf-life and high production cost.

The present manuscript will review the state of current knowledge and likely future directions of research into a vaccine against *N. caninum*, with particular emphasis on preventing abortion in cattle due to neosporosis.

## 2. *Neospora caninum*

### *2.1. Life cycle and epidemiology*

*Neospora caninum* infections entail a life cycle between a definitive host (dogs and some other canids (McAllister et al., 1998)) and the intermediate host (predominantly cattle, possibly sheep (Dubey et al., 2007)). Dogs excrete oocysts that become infectious within a couple of days. These may infect cattle and lead to either infection with no discernible clinical symptoms, or induce abortions two to 3 weeks later, usually at about 5-7 months of gestation (Dubey et al., 2006) (Fig. 1). On the other hand, oral ingestion of oocysts of *N. caninum* does not, in itself, reliably lead to infection and/or abortion in cattle (Trees et al., 2002), suggesting that preventing infection via the faecal-oral route might not be the critical step in preventing *Neospora* abortions.

Infected dams that have not aborted may, however, infect their offspring in utero, a mode of transmission that appears to be highly efficient (~80-95% (Paré et al., 1996; Davison et al., 1999)) and is thought to cause the majority of infections in the field (Trees and Williams, 2005). Abortion may thus be the exception rather than the norm, yet abortions often involve a large proportion of the cows at risk (i.e. in gestation) and can be of epidemic proportions (Thornton et al., 1994; Thurmond et al., 1997).

Post-natal transmission has been reported to be of generally low frequency (around 1% per cow year (Davison et al., 1999; Hietala and Thurmond, 1999)) and thus appears to only rarely contribute to the perpetuation of infection in the cattle population.

Epidemiological analysis implicates the presence of dogs on farms in abortion epidemics (Sawada et al., 1998; Wouda et al., 1999b), which would suggest that cattle

104 become infected by sporulated oocysts. Other scenarios suggest that a recrudescence of
105 the parasite in already, chronically, infected dams leads to abortion (Wouda et al., 1999a).

106 *2.2. Immunity and N. caninum infection*

107 Neosporosis in cattle is predominantly a disease of the pregnant dam that results
108 in either abortion or vertical transmission of the parasite to the foetus. Both humoral
109 (antibody) and cell-mediated immune (CMI) responses have been observed in
110 experimentally and naturally infected animals (Andrianarivo et al., 2005). Strong
111 antibody responses are produced by natural infection, vaccination with whole inactivated
112 tachyzoites of *N. caninum* (including the commercially available vaccine) (Andrianarivo
113 et al., 2005) or lysates of *N. caninum* tachyzoites in cattle (Williams et al., 2007) and in
114 the murine model (Miller et al., 2005). There is doubt, however, that the antibody-
115 responses are protective against abortion or vertical transmission (Innes et al., 2002). It
116 has been argued that it is the modulation of the CMI response as part of the naturally
117 occurring immune-modulatory changes during pregnancy that are at the core of the
118 abortions often associated with *N. caninum* infections (Quinn et al., 2002a).

119 A Th1-type immune response (with, for example, strong IFN-γ and IL-12
120 responses (Williams et al., 2007; Rosbottom et al., 2008)) is usually induced by *N.*
121 *caninum* infections, but this may interfere with the successful maintenance of pregnancy
122 and lead to abortion if the infection occurs early in pregnancy (Williams et al., 2000). In
123 the later phases of gestation the success of pregnancy relies on a switch to a Th2-type
124 immune response at the maternal-foetal interface (in order to allow the dam's immune
125 system to accept the foetal allograft). This, in turn, might favour the proliferation of the

126 parasite and facilitate its transmission across the placenta to the foetus (Innes et al.,
127 2005).

128 The foetus' own stage of developing immune-competence will then influence the
129 outcome; with early gestation infection leading invariably to abortion, and infections later
130 in pregnancy encountering an increasingly immune-competent foetus that can effectively
131 contain the *N. caninum* invasion resulting in congenitally infected, yet clinically normal,
132 calves being born (Williams et al., 2000).

133 Because of the peculiarities of its life cycle, a number of questions arise regarding
134 the epidemiological and immunological events that drive infection and determine
135 outcomes, i.e. abortion versus congenital infection.

136 Epidemiological information suggests that post-natal infection is at the root of
137 abortion epidemics and that the presence of dogs increases the risk of those epidemics
138 occurring (Bartels et al., 1999; Schares et al., 2004; Hobson et al., 2005). The timing of
139 infection also appears to be critical: experimental (de novo) infection with tachyzoites of
140 a foetopathic strain of *N. caninum* leads to abortion after about 3 weeks in pregnant cattle
141 that were infected on Day 70 of gestation. Infection on Day 210, however, results in the
142 birth of clinically normal, yet invariably, congenitally infected calves (Rosbottom et al.,
143 2008).

144 Field experience suggests that *N. caninum* abortions occur mostly between 5 – 7
145 months of gestation (Dubey et al., 2006), and may affect a large proportion (up to 56%)
146 of the cows at risk (Wouda et al., 1999a).

147 One of the interesting aspects about *N. caninum* is its ability to repeatedly cause
148 abortions in the same dam (Thurmond and Hietala, 1997b), although that risk appears to

reduce in subsequent gestations (Thurmond and Hietala, 1997b). The parasite is even more efficiently transmitting congenital infection from generation to generation (Paré et al., 1996), suggesting that the immunity developing after natural infection is either never very strong, or is sufficiently down-regulated during gestation to allow a recrudescence of the parasite (Innes et al., 2002). While abortions are generally reported from early and mid-gestation, congenital infection appears to occur later in gestation (from mid-gestation to the third trimester) (Guy et al., 2001).

Both scenarios (repeat abortions and repeated congenital infection of offspring) would, arguably, not augur well for the possibility of any successful vaccine development.

*2.3. Implications and requirements for a successful/efficacious N. caninum vaccine*

The challenge for an *N. caninum* vaccine appears to be thus either to be able to prevent infection of the dam, i.e. a vaccine that may be able to prevent a de novo infection in a previously naïve animal or, in the case of the already (congenitally or post-natally) infected dam to prevent (or ameliorate) a recrudescence of the parasite. That might prevent abortion (storms) from occurring, and/or prevent (possibly) repeated congenital infection of subsequent generation(s).

If the objective is to prevent abortion storms, then the primary infection of an early to mid-term in-calf cow needs to be prevented. Arguably the currently available vaccine is targeting early to mid-term of gestation, with vaccination regimes suggesting application before breeding commences, or early in the first trimester (http://www.neosporosis.com/product-description.asp). This approach might thus be

171 focussing on preventing infection from the ingestion of the sporulated, infective oocyst
172 stage excreted by the dog.

173 If, however, one assumes that abortions are triggered by a recrudescence of a
174 latent *N. caninum* infection, then the transformation of bradyzoites into the fast-
175 replicating tachyzoite stage needs to be curtailed. That alternative vaccine approach
176 could be aimed at preventing abortion by enhancing both humoral and cell-mediated
177 immune responses sufficiently to prevent the recrudescence of an *N. caninum*
178 parasitaemia late in gestation in an already infected animal. This latter approach may
179 also be successful in preventing (recurrent) congenital infection.

180 In the face of all these scenarios, what form should a vaccine to prevent *N.*
181 *caninum* infection and/or abortion in cattle take? There is substantial evidence to suggest
182 that vaccines can be developed to protozoan parasites that prevent clinical disease, rather
183 than vaccines that prevent infection, which is a more daunting task.

184

185 **3. 3. Existing (protozoal) parasite vaccines**

186 A number of parasite vaccines are now commercially available, and of particular
187 interest is the fact that many of these are against protozoa closely related to *N. caninum*.
188 Most of them are live vaccines; the first described was a *Besnoitia* vaccine, a live
189 attenuated vaccine for cattle based on a naturally occurring isolate from a wildebeest
190 (Bigalke et al., 1974). In the case of *T. gondii*, a commercial vaccine for sheep utilises a
191 strain of the parasite (S-48) that had lost its ability to form cysts after long-term passage
192 in mice (Buxton, 1993). A live cattle vaccine for *Babesia bigemina* and *Babesia bovis*
193 ('Tick fever") has been employed successfully in Australia for a number of years (Bock

et al., 2004). Live attenuated vaccines for poultry coccidiosis are also distributed commercially (Shirley et al., 2005). However, these (live) vaccines all suffer from several disadvantages; there is the risk of contamination with other animal pathogens (Bovine Viral Diarrhoea, Enzootic Bovine Leukosis, to name but a few), the higher cost of production (as they are usually prepared in cell culture or even in live animals) and a limited shelf-life with associated stability issues. For that latter reason, these types of vaccines are generally only produced to order, limiting their more general appeal and distribution.

Inactivated vaccines based on whole parasites or modified fractions exist for *Giardia* in dogs (GiardiaVax®) (Olson et al., 2000), *Sarcocystis neurona* (the cause of Equine protozoal myeloencephalitis) in horses (Marsh et al., 2004) and *Leishmania donovani* in dogs (Leishmune®) (Borja-Cabrera et al., 2002, 2004).

Wide-scale attempts to develop vaccines based on individual or combinations of proteins, including those expressed from a vector system, have not met with great success. Within the world of protozoology, arguably the most successful one is a subunit vaccine, based on native gametocyte antigens, that was introduced to the commercial world for the control of *Eimeria* in poultry (Wallach, 1997). This vaccine has now been used in millions of broilers around the world (Wallach et al., 2008). Its manufacture, however, is technically demanding requiring the infection of chickens, along with purification of parasites and proteins. A recombinant version would greatly improve the ease of manufacture.

Recombinant vaccines to malaria, despite enormous investment, have failed to deliver the promise, despite the wide range of candidates that have been evaluated.

Recent effort has come full circle and live and killed vaccines are now again under evaluation (Good, 2005). Progress in the creation of knock-out malarial parasites show that clinical signs of malaria can be prevented in a mouse model (Ting et al., 2008).

Successful recombinant vaccines have, however, been generated for some parasites. A highly efficacious (close to 100%), and in recombinant terms simple, *Escherichia coli*-expressed, vaccine against *Echinococcus granulosus* (Eg95) has been described for a number of years now (Heath et al., 2003; Lightowlers and Heath, 2004) yet has found little commercial application. This vaccine's approach has since been expanded to include other cestodes, such as *Taenia ovis*, *Taenia saginata* and *Taenia solium* (Lightowlers, 2006).

In contrast, "hidden (gut)" antigens of the helminth *Haemonchus contortus* are highly efficacious in their native form, yet many attempts at producing recombinant versions of similar efficacy have failed (Newton and Meeusen, 2003).

Recombinant vaccines against *Boophilus microplus* target a mid-gut protein and demonstrate varying efficacies around 50% (Andreotti, 2006). Bm86 homologues in other *Boophilus* species are now also being targeted (Odongo et al., 2007).

Clearly the transition from native to recombinant vaccine is an enormous step, yet to be achieved reliably in the field of anti-parasite vaccines. Nevertheless, the success of these developments potentially heralds the beginning of a new era in vaccine development focussed on parasitic organisms.

## 4. Economics of control

The magnitude of the economic losses incurred by primary producers around the world due to *Neospora*-associated abortion has been estimated to exceed hundreds of millions of dollars per year (Dubey et al., 2007). In Australia and New Zealand the estimates for annual losses due to abortions only are AUD $85 million for the dairy industry in Australia, $25 million in the beef industry and between NZ $17.8 million and 28.4 million annually to the dairy industry in New Zealand (Reichel, 2000; Reichel and Ellis, 2006). In Switzerland the median annual loss to the dairy industry was estimated to be Euro 9.7 million, which included the cost of abortion, the cost of curtailed milk production, increased veterinary costs and premature culling (Häsler et al., 2006a).

Losses are incurred by a range of aspects of the infection: abortions are the most prominent effects of *N. caninum* infection and can be devastating (and costly) when a large proportion of the at-risk (i.e. in-calf) cow population in a herd may abort (Thornton et al., 1994; Pfeiffer et al., 2002). In dairy cattle, the impact on milk production can add to these economic losses (Thurmond and Hietala, 1997a; Romero et al., 2005), although others have failed to record a correlation between infection and reduced production (Pfeiffer et al., 2002). *Neospora caninum*-infected cows are also more likely to be culled early (Thurmond and Hietala, 1996) and in beef cattle, weight gains and final body weight were negatively affected in serologically positive individuals, and an increase in veterinary treatment cost observed (Barling et al., 2000).

Previous economic modelling by the authors suggested that the threshold for intervention to be economical might be reached at within-herd prevalences of *N. caninum*

infection ranging between 18% to 21%. Below that threshold of infection it was more economical for the primary producer to live with the infection and risk of abortions. Dramatic increases in dairy livestock and dairy products can reduce those thresholds to between 10.8 to 12.6% (Reichel and Ellis, 2008). A live vaccination approach, possibly requiring only one vaccination in a cow's commercial life-time, will reduce this threshold even further, to a within-herd prevalence of *N. caninum* infection below 3%.

*4.1. Market need*

While there have been a number of previous studies that have quantified the cost of *N. caninum* infection, it has only been in the last couple of years that the cost of control has also been estimated and the two compared (Häsler et al., 2006a, 2006b; Reichel and Ellis, 2006).

There are several options for controlling neosporosis: one aims at eliminating or decreasing the risk of *N. caninum* infection at the farm level and consists of either:

- identification and elimination of either only the (*N. caninum*) aborted dams, selective non-breeding from aborted and/or all sero-positive dams or culling of all sero-positive cattle from the herd (predicated on the assumption that the major route of transmission in the herd is vertical)
- treatment of all (or all replacement) *N. caninum*-infected animals in the herd with a coccidiostat

Another approach might aim at preventing infection (and thus post-natal infection) with *N. caninum* at the farm level through:

- increased biosecurity (fences, testing of all incoming stock, restrictions placed on dogs, such as muzzling), in order to reduce the risk of post-natal (horizontal) infection, or
- vaccination of all susceptible stock, including both sero-positive and sero-negative cattle present in a herd.

These options, when fully costed in an economic model, suggested that in Australasia vaccination is the preferred control option. With increasing livestock prices, intervention is economically preferable over continuing to live with the disease (Reichel and Ellis, 2008), at the demonstrated national level of sero-prevalence of *N. caninum* (Reichel, 1998). This was even true when the commercially available vaccine (Neoguard®), which shows only limited efficacy was assumed in the model (Fig. 2). The benefits, in terms of the cost-benefit ratio, are even more convincing if a fully efficacious vaccine could be employed (Reichel and Ellis, 2006).

In the Swiss economic model (Häsler et al., 2006a), treatment of all calves with a cocciodiostat was the economically preferred outcome. That option, however, is still only hypothetically available.

An (efficacious) vaccine for neosporosis thus appears to be economically viable and justified.

*4.2. Customer expectations and concerns*

To the primary producer, the most noticeable and most devastating impact of *N. caninum* in the herd are abortions, particularly those of epidemic proportions. While major inroads have been made into the accurate diagnosis of these abortions, little has been offered in terms of practical management strategies in either the face of an outbreak, as a way of minimising the risk of an outbreak, or the repeat of such an occurrence. Primary producers would want to see a solution that gives them reasonable guarantees of solving the issue, preventing a (repeat) abortion outbreak in the future, with a rate of return that makes that option economically viable.

Treatment with a coccidiostat is only hypothetically available at the present time, while test-and-cull options are expensive, and only viable if uninfected stock can reliably be bought to replace the culled, infected ones.

Vaccination with the presently available commercial vaccine (Neoguard®) will interfere with the serological diagnosis of infection, i.e. vaccinated animals cannot be distinguished from naturally-infected cattle by serological testing (although molecular testing might be available, at a price, to test for the presence of the parasite in naturally infected animals viz absence in Neoguard® vaccinated ones). At the individual farm level, this may not present a problem, however if animals are traded, the differentiation of infection versus vaccination status may become an issue.

Neosporosis, however, is not a notifiable disease in most countries (although it is in Switzerland (Häsler et al., 2006a)), and thus very few countries would require testing for *N. caninum* infection in international trade. There is thus also no impediment to live vaccination, as long as assurances can be given (as would be required as part of the

regulatory process) against the possibility of a reversion to virulence, or wider dissemination of the vaccine parasite population into the wider target population.

Live vaccination may also present a problem with the timely delivery of vaccine doses, as storage and distribution of such a vaccine will be dependent on the provision of an efficient cold chain. In countries or regions with marked seasonal calving patterns this may put particular strains on production capacities (for example New Zealand, and Switzerland to an extent). On a positive note, however, seasonal calving systems may greatly enhance vaccine uptake because of the potential ease of scheduling vaccine production prior to need.

## 5. Animal models

Proof of concept for a vaccine requires a pathway of evaluation that ultimately relies on animal models. Mouse models, despite their limitations, are a helpful first screen, however the cow is the target species and it is necessary to carry out vaccination protocols in that species. In the case of neosporosis, mouse and cattle models exist through which vaccine approaches can and have been evaluated.

A number of trials with putative *N. caninum* vaccine candidates have involved the use of mice as an initial screen for the potential of the candidates. Mouse models for both CNS disease (cerebral neosporosis) and for transplacental transmission, exist and have been extensively validated (Atkinson et al., 1999; Miller et al., 2002). A model mimicking the abortion effect of *N. caninum* appears to be more difficult to reproduce in mice (Quinn et al., 2002b), although other researchers have persisted with this approach, seemingly with success (e.g. Lopez-Perez et al., 2008). Both viable and dead foetuses

350 can be identified in pregnant mice infected by *N. caninum* (Long and Baszler, 1996), and
351 so determining foetal loss is an attractive method for determining vaccine efficacy in the
352 mouse (Quinn et al., 2002b).

353 A model for cerebral (CNS) disease based on the BALB/c mouse is attractive
354 because of the florid brain pathology (Lindsay et al., 1995a) produced as a result of
355 inflammation that may also be associated with other clinical signs of disease such as head
356 tilting, limb paralysis and circling motion (Fig. 3). Monitoring weight loss over the 30
357 days p.i. provides a reliable way to monitor the outcome of infection, as a decrease in
358 body weight by 20% (a humane experimental end point) is normally associated with
359 subsequent death in this model.

360 Changes in clinical signs of infection in mice is also the basis of a scoring system
361 suggested by Bartley and colleagues (Table 1) (Bartley et al., 2006). Infection of mice by
362 *N. caninum*, like strains of *T gondii*, induces disease that is reflected by the natural
363 progression of clinical signs such as ruffled coat (Fig. 4), inactivity and weight loss, and
364 so by scoring the appearance of these, an acceptable end point for the experiment can be
365 reached (in the absence of death due to disease).

366 C57BL/6 mice are also recognised as a way to evaluate vaccines (Ramamoorthy
367 et al., 2007a). The lethal challenge model described would be unacceptable to most
368 developed countries of this world, since death is not recognised as a humane end point.
369 Mice that die from a lethal dose of tachyzoites do so probably from peritonitis and organ
370 failure. In contrast, mice given a sub-lethal dose show a range of pathological features
371 upon which a scoring system and a method for quantization of levels of brain pathology
372 was developed (Table 2) (Ramamoorthy et al., 2007a). This is a useful development,

373 although assessment of brain pathology even with this scoring system is still a very
374 subjective measure of infection and the responses to it.

375 Real-time PCR can also be used to measure parasite burden in tissues (Collantes-
376 Fernandez et al., 2006), and so can be used to monitor quantitative changes amongst
377 vaccinated and non-vaccinated groups (Vemulapalli et al., 2007). Real-time PCR
378 therefore does have a role to play in evaluating vaccine efficacy where there is a need to
379 determine parasite numbers (such as in mouse experiments, Table 3).

380 C57BL/6 mice have also been used in studies on transplacental transmission to
381 evaluate vaccines (Ramamoorthy et al., 2007c). Since these mice (like the BALB/c) are
382 susceptible to infection and the dam is likely to experience a life threatening infection, the
383 interpretation of data on transmission in utero may be difficult. Under conditions of such
384 an infection mice typically resorb their foetuses (depending on tachyzoite dose and time
385 of infection), and so the extent of the data generated may not be sufficiently rigorous for
386 vaccine studies. The study by Ramamoorthy et al. (2007c) is therefore highly significant
387 because the vaccination data suggest that susceptible animals can be used in vaccine
388 trials, since it may provide extra levels of selection for protective immunity (dam
389 survival, foetal survival (Fig. 5) and transplacental transmission). In the BALB/c mouse,
390 mortality is also the main outcome of post-natal development of pups infected with *N.*
391 *caninum* during pregnancy (Lopez-Perez et al., 2008).

392 We pioneered the use of the Quackenbush (Qs) mouse as a model of
393 transplacental transmission. The Qs mouse is a large mouse that is innately resistant to *N.*
394 *caninum*-induced pathology in the adult (Fig. 6). Infections of *N. caninum* given during
395 pregnancy are transmitted with high efficiency to the foetuses in utero. Of special

396 mention is the large litter sizes associated with this mouse type, with litters up to 15-20
397 being common. Consequently quality data on transplacental transmission (Table 4) can
398 be generated from the number of pups that are infected during vaccine
399 (immunisation/challenge) evaluation-style experiments (Miller et al., 2005).

400 Despite all these advances in using mice as a strategic screen for vaccine efficacy,
401 one ultimately needs to do cattle trials with putative vaccine candidates. Trials with cattle
402 are more expensive, and also hampered by the longer gestational period of cattle. They
403 present, however, the target species for any commercially successful vaccine and need to
404 be carried out, if only to satisfy the regulatory processes and to demonstrate efficacy.

405 A vertical transmission model was initially reported by Innes et al. (2001b)
406 (challenging cattle in mid-gestation at Day 140), who demonstrated the efficacy of a live
407 tachyzoite vaccination in preventing congenital transfer to the foetus (Table 5). Currently
408 the cattle immunisation/challenge model (Williams et al., 2007) is the most valuable
409 addition to the repertoire of approaches available for vaccine evaluation. The limitation
410 of this approach is clearly the ability of the challenge strain to reproducibly induce foetal
411 death/abortion. A number of factors may affect the ability of the selected strain to do
412 this. Probably the most important to consider is that long-term passage of *N. caninum*
413 may potentially ameliorate parasite virulence, thereby limiting the effectiveness of a
414 cultured organism to cause clinical disease (Bartley et al., 2006).

415 The same study (Williams et al., 2007) also demonstrated that live tachyzoite
416 vaccination can be highly efficacious in preventing abortions (against a challenge at Day
417 70 of gestation). While foetuses were protected, the dams also, apparently, did not
418 become persistently infected, as PCR could not detect any parasite DNA in their brains.

This would suggest some promise for a commercial vaccine, as persistence or even spread of the live vaccine in the target population does not appear to be a concern (as it might be, from a regulatory point of view, for live vaccines).

Sheep are also highly susceptible to *N. caninum* infections and have been used as a model to facilitate the study of *Neospora* pathology, as well as the effects of vaccination (Buxton et al., 1998, 2001; Innes et al., 2001a; Jenkins et al., 2004c), as have pygmy goats (Lindsay et al., 1995b).

## 6. Evaluation of vaccine efficacy in field trials

Similarly the conduct of field trials using vaccines to *N. caninum* is equally complex. Reproductive loss, by its very nature, can result from a variety of causes and a number of published studies to date have often concentrated on monitoring changes in abortion levels per se ( Muñoz Bielsa, J., Romero, J.J., Heuer, C., 2004. Control of neosporosis in cattle with Bovilis® Neoguard: the field experience. In, World Buiatrics Conference, Quebec; Romero et al., 2004), rather than specifically targeting *Neospora*-associated abortion. On the other hand, *N. caninum* lesions are quite distinct (Dubey et al., 2006), and diagnosis of specific *Neospora*-abortions is potentially feasible and necessary. Future field trials may be advised to monitor these as a specific measure of a vaccine's efficacy.

## 7. Alternative approaches for vaccine development

Within the context of vaccine development, it is worthwhile here to document current knowledge on potential methods and molecules that may form the basis of a commercial vaccine to neosporosis and associated abortion in cattle. There is a wide

443 range of approaches being evaluated, with live vaccination being the most advanced in
444 development.

445 *7.1. Inactivated vaccines*

446 Irradiated tachyzoites of *N. caninum* have been used to protect mice from an
447 otherwise lethal challenge (Ramamoorthy et al., 2006). This approach has also been used
448 successfully in the case of a commercial vaccine, which utilises 1,000 irradiated L3s,
449 inoculated twice, 4 weeks apart, for the cattle lungworm *Dictyocaulus viviparous*
450 (Intervet, Huskvac
451 http://www.intervet.co.uk/Products_Public/Bovilis_Huskvac/090_Product_Datasheet.asp
452 ).

453 The only commercially available vaccine against *N. caninum* (Neoguard®,
454 Intervet) contains $3 \times 10^6$ inactivated tachyzoites (and Havlogen as the adjuvant) and is
455 applied prior to breeding or early in the first trimester of gestation twice (formulated in a
456 5 mL dose), 4 weeks apart, with one or two annual booster vaccinations. The induced
457 immunological response appears to be mainly humoral in nature (Andrianarivo et al.,
458 2000). It has however been demonstrated that cell-mediated responses are instrumental
459 in effective protection against infection/abortion (Innes et al., 2002; Williams and Trees,
460 2006).
461

462 *7.2. Live vaccines*

463 A commercial vaccine available for *T. gondii* (Ovilis Toxovax®, Intervet) uses the
464 S-48 strain, and relies on that vaccine strain not being able to encyst in the primary host,

465 the sheep (Buxton, 1993). This prevents a persistent infection and subsequent
466 recrudescence, as appears to be sometimes possible with field infections (Buxton et al.,
467 2007). The development of this vaccine is, however, important in that it shows that a
468 vaccine can be produced that prevents abortion due to a parasitic infection of a livestock
469 animal. In addition, it shows admirably that manufacturing and distribution issues
470 surrounding the sale of a live vaccine are surmountable, when there is a market driving
471 such a vaccine. Toxovax® is distributed in New Zealand, for example, where millions of
472 doses/year are regularly sold.

473 Live vaccines for *N. caninum* can take on many forms, but are likely to be based
474 on populations of parasites that are attenuated in one or more of their phenotype
475 characteristics. Temperature –sensitive mutants and irradiated tachyzoites represent two
476 such populations which have been reported (Lindsay et al., 1999; Teixeira et al., 2005;
477 Ramamoorthy et al., 2006). Both types are successful at inducing immunity in a mouse
478 that reduces or completely prevents the onset of clinical signs of disease, as well as brain
479 pathology associated with infection. There are no reports yet of trialling these attenuated
480 parasites in pregnant cattle.

481 Naturally attenuated wild-type populations of *N. caninum* are currently under
482 evaluation as live vaccines and progress has been rapid. Specifically, a live vaccine
483 approach that prevents abortion in cattle, based on tachzyoites isolated from naturally
484 infected animals that do not show signs of neosporosis, is currently being explored. The
485 extensive observations that *N. caninum* can exist in cattle without them showing any
486 clinical signs of disease may represent the ease through which this parasite can adapt to
487 life in cattle. Cattle that previously aborted due to *N. caninum* have a much lower risk of

488 abortion and typically carry their calves to term, leading to the birth of a normal calf
489 (Barr et al., 1993). Whether this really represents adaptation of the parasite to its host or
490 other scenarios such as induction of immunity is really not known. However it is clear
491 that tachyzoites derived from asymptomatic calves may be attenuated in their ability to
492 cause disease in the mouse and therefore represent leads for a cattle vaccine. Several
493 lines of evidence show this to be true.

494 NC-Nowra was first isolated from a calf without any clinical signs of infection,
495 and it was identified through a comparison with NC-Liverpool as attenuated in its ability
496 to induce clinical signs of disease and brain pathology in mice (Miller et al., 2002).
497 Vaccine trials in mice (Miller et al., 2005) showed that infection of mice with NC-Nowra
498 before pregnancy resulted in a dramatic reduction in transplacental transmission of a
499 challenge strain given during pregnancy. Miller et al. (2005) used live tachyzoites of
500 NC-Nowra to vaccinate outbred Qs mice before pregnancy and this reduced vertical
501 transmission by more than 80-90%. The application of multiplex PCR confirmed the
502 identity of the *N. caninum* in the remaining infected pups as the challenge strain NC-
503 Liverpool (Al-Qassab et al., 2009). Subsequent inoculation of $10^7$ tachyzoites of NC-
504 Nowra into cattle prior to breeding protected 100% of foetuses from death by an
505 otherwise lethal challenge by NC-Liverpool at Day 70 of gestation (Williams et al.,
506 2007). In the same study lysates of NC-Nowra also failed to protect cattle from abortion
507 (Williams et al., 2007). Such data clearly calls for further evaluation of the live vaccine
508 approach, although this strategy may warrant concerns regarding the possibility of the
509 inoculum persisting in the host. In the study by Williams et al. (2007) the inoculated
510 dams were found to be free of any parasitic DNA. Reversion in virulence may also be

another concern undermining the live vaccine approach, but as yet there are no reports of this occurring from animal experiments. Finally, another concern may be whether *N. caninum* is responsible for early foetal loss in cattle. In mice, prior infection with *N. caninum* was reported to reduce the number of pups/litter, and field work with cattle provided anecdotal evidence for early foetal loss (in that sero-positive cattle required more attempts by artificial insemination that sero-negative cows to generate a pregnancy) (Hall et al., 2005).

Other isolates are now starting to emerge with biological properties that, like NC-Nowra, are attenuated in their ability to cause disease in mice. Nine isolates were made from asymptomatic calves in Spain (Regidor-Cerrillo et al., 2008), and one of them (Nc-Spain-1H) failed to induce clinical signs in a BALB/c mouse, grew slowly in vitro, and provided protection against foetal death in a pregnant mouse model (Rojo-Montejo et al., 2009). Isolates from Spain, a country where bovine spongiform encephalopathy in cattle is increasing in incidence, are unlikely to find global appeal for live vaccine development, especially for big dairy markets such as the USA, where safety concerns for food-borne diseases are high on the agenda.

The potential short shelf-life of a live vaccine product means that the manufacture and distribution of the live vaccine to meet the potentially large global market that exists in cattle producing countries requires further consideration. Experience with Toxovax® shows, however, that these potential problems with the distribution of a live formulation can be overcome.

The manufacture of a live vaccine also requires that several issues relating to the quality control of a commercial product be addressed. *Neospora caninum* grows well in

534 tissue culture and the viability of tachyzoites can be easily assessed by in-vitro culture.
535 The growth of tachyzoites can be observed simply through changes in cell number (Lei et
536 al., 2005b). PCR also allows the monitoring of tachyzoite viability (Strohbusch et al.,
537 2008). While thus far used for the evaluation of the efficacy of compounds for the
538 treatment of *N. caninum* tachyzoites, such PCR approaches may also be of value in the
539 future for the quality control of tachyzoite numbers in commercial production of any live
540 vaccine.

541 *7.3. Subunit (and recombinant) vaccines*

542 Most studies published to date have focussed on the evaluation of tachyzoite
543 proteins for their vaccine potential despite the fact that it is still not clear whether this life
544 cycle stage contains protective antigens. That tachyzoites contain molecules that confer
545 protective immunity is debateable, since lysates derived from them have shown varying
546 ability to induce immunity. For example, Liddell et al. (1999a) showed that a single
547 injection of a crude lysate into mice before pregnancy completely prevented vertical
548 transmission in BALB/c mice (Liddell et al., 1999a). In contrast, Miller et al. (2005)
549 showed that an immune response generated to a lysate from NC-Nowra did not prevent
550 transplacental transmission of a challenge strain given during early gestation (Miller et
551 al., 2005). Unfortunately, no comparison has yet been made between the vaccination
552 outcomes using lysates made from different isolates, but such a comparison may be
553 helpful in overcoming the doubt that exists on lysates as a vaccine. Proteomic analyses
554 have shown that tachyzoites from two different isolates of *N. caninum* are not identical in
555 their molecular composition, suggesting that natural variation exists amongst natural
556 populations (Lee et al., 2005; Shin et al., 2005b). Whether such differences contribute to

557 the ability of a tachyzoite preparation to act as an effective vaccine is unknown;
558 nevertheless, the study by Liddell et al. (1999a) gave hope that a subunit vaccine was
559 potentially feasible (Liddell et al., 1999a).

560 The molecules present in the Excreted Secreted (ES) fraction are now being more
561 thoroughly defined (Jenkins et al., 2004b; Liao et al., 2006). This fraction, although
562 difficult to produce in terms of quantity and quality, contains many of the molecules that
563 are now recognised as important antigens of *N. caninum* (some of which are described
564 below). In its own right, this fraction may be worth evaluating in a cell-free vaccine
565 formulation.

566 Generic approaches for the discovery, identification and subsequent
567 characterisation of vaccine candidates from *N. caninum* have previously been
568 documented (Hemphill et al., 1999; Jenkins, 2001; Ellis et al., 2003). A large number of
569 proteins are known to exist in *N. caninum* (Lee et al., 2003, 2004) and, similar to current
570 research on other Apicomplexa, the choice of candidates for vaccine evaluation has
571 focussed on those that are likely to be located on the parasite surface (such as membrane
572 proteins) or involved in parasite-host interaction. In the case of the latter, those
573 molecules present and secreted from organelles such as micronemes and dense granules
574 during host cell invasion are a common choice (Mercier et al., 2005). Such molecules are
575 also typically found in the ES fraction that can be produced from parasites maintained in
576 vitro. Many such molecules, identified initially because of their antigenic properties,
577 have now been characterised from *N. caninum*.

578 From first principles it appears irrational to suggest that molecules found in *N.*
579 *caninum*, and that are also highly conserved amongst other species of cyst-forming

580 coccidian, are good vaccine candidates. Vast differences exist in the biology of these
581 species, which suggests that a common mechanism of immunity is unlikely. Indeed a
582 model of common (e.g. the importance of IFN-γ as a central defence mechanism) and
583 species-specific host responses (eg antibody production) is obviously applicable to the
584 Toxoplasmatinae. For example, infections by *T. gondii* and *N. caninum* do not induce
585 cross-protective immunity (Lindsay et al., 1990; Innes et al., 2001a). However, extensive
586 studies on the composition of the Toxoplasmatinae (and Apicomplexa more generally)
587 show that a wide variety of taxa share many molecules. Consequently, it is difficult to
588 ignore the vast amount of vaccine-related research that has occurred in malaria and other
589 Apicomplexa, where proteins that are homologous amongst species are easily identifiable
590 (Wan et al., 1996; Ajioka, 1998; Ajioka et al., 1998; Ellis et al., 2003; Li et al., 2003).
591 This research may clearly provide useful pointers to those molecules that may determine
592 or direct the host-parasite relationship that can be the focus of vaccine development.

593 The ability of proteins to interact and direct the host immune response (antigenic
594 in the broadest sense) represent one criterion by which *N. caninum* molecules can be
595 selected for evaluation as vaccines. Those molecules that are antigens have now been
596 extensively studied, and indeed detection of antibody to such molecules was one of the
597 first approaches to define the immunodominant molecules of *N. caninum* (Hemphill and
598 Gottstein, 1996; Lally et al., 1996). More recently, proteomic approaches were used to
599 define more broadly the immune-dominant molecules present in tachyzoites of *N.*
600 *caninum*, as defined by IgG, IgA and IgE (Lee et al., 2004; Shin et al., 2004, 2005a,
601 2005b).

One useful approach that has emerged for the rapid assessment of a molecule's vaccine potential is the evaluation of antibodies raised against the molecules to prevent in vitro attachment and invasion. Several studies have shown that antibodies raised to recombinant proteins can reduce cell invasion in vitro, suggesting that induction of antibodies in vivo by vaccination is worth further evaluation (Augustine et al., 1999; Zhang et al., 2007b; Debache et al., 2008).

Cyclophilin, first identified in *N. caninum* as an expressed sequence tag (Hemphill and Gottstein, 2000), is a fine example where common knowledge from the *T. gondii* discipline has helped shape research into *N. caninum.* In *T. gondii*, cyclophilin is an 18 kDa protein that is a potent stimulator of IFN-γ (Aliberti et al., 2003; Golding et al., 2003; Yarovinsky et al., 2004). Since IFN-γ is a central mediator of immunity to *N. caninum* and other parasitic protozoa (Innes et al., 1995; Quinn et al., 2002a), such molecules that stimulate production of this important cytokine must rank high on the list of vaccine candidates for evaluation.

Molecular function and cell location are other important criteria to consider during vaccine development. The literature in parasitology is full of references to molecules that have important roles in cell structure, metabolism, respiration, as well as many other cellular activities. Should one focus on identifying essential functions as targets for vaccine development? Clearly there is some evidence that this approach may be an effective strategy since targeting of hidden antigens would appear to be a worthwhile aim in protozoology (Knox, 2000; Nuttall et al., 2006). Molecular function is also closely tied to cellular location; hence the focus on surface proteins in many vaccine programs.

As with *T. gondii*, the surface of the *N. caninum* tachyzoite is dominated by the glycosylphosphatidylinositol (GPI) anchored protein SAG1 homologue (Lei et al., 2005a), also known as p29 (Howe et al., 1998). Although other molecules are present and detectable in membrane preparations by Western blotting, the relative abundance of these is significantly lower (Lei et al., 2005a). Previously we reported the presence of a 22 kDa protein on the surface of *N. caninum* tachyzoites (Lei et al., 2005a), but subsequent protein analyses showed this was not an *N. caninum* protein and so was not pursued further (unpublished data). Immunisation of mice with recombinant SAG1 shows significant protection against cerebral infection by *N. caninum* (Cannas et al., 2003a).

The surface of the *T. gondii* tachyzoite also contains members of a family of SAG related sequences (SRS) (Jung et al., 2004). A homologue of the SRS2 surface protein of *T. gondii* is known to be present on the surface of *N. caninum* (Hemphill and Gottstein, 1996; Hemphill et al., 1997; Howe et al., 1998) and is found on both tachyzoites and bradyzoites (Hemphill, 1996; Fuchs et al., 1998). Antibodies raised to SRS2 can partially inhibit tachyzoite attachment and invasion of host cells (Hemphill, 1996; Nishikawa et al., 2000c; Cho et al., 2005; Haldorson et al., 2006). Recent evidence suggests this molecule is also a strong vaccine candidate. Mice immunised with iscoms containing recombinant SRS2 had lower amounts of *N. caninum* DNA in their brains compared to a control group (Pinitkiatisakul et al., 2005, 2007) and improved gerbil survival (Cho et al., 2005). Another study demonstrated that inoculation of native SRS2 into mice induced immunity that prevented transplacental transmission in mice (Haldorson et al., 2005). The immune response induced was of the Th2 type, which suggests a Th1 response alone

647 may be insufficient to prevent transplacental transmission of *N. caninum*. NcSRS2
648 coupled to palmitic acid (giving a lipoprotein), when injected into cattle with Freund's
649 adjuvants, induced T-cell activation and IFN-γ secretion, similar to that induced by a live
650 *N. caninum* infection (Staska et al., 2005; Baszler et al., 2008). Such observations
651 suggest that vaccine trials with SRS2 in cattle are warranted.

652 Dense granules (DG) are secretory organelles found in cyst-forming coccidia. The
653 contents of DG are typically secreted into the parasitophorous vacuole (PV) during the
654 invasion process, and appear important for the establishment and functioning of the
655 vacuole. NCDG1 was the first DG antigen reported from *N. caninum* and is 43%
656 identical to TgGRA7 (Lally et al., 1997). Subsequently NCDG2, similar to TgGRA6,
657 was identified (Liddell et al., 1998). Both of these proteins are immunogenic in cattle
658 since they were subsequently used to demonstrate antibody responses in cattle (Lally et
659 al., 1996). Other well characterised DG proteins are nucleoside triphosphate hydrolase
660 (NTPase) (Asai et al., 1998) and GRA2 (Ellis et al., 2000), the later which is 50% similar
661 to TgGRA2. NCGRA7 was recently identified as the 17 kDa immune-dominant antigen
662 of tachyzoites (Alvarez-Garcia et al., 2007). Previous DNA vaccination studies showed
663 prevention of foetal infection when dams were immunized with NcGRA7 (plus CPG)
664 (Liddell et al., 2003; Jenkins et al., 2004a). Dense granules also produce novel protease
665 inhibitors that are discharged during infection into the PV (Morris et al., 2004).

666 Other subcellular organelles are the micronemes and rhoptries. Evidence suggests
667 that injection of NcMIC1 or NcMIC3 into mice prevents a subsequent cerebral infection
668 by an *N. caninum* challenge (Cannas et al., 2003b; Alaeddine et al., 2005). NcMIC10, a
669 homologue of TgMIC10, has been described (Atkinson et al., 2001; Hoff et al., 2001) but

proved of no value in vaccination studies using mice (Ellis et al., 2008). An *E. coli* expressed rhoptry antigen (Debache et al., 2008) confers protection in mice in the cerebral disease model (preventing development of clinical signs completely and reduced parasite loads significantly) (Debache et al., 2008). In vitro studies with antibodies to NcROP2 also demonstrated that these prevented host cell invasion.

Apical membrane protein 1 (AMA-1) is one of the lead vaccine candidates in malaria (Remarque et al., 2008). It is a merozoite protein that plays a role in the early invasion process. AMA-1 appears on the surface of merozoites after release from rhoptries and subsequent processing events. The extent of the vaccine related work on AMA1 in malaria is worthy of mentioning here, since these studies have progressed from laboratory-based studies to proof-of-concept for a vaccine in the field. The discovery pathway is therefore of historical interest. Initially it was observed that monoclonal antibodies to the *Plasmodium knowlesi* protein prevented merozoites from invading erythrocytes (Deans et al., 1982). Immunisation with native AMA-1 protected rhesus monkeys against homologous challenge (Deans et al., 1988). Subsequent studies demonstrated mice immunised with AMA-1 from *Plasmodium chabaudi* displayed high levels of protection (Crewther et al., 1996; Anders et al., 1998). Only a low degree of similarity exists between AMA-1 of *Plasmodium* species and TgAMA-1 of *T. gondii* (Hehl et al., 2000), however 12 of the 16 cysteines that are invariant in *Plasmodium* are conserved in TgAMA-1, suggesting that folding of the protein is conserved. In the cyst-forming coccidia this molecule is produced during tachyzoite replication and is located in the micronemes. TgAMA1 is predicted to be a type-1 transmembrane protein that is proteolytically processed into at least two fragments: a 53 kDa N-terminal fragment

693 which is released from the parasite and a 12 kDa C-terminal fragment that remains
694 associated with the tachyzoite (Donahue et al., 2000). Mouse antiserum to TgAMA-1
695 blocked tachyzoite invasion of host cells by approx 40% (Hehl et al., 2000). NcAMA-1
696 shows 73% identity to TgAMA-1 (Zhang et al., 2007a) and a 57 kDa product is released
697 into the excreted secreted fraction of *N. caninum*. Antibodies to NcAMA-1 inhibited host
698 cell invasion by 67%. AMA1 may therefore warrant further evaluation as a vaccine
699 candidate.

700 Many of the *N. caninum* proteins summarised in Tables 3 and 4 were produced in
701 either bacterial or eukaryotic expression systems and either recombinant DNA or purified
702 protein evaluated as vaccines in mice. It is unfortunate that native protein was not used in
703 most of the trials, since there are numerous issues associated with using recombinant
704 protein (see Section 3 for example) that may prevent a molecule truly acting as a vaccine.
705 As mentioned above, native SRS2 showed promise as a vaccine (Haldorson et al., 2005).

706 *7.4. Vector vaccines*

707 Vaccinia virus has been used to deliver NcSRS2 to mice (Nishikawa et al., 2000b).
708 The choice of this vector system was based on the arguments that it had a wide host range
709 (and so can be used in cattle) and the capacity to induce both humoral and cellular
710 immunity. The available evidence from vaccination using recombinant vaccinia
711 expressing SRS2 shows that an IgG1 antibody was produced in response to vaccination
712 as well as IFN-γ (Nishikawa et al., 2001a, 2001b). Vaccination with these vaccinia
713 constructs was able to reduce the load of *N. caninum* in the brains of mice (Nishikawa et
714 al., 2001a) as well as the transplacental transmission of *N. caninum* in utero (Nishikawa
715 et al., 2001b). NcSRS2 has also been expressed in a canine herpes virus vector and

delivery of virus intranasally to dogs resulted in the induction of antibody that detected *N. caninum* antigen by Western blotting (Nishikawa et al., 2000a).

Others have used *Brucella abortus* with success as a delivery system for a number of *N. caninum* antigens, namely MIC1, MIC3, GRA2, GRA6 and SRS2 (Ramamoorthy et al., 2007b, 2007c). MIC 1 and GRA 6 conferred complete protection from lethal infection. However, while promising, the use of *B. abortus* as a vector might not be acceptable in the cattle populations of countries that want also to demonstrate freedom from bovine brucellosis. In passing we note that the RB51 strain used in the above-mentioned studies does allow the differentiation of vaccinated versus cattle naturally infected with *B. abortus* via appropriate testing. It therefore appears a potentially valuable system with which to investigate the vaccine potential of *N. caninum* molecules.

## 8. Discussion

The development of vaccines to *Neospora*-associated abortion in cattle represents an interesting proposition to consider. It is tempting to hypothesise, as suggested by others (Innes et al., 2002; Williams and Trees, 2006), that prevention of infection in cattle ultimately will be the main strategy to prevent abortion. However there is no evidence that natural sterilising immunity occurs in cattle to *N. caninum*, leading to cattle free of infection. In contrast, the development of an anti-disease vaccine that prevents abortion appears achievable. The demonstration that infection of cattle prior to pregnancy with *N. caninum* induces immunity that prevents subsequent foetal death is an important advance in this discipline (Williams et al., 2003, 2007) upon which to build.

738 The development of anti-disease vaccines have been previously highlighted by the
739 advances in malaria (Schofield, 2007) where malarial GPI anchors play a major role in
740 the induction of pathology and pro-inflammatory responses via a mechanism involving
741 TNF production. Glycosylphosphatidylinositols have been reported from *N. caninum*
742 (Schares et al., 2000) but it is unknown whether they contribute to the pathologic process
743 occurring during infection. Recent studies on the placenta of cattle have shown a
744 correlation of placental pathology (Maley et al., 2006; Gibney et al., 2008) and cytokine
745 responses (Rosbottom et al., 2008) with foetal loss, and so it may be reasonable to focus
746 on the characterisation of *N. caninum* molecules that are associated with the induction of
747 these pathologic processes.

748 The mouse model for cerebral neosporosis (Atkinson et al., 1999; Bartley et al.,
749 2008) has been used extensively for evaluation of vaccine candidates. There are several
750 generalisations that can be made from consideration of such studies (summarised in
751 Tables 3 and 4). Induction of non-specific immunity by, for example, the adjuvant used
752 in the trials (e.g. RIBI (Alaeddine et al., 2005) or the vector RB51 (Ramamoorthy et al.,
753 2007b)) can prevent induction of clinical signs of disease, typically associated with
754 neosporosis in a mouse. In some cases, this makes assessment of protection very
755 subjective in the way the levels of protection are calculated. Mouse survival and
756 prevention of weight loss can also occur in the presence of variable numbers of parasites
757 (as judged by PCR) or pathology detectable in the brain. Immunohistochemistry is a poor
758 indicator of protection as parasites are rarely seen in sections (Cannas et al., 2003a,
759 2003b; Alaeddine et al., 2005). The method of measuring protection in the cerebral
760 model is therefore subject to significant differences in interpretation. For example, in one

761 study (Vemulapalli et al., 2007) protection was measured by the number of animals not
762 returning a positive PCR result, or by comparing mean results from the group. Quite
763 different levels of protection can be calculated depending on the criterion used. The
764 vertical transmission model is less subject to interpretation, although PCR sensitivity is
765 an obvious limitation in the comparison of data from different labs.

766 The development of new animal models for investigating vaccines is also needed.
767 Some types of mice tested do not show satisfactory levels of foetal loss, and thus are
768 limiting in their capacity as predictive systems for the development of a vaccine against
769 bovine abortions. Implantation analysis in mice (based on foetal viability) is relatively
770 simple to conduct and is an attractive approach to adopt. The use of sheep or other
771 ruminants in trials, when cattle are the main target species, would appear to have limited
772 value.

773 Live vaccination is currently the most advanced technology available for the
774 control of abortion in cattle due to *N. caninum*, with the greatest chance of success, if
775 efficacy (100% or close to it) is considered as a measure of commercial and economic
776 success. The evidence to date shows that vaccination with a naturally-attenuated strain of
777 *N. caninum* (such as NC-Nowra) can prevent foetal death. It also did not persist to any
778 detectable level in the vaccinated dam; nor did it pass via congenital transmission to the
779 surviving foetus (Williams et al., 2007). This advance can be regarded as the Mark I
780 prototype version of an efficacious *Neospora* vaccine. There are of course a number of
781 remaining issues to address during further development of this vaccine; safety is a
782 paramount concern, although prescribing such a vaccine for use in the non-pregnant
783 animal may reduce any risk associated with infection. Persistence of the vaccine strain

784 also needs to be further investigated as well as dissemination of the strain to native fauna.
785 The information available to date, however, indicates that NC-Nowra does not persist or
786 transmit to subsequent generations (Williams et al., 2007). NC-Nowra also derives from
787 a calf born in a country (Australia) recognised to be free of BSE. *Neospora caninum*,
788 unlike *T. gondii*, is not recognised as a human pathogen and evidence to date shows that
789 *N. caninum* is rarely detected by serology in the human population (Petersen et al., 1999;
790 Tranas et al., 1999), hence spread to the human population (possibly through meat
791 consumption) may not be a concern. In addition, the short shelf-life of live vaccines such
792 as Toxovax® shows the need for advances in technology behind cell storage and survival.
793 A live vaccine based on an attenuated strain of *N. caninum*, such as NC-Nowra, could be
794 registered quickly, over a period of four to 5 years and brought to the market.

795 There is still wide scope for evaluation of killed formulations of *N. caninum* as
796 vaccines, potentially limited only by the availability of acceptable adjuvants. Although
797 the immunodominant antigens of tachyzoites are at an advanced stage of definition and
798 characterisation, those from other life cycle stages, such as bradyzoites (Fernandez-
799 Garcia et al., 2006; Risco-Castillo et al., 2007), are not. Similarly, it is not clear whether
800 they are involved in the generation of immunity and the role they may play in this
801 process. Recombinant antigens have been produced in both prokaryotic and eukaryotic
802 expression systems (Cannas et al., 2003a, 2003b; Ellis et al., 2008), however *T. gondii*
803 may well be the best system to explore for production of *N. caninum* protein.

804 Genetic manipulation of *T. gondii* is feasible and available technology could
805 easily lead to the development of a novel class of live vaccines where *N. caninum*
806 molecules are expressed in *T. gondii*. Public concerns about the release of genetically

807 manipulated organisms, however, probably limits this approach to one of scientific
808 curiosity, although one can imagine a plethora of novel methods for identification of
809 potential vaccine targets.

810 Rapid advances are also being made in "reverse" vaccinology (Rappuoli, 2001;
811 Mora et al., 2003). Analyses of the genome and transcriptome sequence data of *N.*
812 *caninum* may identify new vaccine candidates (possibly for insertion into a suitable
813 delivery system, such as a potent vector); the currently available knowledge on parasitic
814 vaccines would tend to suggest an approach that favours the identification of novel
815 "hidden" or surface antigens for future evaluation

816 A vaccine for dogs has also not been considered in detail. The unclear role of
817 canids in post-natal infection of cattle has delayed debate about whether a transmission-
818 blocking vaccine would be worthwhile for the farm setting. Oocyst production by a
819 definitive host may be targeted through vaccines that affect sexual stages (Wallach et al.,
820 1995, 2008) but there has been no progress in defining those in *N. caninum*. However
821 this remains a potentially fruitful area of research for the future.

822 The future for the development of a vaccine that prevents abortion in cattle due to
823 neosporosis is clearly bright, with many opportunities open for evaluation. There are
824 many leads to follow, and a bottleneck in the discovery process is the costs and time-
825 frame associated with conducting cattle trials.

826 In summary, *N. caninum* infection and abortion in cattle is the cause of significant
827 economic loss to the primary producer. Economic analysis suggests huge up-side and
828 potential for vaccine manufacturers, with efficacious vaccines having the potential to
829 reduce the cost of *N. caninum* infection by over 90% over the long-term. These facts

830 establish a clear need and market for an efficacious *N. caninum* vaccine and development
831 efforts should rapidly follow in this area.
832
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**Figure legends**

**Fig. 1.** Mid-term aborted bovine foetus from a farm with chronic, sporadic *Neospora caninum* abortions (from the study by Hall et al., 2005).

**Fig. 2.** Example of a decision-tree analysis of the cost of *Neospora caninum* infection (do nothing option/live with the infection equals NZD 97,154.79) in an average-sized dairy herd in New Zealand versus the cost of various control options viewed over a 5 year horizon (for further details see also Reichel and Ellis (2006)). A once-a-lifetime vaccination with an attenuated (but highly efficacious) vaccine (utilising the NC-Nowra Australian isolate) for *N. caninum* would cost the average *N. caninum*-infected herd just NZD 8,248.74 over 5 years.

**Fig. 3.** Brain lesions in a *Neospora caninum*-infected mouse. A) Perivascular cuffing; B) necrosis; C) necrosis with mineralisation; D) meningitis.

**Fig. 4.** Ruffled coat in a BALB/c mouse after experimental *Neospora caninum* infection.

**Fig. 5.** Foetal death at implantation sites in the uterus due to *Neospora caninum* infection. The arrows point to the dead/dying implantation sites which are discoloured.

**Fig. 6.** A comparison of the Quackenbush (Qs) (left) and BALB/C mouse (right) showing the Qs as a much larger mouse, typically 20 g at 5 weeks of age.

Table 1. Assessment of mouse morbidity (modified from Bartley et al., 2006).

| Category | Features | Score |
|---|---|---|
| **A** | Sleek glossy coat | 0 |
| Febrile response | Ruffled coat | 1 |
| | Stary stiff coat | 2 |
| **B** | | |
| Dehydration/loss of appetite | Weight maintained at pre-infection level | 0 |
| | 10% weight loss | 1 |
| | 20% weight loss | 2 |
| **C** | Bright and active | 0 |
| Demeanor | Hunched appearance | 1 |
| (accumulative scoring in C) | Tottering gait | 1 |
| | Reluctance to move | 1 |

Total score = A+B+C

Table 2. Assessment of lesion scores in brain tissue of mice infected with a sub-lethal dose of *Neospora caninum* tachyzoites (modified from Ramamoorthy et al., 2007a).

| Lesion Score | Pathologic description |
|---|---|
| 0 | No lesions present |
| 1 | Minimal number of lesions present limited to lymphoplasmacellular meningitis and perivasculitis |
| 2 | Mild lesions present including meningitis, perivasculitis and focal glial cell activation |
| 3 | Moderate lesion including meningitis, perivasculitis, glial cell activation and rarefaction of the neuropil with macrophage infiltration |
| 4 | Moderate lesion including meningitis, perivasculitis, glial cell activiation and rarefaction of the neuropil with macrophage infiltration, and focally extensive necrosis |

Total number of lesions per sample/number of sections counted for that sample
Pathology score = average number of lesions per section for the sample x lesion score

1441

1442 Table 3. Efficacy of candidate vaccines/antigens in laboratory animals (cerebral
1443 neosporosis model). For standardization, the best estimates are presented for each study
1444 after recalculation compared to the relevant controls presented.

1445

1446

| Vaccine | Protection (% efficacy)[a] | Protection criterion | Animal Model | Reference |
|---|---|---|---|---|
| Live | 70-90; 90+; 14-44; 28-44 | Mouse survival; morbidity score; PCR; pathology | BALB/c | (Bartley et al., 2008) |
| Lysate | 64.7 | Gerbil survival | Gerbil | (Cho et al., 2005) |
| Irradiated tachyzoites | 100; 0 | Mouse survival; pathology | C57BL/6 | (Ramamoorthy et al., 2006) |
| NcMIC1 | 40-100;18-100[b]; ns but lower or higher than controls | Clinical signs; PCR; IHC | C57BL/6 | (Alaeddine et al., 2005) |
| RIBI adjuvant | 50 | Clinical signs | C57BL/6 | (Alaeddine et al., 2005) |
| NcMIC3 | 100;71;ns | Clinical signs; PCR; IHC | C57BL/6 | (Cannas et al., 2003b) |
| NcMIC4 [c] (native) | Significant reduction [c] | PCR | C57BL/6 | (Srinivasan et al., 2007) |
| NcROP2 | 100;75 - 93 | Clinical signs; PCR | C57BL/6 | (Debache et al., 2008) |
| cDNA + recSRS2 or recSAG1 | ns, ns, ns | Mouse survival; PCR; IHC | C57BL/6 | (Cannas et al., 2003a) |
| MIC1, MIC3, GRA2, GRA6 and SRS2 in RB51 | 0-100[d]; 88 (SRS2) 53 (MIC1) | Mouse survival; lesion scores | C57BL/6 | (Ramamoorthy et al., 2007b) |
| RB51 control | 69 | Mouse survival | C57BL/6 | (Ramamoorthy et al., 2007b) |
| RB51/SRS2 | 60-85[e] | PCR | BALB/c | (Vemulapalli et al., 2007) |
| NcSAG1, NcSRS2, NcDG1, or NcDG2 | 30-61 | Gerbil survival | Gerbil | (Cho et al., 2005) |
| NcSRS2/vaccinia | 33-42 | PCR | BALB/c | (Nishikawa et al., 2001a) |
| NcSRS2 iscoms | 0; reduction in parasite DNA present by 1.5 logs) | Clinical signs; PCR | BALB/c | (Pinitkiatisakul et al., 2005) |
| NcSRS2 iscoms | 0;30-100 | Clinical signs; PCR | BALB/c | (Pinitkiatisakul et al., 2007) |

1447 [a] Estimated from a comparison of treatment and control groups;
1448 [b] Data from adjuvant control confounds calculations;
1449 [c] Injection causes increase in mouse mortality. Not possible to calculate protection from
1450 data presented.
1451 [d] Data from RB51 vector controls confounds calculations;

1452 [e] Three mice (out of five) contained no parasite DNA (60% efficacy), and the other two
1453 had very low amounts giving an overall level of protection significantly higher than
1454 control groups (85%).

1455 Table 4. Efficacy of candidate vaccines/antigens in laboratory animals (mouse vertical
1456 transmission model). For standardization, the best estimates are presented for each study
1457 after recalculation compared to the relevant controls presented.

1458
1459

| Vaccine candidate | Protection (%)[a,b,c] | Protection assessment | Mouse type | Reference |
|---|---|---|---|---|
| Live | 89.5 | PCR | Qs | (Miller et al., 2005) |
| Lysate | 17.1-20.8 | PCR | Qs | (Miller et al., 2005) |
| Lysate | 100 | PCR | BALB/c | (Liddell et al., 1999b) |
| MIC10 | 0 - 13.2 ns | PCR | Qs | (Ellis et al., 2008) |
| GRA1 | 14.9 ns | PCR | Qs | (Ellis et al., 2008) |
| GRA2 | 5.4 ns | PCR | Qs | (Ellis et al., 2008) |
| MIC 10 | 13.5 ns | PCR | Qs | (Ellis et al., 2008) |
| p24B | 7.9 - 18.9 ns | PCR | Qs | (Ellis et al., 2008) |
| MIC10 + p24B | -2.7 ns - 32.9 | PCR | Qs | (Ellis et al., 2008) |
| MIC1, MIC3, GRA2, GRA6 and SRS2 in RB51 | 6-38*/60.4 - 93.5 | PCR/parasite burden | C57BL/6 | (Ramamoorthy et al., 2007c) |
| RB51 | 25/84.7 | PCR/parasite burden | C57BL/6 | (Ramamoorthy et al., 2007c) |
| GRA7, HSP33 | 47, 54 | PCR | BALB/c | (Liddell et al., 2003) |
| GRA7 | 84.6 | PCR | BALB/c | (Jenkins et al., 2004a) |
| NcSRS2/vaccinia | 100/77.3 | PCR/surviving pups | BALB/c | (Nishikawa et al., 2001b) |
| NcSRS2 | 61.3 | PCR | BALB/c | (Haldorson et al., 2005) |

1460
1461 [a] Protection measured as a reduction in the transmission of the *Neospora caninum*
1462 challenge compared to the control group.
1463 [b] ns – observation not statistically significant
1464 [c]But no reduction compared to RB51 control

1465 Table 5. Efficacy of candidate vaccines/antigens in ruminants.
1466

| Vaccine candidate | Host species | Efficacy (%) | Protection criterion | Reference |
|---|---|---|---|---|
| Live | Cattle | 100 | Abortion | (Williams et al., 2007) |
| Live | Cattle | 100 | Transmission | (Innes et al., 2001b) |
| Lysate | Cattle | 0 | Abortion | (Williams et al., 2007) |
| Killed tachyzoites | Sheep | 85.7 | Abortion | (Jenkins et al., 2004c) |

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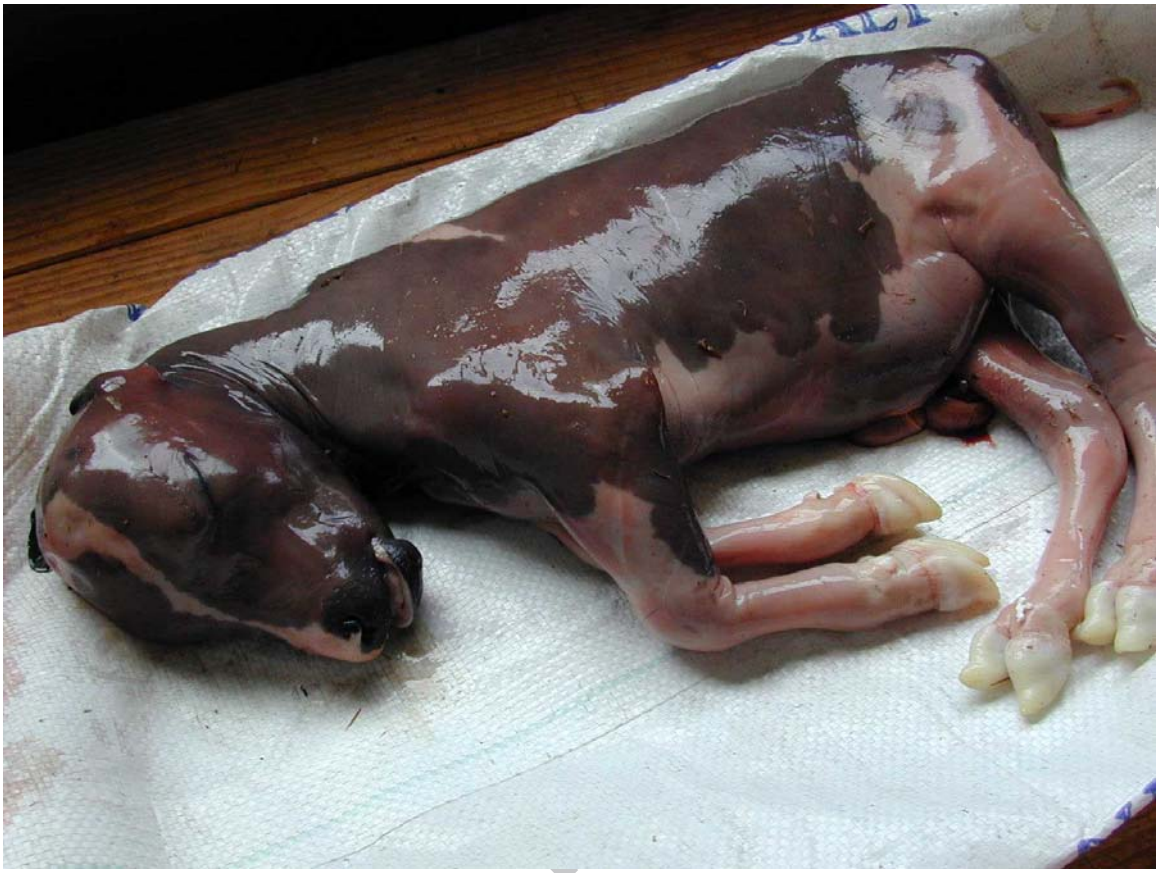

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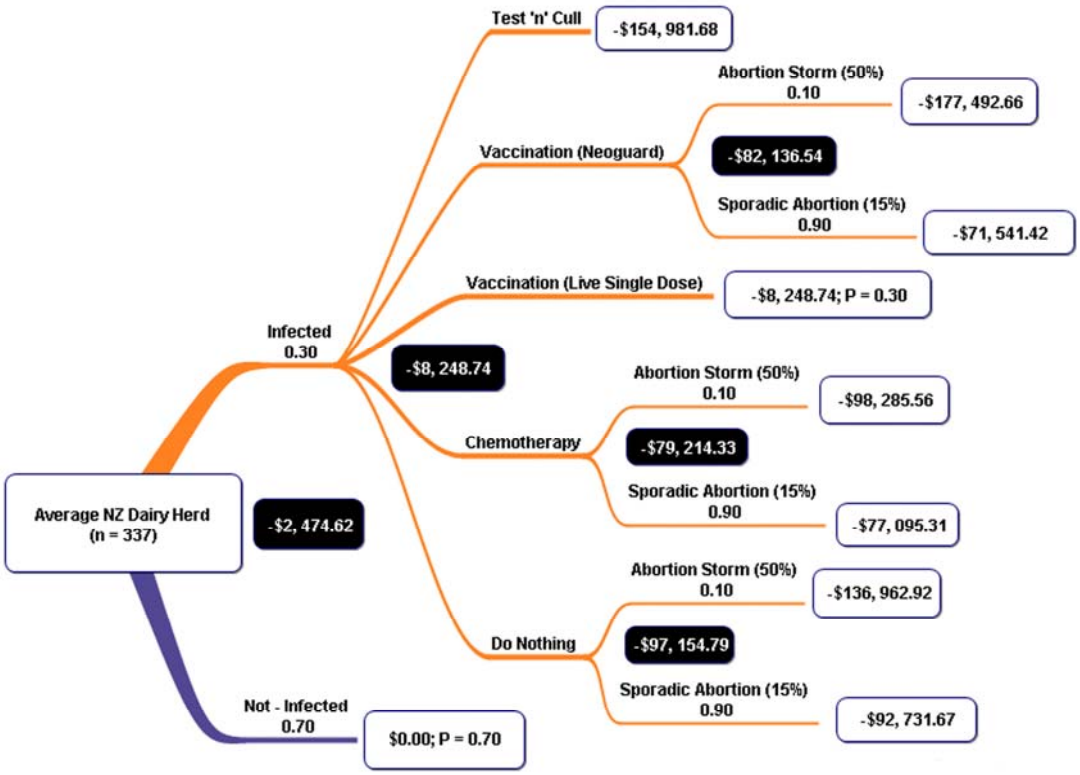


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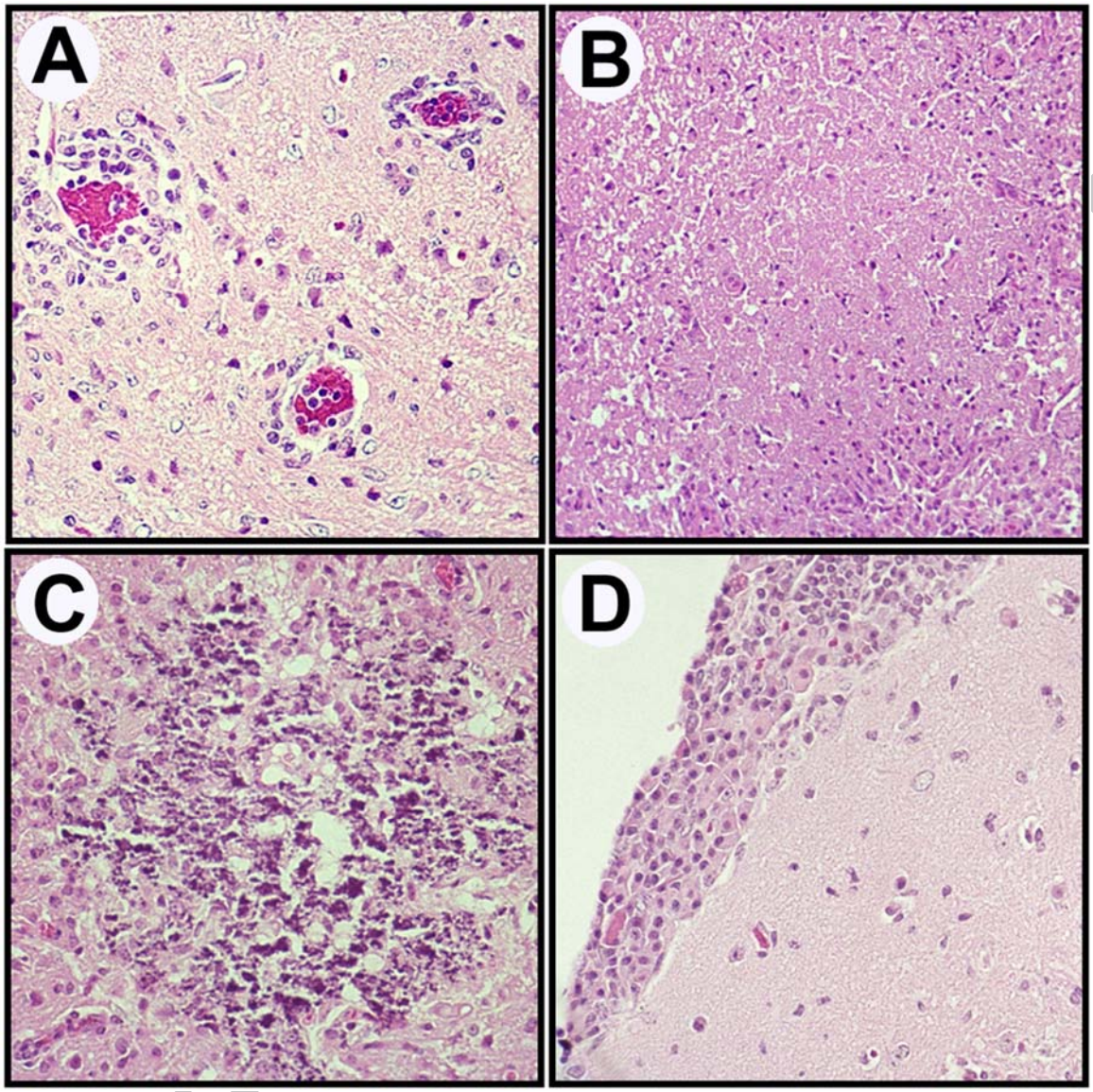


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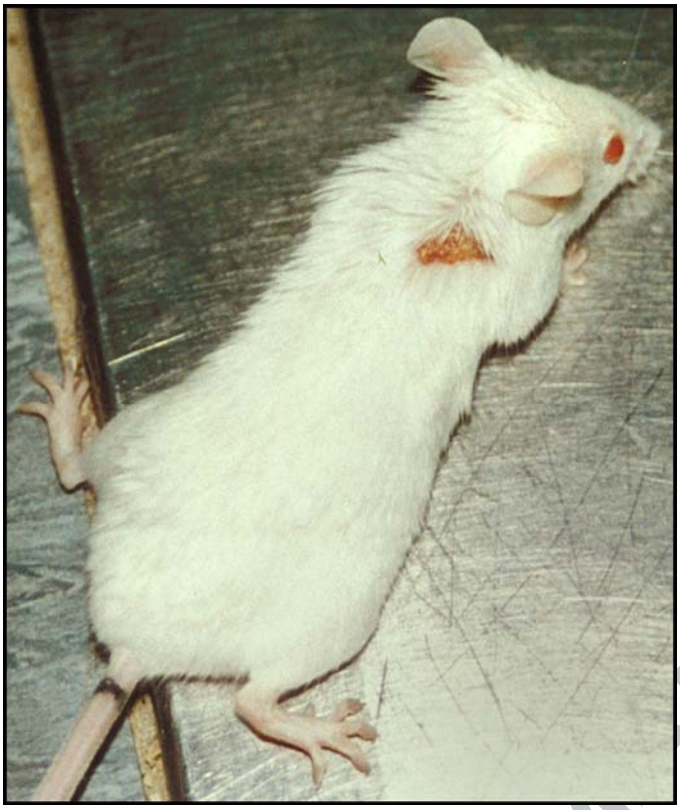

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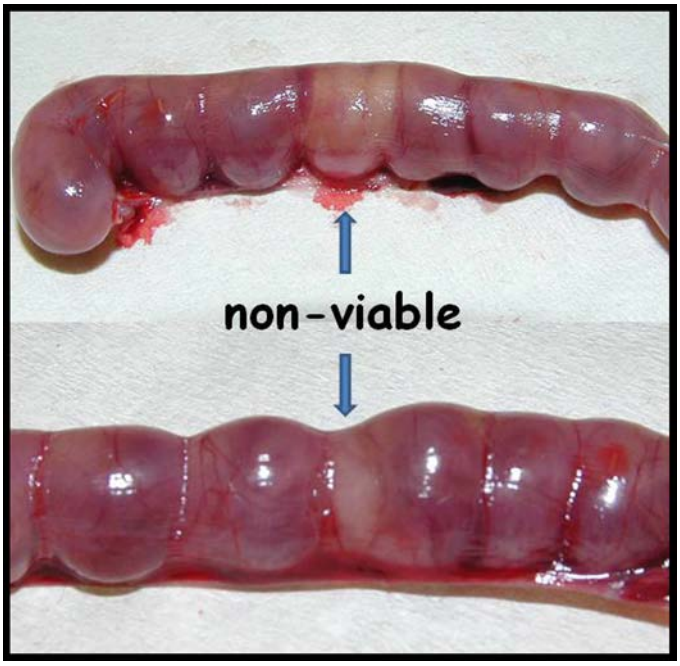


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