EXPERIMENTAL RUMINANT MODELS FOR BOVINE NEOSPOROSIS: WHAT IS KNOWN AND WHAT IS NEEDED

Julio Benavides[1], Esther Collantes-Fernández[2], Ignacio Ferre[2], Valentín Pérez[1], Carlos Campero[3], Rinaldo Mota[4], Elisabeth Innes[5] & Luis M. Ortega-Mora[2]*

1. Departamento de Sanidad Animal, Instituto de Ganadería de Montaña (CSIC-ULE), Facultad de Veterinaria, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain

2. SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain.

3. Instituto Nacional de Tecnología Agropecuaria (INTA), 7620 Balcarce, Argentina

4. Departamento de Medicina Veterinaria, Universidade Federal Rural de Pernambuco, Brazil

5. Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh, EH26 0PZ, United Kingdom

Running head: EXPERIMENTAL RUMINANT MODELS FOR BOVINE NEOSPOROSIS

*Corresponding author: Luis M. Ortega-Mora. *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain.* Tel: +34 913944069 Fax: +34 913943908. Email address: luis.ortega@vet.ucm.es

SUMMARY:

At present, bovine neosporosis is an important worldwide concern because of its wide geographic distribution and economic impact. Abortion is the main clinical sign of bovine neosporosis in both dairy and beef cattle. Ruminant challenge models are critical to evaluate potential vaccine candidates to help tackle bovine neosporosis and to study pathogenesis and host responses to infection. Several research groups have developed ruminant models of *N. caninum* infection independently of others, resulting in a high degree of variability due to the use of different species of animals, breeds, strain/isolate of *N. caninum*, doses, route and time of inoculation. Standardization is greatly needed to advance research in a more collaborative, timely and efficient manner. In the absence of widely-accepted international guidelines, this manuscript serves to summarize and discuss the different models and parameters currently in use. Parameters essential for the development of non-pregnant and pregnant ruminant models are outlined and the main knowledge gaps are identified. This information could act as the basis to develop a consensus for international standard guidelines for ruminant models of neosporosis that would be helpful for researchers in this field worldwide.

Keywords: bovine neosporosis, ruminant models, harmonization, knowledge gaps

INTRODUCTION

Neospora caninum is a heteroxenous, cyst-forming coccidian closely related to *Toxoplasma gondii* that has been recognized as a major cause of abortion and reproductive failure in cattle (reviewed by Dubey *et al.* 2007). At present, bovine neosporosis is an important worldwide concern because of its wide geographic distribution (reviewed by Dubey *et al.* 2007 and Dubey and Schares, 2011) and economic impact (Trees *et al.* 1999; Reichel *et al.* 2013). Serologic prevalence of *N. caninum* antibodies in cattle indicates that there are considerable differences in prevalence of infection among countries, within countries, between regions, and between beef and dairy cattle (reviewed by Dubey and Schares, 2011). Major economic losses in livestock operations are associated to lengthened calving interval, reduced stock value and increased culling rate (Trees *et al.* 1999). In a recent review and economic analysis by Reichel et al. (2013), the impact of *N. caninum* abortions in just ten countries was estimated to be on average, a billion US dollars.

The life cycle of *N. caninum* has three described infectious stages: tachyzoites, tissue cysts bearing bradyzoites, and oocysts (Fig. 1). Intermediate hosts harbor intracellular tachyzoites and bradyzoites. Tissue cysts are primarily found in the central nervous system of intermediate hosts, but this stage has also been detected in cattle muscle (Peters *et al.* 2001). Definitive hosts excrete environmentally-resistant oocysts in an un-sporulated stage. The finding of *N. caninum* oocysts in the faeces of naturally infected dogs and grey wolves has allowed identifying them as natural definitive hosts for the parasite (Basso *et* al. 2009; Dubey *et al.* 2011). The shedding of oocysts in faeces has also been proven in experimentally infected dogs, coyotes and dingoes (reviewed by Dubey *et al.* 2006 and Dubey and Schares, 2011). Serological evidence in

domestic, wild and zoo animals indicates that many species have been exposed to this parasite, but viable *N. caninum* has only been isolated from a few host species such as cattle, sheep, water buffalo, dog, horse, bison, and white-tailed deer (reviewed by Dubey and Schares, 2011).

The routes of *N. caninum* transmission in cattle include transplacental infection from the dam to the foetus during gestation through tachyzoites, (vertical transmission) and ingestion of sporozoite-containing oocysts shed by a definitive host (horizontal transmission). Exogenous transplacental transmission occurs following primary oocyst-derived infection of pregnant dams, while endogenous transplacental transmission occurs following recrudescence of infection in persistently infected cows during pregnancy (Trees and Williams, 2005). Transmission of the parasite from dam to offspring seems to be highly efficient on the basis of precolostral serological data, ranging from 48–95% (Schares *et al.* 1998; reviewed by Dubey *et al.* 2007). To date, cow to cow horizontal transmission of *N. caninum* has not been observed and the ingestion of sporulated *N. caninum* oocysts from the environment is the only demonstrated natural mode of infection of cattle after birth (McCann *et al.* 2007). Post-natal transmission is responsible for less than 15% of infections in the herd (Hall *et al.* 2005), although horizontal transmission could be important in some herds in high prevalence areas (Eiras *et al.* 2011). In this study, a large sample size was investigated showing high herd (80.6%) and within herd (25.1%) seroprevalence and a significant increase of seroprevalence with age (11, 14.4 and 27.5 in animals younger than 24 months, 25-36 months and higher than 36 months respectively). Sero-positivity is assumed to persist for life (reviewed by Dubey and Schares, 2006), although fluctuation in antibody responses may result in some animals becoming sero-negative

again (Pereira-Bueno *et al.* 2003). Seropositive animals have a higher risk of abortion, ranging between 1.7 and 7.4-fold but the risk decreases over time with increasing parity (Paré *et al.* 1997; Thurmond and Hietala, 1997; Wouda *et al.* 1998). Lactogenic and venereal routes are also other suggested modes of transmission. *N. caninum*-DNA has been detected in milk and colostrum (Moskwa *et al.* 2007) and neonatal calves may become infected after ingestion of milk experimentally spiked with tachyzoites (Davison *et al.* 2001), but the lactogenic transmission of *N. caninum* was not demonstrated in natural conditions (Dijkstra *et al.* 2001). Although the presence of *N. caninum*-DNA has been detected in semen (Ortega-Mora *et al.* 2003), dams naturally bred with experimentally infected bulls failed to seroconvert (Osoro *et al.* 2009).

Control measures for bovine neosporosis such as management strategies, chemotherapy and vaccination have been reviewed in Dubey *et al.* (2007) and Reichel and Ellis (2002). However, a general strategy to control neosporosis worldwide is not applicable because of regional differences in the epidemiology of bovine neosporosis. Standard biosecurity measures are paramount to avoid the introduction of infection in *N. caninum*-free herds. In *N. caninum*-infected herds, control programs are based on decreasing the vertical transmission in herd by reduction of the number of seropositive cattle and/or decreasing the risk of horizontal transmission of *N. caninum* mainly by controlling the definitive host population as a source of oocyst contamination (reviewed by Dubey *et al.* 2007). Test and cull of *N. caninum* infected cattle seems to be an efficacious but costly option. Other practical alternatives for the control of neosporosis would be the selective breeding of only seronegative cows, breeding of seropositive cows only to beef, and the culling of those cows that actually aborted. In high prevalence areas, the difficulties that need to be overcome in establishing a

control programme for neosporosis include the limited number of seronegative animals available for breeding replacement animals, reducing selection by genetic merit and an increase in replacement costs (Eiras *et al.* 2011).

Initiatives for model harmonization in N. caninum *infection*

During the 2011 meeting of the World Association for the Advancement of Veterinary Parasitology held in Buenos Aires, a workshop dealing with "Perspectives for control for cattle reproductive diseases caused by protozoans" stressed the urgency to standardize pre-clinical and clinical models for *N. caninum*. These models are essential to study host-pathogen interactions, host immunity at the local and systemic level, and for evaluating vaccine candidates and therapeutics (Campero and Ortega-Mora, personal communication). The following year during the first "Apicowplexa" meeting in Lisbon (Apicomplexans in farm animals, http://www.apicowplexa.net/), a presentation including "pros" and "cons" of *in vitro* and *in vivo* models used for *N. caninum* vaccine testing was presented. The necessity of consensus guidelines including isolates/strains of *N. caninum* used, challenge dose, time and route of challenge, preparation of inoculum, animal model (mice versus cattle; sheep versus cattle), and other parameters were discussed and a proposal to develop international standard guidelines that would be an aid to researchers in this field and gain acceptance worldwide was put forward (reviewed by Ortega-Mora et al. 2012).

Several recent reviews have discussed *in vitro* and *in vivo* laboratory animal (mainly mice) models of *N. caninum* infection. *In vitro* culture systems represent powerful tools for the study of apicomplexan parasites like *Neospora* and have enabled studies on host-parasite interactions including pathogen virulence, host factors involved in innate

resistance, parasite stage conversion and differentiation, genetics and transfection technology and vaccine candidates and drug effectiveness (reviewed by Muller and Hemphill, 2013). Notably, a recent comparative analysis of the invasive and proliferative capacities of bovine *N. caninum* isolates obtained from asymptomatic calves revealed intra-species variability, which was associated with disease severity in a pregnant mice model (Regidor-Cerrillo *et al.* 2011). *In vitro* invasion rate and tachyzoite yield were confirmed as traits associated with the virulence phenotype for these *N. caninum* isolates (Regidor-Cerrillo *et al.* 2012).

Mouse models are a suitable tool for an initial screening of vaccine candidates to assess their *in vivo* efficacy, (reviewed by Innes and Vermelulen, 2006). Indeed, cerebral and congenital mouse models of *N. caninum* infections associated with exogenous transplacental transmission have been successfully developed (Collantes-Fernández *et al.* 2006; López-Pérez *et al.* 2006, 2008) and afterwards, employed in vaccine efficacy assays (Aguado-Martínez *et al.* 2009; Rojo-Montejo *et al.* 2011). On the other hand, low rates of reactivation of *N. caninum* infection in both persistently and congenitally infected mice confirm that the mouse is not an appropriate or suitable species for inducing the endogenous transplacental transmission that frequently occurs in naturally infected cattle (Jiménez-Ruiz *et al.* 2013*a, b*).

Ruminant challenge models are critical to evaluate potential vaccine candidates to help confront bovine neosporosis and to study pathogenesis and host responses to infection. Several research groups have developed ruminant models of *N. caninum* infection independently of others, resulting in a high degree of variability due to the use of different species of animals, breeds, strain/isolate of *N. caninum*, doses, route

and time of inoculation. Standardization is greatly needed to advance research in a more collaborative, timely and efficient manner. These models will be useful to study host–pathogen interactions, host immunity at the local and systemic level, and for evaluating diagnostics, vaccines, and therapeutics. In the absence of widely-accepted international guidelines, this manuscript serves to summarize and discuss the different models and parameters currently in use. Methodologies essential for the development of non-pregnant and pregnant ruminant models are outlined and the main knowledge gaps are identified. This information could act as the basis to develop a consensus for internationally accepted standard guidelines for ruminant models of neosporosis that would be helpful for researchers in this field worldwide.

NEOSPORA CANINUM INFECTION DYNAMICS IN CATTLE

Abortion is the main clinical sign of bovine neosporosis in both dairy and beef cattle. Despite many studies in experimentally-infected pregnant cows, only limited information is available in naturally occurring infection. It is unclear which factors play a major role in determining the outcome of the infection, these include: parasite virulence, routes of *Neospora* transmission (vertical or horizontal), type of infection (primary infection, recrudescence and re-infection), the efficacy of maternal and foetal immune response and the stage of gestation at which the dam is infected.

Cows of any age may abort from 3 months of gestation to term, with most abortions observed at 5-7 months of gestation (reviewed by Dubey *et al.* 2007 and Almería and López-Gatius, 2013). Persistent *N. caninum* infection prior to pregnancy is not responsible for abortion during the early foetal period, but does exert a significant

abortifacient effect after 90 days of pregnancy (López-Gatius *et al.* 2004). *Neospora* induced abortions may occur year-round. Both dairy and beef cattle with antibodies to *N. caninum* can have a 7.4-fold-increased risk of abortion compared to seronegative cows (Paré *et al.* 1997, Thurmond and Hietala, 1997; Wouda *et al.* 1998). Several studies showed that *N. caninum* antibody titers were significantly correlated with abortion in parous cows (López-Gatius *et al.* 2005; Thurmond and Hietala, 1997). Irrespective of the herd level of *Neospora*-seroprevalence, the individual serum antibody titer against *N. caninum* is a good indicator of the risk of abortion (Quintanilla-Gozalo *et al.* 1999). Increasing *N. caninum* antibody titers in the second and third trimester of gestation have been associated with abortion and birth of congenitally infected calves, respectively (Stenlund *et al.* 1999; Quintanilla-Gozalo *et al.* 1999; Williams *et al.* 2000).

Foetuses in *Neospora* infected dams, may die *in utero* and be reabsorbed, mummified, autolyzed, or stillborn. Infections after mid-pregnancy may produce calves born alive but with clinical signs or apparently clinically normal but persistently infected with the parasite. In aborted foetuses, the most common lesions are multifocal non-suppurative necrotizing encephalitis and myocarditis (Wouda *et al.* 1997). In addition, multifocal areas of necrosis surrounded by inflammatory cells may be observed in spinal cord, liver, lung, and placenta (Barr *et al.* 1991). It has been indicated that the age of foetus is an important factor which appears to influence the histological features and distribution of lesions. Frequency and severity of lesions appears to be higher in foetal tissues during the first and second trimesters of gestation rather than the last trimester, and this is likely to be a consequence of to the ontogeny of the

foetal immune response which progressively matures throughout gestation (Maley *et al.* 2003; Macaldowie *et al.* 2004; Gibney *et al.* 2008; Benavides *et al.* 2012).

In cattle, *N. caninum* is an abortifacient and abortions follow two main patterns: endemic and epidemic abortions. The epidemic abortions show a storm-like pattern with a large proportion (>10%) of at risk cows (in-calf) aborting over a short period of time (reviewed by Dubey *et al.* 2007). In contrast, endemic abortion persists in the herd for several months or years. Epidemic and endemic abortion patterns are related to horizontal and vertical routes of transmission of *N. caninum* infections, respectively. The immunological context of the initial priming of the immune system by the parasites may have profound consequences for the risk of disease and transmission of *N. caninum*. It appears that infection in cattle originally infected *in utero* when their immune system is still developing behave differently from infection in post-natally infected cattle (reviewed by Innes and Vermeulen, 2006 and Williams *et al.* 2009). Cattle infected *in utero* (transplacental transmission) remain persistently infected and can experience recrudescence of infection (Williams *et al.* 2003). Heifers can abort or transmit the infection in successive gestations and immunity after *in utero* infection protects against an exogenous challenge (concomitant immunity) (McAllister *et al.* 2000; Innes *et al.* 2001; Williams and Trees, 2006; Williams *et al.* 2007). With post-natal transmission, some animals seem to clear the infection and they do not present with abortion or transmission in successive gestations (or rarely), developing a good protective immunity against an exogenous challenge (Dijkstra *et al.* 2008; McCann *et al.* 2007). These facts are important for vaccine development.

RUMINANT MODELS FOR BOVINE NEOSPOROSIS

Purposes and endpoints of the models

Ruminant experimental models have been developed to study the main consequences of *N. caninum* infection: foetal death or parasite transmission to the offspring. These models have been used to understand the host-pathogen interactions enabling the study of immune responses and disease pathogenesis during gestation. In addition, *in vivo* models enable studies to compare virulence between isolates, therapeutics and to test the ability of vaccine formulations to prevent abortion or transmission by immunization trials and challenge.

Different endpoints have been evaluated to provide a reliable way to monitor foetopathy or parasite dissemination and transmission, which include clinical observations, detection of *N. caninum* presence, lesions and immune responses. With regard to clinical observations, foetal viability is the most significant parameter to be evaluated in the abortion model and this can be monitored by trans-rectal ultrasonography during gestation. If foetal death occurs, this is normally observed between 26 and 56 days after intravenously (iv) inoculation of live tachyzoites at 70 days of gestation infection (Williams *et al.* 2000, 2003, 2007; Macaldowie *et al.* 2004; Gibney *et al.* 2008; Caspe *et al.* 2012; Rojo-Montejo *et al.* 2009, 2013; Regidor-Cerrillo *et al.* 2014) showing a mean time to foetal death of 24 days (Innes *et al.* 2001). However, abortion can be also observed later (about 80 days pi) (Rojo-Montejo *et al.* 2013). These differences could be due to the inoculum size, inoculation route or differences in virulence between parasite isolates. Additionally, another clinical parameter associated with the infection is rectal temperature. In accordance with previous reports, a transient rise in the body temperature was recorded after infection

during the first week pi, which is likely to be due to tachyzoite inoculation and the first cycles of replication of the parasite in host tissues (Innes *et al.* 2001; Maley *et al.* 2003; Macaldowie *et al.* 2004; Williams *et al.* 2000; Caspe *et al.* 2012; Regidor-Cerrillo *et al.* 2014). A biphasic temperature response, with a second lower temperature peak at day 7 pi, was also recorded in some studies (Maley *et al.* 2003; Macaldowie *et al.* 2004; Benavides *et al.* 2012; Regidor-Cerrillo *et al.* 2014).

Other key indicators are those used to measure the dissemination of the parasite to different locations by means of PCR or immunohistochemistry, parasite loads by quantitative real-time PCR or presence of lesions by histopathological evaluation, which can be categorized using a severity scoring system. In models where abortion is studied, the occurrence of severe pathological changes with simultaneous parasite detection and high parasite DNA levels in target foetal tissues (brain, heart and liver) and placenta, are thought to play a role in foetal death (Barr *et al.* 1994; Maley *et al.* 2006; Macaldowie *et al.* 2004; Gibney *et al*. 2008; Rojo-Montejo *et al.* 2009, 2013; Regidor-Cerrillo *et al.* 2014). On the other hand, if the goal is to study vertical transmission, this is normally identified in calves after birth by the presence of *Neospora*-specific antibodies in pre-colostral blood and detection of parasite DNA or lesions in tissue samples from calves. However, in newborn calves, parasite DNA is sometimes difficult to detect and histological lesions are rare, mild and limited to brain tissue from these calves, which is probably due to the control of parasite multiplication by a competent foetal immune system which develops between mid to late gestation (Innes *et al.* 2001; Gibney *et al.* 2008; Almería *et al.* 2010; Rosbottom *et al*. 2011; Benavides *et al.* 2012; Bartley *et al.* 2013; Rojo-Montejo *et al.* 2013). Additional measurements which could provide complementary data are detection of *N. caninum*

DNA in dam blood and microsatellite analysis in foetal tissues. The detection of circulating parasite in the maternal blood is difficult and interpretation of the parasitaemia results are sometimes hard to assess, mainly because of low parasite numbers or short periods of detectable parasitaemia (Maley *et al.* 2003; Macaldowie *et al.* 2004; Serrano *et al.* 2006; Rojo-Montejo *et al.* 2009; Benavides *et al.* 2012). On the contrary, microsatellite analysis was very useful to distinguish the endogenous infection from any experimentally administered challenge (Benavides *et al.* 2012) and to rule out the involvement of the vaccine isolate in foetopathy during vaccine assays (Rojo-Montejo *et al.* 2013).

Immune responses have been also evaluated in bovine models but currently are not precise to be used as definitive indicators for disease or protection. Bovine experimental models have been used to study early innate and adaptive immune responses and those involved in immunopathology, which are critical to understand foetal losses, parasite transmission or protective immunity. Standard tools (such as commercially available ELISA or in-house ELISA tests) have been used to evaluate humoral immune responses (specific IgG or IgG1, IgG2 serotypes) (Williams *et al.* 2000; Bartley *et al.* 2004; Macaldowie *et al.* 2004; Serrano *et al.* 2006; Rojo-Montejo *et al.* 2009, Regidor-Cerrillo *et al.* 2014). In addition, avidity *N. caninum* ELISA tests (Björkman *et al.* 2006; Williams *et al.* 2007) and stage-specific antigen-based ELISAs employing recombinant NcGRA7 and NcSAG4 proteins, can be also used for discrimination between primoinfection and persistent infection, and also detection of recrudescence, even at the individual level (Aguado-Martínez *et al.* 2008). Several techniques have been also employed to analyze *N. caninum* associated cellular immune responses in cattle, such as phenotypic analysis of lymphocyte populations by

flow cytometry, lymphoproliferative responses following antigen specific stimulation, as well as the measurement of bovine cytokines in cell free supernatants by ELISA tests or cytokine gene expression by real time RT-PCR in both peripheral blood mononuclear cells, dam and foetal tissues (spleen and lymph nodes) or at the foetal-maternal interface (Gibney *et al.* 2008; Bartley *et al.* 2004, 2012, 2013; Almeria *et al.* 2003, 2011; Rosbottom *et al.* 2007, 2008; Regidor-Cerrillo *et al.* 2014). Little is known regarding the role of innate immune responses in bovine neosporosis. Cytotoxic NK cells seem to play a role in the control of the disease through both cytotoxic and interferon gamma (IFNγ) mediated mechanisms (Boysen *et al.* 2006; Klevar *et al.* 2007). Recently, it has been shown that the immune response in inoculated dams at early gestation appeared to have lead to superior priming of a cell mediated immune response in dams carrying live foetuses *vs.* dams carrying dead foetuses (Bartley *et al.* 2012) and there was also evidence of innate immunity, with significant increases in expression of toll like receptors (TLR)-2 and TLR-9 in spleen and lymph nodes in cattle challenged at day 210 of gestation (Bartley *et al.* 2013). Intracellular protozoan parasites normally induce and are controlled by cellular immune responses. Data from several studies have demonstrated that experimental infection in cattle is accompanied by lymphocyte proliferation and IFNγ responses (Lunden *et al.* 1998; Marks *et al.* 1998; Andrianarivo *et al.* 2001; Williams *et al.* 2003), and that these responding immune cells tend to be CD4+ T lymphocytes (Marks et al 1998; Staska *et al.* 2003; Tuo *et al.* 2005; Rosbottom *et al.* 2007; Rocchi *et al.* 2011), simultaneously with a strong anti-*N. caninum* humoral response (Williams *et al.* 2000; Bartley *et al.* 2004; Macaldowie *et al.* 2004; Serrano *et al.* 2006; Rojo-Montejo *et al.* 2009, 2013; Regidor-Cerrillo *et al.* 2014). Additionally, an increase of both Th1 and Th2 cytokines expression in placenta has been associated

with foetal death after infection in the first third of gestation (Maley et al 2006; Rosbottom *et al.* 2008; Regidor-Cerrillo *et al.* 2014). On the other hand, minor fold-change differences in expression of these cytokines were detected after experimental infection during the second and third trimester of gestation, when parasite transmission to foetus occurs but the calf is born apparently healthy (Almeria *et al.* 2011; Rosbottom *et al.* 2008). Therefore, bovine immunological studies have showed that immune cytokines may be either beneficial or detrimental to the host depending on their concentration, pregnancy period or tissue location and could have a role in the transplacental transmission of the parasite and/or mediate tissue damage, specifically at the dam-foetal interface.

Pregnant ruminant models of N. caninum *infection*

Ruminant species. Although there is serological evidence of natural contact with *N. caninum* in a wide range of species, the isolation of the viable agent and experimental reproduction of the disease have been made mostly in ruminants (reviewed by Dubey *et al.* 2007 and Dubey and Schares, 2011). The establishment of exogenous *N. caninum* infection and the occurrence of vertical transmission to the foetus or abortion have been mainly reproduced in cattle but also, to lesser extent, in sheep, goat and water buffalo (Tables 1 and 2). The descriptions of the clinical presentation and consequences of experimental exogenous neosporosis are similar in all these species, mainly consisting of abortions or the *in utero* vertical transmission of the infection to the foetus. Congenitally infected animals may be born clinically normal or show weakness and clinical signs involving the CNS. The target organs in the foetus (placenta

and foetal brain, heart, lung, liver or muscle) and histological lesions, characterized by multifocal necrosis and non suppurative inflammation, are also similar in all ruminant species, although it has been suggested that focal leukomalacia would be more frequent in the ovine foetuses than in the other species. However, it is not known whether this reflects different pathogenesis of the lesions depending on the species (Buxton *et al.* 1998*).*

Breed. Epidemiological studies, based on serological evaluation of a high number of naturally infected cows have suggested that certain breeds, mainly dairy (i.e. Holstein-Freisian), are more susceptible to infection than others, i.e. Limousin (Lopez-Gatius *et al.* 2005; Armengol *et al.* 2007). However, there is no experimental evidence for a different susceptibility to infection or disease depending on the breed of the host, and the differences observed may also be related to the different production systems used for a variety of breeds (Bartels *et al.* 2006). Similarly, other epidemiological studies have found that the management practices have more influence in *N. caninum* infection than the genetic background (Pan *et al*. 2004). On the other hand, using beef bull semen has epidemiologically proven to reduce the risk of suffering abortion by dairy dams when compared with dairy bull semen (Lopez-Gatius *et al.* 2005). Bovine neosporosis has been reproduced in both beef and dairy breeds, although never in parallel in a comparative study. Ovine breeds used for experiment have been mainly those for meat or mixed production, while only Pigmy or Boer goats have been used for reproducing caprine neosporosis (Table 2).

Stage of gestation. There is a significant relationship between the stage of gestation when infection occurs and the clinical consequences of infection. This has been experimentally proved, with similar outcomes in the four ruminant species where

exogenous neosporosis has been reproduced (Lindsay *et al*. 1995; Buxton *et al*. 1998; Maley *et al*. 2003; Gondim *et al*. 2004; Macaldowie *et al.*2004; McCann *et al*. 2007; Rosbottom *et al.* 2007; Benavides *et al.* 2012; Konrad *et al.* 2012). Reactivation of infection and abortion after exogenous, experimental infection, before pregnancy has been attempted in cattle and goats but it has not been yet achieved (Lindsay *et al.* 1995; McCann *et al*. 2007).

Infection during early gestation (first term) is generally associated with parasite colonization of the placenta and the development of severe necrotic and inflammatory lesions, with subsequent death and reabsorption, autolysis or mummification of the foetus (Dubey *et al*. 1992; Barr *et al.* 1994; Macaldowie *et al*. 2004; Rojo-Montejo *et al*. 2009*;* Williams *et al*. 2012). However, infection of cattle at this stage of pregnancy does not always result in foetal death and dams infected during the first trimester may carry live foetuses, with no lesions, detectable parasite or serological evidence of infection in the foetus at the time of calving, while other animals, infected with the same dose and strain of *N. caninum* would abort (Barr *et al*. 1994; Williams *et al.* 2000; Gondim *et al.* 2004; Macaldowie *et al*. 2004; McCann *et al*. 2007; Caspe *et al*. 2012). The fact that infection during the first trimester resulted in either foetal death or complete absence of detectable infection in the foetus was described as an “all or nothing” response where, in some dams, the parasite inoculated at early gestation did not invade the placenta as opposed to those dams where infection is extended to the foetus and showed severe placentitis and foetal death. The occurrence of the “all or nothing” response could be related to the dose inoculated to the animals, as it was more frequent in the subcutaneously inoculated group, where fewer parasites are thought to reach the placenta when compared to animals where parasites are directly

inoculated into the bloodstream (Macaldowie *et al*. 2004). Dams carrying live foetuses also showed stronger IFNγ production and cell proliferation in lymphostimulation assays from local lymph nodes compared to those dams where the foetus was dead, suggesting that the quality of the maternal immune response triggered by the infection could influence the parasite dissemination, preventing the colonization of the placenta and foetus (Bartley *et al.* 2012). Interestingly, the "all or nothing" response has not been observed in small ruminants, where all sheep and goats infected during the first term of gestation suffered foetal death (Lindsay *et al*. 1995; McAllister *et al*. 1996; Buxton *et al*. 1998). Similarly, all but one out of ten foetuses from pregnant buffalos inoculated in early pregnancy were infected and had histological lesions of neosporosis (Konrad *et al*. 2012). However, number of studies in these three species (buffalo, sheep and goats) are not enough to elucidate whether the absence, or not, of an "all or nothing" response is associated to the species, and the probable influence of the isolate, dose or route should be investigated to further characterize this response.

Although experimental infections during mid pregnancy (second third of gestation) may also result in abortion (Dubey *et al.* 1992), the occurrence of transplacental transmission with the delivery of live, although congenitally infected calves are the most common outcomes. These calves are mainly born without any obvious clinical signs, but neurological signs were occasionally observed (Innes *et al*. 2001; Barr *et al*. 1994; Almeria *et al*. 2003; Gondim *et al*. 2004). The histological lesions found in the placenta and foetuses following infection at mid-gestation were milder than those observed after infection earlier in pregnancy (Maley *et al*. 2003). The only study carried out in goats described one abortion and the birth of one live, healthy kid. No parasites or lesions were found in the live kid, but congenital transmission could not be

ruled out as no serological analysis or identification of the parasite by PCR were attempted (Lindsay *et al*. 1995). In contrast to the clinical outcome in cattle, infection during mid pregnancy in sheep resulted in more dramatic consequences, as most of the animals aborted or, less frequently, produced weak lambs. Only a few lambs were born alive, and parasite cysts were found in one third of them (McAllister *et* al. 1996; Buxton *et al*. 1998).

Transplacental transmission of the infection and delivery of congenitally infected live calves with no obvious clinical signs have been a common consequence of experimental exogenous *Neospora* infection during late gestation (last term) in cattle (Williams *et al*. 2000; McCann *et al*. 2007). Furthermore, histological lesions in the placenta and foetus were the mildest, and parasite burdens the lowest, when compared to infections during the first and second trimesters of gestation (Gibney *et al*. 2008; Benavides *et al*. 2012). Infection in the last third of gestation in sheep and goats has a similar outcome to that seen with cattle, where kids and lambs are born alive. The only study carried in goats showed the kids to be born weak, although the parasite was not detected, while the study done in sheep described the delivery of clinically healthy lambs, some of them congenitally infected (Lindsay *et al*. 1995; McAllister *et al*. 1996).

Route, parasite stage and dose of inoculation. N. caninum oocysts, the infectious stage for ruminants in natural infection, are difficult to obtain, as experimentally infected dogs shed very few (reviewed by Dubey and Schares, 2011). For this reason, most of the experimental studies on ruminants have been carried out with tachyzoites as the parasite stage of the infectious inoculums (Tables 1 and 2). Because *N. caninum*

tachyzoites are susceptible to digestion, and thus destruction in the gastric environment, they have usually been inoculated through a parenteral route, namely subcutaneous, intravenous or intramuscular. All of these routes have been shown to be effective in infecting cattle and sheep while only some of them have been used in goats and water buffalos. When comparing the intravenous and subcutaneous routes, which are the most frequent routes of inoculation used, the former is associated with a more severe clinical presentation, i. e. higher peak temperature response and more frequent foetal mortality (Macaldowie *et al*. 2004).

As was explained previously, similar doses of the same parasite strain may lead to different clinical outcomes depending on the stage of gestation. However, the main effect of the dose of infection of tachyzoite-based inoculums is closely related to the route of administration and the host species. Intravenous doses administered to cattle have ranged from $1.2x10^6$, which caused infection in all inoculated dams at day 76 of gestation and 75% of abortions (Weber *et al*. 2013), to $5x10^8$, which cause both infection and abortion in all cows infected on day 70 of gestation (Macaldowie *et al.* 2004). An intermediate dose of 10^7 has caused variable results: while in one study it caused foetal death in all dams inoculated (Gibney *et al*. 2008), in other experimental infections only 3 out of 5 dams showed foetal death, although all were infected (Rojo-Montejo *et al*. 2009). A dose-titration study in sheep showed that no abortions or vertical transmission of the parasite occurred after intravenous inoculation of 50 tachyzoites, while $5x10^3$ caused abortion in 50% of infected animals, and with a dose of 10^7 or 10^8 tachyzoites all ewes aborted, although the latter was associated with a longer period of fever (Weston *et al*. 2009). An intravenous dose of 10^4 tachyzoites caused abortion in all infected goats, and was associated with a later detection of the

parasitemia by ELISA when compared with a higher dose, i.e. 10^6 (Yin *et al*. 2012). Pregnant buffalos inoculated intravenously with 10^8 tachyzoites during the first trimester of gestation showed foetal and placental lesions after transplacental transmission of the disease in all cases (Konrad *et al*. 2012).

Subcutaneous inoculation of tachyzoites has also been used in other studies, as it was suggested that, although it is still an artificial route of inoculation, it may model the natural infection better than the intravenous route of inoculation (reviewed by Dubey and Schares, 2006). Subcutaneous inoculation of cattle with 10^7 and $5x10^8$ caused transplacental transmission and lesions of the foetus when inoculated at the second trimester of gestation, although the higher dose was associated with earlier and more severe histopathological changes (Maley *et al*. 2003). Inoculation of sheep with a dose of 10^6 caused vertical transmission, histological lesions in the foetuses and a high percentage of abortion, i.e. 75 to 100% (Buxton *et al*. 1997, 1998), although a higher dose of $5x10^6$ only resulted in abortion of 6 out of 11 sheep (Jenkins *et al*. 2004). The reduction in the percentage of animals aborting could have been due to the influence of other parameters, such as the isolate or host breed, as a further study by Buxton *et al*. confirmed that high doses, i.e. 10^7, caused abortions in 100% of the sheep (Buxton *et al*. 2001). The same dose has been employed in subcutaneous inoculations of goats, confirming the occurrence of abortion or transplacental transmission depending on the term of gestation (Lindsay *et al*. 1995).

Inoculation of tachyzoites through the conjunctiva, at doses of 1 and $2.5x10^8$, resulted in infection and induction of specific immune response in pregnant cattle without causing transplacental transmission of the parasite to the foetus (Yaniz *et al*. 2007; Moore *et al*. 2014). Cows infected with the lower dose even reverted to seronegative

status four months after infection (Yaniz *et al*. 2007). Intramuscular inoculation of tachyzoites has not been commonly employed. As the sole route of inoculation, it has been tried directly into the foetus at day 118 of gestation, causing foetal death 18 days after infection (Barr *et al*. 1994). It has also been used in other studies, but only in addition to intravenous or subcutaneous inoculations on pregnant cows or sheep, where the consequences varied according to the term of gestation (Dubey *et* al. 1990, 1992; Conrad *et* al. 1993; Barr *et al*. 1994; Adrianarivo *et al*. 2000, 2001).

The possibility of venereal transmission of neosporosis has been investigated after N. *caninum* DNA was found in semen from naturally infected bulls (Ortega-Mora *et al*. 2003). Experimental studies have shown that heifers are highly susceptible to intrauterine inoculation of semen spiked with tachyzoites at different doses, from 10^2 to 10^7, where persistent specific serum antibody responses were detected with doses from $5x10^4$ tachyzoites. There was also a direct relation between the inoculated dose and fertility reduction (Serrano-Martinez *et al*. 2007*a*, 2007*b*). Adult cows were less susceptible to becoming infected using this route of inoculation, although one animal inoculated with $5x10^5$ tachyzoites developed serological antibodies against *N. caninum* (Canada *et al*. 2006; Serrano-Martinez *et al*. 2007*b*). Further studies failed to demonstrate venereal transmission of *N. caninum* infection from experimentally infected bulls to heifers, either through artificial insemination with semen pooled from these bulls (Ferre *et al*. 2008) or through natural breeding (Osoro *et al*. 2009). A similar approach was investigated in sheep, where experimentally infected rams did not transmit the infection to adult ewes after natural breeding (Syed-Hussain *et al*. 2013).

Even though sporulated oocysts purified from infected dogs are difficult to obtain, they have been employed in a number of studies to reproduce the disease (Table 1). These

studies have found that infection of pregnant cattle after oral inoculation of sporulated oocysts is possible. Similarly to what has been described in tachyzoite inoculated animals, the establishment of the infection and the occurrence of abortions or transplacental transmission depends very much on the combination between the dose administered and the stage of gestation (Trees *et al*. 2002; Gondim *et al.* 2004; McCann *et al*. 2007). Vertical transmission of the infection is more likely to occur in the second half of gestation, as a dose of $1.5x10^3$ oocysts at day 169 of gestation in cattle effectively caused transplacental transmission to the foetus while a higher dose, of $7x10^4$, at day 70 of gestation failed to cause foetal infection (Gondim *et al*. 2004). However, comparisons between different studies using oocysts are difficult, as there might be significant variability in the viability of oocysts, so the verification of the infectivity by bioassay was recommended as a way to standardized protocols (reviewed by Dubey, 2009).

Parasite strain/isolate. Clear differences in virulence among isolates of *N. caninum* were shown by variations between isolates in their ability to produce lesions, vertical transmission and immunological effects in a murine experimental model (Regidor-Cerrillo *et al*. 2010). In the numerous experimental infections carried out in pregnant ruminants, several different *Neospora* isolates have been employed (Tables 1 and 2). However, it is difficult to compare virulence between the different isolates used in these studies, as the experimental design varied considerably among them, including the host species employed, the stage of gestation and route or dose of inoculation. All of these factors will have an effect upon clinical outcome of the infection. Due to economic and infrastructure constraints involved in experimental infection of large animals, especially cattle, only a few studies have been conducted to compare

infection with different isolates. In these studies, the isolate Nc 1, was used as a virulent strain to which compare other isolates/strains. Caspe *et al.* (2012) found that Nc Spain 7 shows similar virulence to Nc 1, when inoculated into cattle at early gestation (day 65) in pregnant cattle, while Nc Spain 1H was clearly less virulent and induced milder lesions in the foetus when inoculated into cattle at the same trimester (day 70) (Rojo-Montejo *et al*. 2009). Nc Spain 7 and 8 had shown similar clinical outcomes (100% abortions) when inoculated intravenously at day 70 of gestation (10^8 tachyzoites), although the Nc Spain 7 isolated showed a wider distribution in the foetus and placenta, determined by quantitative PCR (Regidor-Cerrillo *et al.* 2014)

The results on isolate virulence are also difficult to compare between different studies as most of them do not state how many passages the parasite has been cultured in vitro, prior to inoculation in the host. Among studies where the passage is recorded, there is variation, from less than 10 to 100 (Williams *et al*. 2000; de Yaniz *et al*. 2007). This information is relevant, as serial passage of parasites through tissue culture for extended periods of time has been shown to attenuate the virulence of *N. caninum* (Bartley *et al*. 2008) therefore, affecting the outcome of the infection.

There was also variability regarding the origin of the isolates employed in the studies. While the "classical" isolates, such as Nc 1or Nc Liverpool were isolated from nervous tissue of congenitally infected dogs (Dubey *et* al.1988; Barber *et al*, 1995), the more recent isolates, originated from congenitally infected calves, such as the different Nc Spain isolates, Nc Illinois or Nc Beef (Gondim *et al.* 2002; Regidor-Cerrillo *et al*. 2010) or from bovine abortions, like Nc BPA1 (Conrad *et al*. 1993). Several isolates have been used to infect sheep. However, no comparison in the virulence of the isolate is feasible since, in order to assure infection, different isolates were mixed within the same

inoculum in studies where more than one isolate were used (McAllister *et al*. 1996; Weston *et al*. 2009).

Non-pregnant ruminant models of N. caninum *infection*

The majority of the studies performed in non pregnant ruminants were aimed to conduct research on the parasite cycle or the possibility of post natal infection (Table 3). Cyclical oral transmission between dog and cattle was proven when dogs were fed meat from calves inoculated with as few as 300 oocysts. Then, new oocysts isolated from faecal samples of one of these dogs were administered to a new calf. In turn, the meat from this calf, fed to a new dog, transmitted the infection and caused the shedding of oocysts (Gondim *et al*. 2002). A similar approach was used to investigate and establish the cyclic transmission between dogs and sheep or goats, also orally inoculated with oocysts shed by dogs (Schares *et al*. 2001).

The possibility of post natal transmission of neosporosis has been addressed on newborn calves. Although tachyzoites are susceptible to digestion in the digestive tract, tachyzoites mixed with colostrum or milk, have been shown to be infectious when administered by feeding bottle through the oral route to neonatal calves (Uggla *et al*. 1998; Davison *et al*. 2001). The reduced production of chloride acid and enzymes in newborn animals, together with the presence of trypsin inhibitors in colostrum, facilitate the survival of tachyzoites (Uggla *et al.* 1998). This mechanism opens the possibility of vertical transmission from infected dams to calves, as *N. caninum* DNA has been identified in colostrum from naturally infected cows (Moskwa *et al.* 2007). However, it is still unknown whether viable parasites capable of infection, are present in colostrum. Attempts to transmit the infection to calves by feeding them milk from

experimentally inoculated dams have been unsuccessful (Davison *et al*. 2001). A placenta homogenate, obtained from seropositive dams aborting or giving birth to congenitally seropositive calves, did not transmit either the infection to calves or adult cows when given orally (Davison *et al*. 2001), although this has shown to be an effective source of infection for dogs, which shed oocyst in the faeces after being fed cotyledons from *Neospora*-infected cows (Dijkstra *et al.* 2001).

Neospora DNA has been found in semen from naturally infected bulls (Ortega-Mora *et al*. 2003), leading to a series of experimental infections to investigate the possibility of venereal transmission of the disease. With this purpose, an experimental model based in the intravenous inoculation of adult bulls with 10^7 to 10^8 of Nc 1 tachyzoites was established to analyse the presence of the parasite in semen. These studies have shown that there is intermittent shedding of low numbers of parasites, less than 20 parasites/ml estimated by quantitative PCR. This burden was estimated by quantitative PCR, so it remains unclear whether there were actual infectious stages (i.e. tachyzoites) or just remains of parasite nucleic acids. The number of parasites found in the semen was below the threshold for venereal transmission, estimated in $5x10^3$ tachyzoites (Serrano-Martinez *et al*. 2007*b*; Ferre *et al*. 2008). This experimental model has been recently transferred to sheep, where a dose titration showed that the frequency and quantity of parasite present in semen is in direct relation with the dose inoculated into the ram. An intravenous inoculum of 10^7 tachyzoites of Nc NZ1 induced one ram to shed as much as 723 tachyzoites/ml, estimated using a quantitative PCR technique. Despite the presence of *Neospora* in semen, breeding with this ram did not transmit the infection to sheep (Syed-Hussain *et al*. 2013).

Numerous experimental studies have been carried out to characterize the immune responses occurring after *N. caninum* infection (Table 3). It was shown that when calves were inoculated orally with sporulated oocysts, or subcutaneously with *N. caninum* tachyzoites, a cellular immune response was triggered and antigen specific cell proliferation could be detected (Marks *et al.* 1998; De Marez *et al.* 1999). This proliferation was evident from day 4-6 post inoculation and is associated with the production of IFNγ induced by *Neospora* antigens (Lunden *et al.* 1998; Tuo *et al.* 2005). IFNγ has been shown to control the intracellular multiplication of the parasite in cell culture (Innes *et al.* 1995) and is mainly produced by CD4+ after infection. CD8+ and NK cells also play a pivotal in controlling the parasite during the early stages of infection (Klevar *et al.* 2007). CD4 positive cytotoxic T lymphocytes have also been shown to play a relevant role in controlling early *N. caninum* dissemination in the host through the specific elimination of parasite infected cells (Staska *et al.* 2003). A specific serological response could be detected from two weeks after inoculation of calves with *Neospora* parasites and fluctuation in the levels of antibodies has been shown to occur, both in non pregnant and pregnant cattle (Conrad *et al.* 1993; Maley *et al.* 2001). Interestingly, the antibody titre was not influenced by the dose of inoculation, as 10^6 and 10^8 produced similar serological titres (Maley *et al.* 2001).

Ruminant models to evaluate pharmacological treatments

Experimental infection of non pregnant ruminants has been also used to evaluate the efficacy of several drugs. Efficacy is difficult to assess because exogenous infection, or recrudescence of a persistent infection in dams, does not produce evident clinical signs until the occurrence of abortion, when it is too late for treatment. It is also difficult to

find clinical evidence of infection if the parasitaemia occurs from mid pregnancy onwards. Field observations have suggested that cows receiving monensin were less likely to abort due to neosporosis (VanLeeuwen *et al*. 2010). Treatment with this ionophore was assessed in an experimental model, where adult cattle were subcutaneously challenged with $5x10^6$ Nc 1 tachyzoites while being treated with a low-release monensin bolus. Although treated animals showed a lower humoral response at certain days, this result was not significant when adjusting for repeated measures on time (VanLeeuwen *et al.* 2011). In a different experiment, designed as a pilot study, calves inoculated subcutaneously and intravenously with a total of $2x10^8$ Nc 1 tachyzoites responded to toltrazuril-sulfone treatment, administered for six days after challenge, with lower body temperature, lower specific serological antibodies and no parasite detectable in muscle samples when compared with non-treated animals (Kritzner *et al.* 2002).

Ruminant models to evaluate vaccines

Controlling ruminant neosporosis, reducing its incidence and vertical transmission are of great global interest. The main efforts in controlling neosporosis are being focused on developing effective vaccines, as they provide a sustainable prevention and control strategy, reduce the use of pharmacological treatments and minimise impact on the environment (reviewed by Innes *et al.* 2011). Field observations suggested that a previously acquired infection by *N. caninum* would protect against abortion in subsequent infections during pregnancy (McAllister *et al*. 2000). Considering these observations, the role of previous and persistent infections as a source of protection has been investigated. Persistently infected seropositive cattle challenged during the

first trimester of gestation through intravenous inoculation of 10^7 tachyzoites of Nc Liverpool did not suffer any abortion, while those seronegative cattle suffered 100% abortion. However persistent infection did not protect against transplacental transmission of the parasite after challenge (Williams *et al*. 2003). Furthermore, a recent study with the same experimental approach found foetal lesions consistent with Neospora infection, although no parasite antigen, in persistently infected seropositive cows inoculated at early gestation with $5x10^7$ tachyzoites of the Nc 6 Argentina isolate (Bacigalupe *et al*. 2013). However, comparisons between these two studies are difficult due to the substantial differences in the experimental designs; thus, the protective effect of persistent infection warrants further investigation.

Several studies have been conducted to assess the protection conferred by inoculation of animals with different strains of *N. caninum* prior to challenge. In an experimental model in sheep, it was shown that infection through subcutaneous inoculation of $1x10^7$ tachyzoites of Nc 1 prior to mating reduced the abortion rate, although the protection was not enough to avoid vertical transmission, after challenging animals with the same *N. caninum* strain, dose and route at mid gestation (Buxton *et al*. 2001). A subsequent study with a similar model applied to cattle showed that cows inoculated with infectious *N. caninum* prior to mating were protected against vertical transmission when challenged at mid gestation (Innes *et al*. 2001). The protection conferred by the previous infection is not specific for the isolate, as further investigations have shown that both virulent, i. e. Nc 6 Argentina, and low virulent isolates, i. e. Nc Nowra (Miller *et al*. 2002) or Nc Spain 1H (Rojo-Montejo *et al*. 2009), used as the vaccination strain before gestation induce variable degrees of protection against abortion and vertical transmission after inoculation of virulent isolates, i. e. Nc

Liverpool or Nc 1, during gestation (Williams *et al*. 2007; Hecker *et al.* 2013; Rojo-Montejo *et al*. 2013; Weber *et al*. 2013). Although these are promising results for vaccine development, there is a risk using live strains as vaccines, as they may evolve to become more virulent (Miller *et al.* 2002). Other issues for the live vaccines based on attenuated strains are the short shelf life and the need for a cold chain to deliver the vaccines, although the current existence of commercially available live vaccines shown that these issues may be overcome. A major concern when using live vaccines to protect against bovine neosporosis is that the vaccine should ideally not persist in the host due to the danger of the live vaccine being vertically transmitted to the foetus *in utero* (reviewed by Innes et al. 2011).

Experimental models have been also used to evaluate the efficacy of vaccines based on killed tachyzoites. While these formulations, inoculated before mating, partially protected sheep against abortion after challenge at mid-late gestation (subcutaneous inoculation of $5x10^6$ tachyzoites of Nc Illinois), they did not prevent vertical transmission of the infection (O'Handley *et al*. 2003; Jenkins *et al.* 2004). Furthermore, a similar formulation of adjuvanted killed tachyzoites, administered at early gestation did not protect pregnant cattle against abortion or vertical transmission when challenged with $4x10^7$ tachyzoites of Nc BPA1 by intramuscular and intravenous route (Andrianarivo *et al*. 2000). Several studies, where relative protection against challenge was achieved through vaccination with live tachyzoites, failed to produced similar protection when vaccinating with killed tachyzoites or antigen formulations from the same isolate used in the challenge (Williams *et al.* 2007; Hecker *et al.* 2013; Weber *et al.* 2013). This suggests that the lack of protection when using antigen or whole tachyzoite formulations was most probably related to the absence of parasite

intracellular multiplication, and hence lack of induction of protective cell-mediated immune responses rather than the lack of cross-recognition of the immunogenic antigens present on the vaccination and challenge isolates. An experimental model of vaccination during gestation, without challenge, has shown that vaccines based on inactivated adjuvanted antigens induced a similar immune response, in terms of serological antibodies, lymphocyte proliferation and IFNγ production, to that found in persistently infected dams during gestation. However, vaccinated and naturally infected animals differed in the predominant IgG isotype (Moore *et al.* 2005).

Regarding vaccination, experimental models in non pregnant ruminants have been employed to assess the immunogenicity of several vaccine candidates, based on the evaluation of the desirable CD4+ T cell activation and IFNγ secretion achieved by the immunization products. A preparation of killed whole *N. caninum* tachyzoites with a variety of adjuvants was subcutaneously inoculated to heifers three times over three months. None of the formulations induced the same levels of serological antibodies, cell proliferation or IFNγ compared to natural infection, but among all the adjuvants tested, Polygen was the one that induced the highest humoral response and IFNγ production. This is a low molecular weight, non-particulate polymer that in solution can form a high molecular weight gel (Andrianaviro *et al.* 1999). A DNA subunit vaccine based on NcSRS2, a surface antigen of *N. caninum*, was effective, after a boost with adjuvanted antigens, in inducing a strong cell-mediated response, measured by T cell activation and IFNγ, similar to that described after infection with live parasites (Baszler *et al.* 2008). Another study showed that the addition of immune stimulation complexes (ISCOMs) to native antigens of *N. caninum* in a vaccine formulation for calves induced

higher titers of serological antibodies and similar production of IFNγ to that observed in calves intravenously infected with live 10^8 Nc 1 tachyzoites (Moore *et al.* 2011).

FUTURE NEEDS

Different research groups have developed ruminant models of *N. caninum* infection independently from others, resulting in a high degree of variability. However, a better definition of such models will enable a more robust comparison of available data. Looking ahead, it is important to accurately define and standardize the animal model depending on the purpose of the study, keeping in mind those variables which play an essential role and have been reviewed in this manuscript (ruminant species, breed, term of gestation, route of inoculation, parasite stage, parasite strain/isolate, doses). In addition, several parameters that may influence the final outcome must also be agreed: inoculum preparation, method of quantifying inoculums, storage of challenge inoculum, selection of animals and statistical procedures. Finally, validation and standardization of analytical techniques used to monitor the infection (clinical data, lesion scores, PCR techniques, immunological techniques) also need to be harmonized in order to be able to compare the data obtained.

Most of the animal models currently available are primo-infection models suitable to evaluate exogenous transplacental transmission. However, under field conditions, endogenous transplacental transmission (following a recrudescence of infection in persistently infected cows during pregnancy) is the major transmission route and thus mainly responsible for the maintenance of the infection and occurrence of abortions associated with neosporosis in cattle (Trees and Williams, 2005; reviewed by Dubey *et al.* 2007). Therefore, the development of a standardized ruminant model for

endogenous neosporosis, in order to study the recrudescence processes of *N. caninum* infection during pregnancy is needed to further increase our understanding of the host-parasite relationship and for testing control strategies. Additionally, an abortion model based on challenge at 70 days of gestation may not be the most appropriate model to mimic the natural infection. *N. caninum*-associated abortion in cattle may occur from 3 months of gestation to term, but field data show that the majority of *N. caninum* abortions are observed between 5-7 months of gestation (reviewed by Dubey *et al.* 2007 and Almería and López-Gatius, 2013). In addition, the primo-infection at 70 days of gestation by inoculating the cattle intravenously with high parasite doses could be too aggressive for testing the efficacy of vaccines; as a result, the vaccine potency could potentially be underestimated.

In summary, experimental ruminant model standardization is urgently required in order to gain knowledge on these relevant protozoan diseases. This is especially relevant when testing vaccine formulations, since the success of developing a vaccine rests not only on identifying the optimal candidate and immunization regimen, but also on devising the best strategy to test vaccine efficacy.

ACKNOWLEDGEMENTS

We gratefully thank members of the SALUVET group, specially Drs. Silvia Rojo, Javier Regidor, Ignacio Ferre, Mercedes Gómez y Gema Alvarez, and Dr. Mark Dagleish (Moredun Research Institute) for the suggestions and constructive criticism of the manuscript.

FINANCIAL SUPPORT

This work was partially supported by research grants from the Spanish Ministry of Economy and Competitiveness (AGL 2010-22191), Junta de Castilla y Leon (LE253U13), Brazilian CNPq (no. 400405/2012-1) and CYTED (Thematic Network 113RT0469-Protozoovac).

REFERENCES

Aguado-Martinez, A., Alvarez-Garcia, G., Fernandez-Garcia, A., Risco-Castillo, V., Arnaiz-Seco, I., Rebordosa-Trigueros, X., Navarro-Lozano, V. and Ortega-Mora, L. M. (2008). Usefulness of rNcGRA7- and rNcSAG4-based ELISA tests for distinguishing primo-infection, recrudescence, and chronic bovine neosporosis. *Veterinary Parasitology* **157**, 182-195. doi: 10.1016/j.vetpar.2008.08.002

Aguado-Martinez, A., Alvarez-Garcia, G., Fernandez-Garcia, A., Risco-Castillo, V., Marugan-Hernandez, V. and Ortega-Mora, L. M. (2009). Failure of a vaccine using immunogenic recombinant proteins rNcSAG4 and rNcGRA7 against neosporosis in mice. *Vaccine* **27**, 7331-7338. doi: 10.1016/j.vaccine.2009.09.050

Almeria, S., Araujo, R., Tuo, W., Lopez-Gatius, F., Dubey, J. P. and Gasbarre, L. C. (2010). Fetal death in cows experimentally infected with *Neospora caninum* at 110 days of gestation. *Veterinary Parasitology* **169**, 304-311. doi: 10.1016/j.vetpar.2009.12.044

Almeria, S., Araujo, R. N., Darwich, L., Dubey, J. P. and Gasbarre, L. C. (2011). Cytokine gene expression at the materno-foetal interface after experimental *Neospora caninum* infection of heifers at 110 days of gestation. *Parasite Immunology* **33**, 517-523. doi: 10.1111/j.1365-3024.2011.01307.x

Almeria, S., De Marez, T., Dawson, H., Araujo, R., Dubey, J. P. and Gasbarre, L. C. (2003). Cytokine gene expression in dams and foetuses after experimental *Neospora caninum* infection of heifers at 110 days of gestation. *Parasite Immunology* **25**, 383-392.

Almeria, S. and Lopez-Gatius, F. (2013). Bovine neosporosis: clinical and practical aspects. *Research in Veterinary Science* **95**, 303-309. doi: 10.1016/j.rvsc.2013.04.008

Andrianarivo, A. G., Barr, B. C., Anderson, M. L., Rowe, J. D., Packham, A. E., Sverlow, K. W. and Conrad, P. A. (2001). Immune responses in pregnant cattle and bovine fetuses following experimental infection with *Neospora caninum*. *Parasitology Research* **87**, 817-825.

Andrianarivo, A. G., Choromanski, L., McDonough, S. P., Packham, A. E. and Conrad, P. A. (1999). Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *International Journal for Parasitology* **29**, 1613-1625.

Andrianarivo, A. G., Rowe, J. D., Barr, B. C., Anderson, M. L., Packham, A. E., Sverlow, K. W., Choromanski, L., Loui, C., Grace, A. and Conrad, P. A. (2000). A POLYGEN-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following i.v./i.m. experimental tachyzoite challenge. *International Journal for Parasitology* **30**, 985-990.

Armengol, R., Pabon, M., Santolaria, P., Cabezon, O., Adelantado, C., Yaniz, J., Lopez-Gatius, F. and Almeria, S. (2007). Low seroprevalence of *Neospora caninum* infection associated with the limousin breed in cow-calf herds in Andorra, Europe. *The Journal of Parasitology* **93**, 1029-1032. doi: 10.1645/GE-1242R.1

Bacigalupe, D., Basso, W., Caspe, S. G., More, G., Lischinsky, L., Gos, M. L., Leunda, M., Campero, L., Moore, D. P., Schares, G., Campero, C. M. and Venturini, M. C. (2013). *Neospora caninum* NC-6 Argentina induces fetopathy in both serologically positive and negative experimentally inoculated pregnant dams. *Parasitology Research* **112**, 2585-2592. doi: 10.1007/s00436-013-3424-1

Barber, J. S., Holmdahl, O. J., Owen, M. R., Guy, F., Uggla, A. and Trees, A. J. (1995). Characterization of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper and Uggla). *Parasitology* **111 (Pt 5)**, 563-568.

Barr, B. C., Conrad, P. A., Dubey, J. P. and Anderson, M. L. (1991). *Neospora*-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. *Journal of Veterinary Diagnostic Investigation* **3**, 39-46.

Barr, B. C., Rowe, J. D., Sverlow, K. W., BonDurant, R. H., Ardans, A. A., Oliver, M. N. and Conrad, P. A. (1994). Experimental reproduction of bovine fetal Neospora infection and death with a bovine Neospora isolate. *Journal of Veterinary Diagnostic Investigation* **6**, 207-215.

Bartels, C. J., Arnaiz-Seco, J. I., Ruiz-Santa-Quitera, A., Bjorkman, C., Frossling, J., von Blumroder, D., Conraths, F. J., Schares, G., van Maanen, C., Wouda, W. and Ortega-Mora, L. M. (2006). Supranational comparison of *Neospora caninum* seroprevalences in cattle in Germany, The Netherlands, Spain and Sweden. *Veterinary Parasitology* **137**, 17-27. doi: 10.1016/j.vetpar.2005.12.016.

Bartley, P. M., Katzer, F., Rocchi, M. S., Maley, S. W., Benavides, J., Nath, M., Pang, Y., Canton, G., Thomson, J., Chianini, F. and Innes, E. A. (2013). Development of maternal and foetal immune responses in cattle following experimental challenge with *Neospora caninum* at day 210 of gestation. *Veterinary Research* **44**, 91. doi: 10.1186/1297-9716-44-91

Bartley, P. M., Kirvar, E., Wright, S., Swales, C., Esteban-Redondo, I., Buxton, D., Maley, S. W., Schock, A., Rae, A. G., Hamilton, C. and Innes, E. A. (2004). Maternal and fetal immune responses of cattle inoculated with *Neospora caninum* at mid-

gestation. *Journal of Comparative Pathology* **130**, 81-91. doi: 10.1016/j.jcpa.2003.08.003.

Bartley, P. M., Wright, S., Chianini, F., Buxton, D. and Innes, E. A. (2008). Inoculation of Balb/c mice with live attenuated tachyzoites protects against a lethal challenge of *Neospora caninum*. *Parasitology* **135**, 13-21. doi: 10.1017/S0031182007003526.

Bartley, P. M., Wright, S. E., Maley, S. W., Macaldowie, C. N., Nath, M., Hamilton, C. M., Katzer, F., Buxton, D. and Innes, E. A. (2012). Maternal and foetal immune responses of cattle following an experimental challenge with *Neospora caninum* at day 70 of gestation. *Veterinary Research* **43**, 38. doi: 10.1186/1297-9716-43-38

Basso, W., Venturini, L., Venturini, M. C., Hill, D. E., Kwok, O. C., Shen, S. K., Dubey, J. P. (2001). First isolation of *Neospora caninum* from the feces of a naturally infected dog. *The Journal of Parasitology* **87**, 612-618.

Baszler, T. V., Shkap, V., Mwangi, W., Davies, C. J., Mathison, B. A., Mazuz, M., Resnikov, D., Fish, L., Leibovitch, B., Staska, L. M. and Savitsky, I. (2008). Bovine immune response to inoculation with *Neospora caninum* surface antigen SRS2 lipopeptides mimics immune response to infection with live parasites. *Clinical and Vaccine Immunology* **15**, 659-667. doi: 10.1128/CVI.00436-07

Benavides, J., Katzer, F., Maley, S. W., Bartley, P. M., Canton, G., Palarea-Albaladejo, J., Purslow, C. A., Pang, Y., Rocchi, M. S., Chianini, F., Buxton, D. and Innes, E. A. (2012). High rate of transplacental infection and transmission of *Neospora caninum* following experimental challenge of cattle at day 210 of gestation. *Veterinary Research* **43**, 83. doi: 10.1186/1297-9716-43-83

Bjorkman, C., Alvarez-Garcia, G., Conraths, F. J., Mattsson, J. G., Ortega-Mora, L. M., Sager, H. and Schares, G. (2006). *Neospora caninum* IgG avidity tests: an interlaboratory comparison. *Veterinary Parasitology* **140**, 273-280. doi: 10.1016/j.vetpar.2006.04.030.

Boysen, P., Klevar, S., Olsen, I. and Storset, A. K. (2006). The protozoan *Neospora caninum* directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. *Infection and Immunity* **74**, 953-960. doi: 10.1128/IAI.74.2.953-960.2006.

Buxton, D., Maley, S. W., Thomson, K. M., Trees, A. J. and Innes, E. A. (1997). Experimental infection of non-pregnant and pregnant sheep with *Neospora caninum*. *Journal of Comparative Pathology* **117**, 1-16.

Buxton, D., Maley, S. W., Wright, S., Thomson K. M., Rae, A. G. and Innes, E. A. (1998). The pathogenesis of experimental neosporosis in pregnant sheep. *Journal of Comparative Pathology* **118**, 267-279.

Buxton, D., Wright, S., Maley, S. W., Rae, A. G., Lunden, A. and Innes, E. A. (2001). Immunity to experimental neosporosis in pregnant sheep. *Parasite Immunology* **23**, 85-91.

Canada, N., Meireles, C. S., Ferreira, P., Correia da Costa, J. M. and Rocha, A. (2006). Artificial insemination of cows with semen in vitro contaminated with *Neospora caninum* tachyzoites failed to induce neosporosis. *Veterinary Parasitology* **139**, 109-114. doi: 10.1016/j.vetpar.2006.02.014.

Caspe, S. G., Moore, D. P., Leunda, M. R., Cano, D. B., Lischinsky, L., Regidor-Cerrillo, J., Alvarez-Garcia, G., Echaide, I. G., Bacigalupe, D., Ortega Mora, L. M., Odeon, A. C. and Campero, C. M. (2012). The *Neospora caninum*-Spain 7 isolate induces placental

damage, fetal death and abortion in cattle when inoculated in early gestation. *Veterinary Parasitology* **189**, 171-181. doi: 10.1016/j.vetpar.2012.04.034

Collantes-Fernandez, E., Lopez-Perez, I., Alvarez-Garcia, G. and Ortega-Mora, L. M. (2006). Temporal distribution and parasite load kinetics in blood and tissues during *Neospora caninum* infection in mice. *Infection and Immunity* **74**, 2491-2494. doi: 10.1128/IAI.74.4.2491-2494.2006.

Conrad, P. A., Sverlow, K., Anderson, M., Rowe, J., BonDurant, R., Tuter, G., Breitmeyer, R., Palmer, C., Thurmond, M. and Ardans, A. (1993). Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. *Journal of Veterinary Diagnostic Investigation* **5**, 572-578.

Davison, H. C., Guy, C. S., McGarry, J. W., Guy, F., Williams, D. J., Kelly ,D. F. and Trees, A. J. (2001). Experimental studies on the transmission of *Neospora caninum* between cattle. *Research in Veterinary Science* **70**, 163-168. doi: 10.1053/rvsc.2001.0457.

De Marez, T., Liddell, S., Dubey, J. P., Jenkins, M. C. and Gasbarre, L. (1999). Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses. *International Journal for Parasitology* **29**, 1647-1657.

de Yaniz, M. G., Moore, D. P., Odeon, A. C., Cano, A., Cano, D. B., Leunda, M. R. and Campero, C. M. (2007). Humoral immune response in pregnant heifers inoculated with *Neospora caninum* tachyzoites by conjunctival route. *Veterinary Parasitology* **148**, 213-218. doi: 10.1016/j.vetpar.2007.06.030.

Dijkstra, T., Barkema, H. W., Eysker, M. and Wouda, W. (2001). Evidence of post-natal transmission of *Neospora caninum* in Dutch dairy herds. *International Journal for Parasitology* **31**, 209-215.

Dijkstra, T., Eysker, M., Schares, G., Conraths, F. J., Wouda, W. and Barkema, H. W. (2001). Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. *International Journal for Parasitology* **31**, 747-752.

Dijkstra, T., Lam, T. J., Bartels, C. J., Eysker, M. and Wouda, W. (2008). Natural postnatal *Neospora caninum* infection in cattle can persist and lead to endogenous transplacental infection. *Veterinary Parasitology* **152**, 220-225. doi: 10.1016/j.vetpar.2007.12.034

Dubey, J. P. (2009). Toxoplasmosis in sheep--the last 20 years. *Veterinary Parasitology* **163**, 1-14. doi: 10.1016/j.vetpar.2009.02.026.

Dubey, J. P., Chapman, J. L., Rosenthal, B. M., Mense, M. and Schueler, R. L. (2006). Clinical *Sarcocystis neurona, Sarcocystis canis, Toxoplasma gondii,* and *Neospora caninum* infections in dogs. *Veterinary Parasitology* **137**, 36-49. doi: 10.1016/j.vetpar.2005.12.017.

Dubey, J. P., Hattel, A. L., Lindsay, D. S. and Topper, M. J. (1988). Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medical Association* **193**, 1259-1263.

Dubey, J. P., Jenkins, M. C., Rajendran, C., Miska, K., Ferreira, L. R., Martins, J., Kwok, O. C., Choudhary, S. (2011) **Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum***. *Veterinary Parasitology*. **181**, 382-387. doi: 10.1016/j.vetpar.2011.05.018.

Dubey, J. P. and Lindsay, D. S. (1990). *Neospora caninum* induced abortion in sheep. *Journal of Veterinary Diagnostic Investigation* **2**, 230-233.

Dubey, J. P., Lindsay, D. S., Anderson, M. L., Davis, S. W. and Shen, S. K. (1992). Induced transplacental transmission of *Neospora caninum* in cattle. *Journal of the American Veterinary Medical Association* **201**, 709-713.

Dubey, J. P. and Schares, G. (2011). Neosporosis in animals--the last five years. *Veterinary Parasitology* **180**, 90-108. doi: 10.1016/j.vetpar.2011.05.031.

Dubey, J. P. and Schares, G. (2006). Diagnosis of bovine neosporosis. *Veterinary Parasitology* **140**, 1-34. doi: 10.1016/j.vetpar.2006.03.035.

Dubey, J. P., Schares, G. and Ortega-Mora, L. M. (2007). Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews* **20**, 323-367. doi: 10.1128/CMR.00031-06.

Eiras, C., Arnaiz, I., Alvarez-Garcia, G., Ortega-Mora, L. M., Sanjuanl, M. L., Yus, E. and Dieguez, F. J. (2011). *Neospora caninum* seroprevalence in dairy and beef cattle from the northwest region of Spain, Galicia. *Preventive Veterinary Medicine* **98**, 128-132. doi: 10.1016/j.prevetmed.2010.10.014.

Ferre, I., Serrano-Martinez, E., Martinez, A., Osoro, K., Mateos-Sanz, A., Del-Pozo, I., Aduriz, G., Tamargo, C., Hidalgo, C. O. and Ortega-Mora, L. M. (2008). Effects of re-infection with *Neospora caninum* in bulls on parasite detection in semen and blood and immunological responses. *Theriogenology* **69**, 905-911. doi: 10.1016/j.theriogenology.2007.11.006.

Gibney, E. H., Kipar, A., Rosbottom, A., Guy, C. S., Smith, R. F., Hetzel, U., Trees, A. J. and Williams, D. J. (2008). The extent of parasite-associated necrosis in the placenta and foetal tissues of cattle following *Neospora caninum* infection in early and late gestation correlates with foetal death. *International Journal for Parasitology* **38**, 579-588. doi: 10.1016/j.ijpara.2007.09.015.

Gondim, L. F., Gao, L. and McAllister, M. M. (2002). Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. *The Journal of Parasitology* **88**, 1159-1163. doi: 10.1645/0022-3395(2002)088[1159:IPONCO]2.0.CO;2

Gondim, L. F., McAllister, M. M., Anderson-Sprecher, R. C., Bjorkman, C., Lock, T. F., Firkins, L. D., Gao, L. and Fischer, W. R. (2004). Transplacental transmission and abortion in cows administered *Neospora caninum* oocysts. *The Journal of Parasitology* **90**, 1394-1400. doi: 10.1645/GE-359R.

Hall, C. A., Reichel, M. P. and Ellis, J. T. (2005). *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. *Veterinary Parasitology* **128**, 231-241. doi: 10.1016/j.vetpar.2004.12.012.

Harkins, D., Clements, D. N., Maley, S., Marks, J., Wright, S., Esteban, I., Innes, E. A. and Buxton, D. (1998). Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and with *Toxoplasma gondii*. *Journal of Comparative Pathology* **119**, 45-55.

Hecker, Y. P., Moore, D. P., Quattrocchi,V., Regidor-Cerrillo, J., Verna, A., Leunda, M. R., Morrell, E., Ortega-Mora, L. M., Zamorano, P., Venturini, M. C. and Campero, C. M. (2013). Immune response and protection provided by live tachyzoites and native antigens from the NC-6 Argentina strain of *Neospora caninum* in pregnant heifers. *Veterinary Parasitology* **197**, 436-446. doi: 10.1016/j.vetpar.2013.07.027.

Innes, E. A., Bartley, P. M., Rocchi, M., Benavidas-Silvan, J., Burrells, A., Hotchkiss, E., Chianini, F., Canton, G. and Katzer, F. (2011). Developing vaccines to control protozoan parasites in ruminants: dead or alive? *Veterinary Parasitology* **180**, 155-163. doi: 10.1016/j.vetpar.2011.05.036.

Innes, E. A., Panton, W. R., Marks, J., Trees, A. J., Holmdahl, J. and Buxton, D. (1995). Interferon gamma inhibits the intracellular multiplication of *Neospora caninum*, as shown by incorporation of 3H uracil. *Journal of Comparative Pathology* **113**, 95-100.

Innes, E. A. and Vermeulen, A. N. (2006). Vaccination as a control strategy against the coccidial parasites *Eimeria, Toxoplasma* and *Neospora*. *Parasitology* **133 Suppl**, S145-68. doi: 10.1017/S0031182006001855.

Innes, E. A., Wright, S. E., Maley, S., Rae, A., Schock, A., Kirvar, E., Bartley, P., Hamilton, C., Carey, I. M. and Buxton, D. (2001). Protection against vertical transmission in bovine neosporosis. *International Journal for Parasitology* **31**, 1523-1534.

Jenkins, M. C., Tuo, W. and Dubey, J. P. (2004). Evaluation of vaccination with *Neospora caninum* protein for prevention of fetal loss associated with experimentally induced neosporosis in sheep. *American Journal of Veterinary Research* **65**, 1404-1408.

Jimenez-Ruiz, E., Alvarez-Garcia, G., Aguado-Martinez, A. and Ortega-Mora, L. M. (2013*a*). Low rates of *Neospora caninum* infection reactivation during gestation are observed in both chronically and congenitally infected mice. *Parasitology* **140**, 220-228. doi: 10.1017/S0031182012001515.

Jimenez-Ruiz, E., Alvarez-Garcia, G., Aguado-Martinez, A. and Ortega-Mora, L. M. (2013*b*). Mice congenitally infected with low-to-moderate virulence *Neospora caninum* isolates exhibited clinical reactivation during the mating period without transmission to the next generation. *Experimental Parasitology* **134**, 244-248. doi: 10.1016/j.exppara.2013.03.002.

Klevar, S., Kulberg, S., Boysen, P., Storset, A. K., Moldal, T., Bjorkman, C. and Olsen, I. (2007). Natural killer cells act as early responders in an experimental infection with

Neospora caninum in calves. *International Journal for Parasitology* **37**, 329-339. doi: 10.1016/j.ijpara.2006.11.002.

Konrad, J. L., Moore, D. P., Crudeli, G., Caspe, S. G., Cano, D. B., Leunda, M. R., Lischinsky, L., Regidor-Cerrillo, J., Odeon, A. C., Ortega-Mora, L. M., Echaide, I. and Campero, C. M. (2012). Experimental inoculation of *Neospora caninum* in pregnant water buffalo. *Veterinary Parasitology* **187**, 72-78. doi: 10.1016/j.vetpar.2011.12.030.

Kritzner, S., Sager, H., Blum, J., Krebber, R., Greif, G. and Gottstein, B. (2002). An explorative study to assess the efficacy of toltrazuril-sulfone (ponazuril) in calves experimentally infected with *Neospora caninum*. *Annals of Clinical Microbiology and Antimicrobials* **1**, 4.

Lindsay, D. S., Rippey, N. S., Powe, T. A., Sartin, E. A., Dubey, J. P. and Blagburn, B. L. (1995). Abortions, fetal death, and stillbirths in pregnant pygmy goats inoculated with tachyzoites of *Neospora caninum*. *American Journal of Veterinary Research* **56**, 1176-1180.

Lopez-Gatius, F., Garcia-Ispierto, I., Santolaria, P., Yaniz, J. L., Lopez-Bejar, M., Nogareda, C. and Almeria, S. (2005). Relationship between rainfall and *Neospora caninum*-associated abortion in two dairy herds in a dry environment. *Journal of Veterinary Medicine. B, Infectious diseases and veterinary public health* **52**, 147-152. doi: 10.1111/j.1439-0450.2005.00831.x.

Lopez-Gatius, F., Pabon, M. and Almeria, S. (2004). *Neospora caninum* infection does not affect early pregnancy in dairy cattle. *Theriogenology* **62**, 606-613. doi: 10.1016/j.theriogenology.2003.11.002.

Lopez-Gatius, F., Santolaria, P., Yaniz, J. L., Garbayo, J. M. and Almeria, S. (2005). The use of beef bull semen reduced the risk of abortion in Neospora-seropositive dairy

cows. *Journal of Veterinary Medicine.B, Infectious diseases and veterinary public health* **52**, 88-92. doi: 10.1111/j.1439-0450.2004.00818.x.

Lopez-Perez, I. C., Collantes-Fernandez, E., Aguado-Martinez, A., Rodriguez-Bertos, A. and Ortega-Mora, L. M. (2008). Influence of *Neospora caninum* infection in BALB/c mice during pregnancy in post-natal development. *Veterinary Parasitology* **155**, 175-183. doi: 10.1016/j.vetpar.2008.05.018.

Lopez-Perez, I. C., Risco-Castillo, V., Collantes-Fernandez, E. and Ortega-Mora, L. M. (2006). Comparative effect of *Neospora caninum* infection in BALB/c mice at three different gestation periods. *The Journal of Parasitology* **92**, 1286-1291. doi: 10.1645/GE-883R.1.

Lunden, A., Marks, J., Maley, S. W. and Innes, E. A. (1998). Cellular immune responses in cattle experimentally infected with *Neospora caninum*. *Parasite Immunology* **20**, 519-526.

Macaldowie, C., Maley, S. W., Wright, S., Bartley, P., Esteban-Redondo, I., Buxton, D. and Innes, E. A. (2004). Placental pathology associated with fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. *Journal of Comparative Pathology* **131**, 142-156. doi: 10.1016/j.jcpa.2004.02.005.

Maley, S. W., Buxton, D., Macaldowie, C. N., Anderson, I. E., Wright, S. E., Bartley, P. M., Esteban-Redondo, I., Hamilton, C. M., Storset, A. K. and Innes, E. A. (2006). Characterization of the immune response in the placenta of cattle experimentally infected with *Neospora caninum* in early gestation. *Journal of Comparative Pathology* **135**, 130-141. doi: 10.1016/j.jcpa.2006.07.001.

Maley, S. W., Buxton, D., Rae, A. G., Wright, S. E., Schock, A., Bartley, P. M., Esteban-Redondo, I., Swales, C., Hamilton, C. M., Sales, J. and Innes, E. A. (2003). The

pathogenesis of neosporosis in pregnant cattle: inoculation at mid-gestation. *Journal of Comparative Pathology* **129**, 186-195.

Maley, S. W., Buxton, D., Thomson, K. M., Schriefer, C. E. and Innes, E. A. (2001). Serological analysis of calves experimentally infected with *Neospora caninum*: a 1-year study. *Veterinary Parasitology* **96**, 1-9.

Marks, J., Lunden, A., Harkins, D. and Innes, E. (1998). Identification of Neospora antigens recognized by CD4+ T cells and immune sera from experimentally infected cattle. *Parasite Immunology* **20**, 303-309.

McAllister, M. M., Bjorkman, C., Anderson-Sprecher, R. and Rogers, D. G. (2000). Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. *Journal of the American Veterinary Medical Association* **217**, 881-887.

McAllister, M. M., McGuire, A. M., Jolley, W. R., Lindsay, D. S., Trees, A. J. and Stobart, R. H. (1996). Experimental neosporosis in pregnant ewes and their offspring. *Veterinary Pathology* **33**, 647-655.

McCann, C. M., McAllister, M. M., Gondim, L. F., Smith, R. F., Cripps, P. J., Kipar, A., Williams, D. J. and Trees, A. J. (2007). *Neospora caninum* in cattle: experimental infection with oocysts can result in exogenous transplacental infection, but not endogenous transplacental infection in the subsequent pregnancy. *International Journal for Parasitology* **37**, 1631-1639. doi: 10.1016/j.ijpara.2007.05.012.

Miller, C. M., Quinn, H. E., Windsor, P. A. and Ellis, J. T. (2002). Characterisation of the first Australian isolate of *Neospora caninum* from cattle. *Australian Veterinary Journal* **80**, 620-625.

Moore, D. P., Alvarez-Garcia, G., Chiapparrone, M. L., Regidor-Cerrillo, J., Lischinsky, L. H., de Yaniz, M. G., Odeon, A. C., Ortega-Mora, L. M. and Campero, C. M. (2014). *Neospora caninum* tachyzoites inoculated by the conjunctival route are not vertically transmitted in pregnant cattle: A descriptive study. *Veterinary Parasitology* **199**, 1-7. doi: 10.1016/j.vetpar.2013.10.006.

Moore, D. P., Echaide, I., Verna, A. E., Leunda, M. R., Cano, A., Pereyra, S., Zamorano, P. I., Odeon, A. C. and Campero, C. M. (2011). Immune response to *Neospora caninum* native antigens formulated with immune stimulating complexes in calves. *Veterinary Parasitology* **175**, 245-251. doi: 10.1016/j.vetpar.2010.10.020.

Moore, D. P., Leunda, M. R., Zamorano, P. I., Odeon, A. C., Romera, S. A., Cano, A., de Yaniz, G., Venturini, M. C. and Campero, C. M. (2005). Immune response to *Neospora caninum* in naturally infected heifers and heifers vaccinated with inactivated antigen during the second trimester of gestation. *Veterinary Parasitology* **130**, 29-39. doi: 10.1016/j.vetpar.2005.03.010.

Moskwa, B., Pastusiak, K., Bien, J. and Cabaj, W. (2007). The first detection of *Neospora caninum* DNA in the colostrum of infected cows. *Parasitology Research* **100**, 633-636. doi: 10.1007/s00436-006-0288-7.

Muller, J. and Hemphill, A. (2013). In vitro culture systems for the study of apicomplexan parasites in farm animals. *International Journal for Parasitology* **43**, 115-124. doi: 10.1016/j.ijpara.2012.08.004.

Nishimura, M., Kohara, J., Hiasa, J., Muroi, Y., Yokoyama, N., Kida, K., Xuan, X., Furuoka, H. and Nishikawa, Y. (2013). Tissue distribution of *Neospora caninum* in experimentally infected cattle. *Clinical and Vaccine Immunology* **20**, 309-312. doi: 10.1128/CVI.00556-12; 10.1128/CVI.00556-12.

O'Handley, R., Liddell, S., Parker, C., Jenkins, M. C. and Dubey, J. P. (2002). Experimental infection of sheep with *Neospora caninum* oocysts. *The Journal of Parasitology* **88**, 1120-1123. doi: 10.1645/0022-3395(2002)088[1120:EIOSWN]2.0.CO;2

O'Handley, R. M., Morgan, S. A., Parker, C., Jenkins, M. C. and Dubey, J. P. (2003). Vaccination of ewes for prevention of vertical transmission of *Neospora caninum*. *American Journal of Veterinary Research* **64**, 449-452.

Ortega-Mora, L. M., Regidor-Cerrillo, J., Rojo-Montejo, S., Collantes-Fernandez, E., Alvarez-Garcia, G. (2012) Vaccine development against bovine neosporosis: present situation and *in* vitro and *in vivo* models to test safety and efficacy**.** In: *Proceedings of the 1st Apicowplexa. Apicomplexa in farm animals meeting*, Lisbon, October 2012. Pp 57.

Ortega-Mora, L. M., Ferre, I., del-Pozo, I., Caetano-da-Silva, A., Collantes-Fernandez, E., Regidor-Cerrillo, J., Ugarte-Garagalza, C. and Aduriz, G. (2003). Detection of *Neospora caninum* in semen of bulls. *Veterinary Parasitology* **117**, 301-308.

Osoro, K., Ortega-Mora, L. M., Martinez, A., Serrano-Martinez, E. and Ferre, I. (2009). Natural breeding with bulls experimentally infected with *Neospora caninum* failed to induce seroconversion in dams. *Theriogenology* **71**, 639-642. doi: 10.1016/j.theriogenology.2008.09.035.

Pare, J., Thurmond, M. C. and Hietala, S. K. (1997). *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. *The Journal of Parasitology* **83**, 82-87.

Pereira-Bueno, J., Quintanilla-Gozalo, A., Perez-Perez, V., Espi-Felgueroso, A., Alvarez-Garcia, G., Collantes-Fernandez, E. and Ortega-Mora, L. M. (2003). Evaluation

by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. *Veterinary Parasitology* **111**, 143-152.

Peters, M., Lutkefels, E., Heckeroth, A. R. and Schares, G. (2001). Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. *International Journal for Parasitology* **31**, 1144-1148.

Quintanilla-Gozalo, A., Pereira-Bueno, J., Tabares, E., Innes, E. A., Gonzalez-Paniello, R. and Ortega-Mora, L. M. (1999). Seroprevalence of *Neospora caninum* infection in dairy and beef cattle in Spain. *International Journal for Parasitology* **29**, 1201-1208.

Regidor-Cerrillo, J., Alvarez-Garcia, G., Pastor-Fernandez, I., Marugan-Hernandez, V., Gomez-Bautista, M. and Ortega-Mora, L. M. (2012). Proteome expression changes among virulent and attenuated *Neospora caninum* isolates. *Journal of Proteomics* **75**, 2306-2318. doi: 10.1016/j.jprot.2012.01.039.

Regidor-Cerrillo, J., Arranz-Solis, D., Benavides, J., Gomez-Bautista, M., Castro-Hermida, J. A., Mezo, M., Pére,z V., Ortega-Mora, L. M. and González-Warleta, M. (2014). *Neospora caninum* infection during early pregnancy in cattle: how the isolate influences infection dynamics, clinical outcome and peripheral and local immune responses. *Veterinary Research* **45**,10. doi: 10.1186/1297-9716-45-10.

Regidor-Cerrillo, J., Gomez-Bautista, M., Del Pozo, I., Jimenez-Ruiz, E., Aduriz, G. and Ortega-Mora, L. M. (2010). Influence of *Neospora caninum* intra-specific variability in the outcome of infection in a pregnant BALB/c mouse model. *Veterinary Research* **41**, 52. doi: 10.1051/vetres/2010024.

Regidor-Cerrillo, J., Gomez-Bautista, M., Sodupe, I., Aduriz, G., Alvarez-Garcia, G., Del Pozo, I. and Ortega-Mora, L. M. (2011). In vitro invasion efficiency and intracellular

proliferation rate comprise virulence-related phenotypic traits of *Neospora caninum*. *Veterinary Research* **42**, 41. doi: 10.1186/1297-9716-42-41.

Reichel, M. P., Alejandra Ayanegui-Alcerreca, M., Gondim, L. F. and Ellis, J. T. (2013). What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. *International Journal for Parasitology* **43**, 133-142. doi: 10.1016/j.ijpara.2012.10.022.

Rettigner, C., Lasri, S., De Meerschman, F., Focant, C., Beckers, J. F. and Losson, B. (2004). Immune response and antigen recognition in non-pregnant ewes experimentally infected with *Neospora caninum* tachyzoites. *Veterinary Parasitology* **122**, 261-271. doi: 10.1016/j.vetpar.2003.12.017.

Rocchi, M. S., Bartley, P. M., Inglis, N. F., Collantes-Fernandez, E., Entrican, G., Katzer, F. and Innes, E. A. (2011). Selection of *Neospora caninum* antigens stimulating bovine CD4+ve T cell responses through immuno-potency screening and proteomic approaches. *Veterinary Research* **42**, 91. doi: 10.1186/1297-9716-42-91.

Rojo-Montejo, S., Collantes-Fernandez, E., Blanco-Murcia, J., Rodriguez-Bertos, A., Risco-Castillo, V. and Ortega-Mora, L. M. (2009). Experimental infection with a low virulence isolate of *Neospora caninum* at 70 days gestation in cattle did not result in foetopathy. *Veterinary Research* **40**, 49. doi: 10.1051/vetres/2009032.

Rojo-Montejo, S., Collantes-Fernandez, E., Perez-Zaballos, F., Rodriguez-Marcos, S., Blanco-Murcia, J., Rodriguez-Bertos, A., Prenafeta, A. and Ortega-Mora, L. M. (2013). Effect of vaccination of cattle with the low virulence Nc-Spain 1H isolate of *Neospora caninum* against a heterologous challenge in early and mid-gestation. *Veterinary Research* **44**, 106. doi: 10.1186/1297-9716-44-106.

Rojo-Montejo, S., Collantes-Fernandez, E., Regidor-Cerrillo, J., Rodriguez-Bertos, A., Prenafeta, A., Gomez-Bautista, M. and Ortega-Mora, L. M. (2011). Influence of adjuvant and antigen dose on protection induced by an inactivated whole vaccine against *Neospora caninum* infection in mice. *Veterinary Parasitology* **175**, 220-229. doi: 10.1016/j.vetpar.2010.10.028.

Rosbottom, A., Gibney, E. H., Guy, C. S., Kipar, A., Smith, R. F., Kaiser, P., Trees, A. J. and Williams, D. J. (2008). Upregulation of cytokines is detected in the placentas of cattle infected with *Neospora caninum* and is more marked early in gestation when fetal death is observed. *Infection and Immunity* **76**, 2352-2361. doi: 10.1128/IAI.01780-06.

Rosbottom, A., Guy, C. S., Gibney, E. H., Smith, R. F., Valarcher, J. F., Taylor, G. and Williams, D. J. (2007). Peripheral immune responses in pregnant cattle following *Neospora caninum* infection. *Parasite Immunology* **29**, 219-228. doi: 10.1111/j.1365-3024.2007.00936.x.

Rosbottom, A., Gibney, H., Kaiser, P., Hartley, C., Smith, R.F., Robinson, R., Kipar, A., Williams, D. J. (2011) Up regulation of the maternal immune response in the placenta of cattle naturally infected with Neospora caninum. *PLoS On* **6**:e15799. doi: 10.1371/journal.pone.0015799.

Schares, G., Heydorn, A. O., Cuppers, A., Conraths, F. J. and Mehlhorn, H. (2001). *Hammondia heydorni*-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. *Parasitology Research* **87**, 808-816.

Schares, G., Peters, M., Wurm, R., Barwald, A. and Conraths, F. J. (1998). The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Veterinary Parasitology* **80**, 87-98.

Serrano, E., Ferre, I., Osoro, K., Aduriz, G., Mateos-Sanz, A., Martinez, A., Atxaerandio, R., Hidalgo, C. O. and Ortega-Mora, L. M. (2006). Intrauterine *Neospora caninum* inoculation of heifers. *Veterinary Parasitology* **135**, 197-203. doi: 10.1016/j.vetpar.2005.10.003.

Serrano-Martinez, E., Ferre, I., Martinez, A., Osoro, K., Mateos-Sanz, A., Del-Pozo, I., Aduriz, G., Tamargo, C., Hidalgo, C. O. and Ortega-Mora, L. M. (2007*a*). Experimental neosporosis in bulls: parasite detection in semen and blood and specific antibody and interferon-gamma responses. *Theriogenology* **67**, 1175-1184. doi: 10.1016/j.theriogenology.2007.01.010.

Serrano-Martinez, E., Ferre, I., Osoro, K., Aduriz, G., Mota, R. A., Martinez, A., Del-Pozo, I., Hidalgo, C. O. and Ortega-Mora, L. M. (2007*b*). Intrauterine *Neospora caninum* inoculation of heifers and cows using contaminated semen with different numbers of tachyzoites. *Theriogenology* **67**, 729-737. doi: 10.1016/j.theriogenology.2006.10.004.

Staska, L. M., McGuire, T. C., Davies, C. J., Lewin, H. A. and Baszler, T. V. (2003). *Neospora caninum*-infected cattle develop parasite-specific CD4+ cytotoxic T lymphocytes. *Infection and Immunity* **71**, 3272-3279.

Stenlund, S., Kindahl, H., Magnusson, U., Uggla, A. and Bjorkman, C. (1999). Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with *Neospora caninum*. *Veterinary Parasitology* **85**, 227-234.

Syed-Hussain, S. S., Howe, L., Pomroy, W. E., West, D. M., Smith, S. L. and Williamson, N. B. (2013). Detection of *Neospora caninum* DNA in semen of experimental infected rams with no evidence of horizontal transmission in ewes. *Veterinary Parasitology* **197**, 534-542. doi: 10.1016/j.vetpar.2013.06.002..

Thurmond, M. C. and Hietala, S. K. (1997). Effect of congenitally acquired *Neospora caninum* infection on risk of abortion and subsequent abortions in dairy cattle. *American Journal of Veterinary Research* **58**, 1381-1385.

Trees, A. J., Davison, H. C., Innes, E. A. and Wastling, J. M. (1999). Towards evaluating the economic impact of bovine neosporosis. *International Journal for Parasitology* **29**, 1195-1200.

Trees, A. J., McAllister, M. M., Guy, C. S., McGarry, J. W., Smith, R. F. and Williams, D. J. (2002). *Neospora caninum*: oocyst challenge of pregnant cows. *Veterinary Parasitology* **109**, 147-154.

Trees, A. J. and Williams, D. J. (2005). Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. *Trends in Parasitology* **21**, 558-561. doi: 10.1016/j.pt.2005.09.005.

Tuo, W., Fetterer, R., Jenkins, M. and Dubey, J. P. (2005). Identification and characterization of *Neospora caninum* cyclophilin that elicits gamma interferon production. *Infection and Immunity* **73**, 5093-5100. doi: 10.1128/IAI.73.8.5093-5100.2005.

Uggla, A., Stenlund, S., Holmdahl, O. J., Jakubek, E. B., Thebo, P., Kindahl, H. and Bjorkman, C. (1998). Oral *Neospora caninum* inoculation of neonatal calves. *International Journal for Parasitology* **28**, 1467-1472.

VanLeeuwen, J. A., Greenwood, S., Clark, F., Acorn, A., Markham, F., McCarron, J. and O'Handley, R. (2011). Monensin use against *Neospora caninum* challenge in dairy cattle. *Veterinary Parasitology* **175**, 372-376. doi: 10.1016/j.vetpar.2010.10.016.

Vanleeuwen, J. A., Haddad, J. P., Dohoo, I. R., Keefe, G. P., Tiwari, A. and Scott, H. M. (2010). Risk factors associated with *Neospora caninum* seropositivity in randomly sampled Canadian dairy cows and herds. *Preventive Veterinary Medicine* **93**, 129-138. doi: 10.1016/j.prevetmed.2009.11.013.

Weber, F. H., Jackson, J. A., Sobecki, B., Choromanski, L., Olsen, M., Meinert, T., Frank, R., Reichel, M. P. and Ellis, J. T. (2013). On the efficacy and safety of vaccination with live tachyzoites of *Neospora caninum* for prevention of neospora-associated fetal loss in cattle. *Clinical and Vaccine Immunology* **20**, 99-105. doi: 10.1128/CVI.00225-12.

Weston, J. F., Howe, L., Collett, M. G., Pattison, R. S., Williamson, N. B., West, D. M., Pomroy, W. E., Syed-Hussain, S. S., Morris, S. T. and Kenyon, P. R. (2009). Dose-titration challenge of young pregnant sheep with *Neospora caninum* tachyzoites. *Veterinary Parasitology* **164**, 183-191. doi: 10.1016/j.vetpar.2009.05.013.

Wiengcharoen, J., Thompson, R. C., Nakthong, C., Rattanakorn, P. and Sukthana, Y. (2011). Transplacental transmission in cattle: is *Toxoplasma gondii* less potent than *Neospora caninum*? *Parasitology Research* **108**, 1235-1241. doi: 10.1007/s00436-010-2172-8.

Williams, D. J., Guy, C. S., McGarry, J. W., Guy, F., Tasker, L., Smith, R. F., MacEachern, K., Cripps, P. J., Kelly, D. F. and Trees, A. J. (2000). *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. *Parasitology* **121 (Pt 4)**, 347-358.

Williams, D. J., Guy, C. S., Smith, R. F., Ellis, J., Bjorkman, C., Reichel, M. P. and Trees, A. J. (2007). Immunization of cattle with live tachyzoites of *Neospora caninum* confers protection against fetal death. *Infection and Immunity* **75**, 1343-1348. doi: 10.1128/IAI.00777-06.

Williams, D. J., Guy, C. S., Smith, R. F., Guy, F., McGarry, J. W., McKay, J. S. and Trees, A. J. (2003). First demonstration of protective immunity against foetopathy in cattle with latent *Neospora caninum* infection. *International Journal for Parasitology* **33**, 1059-1065.

Williams, D. J., Hartley, C. S., Bjorkman, C. and Trees, A. J. (2009). Endogenous and exogenous transplacental transmission of *Neospora caninum* - how the route of transmission impacts on epidemiology and control of disease. *Parasitology* **136**, 1895-1900. doi: 10.1017/S0031182009990588.

Wouda, W., Moen, A. R. and Schukken, Y. H. (1998). Abortion risk in progeny of cows after a *Neospora caninum* epidemic. *Theriogenology* **49**, 1311-1316. doi: 10.1016/S0093-691X(98)00078-8.

Wouda, W., Moen, A. R., Visser, I. J. and van Knapen, F. (1997). Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. *Journal of Veterinary Diagnostic Investigation* **9**, 180-185.

Yin, J., Qu, G., Cao, L., Li, Q., Fetterer, R., Feng, X., Liu, Q., Wang, G., Qi, D., Zhang, X., Miramontes, E., Jenkins, M., Zhang, N. and Tuo, W. (2012). Characterization of *Neospora caninum* microneme protein 10 (NcMIC10) and its potential use as a

diagnostic marker for neosporosis. *Veterinary Parasitology* **187**, 28-35. doi: 10.1016/j.vetpar.2012.01.003.

1 TABLES

1 Table 1. Published studies based on *Neospora caninum* infections in pregnant cattle and buffalo

Reference	Breed	Age	DOG	Strain/isolate	Passage	ROI	Dose	Parasite stage
Dubey et al. 1992	Jersey	3-5 yrs	81-129	Nc1, Nc2 and Nc3	ns	im and sc	$3x10^7$ and ns	tz & tc
Conrad et al. 1993	ns	Heifers	120	Nc BPA1	ns	im & iv	$3x10^6$ & $5.x10^6$	tz
Barr et al. 1994	Simmental-cross	Heifers	138; 161	Nc BPA1	13	im foetal	$0.5\text{-}10^6$	tz
Barr et al. 1994	Simmental-cross	Heifers	118	Nc BPA1	13	im & iv	$3x10^6$ & $5x10^6$	tz
Barr et al. 1994	Simmental-cross	Heifers	80-95	Nc BPA1	13	im & iv	$3x10^6$ & $5x10^6$	tz
Barr et al. 1994	Simmental-cross	Heifers	115-120	Nc BPA1	13	im & iv	$3x10^6$ & $5x10^6$	tz
Andrianarivo et al. 2000	ns	Heifers	91	Nc BPA1	ns	im & iv	$2x10^7$ + $2x10^7$	tz
Williams et al. 2000	Holstein-Friesian	Heifers	-63; 70; 210	Nc Liverpool	9	iv	10^7	tz
Andrianarivo et al. 2001	beef	Heifers	159-169	Nc BPA1	ns	im & iv	$3x10^7$+ $5x10^7$	tz
Innes et al. 2001	Holstein-Frisian	Heifers	140	Nc1	ns	sc	$5x10^8$	tz
Trees et al. 2002	Hereford/Fresian cross	10-12 yrs	70	Nc Liverpool	ns	oral	$6x10^2$	oocy
Almeria et al. 2003	Angus	Heifers	110	Nc illinois	ns	iv	10^7	tz
Maley et al. 2003	Holstein-Friesian	Heifers	140	Nc1	ns	sc	10^7; $5x10^8$	tz
Williams et al. 2003	Holstein-Friesian	Adults	70	Nc natural; Nc Liverpool	ns	iv	10^7	tz
Macaldowie et al. 2004	Holstein-Friesian	1-4 yrs	70-176	Nc1	ns	iv or sc	$5x10^8$	tz
Gondim et al. 2004	beef cross	Adults	70	Nc-beef, Nc-Illinois, Nc2	ns	oral	$1.5x10^3$ - $1.15x10^5$	oocy
Canada et al. 2006	Holstein-Friesian	3-6 yrs	mating	ns	ns	it	$1.63x10^7$ + $4.5x10^6$	smn cnt
Serrano et al. 2006	Asturiana de los Vallles	Heifers	mating	Nc 1	ns	it	10^7	smn cnt
De Yaniz et al. 2007	beef cross	heifers	150	Nc1	100	cnj; iv	10^8	tz
McCann et al. 2007	Holstein-Friesian	Heifers	70; 120; 210	Nc Liverpool	ns	oral	$4x10^4$	oocy

¥: study carried out in water buffalo; DOG: day of gestation when infected; ROI: route of inoculation; ns: not stated; iv: intravenous; im: intramuscular; sc: subcutaneous; it: intrauterine; cnj: conjuntival; tz: tachyzoites; tc: tissue cysts; oocy: sporoulated oocysts; smn cnt: semen spiked with tachyzoites;

2 Table 1 (cont). Published studies based on *Neospora caninum* infections in pregnant cattle and buffalo

Reference	Breed	Age	DOG	Strain/isolate	Passage	ROI	Dose	Parasite stage
Rosbottom et al. 2007	Holstein-Friesian	Heifers	70; 210	Nc1	ns	iv	10^7	tz
Serrano-Martinez et al. 2007	Asturiana de los Vallles	Heifers	mating	Nc1	ns	it	10^2- $5x10^5$	smn cnt
Serrano-Martinez et al. 2007	Asturiana de los Vallles	3-18 yrs	mating	Nc1	ns	it	10^2- $5x10^5$	smn cnt
Williams et al. 2007	Holstein-Friesian	Heifers	70	Nc Nowra; Nc Liverpool	ns	iv	10^7	tz
Ferre et al. 2008	Asturiana de los Vallles	Heifers	mating	Nc1	ns	it	0-20	smn infc
Gibney et al. 2008	Holstein-Friesian	Heifers	70; 210	Nc Liverpool	ns	iv	10^7	tz
Osoro et al. 2009	Asturiana de los Vallles	heifers	mating	Nc1 or Nc Spain7	ns	it	natural mating	smn infc
Rojo-Montejo et al. 2009	Holstein-Friesian	16-24 months	70	Nc1; Nc Spain 1H	12; 10	iv	10^7	tz
Almeria et al. 2010	Angus	Heifers	111	Nc illinois	ns	iv	10^7	tz
Wiengeharoen et al. 2011	ns	Heifers	150	Nc K9WA	ns	sc	$3x10^8$	tz
Benavides et al. 2012	beef breeds	Heifers	210	Nc1	ns	sc	$5x10^8$	tz
Caspe et al. 2012	Aberdeen Angus	Heifers	65	Nc Spain7; Nc1	ns	iv	10^8	tz
Konrad et al. 2012	Mediterranean¥	4-14 yrs	70; 90; 90	Nc1	ns	iv	10^8	tz
Weber et al. 2013	ns	Heifers	65	Nc Nowra; Nc S197	10	iv	$1.2\ x10^6$	tz
Hecker et al. 2013	Angus	Heifers	70	Nc6: Nc1	ns	iv	$4.7\ x10^7$	tz
Bacigalupe et al. 2013	Aberdeen Angus	ns	-60; 60	Nc6 Argentina	ns	iv	$5x10^7$	tz
Ribas-Pereira et al. 2013	Hereford cross	Heifers	-80; 70; 135	Nc 1	ns	iv	10^6	tz
Rojo-Montejo et al. 2013	Holstein-Friesian	Heifers	76	Nc Spain 1H; Nc 1	ns	sc; iv	10^7; $4x10^8$	tz
Moore et al. 2014	Aberdeen Angus	4-6 yrs	211	Nc 1	ns	iv; cnj	$2.5x10^8$	tz
Regidor-Cerrillo et al. 2014	Holstein-Friesian	Heifers	70	Nc Spain7; Nc Spain8	8; 13	iv	10^7	tz

DOG: day of gestation when infected; ROI: route of inoculation; ns: not stated; iv: intravenous; im: intramuscular; sc: subcutaneous; it: intrauterine; cnj: conjuntival; tz: tachyzoites; tc: tissue cysts; oocy: sporoulated oocysts; smn cnt: semen spiked with tachyzoites; smn infc: semen from infected bulls

Table 2. Published studies based on *Neospora caninum* infections n pregnant goat and sheep.

Reference	Breed	Age	DOG	Strain/isolate	Passage	ROI	Dose	Parasite stage
Dubey et al. 1990	ns	1 yr	90	Nc1	ns	im & iv	$1.5x10^7$	tz
Lindsay et al. 1995	Pygmy Goat¥	Adults	51; 85; 127	Nc1	ns	sc	10^7	tz
McAllister et al. 1996	Rambouillet	6 yrs	65; 90; 120	Nc2 and Nc Liverpool	ns	iv	1.7.E+05 or $1.7x10^6$	tz
Buxton et al. 1997	Scottish Blackface cross	4-5 yrs	90	Nc Liverpool	ns	sc	10^6	tz
Buxton et al. 1998	Scottish Blackface	2-4 yrs	45; 65; 90	Nc 1	ns	sc	10^6	tz
Harkins et al. 1998	ns¥	4 yrs	65	Nc 1	ns	sc	10^6	tz
Innes et al. 2001	Scottish blackface	2-4 yrs	90	Nc 1	ns	sc	10^7	tz
Buxton et al. 2001	Scottish Blackface	2-4 yrs	90	Nc 1	ns	sc	10^7	tz
O'Handley et al. 2003	Dorset	Adults	68-108	Nc illinois	ns	sc	$5.x10^6$	tz
Jenkins et al. 2004	Dorset	Adults	30-75	Nc illinois	ns	sc	$5x10^6$	tz
Weston et al. 2009	Rommey cross	7-8 months	73-90	Nc NZ1, Nc NZ2 and Nc Z3	ns	iv	50; $5.-5x10^8$	tz
Yin et al. 2012	Boer¥	ns	45-90	Nc illinois	ns	iv	10^4; 10^6	tz
Syed-Hussain et al. 2013	Meat breeds	4 yrs	mating	Nc NZ1	ns	it	natural mating	smn infc.

¥: study carried out in goats; DOG: day of gestation when infected; ROI: route of inoculation; ns: not stated; iv: intravenous; im: intramuscular; sc: subcutaneous; it: intrauterine; tz: tachyzoites; smn infc: semen from infected rams

Table 3. Published studies based on *Neospora caninum* infections in non pregnant ruminants

Reference	Specie	Breed	Age	Strain/isolate	Passage	ROI	Dose	Parasite stage
Buxton et al. 1997	Sheep	Scottish Blackface	1 yr	Nc Liverpool	ns	sc	10^8; 10^6; 10^4	tz
Harkins et al. 1998	Cattle	ns	8 months	Nc 1	ns	sc	10^8	tz
Harkins et al. 1998	Sheep	Scottish blackface	1 yr	Nc 1	ns	sc	10^6	tz
Lunden et al. 1998	Cattle	ns	2-4 moths	Nc 1	ns	sc	$2.5x10^8$	tz
Uggla et al. 1998	Cattle	Swedish Red and White	3-5 months	Nc SweB1	ns	oral	$3\text{-}10^7$	tz
De Marez et al. 1999	Cattle	Holstein-Friesian	2.5 months	Nc 2	ns	oral	$0.1\text{-}10^5$	oocy
Davison et al. 2001	Cattle	Holstein-Friesian	Newborn	ns	42-50	oral	ns	mlk infc.
Davison et al. 2001	Cattle	Holstein-Friesian	Newborn; Adult	NC Liverpool B2	ns	oral	ns	placent.
Maley et al. 2001	Cattle	Beef & dairy	6 months	Nc 1	ns	sc	$5x10^6$; $5x10^6$	tz
Schares et al. 2001	Goat	ns	5-9 months	*H. heydorni* (Berlin 1996)	ns	oral	$10^3\text{-}4x10^5$	oocy
Schares et al. 2001	Sheep	ns	5-9 months	*H. heydorni* (Berlin 1996)	ns	oral	$10^3\text{-}4x10^5$	oocy
Gondim et al. 2002	Cattle	ns	Newborn	Nc-beef, Nc-Illinois, Nc 2	ns	oral; iv	300-1400; $2x10^6\text{-}10^7$	oocy; tz
Kritzner et al. 2002	Cattle	ns	42-98 days	Nc 1	ns	sc & iv	$2x10^8$	tz
O'Handley et al. 2002	Sheep	ns	3-4 months	Nc 2	ns	oral	10^4	oocy
Staska et al. 2003	Cattle	Holstein-Frisian	5-7 yrs	Nc 1	ns	im & iv	$10^7 + 5x10^6$	tz
Rettigner et al. 2004	Sheep	Préalpes du Sud	6 months	Nc 1	ns	sc	$2.5x10^6$	tz

DOG: day of gestation when infected; ROI: route of inoculation; ns: not stated; iv: intravenous; im: intramuscular; sc: subcutaneous; it: intrauterine; cnj: conjuntival; tz: tachyzoites; tc: tissue cysts; oocy: sporoulated oocysts; mlk infc: milk spiked with tachyzoites smn cnt: semen spiked with tachyzoites; smn infc: semen from infected sires

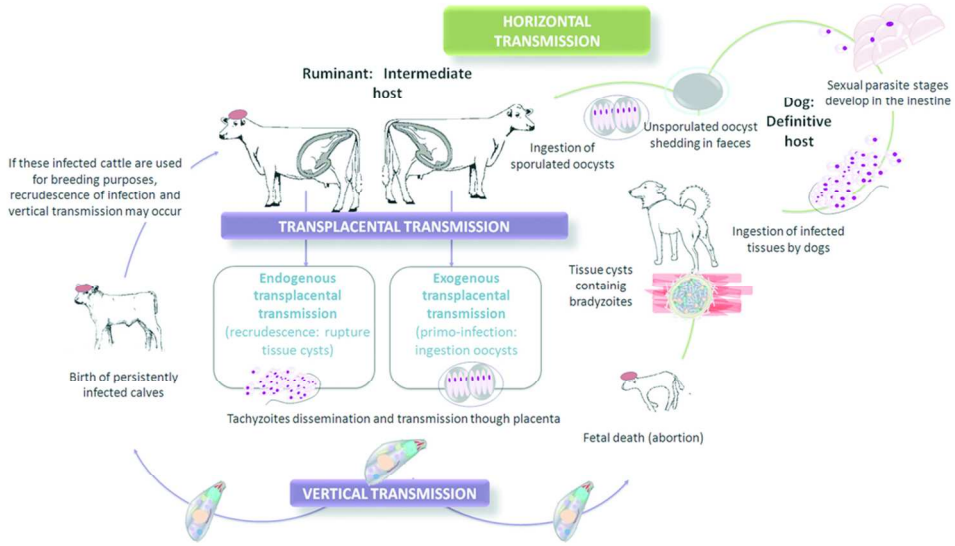
Table 3 (cont). Published studies based on *Neospora caninum* infections in non pregnant ruminants

Reference	Specie	Breed	Age	Strain/isolate	Passage	ROI	Dose	Parasite stage
Klevar et al. 2007	Cattle	Norwegian Red	3 months	Nc Liverpool	9	iv	10^7	tz
Serrano-Martinez et al. 2007	Cattle	Asturiana de los Vallles	3.4-6-5 yrs	Nc 1	ns	iv	10^7	tz
Ferre et al. 2008	Cattle	Asturiana de los Vallles	3-13 yrs	Nc 1	ns	iv	2×10^8; 10^8	tz
Osoro et al. 2009	Cattle	Asturiana de los Vallles	1.5-2 years	Nc1 or Nc Spain7	ns	iv	10^8	tz
Davison et al. 2011	Cattle	Holstein-Friesian	newborn	Nc Liverpool B1	42-50	oral	$4x10^8$	tz
Moore et al. 2011	Cattle	Aberdeen Angus	5 months	Nc 1	ns	iv	10^8	tz
Rocchi et al. 2011	Cattle	ns	2 months	Nc 1	ns	sc	4×10^9	tz
Vanleeuwen et al. 2011	Cattle	Holstein and Jersey	Adults	Nc 1	ns	sc	$2.5x10^6$	tz
Nishimura et al. 2013	Cattle	Holstein-Friesian	2-4 moths	Nc 1	ns	iv	10^6 or $5x10^7$	tz
Syed-Hussain et al. 2013	Sheep	Dairy & milk	6 months	Nc NZ1	ns	iv	50; 10^3; 10^7	tz

ROI: route of inoculation; ns: not stated; iv: intravenous; im: intramuscular; sc: subcutaneous; it: intrauterine; cnj: conjuntival; tz: tachyzoites; tc: tissue cysts; oocy: sporoulated oocysts

FIGURES.

Figure 1. Transmission routes of *Neospora caninum* infection in cattle



Transmission routes of Neospora caninum infection in cattle
94x53mm (300 x 300 DPI)