Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Examination of a herd experiencing Neospora caninum-associated abortion by a mouse monoclonal antibody-based competitive ELISA

João Afonso Coutinho Crisóstomo

Orientador(es) | Helder Cortes

Gereon Rudolf Matthias Schares

Évora 2024

Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Examination of a herd experiencing Neospora caninum-associated abortion by a mouse monoclonal antibody-based competitive ELISA

João Afonso Coutinho Crisóstomo

Orientador(es) | Helder Cortes

Gereon Rudolf Matthias Schares

Évora 2024

A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente	|	Ricardo Jorge Romão (Universidade de Évora)
Vogais	|	Andrew Edward Hemphill (Universität Bern) (Arguente) Helder Cortes (Universidade de Évora) (Orientador)

Évora 2024

Acknowledgements

I would like to express my gratitude and appreciation to those who in any way have contributed to this work, and especially:

To my supervisor Dr. Gereon Schares, for his continuous support during my internship at the Institute of Epidemiology, Friedrich-Loeffler-Institut (FLI), for his patience, availability, and kind advice during our scientific discussions, and for all the guidance and valuable suggestions in the writing of this work.

To my supervisor Prof. Dr. Helder Cortes, for challenging me with this opportunity, and for all the support, encouragement, and kind words.

To Dr. Jana Schulz for her interest in this work, for introducing me to Bayesian statistics and for the guidance and support in the development of the Bayesian latent class analysis (BLCA) model.

To the members of the Institute of Epidemiology at the FLI, especially Prof. Dr. Carola Sauter-Louis, Head of the Institute of Epidemiology, for her kindness and always making me feel welcome.

To the technicians in the parasitological working group, especially Andrea Bärwald, Alrik Kunisch and Martina Abs for the great technical assistance, patience, and hospitality.

To the Erasmus + Programme and the Förderverein of the FLI for the financial support to cover a part of my costs during my stay in Germany.

To my parents and brother for their encouragement, support, and unconditional love and to Leonor for her love, relentless support and for always believing in me. I'm deeply grateful.

Examination of a herd experiencing *Neospora caninum*-associated abortion by a mouse monoclonal antibody-based competitive ELISA

Abstract

In the present study, a competitive enzyme-linked immunosorbent assay (cELISA), based on the 3.10.5 mouse monoclonal antibody, was developed to detect bovine antibodies to *Neospora caninum*. Sera from cattle infected with *N. caninum*, *Besnoitia besnoiti,* and *Sarcocystis* spp. were used to validate the test. The sera were also analysed by a p38-ELISA, Western blot, and immunofluorescence antibody test (IFAT). A Bayesian latent class analysis (BLCA) was performed using the Gibbs sampler to estimate the diagnostic characteristics of the cELISA. The results revealed a sensitivity of 98.5% (95% CI: 96.5% - 99.9%) and a specificity of 98.4% (95% CI: 97.1% - 99.4%).

The cELISA was used to analyse cattle sera from a herd with suspected *N. caninum*-associated abortions and determine whether these abortions could be attributed to neosporosis. No definitive conclusions could be taken regarding the origin of the epidemic outbreak of abortion that occurred in the studied herd.

Keywords: *Neospora caninum*, Competitive ELISA, Monoclonal antibodies, Abortion, Diagnosis.

Análise de uma exploração com suspeita de abortos relacionados com infeção por *Neospora caninum* por um ELISA de competição baseado em anticorpos monoclonais de rato

Resumo

No presente estudo foi desenvolvido um enzyme-linked immunosorbent assay (ELISA) de competição, baseado no anticorpo monoclonal de rato 3.10.5, para a deteção de anticorpos anti-*Neospora caninum*. Soros provenientes de animais infetados com *N. caninum*, *Besnoitia besnoiti,* e *Sarcocystis* spp. foram utilizados para a validação do teste. Estes soros foram testados por p38-ELISA, Western blot, e immunofluorescence antibody test (IFAT). Um modelo baseado em Estatística Bayesiana, usando o método de amostragem Gibbs, foi usado para estimar os parâmetros de diagnóstico do cELISA. A sensibilidade estimada foi de 98.5% (95% IC: 96.5% - 99.9%) e a especificidade de 98.4% (95% IC: 97.1% - 99.4%).

O ELISA desenvolvido foi utilizado para analisar soros provenientes de uma exploração com suspeita de abortos relacionados com infeção por *N. caninum*, de forma a determinar se estes poderiam ser atribuídos a neosporose. A origem dos abortos epidémicos ocorridos na exploração em estudo não foi definitivamente determinada.

Palavras-chave: *Neospora caninum*, ELISA de competição, Anticorpos monoclonais, Aborto, Diagnóstico.

Table of Contents

List of abbreviations

°C	Degrees centigrade
µL	Microlitre
µm	Micrometre
APS	Ammonium persulfate
B. besnoiti	*Besnoitia besnoiti*
BLCA	Bayesian latent class analysis
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
cELISA	Competitive inhibition ELISA
CI	Confidence interval
CNS	Central nervous system
CO_2	Carbon dioxide
DAT	Direct agglutination test
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fluorescence assay rinse buffer
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FLI	Friedrich Loeffler-Institut, Germany
g	Force of gravity
g	Gram
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HAT	Hypoxanthine, aminopterin, and thymidine
HCl	Hydrochloride
HGPRT	Hypoxanthine guanine phosphoribosyltransferase
HIT	Herkunftssicherungs- und Informationssystem für Tiere
IB	Immunoblotting
IDA	Immunodominant antigen
iELISA	Indirect ELISA
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
ISCOM	Immune stimulating complex

JAGS	Computer program Just Another Gibbs Sampler (Plummer, 2003)
kDa	Kilodalton
M	Molar
mAb	Monoclonal antibody
mg	Milligram
min	Minutes
mM	Millimolar
Mr	Relative molecular weight
N. caninum	*Neospora caninum*
NAT	*Neospora* agglutination test
NC	Negative control
NcGRA7	*Neospora caninum* dense-granule protein 7
NcSAG4	*Neospora caninum* surface antigen 4
NcSRS2	*Neospora caninum* SAG1-related sequence 2
NK	Natural Killer
OD	Optical density
PBS	Phosphate-buffered saline
PBS-T	Phosphate buffered saline with 0.05% (v/v) Tween 20
PBS-T-G	PBS with 0.05% (v/v) Tween 20 and 2% (v/v) liquid fish gelatin
PC	Positive control
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
s	Second
SCI	Sample-to-Control-Inhibition
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sp	Specificity
Ss	Sensitivity
T. gondii	*Toxoplasma gondii*
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.05% (v/v) Tween 20
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TG-ROC	Two-graph receiver operating characteristics
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
Tris	Tris(hydroxymethyl)aminomethane
V	Volt
v/v	Volume per volume
w/v	Weight per volume
βME	β-mercaptoethanol

List of figures

List of tables

1. Literature Review

1.1 Neospora caninum: Historical Introduction

Neospora caninum is a coccidian parasite, structurally similar to the closely related *Toxoplasma gondii* (Speer et al., 1999). Like other apicomplexan parasites, *N. caninum* is an obligate intracellular pathogen (Hemphill, 1999). Its first recognition occurred in Norwegian dogs with encephalomyelitis and myosis (Bjerkås et al., 1984). In 1988, a similar parasite causing clinical disease in dogs was identified in the USA and a novel genus and species, different from *T. gondii,* was proposed (Dubey et al., 1988a). In the same year, *N. caninum* was isolated in culture and mice and Koch's postulates were fulfilled (Dubey et al., 1988b). Serological and immunohistochemical tests were developed (Dubey et al., 1988b; Lindsay and Dubey, 1989a) which allowed to confirm that the parasites identified in 1984 by Bjerkås and colleagues were *N. caninum* (Bjerkås and Dubey, 1991). Later it was described in calves with encephalomyelitis (Dubey et al., 1989; Parish et al., 1987). Retrospective studies confirmed the existence of the parasite at least since 1957 (Dubey et al., 1990). Neosporosis is considered a major cause of abortion worldwide (Dubey, 2003) in both dairy and beef cattle (Dubey and Schares, 2006) and recognized as an economically important disease (Reichel et al., 2013).

Experimental infection of pregnant rhesus macaques (*Macaca mulatta*) resulted in transplacental transmission and foetal infection, which raised concerns about the zoonotic potential of *N. caninum* (Barr et al., 1994). However, there is no evidence that *N. caninum* is capable of infecting humans (Calero-Bernal et al., 2019; McCann et al., 2007).

1.2 Life cycle, infectious stages and host range

Like other cyst-forming coccidia parasites *N. caninum* has a heteroxenous life cycle (Figure 1) with three infectious stages: tachyzoites, tissue cysts and oocysts (Dubey, 2003). Both tachyzoites and tissue cysts are found in intermediate hosts and constitute asexually proliferating stages (Dubey et al., 2007; Wouda, 2007). Tachyzoites (from the Greek *tachos*, meaning speed) are ovoid, lunate or globular (depending on the stage of division) and have a size of approximately 3–7 x 1–5 µm (Dubey et al., 2002; Wouda, 2007). They represent the rapid multiplying stage of the parasite and proliferate intracellularly by endodyogeny within a parasitophorous vacuole (Hemphill, 1999; Hemphill et al., 1999). The proliferating tachyzoites cause host cell lysis, infecting neighbouring cells (Hemphill, 1999) or disseminating via the blood to infect distant cells (Lindsay and Dubey, 2020). Tachyzoites are known to proliferate in almost all nucleated cells, including neural cells, endothelial cells, dermal cells, retinal cells, macrophages, hepatocytes, and fibroblasts (Dubey et al., 2017a). The tachyzoite stage is responsible for tissue damage, dissemination of the infection in the intermediate host and transplacental transmission to the foetus (Lindsay and Dubey, 2020). After the initial phases of the infection, in which tachyzoite proliferation occurs, the host immune response is triggered, which in association with other physiological factors is believed to induce tachyzoites to differentiate into bradyzoites (Buxton et al., 2002; Hemphill et al.,

2006). The occurrence of disease results from a balance between the ability of the tachyzoites to multiplicate, leading to cell destruction, and the response of the host inhibiting parasite multiplication (Buxton et al., 2002). Thus, if clinical disease occurs, it is caused by tachyzoites (McAllister, 2016).

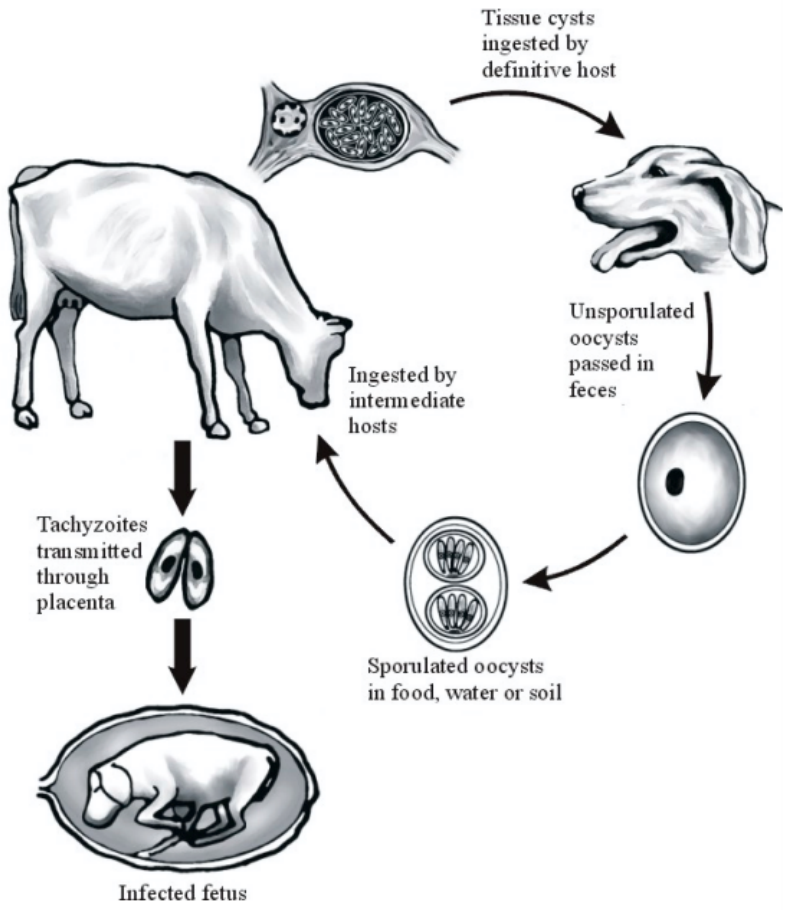

Figure 1. The life cycle of *N. caninum*. Adapted from Dubey (1999) and Stenlund (2000).

Bradyzoites represent the quiescent stage of the parasite and form intracellular tissue cysts (Hemphill et al., 2006). The bradyzoites are located within the tissue cysts and measure approximately 8 x 2 µm (Dubey et al., 2002). The tissue cysts occur primarily in the central nervous system (Dubey and Lindsay, 1996), but have also been found in the skeletal muscles of naturally-infected cattle and dogs (Peters et al., 2001). Tissue cysts can persist in the intermediary host for years without causing evident clinical signs (Hemphill et al., 1999). They are characterized by an oval shape up to 107 µm long, depending on the number of bradyzoites within them (Dubey, 2003; Dubey et al., 2007, 2002). The cyst wall is up to 4 µm thick, depending on how long the infection has existed (Dubey and Lindsay, 1996). The function of the cyst wall is to protect the parasites from immunological and physical reactions mounted by the host, maintaining chemical and physiological stability inside the cyst (Hemphill, 1999; Hemphill et al., 1999).

The sexual phase of the cycle is assumed to occur in the entero-epithelial cells of definitive hosts, preceding oocyst formation. However, the schizogonic and

gametogenic stages have not yet been documented (Dubey et al., 2017a). The oocysts represent the environmentally resistant stage of the parasite and are excreted unsporulated by the definitive hosts (Dubey et al., 2011; Gondim et al., 2004c; McAllister et al., 1998). The sporulation of the oocyst occurs in the environment (in as few as 24 hours) after the excretion in the faeces (Lindsay et al., 1999a). Unsporulated oocysts are approximately 10-11 µm in diameter (Dubey et al., 2002; Schares et al., 2005b), and sporulated oocysts are slightly bigger, 11.7 x 11.3 µm (Lindsay et al., 1999b). Sporulated oocysts contain two sporocysts, each with four sporozoites (McAllister et al., 1998). Although only limited data are available, the environmental resistance of *N. caninum* oocysts seems to be identical to other coccidian parasites (Alves Neto et al., 2011; Dubey and Schares, 2011; Uzêda et al., 2007).

N. caninum has a restricted number of canids as its definitive hosts. The domestic dog and the Australian dingo (both *Canis familiaris*), coyote (*Canis latrans*), and grey wolf (*Canis lupus*) were recognized experimentally as its definitive hosts (Dubey et al., 2011; Gondim et al., 2004c; King et al., 2010; McAllister et al., 1998). However, viable oocysts were only demonstrated in faeces of naturally-infected dogs (Basso et al., 2001), naturally-infected grey wolfs (Dubey et al., 2011) and more recently naturally-infected dingoes (Davidson et al., 2022). The European red fox (*Vulpes vulpes*) failed to shed oocysts after experimental infection, which suggests that the red fox is not a definitive host for *N. caninum* (Schares et al., 2002b).

A wide range of intermediate hosts have been identified (Dubey et al., 2017a; Wouda, 2007). Viable *N. caninum* parasites were isolated from axis deer (*Axis axis)*, cattle (*Bos taurus*), dog (*Canis familiaris)*, European bison (*Bison bonasus*), grey wolf (*Canis lupus)*, sheep (*Ovis aries*), water buffalo (*Bubalus bubalis*) and white-tailed deer (*Odocoileus virginianus*) (Basso et al., 2014; Bień et al., 2010; Conrad et al., 1993a; Dubey et al., 2014; Koyama et al., 2001; Rodrigues et al., 2004; Vianna et al., 2005).

1.3 Transmission

N. caninum can be transmitted horizontally (postnatal infection) and vertically (transplacental infection) and all three stages can be involved. Carnivores become horizontally infected by ingestion of tissues containing cysts (Dubey et al., 2007). The wall of the cyst is regarded resistant to acid pepsin digestion in the stomach, which allows the bradyzoites to be released in the gut (Lindsay and Dubey, 1990). It is not known whether tissues containing only tachyzoites can be orally-infectious (Dubey and Schares, 2011). It seems unlikely given that tachyzoites treated with acidic pepsin are inactivated and thus non-infectious for cell cultures (Lindsay and Dubey, 1990). However, in one study mice were successfully infected by oral inoculation of *N. caninum* tachyzoites (Lindsay and Dubey, 1990). The only demonstrated natural mode of postnatal infection in herbivores is the ingestion of sporulated oocysts from the environment (Dubey et al., 2007).

Vertical transmission occurs from infected dams to their offspring and appears to be the most important natural route of infection in cattle (Wouda, 2007). This form of transmission was documented to occur naturally in cattle, sheep, goats, dogs and

various species of deer (Dubey, 2003). Two forms of vertical transmission have been described in cattle (Trees and Williams, 2005) and sheep (González-Warleta et al., 2018): The exogenous transplacental transmission, after a primary, oocyst-derived, infection of a pregnant dam; and the endogenous transplacental transmission in chronically infected dams after reactivation (recrudescence) of the infection during pregnancy.

1.3.1 Transmission in Cattle

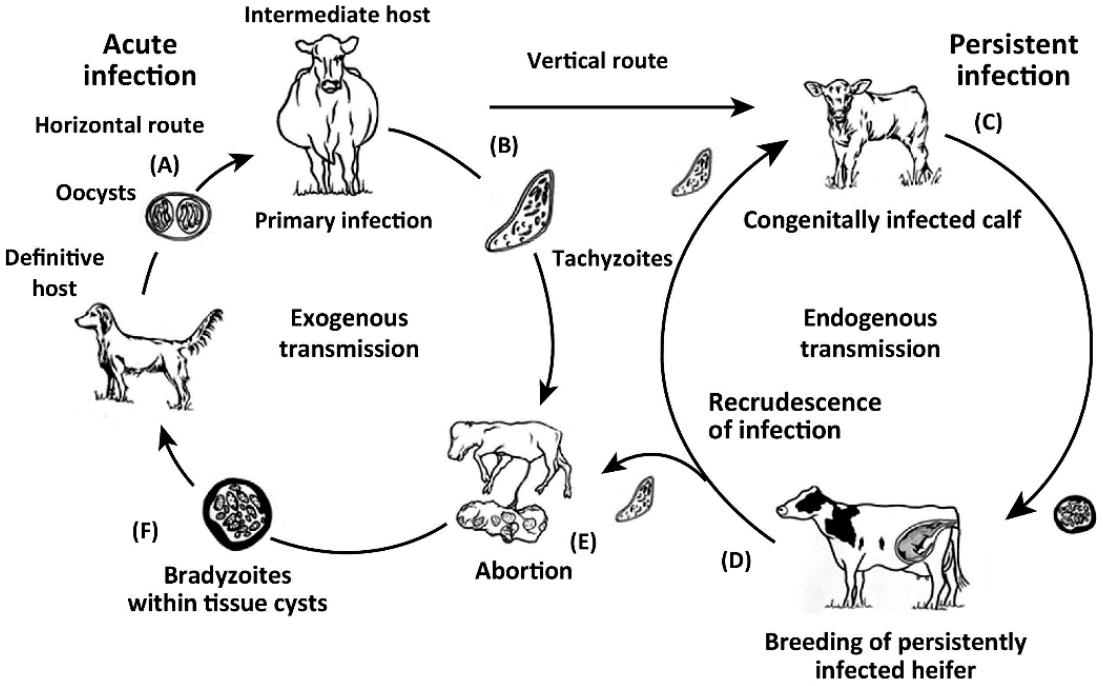

Figure 2. Transmission routes for *N. caninum* in cattle. (A) Oocysts are shed in the faeces by a suitable definitive host and their subsequent ingestion by a susceptible pregnant cow leads to postnatal (horizontal) infection of the dam; (B) and infection of the foetus through the placenta (exogenous transplacental transmission). (C) The rapid multiplying stage (tachyzoite) differentiates into the quiescent stage (bradyzoite), forming intracellular tissue cysts, and the intermediary host can remain infected into adulthood (and even for life). (D) During pregnancy, recrudescence of the infection may occur, resulting in stage conversion of bradyzoites into tachyzoites, which can cross the placenta and infect the foetus (congenital infection). (E) Recrudescence of the infection may occur in several consecutive gestations or intermittently and result in the birth of a congenitally infected calf or abortion. (F) Ingestion of tissues containing *N. caninum* bradyzoites is the natural route of infection of the definitive hosts. Adapted from Guido et al. (2016) and Dubey et al. (2017b).

1.3.1.1 Transplacental (vertical) transmission

Healthy but persistently infected cattle can transmit the infection transplacentally to their offspring (Anderson et al., 1997) in consecutive pregnancies (Fioretti et al., 2003) or intermittently (Boulton et al., 1995; Guy et al., 2001; Wouda et al., 1998b). In this type of transmission, the endogenous transplacental transmission (Figure 2), bradyzoites are believed to differentiate into tachyzoites, which are able to cross the placenta and may infect the foetus (Trees and Williams, 2005).

Several epidemiological studies provided compelling evidence that persistently infected cattle can transmit *N. caninum* very efficiently via endogenous transplacental transmission through successive generations and, at least in some herds, this transmission route seems to be the major mode of transmission (Bergeron et al., 2000; Björkman et al., 1996; Davison et al., 1999a; Dijkstra et al., 2001a; Frössling et al., 2005;

Hall et al., 2005; Schares et al., 1998). Evidence for the efficient endogenous transplacental transmission can also be found in prospective studies comparing the serological status of dams and the precolostral antibody status of their newborn progeny (Anderson et al., 1997; Davison et al., 1999b; Paré et al., 1996). The presence of specific *N. caninum* antibodies in precolostral serum indicates that those antibodies were produced in utero by the foetus because in cattle there is no transfer of antibodies from dam to their foetus during gestation (Dubey et al., 2007; Osburn et al., 1982). It is thus a clear indication of transplacental transmission (Dubey et al., 2017b).

The endogenous transplacental transmission is probably the most important natural route to maintain the parasite within a herd population (Williams et al., 2009). However, mathematical models by French et al. (1999) demonstrated that the prevalence of infection decreases over time because the rate of endogenous transplacental transmission is lower than 100%. Therefore, at least a low rate of horizontal transmission may be necessary to maintain the infection within the infected herds (Dubey et al., 2007).

The efficiency of endogenous transplacental transmission – characterized by the percentage of pregnancies of persistently infected dams in which vertical transmission of *N. caninum* occurs – seems to vary among cattle herds (Dubey and Schares, 2011; McAllister, 2016). Up to 95% of pregnancies of persistently infected cattle may result in the birth of congenitally infected calves (Davison et al., 1999b; Paré et al., 1996). A Dutch study, adjusting probabilities to take into account imperfect test characteristics and horizontal infections, obtained a 45% rate of endogenous transplacental transmission in 96 herds analysed (Bartels et al., 2007). McAllister (2016) combining eight studies, without adjusting for horizontal transmission, reached an average of 63% efficiency of endogenous transplacental transmission.

The risk of endogenous transplacental transmission seems to decrease with increased parity of the dam (Dijkstra et al., 2003; López-Gatius et al., 2005a; Romero et al., 2002). It has been suggested that some degree of immunity might be developed over time, which could prevent the recrudescence of chronic infections (Dijkstra et al., 2003; Romero et al., 2002). Another possible explanation is that control strategies implemented by the farmers – such as selective culling of seropositive cows or aborting animals – could result in a reduced number of seropositive dams in the farm, reducing the probability of the progeny being positive (López-Gatius et al., 2005a; Romero et al., 2002).

Another form of transplacental transmission described in cattle is the exogenous transplacental transmission (Trees and Williams, 2005). This form of transmission (Figure 2) occurs when naïve cattle are infected during pregnancy by ingesting oocysts (the exogenous source of infection), and the tachyzoites, which differentiate from the sporozoites, may cross the placenta infecting the foetus (Williams et al., 2009). Thus, both the dam and the offspring become infected from the same single event (McAllister, 2016). Evidence for this transmission route comes from experimental studies, which demonstrated that infection with oocysts through oral inoculation can result in

(exogenous) transplacental transmission and abortion (Gondim et al., 2004a; McCann et al., 2007). Epidemiologically, oocyst exposure is believed to be the cause of *N. caninum*-associated abortion storms (Dijkstra et al., 2001, 2002; McAllister et al., 2000, 1996).

1.3.1.2 Postnatal (horizontal) transmission

Cattle can become postnatally infected in nature (Figure 2) by ingestion of fodder or water contaminated with sporulated oocysts (De Marez et al., 1999; Gondim et al., 2004a; Trees et al., 2002). Several seroepidemiological studies show evidence for the occurrence of horizontal transmission. In herds experiencing *N. caninum*-associated abortion storms (epidemic abortion), no association between serostatus of dams and their progeny could be found, suggesting transmission of *N. caninum* after birth (Dijkstra et al., 2001a; Kashiwazaki et al., 2004; Patitucci et al., 1999; Schares et al., 1999a; Thurmond et al., 1997). A high rate of seroconversions and the presence of low avidity antibodies in seroconverted animals also constitute an indication of horizontal infections (Dijkstra et al., 2002a; Piagentini et al., 2012). The presence of definitive hosts was consistently associated with an increased risk of seropositivity, showing the importance of the faecal-oral route of infection. Particularly, the presence of farm dogs (Blumröder et al., 2006; Paré et al., 1998), or the number of farm dogs (Corbellini et al., 2006; Mainar-Jaime et al., 1999; Schares et al., 2004b) were identified as risk factors for seropositivity in dairy herds. In one study, the abundance of coyotes was associated with the seroprevalence of *N. caninum* in beef calves (Barling et al., 2000). The sylvatic cycle (deer-wild canids) may thus be important to maintain the domestic cycle (cattle-dog) in some regions (Gondim et al., 2004b; King et al., 2011; Melendez et al., 2021; Rosypal and Lindsay, 2005).

Other routes of postnatal transmission have been proposed, but not proven to occur naturally (Dubey et al., 2007). Anderson et al. (1997) housed seronegative heifers together with seropositive heifers since birth. Both heifers and progeny were evaluated for the presence of specific *N. caninum* antibodies. Seronegative heifers remained seronegative during the study and gave birth to seronegative calves. Seropositive heifers gave birth to clinically normal but congenitally infected calves. Consequently, there is no evidence of cow-to-cow transmission (Dubey, 2003). Placentophagia has been purposed as an alternative transmission route (Modrý et al., 2001). However, there is no evidence that it plays an important role in *N. caninum* transmission (Schares and Conraths, 2001).

The hypothesis that *N. caninum* could be transmitted by semen – via artificial or natural insemination – seems unlikely (Dubey and Schares, 2011). In three studies *N. caninum* DNA was detected in the semen of bulls with naturally-acquired *N. caninum* infections (Caetano-da-Silva et al., 2004; Ferre et al., 2005; Ortega-Mora et al., 2003). However, only a low parasite load (less than 15 parasites/ml of semen) was observed, which represents an ejaculate containing approximately 100 parasites (Ferre et al., 2005; Serrano-Martínez et al., 2007a). Under experimental conditions, much larger numbers of tachyzoites were necessary to cause infection in heifers by intrauterine inoculations of experimentally-contaminated semen (Serrano et al., 2006; Serrano-Martínez et al., 2007b). In one experiment heifers failed to seroconvert after being

naturally bred with experimentally infected bulls (Osoro et al., 2009). Another experiment in which cows were artificially inseminated with frozen and thawed semen experimentally contaminated with *N. caninum* tachyzoites failed to cause infection (Canada et al., 2006).

Transmission by embryo transfer seems unlikely as well (Dubey et al., 2006). Embryo transfer has been recommended as a method to prevent endogenous transplacental transmission in cattle (Baillargeon et al., 2001). Further studies confirmed that when embryos are transferred from seropositive donors into seronegative recipients using standard methods vertical transmission of *N. caninum* does not occur (Landmann et al., 2002; Moskwa et al., 2008).

Lactogenic transmission of *N. caninum* has been demonstrated experimentally in newborn calves by oral inoculation of colostrum or milk experimentally contaminated with tachyzoites (Davison et al., 2001; Uggla et al., 1998). Additionally, *N. caninum* DNA has been recognized in milk and colostrum of seropositive dams (Moskwa et al., 2007, 2003). The possibility of transmission through ingestion of colostrum seems unlikely due to the high level of specific *N. caninum* antibodies present in the colostrum of seropositive cattle (Moskwa et al., 2007). Finally, experiments failed to cause infection of calves given milk or colostrum from naturally infected cows (Davison et al., 2001). Thus, there is no evidence that lactogenic transmission of *N. caninum* occurs naturally (Davison et al., 2001; Dubey et al., 2007).

1.3.2 Sources of infection for dogs and oocyst shedding

Dogs have been shown to shed oocysts after ingestion of placentas from freshly calved seropositive dams (Bergeron et al., 2001b; Dijkstra et al., 2002b, 2001b), and other tissues such as brain, heart, liver, and masseter muscle from naturally-infected calves (Cavalcante et al., 2011). Gondim et al. experimentally fed dogs with a pool of different bovine tissues – including the brain, vertebral column, heart, liver, kidney, tongue, diaphragm, and other muscles – from experimentally-infected calves, resulting in oocyst shedding. However, it is not known which tissue(s) was the source of infection for the dogs. Dogs fed aborted foetuses failed to shed oocysts (Bergeron et al., 2001a; Cedillo et al., 2008). This result may be explained by the autolysis of the foetal tissues, which may compromise parasite survivability (Dubey et al., 2007). Carnivorism seems to be a more important route of infection than faecal transmission in dogs (Dubey and Schares, 2011). Bandini et al. (2011) after feeding dogs with sporulated oocysts found no evidence of patent infections (none of the dogs shed oocysts).

In experimentally infected dogs, oocyst shedding was usually initiated five to seven days after ingestion of *N. caninum*-infected tissues (Schares and Losson, 2007). Most dogs shed oocysts until thirteen days after oral infection, and after this period some dogs intermittently shed oocysts for up to 30 days post-ingestion (Cavalcante et al., 2011; Dijkstra et al., 2001b; Gondim et al., 2002; Lindsay et al., 1999a; McAllister et al., 1998). So, the period of shedding oocysts is probably brief and, as a consequence, the prevalence of dogs excreting oocysts at a moment is low (Dubey et al., 2017c; Schares et al., 2005b). Repeated excretion of oocysts is possible after a new infection (Gondim

et al., 2005; McGarry et al., 2003). A refractory period after primoinfection, in which dogs do not shed oocysts after re-exposure to *N. caninum*-infected bovine tissues, was observed (Gondim et al., 2005).

Remarkably, in experimental studies, the majority of dogs shedding oocysts did not show *N. caninum*-specific anti-tachyzoite surface antibodies (Dijkstra et al., 2001b; Gondim et al., 2002; Lindsay et al., 1999a; McAllister et al., 1998; Schares et al., 2001). Likewise, naturally infected dogs shedding oocysts did not seroconvert (Schares et al., 2005b). Thus, a negative serologic result does not confirm a dog as free of a previously occurring patent infection with *N. caninu*m (Lindsay et al., 1999a; Schares et al., 2005b). For this reason, the hypothesis that during the intestinal stages of the cycle resulting in oocyst production, no or only a few extra-intestinal stages of *N. caninum* develop was raised, which could explain the lack of an antibody response against tachyzoite antigens in dogs shedding oocysts (Gondim et al., 2005; Schares et al., 2001). Coprophagia is a possible reason for seroconversion in some dogs (Schares et al., 2001). However, in one experimental study high doses of sporulated oocysts were necessary to induce seroconversion in dogs (Bandini et al., 2011), raising questions regarding the efficiency of this route (Dubey et al., 2017c). Thus, the presence of *N. caninum*-specific antibodies in a dog is indicative of exposure to the parasite but not oocyst excretion (Dubey et al., 2017c).

A sylvatic cycle has been described in North America which is maintained by white-tailed deer preyed upon by wolves and coyotes (Dubey et al., 2011; Gondim et al., 2004b; Lindsay and Dubey, 2020; Rosypal and Lindsay, 2005). Experimentally, dogs were able to shed *N. caninum* oocysts after ingestion of brains of naturally infected white-tailed deer, thus transmission between wild and domestic animals is possible (Gondim et al., 2004b). Likewise, it has been suggested that dingoes and other “wild dogs” may play a role in the epidemiology of *N. caninu*m in Australia (King et al., 2011).

1.4 Epidemiologic patterns in cattle

Two major patterns of *N. caninum*-associated abortion have been described in cattle: temporary outbreaks of abortion (epidemic pattern), and increased annual abortion losses (endemic pattern) (McAllister, 2016; Thurmond et al., 1997). Abortion outbreaks can be regarded as epidemic if the outbreak is temporary and if 15% of the cows at risk abort within 4 weeks (Schares et al., 1999c), 12.5% of the cows at risk abort within 8 weeks (Wouda et al., 1999), or 10% of the cows at risk abort within 6 weeks (Moen et al., 1998). Conversely, *N. caninum*-associated abortion problems may be considered endemic if they occur intermittently for months or years (Dubey et al., 2017b). These two patterns of *N. caninum* associated-abortion are thought to be related to different routes of transmission i.e., the endogenous or exogenous transplacental transmission (Trees and Williams, 2005; Williams et al., 2009).

1.4.1 Epidemic abortion

Epidemic abortion seem to result from the primary infection of naïve pregnant dams (postnatal infection), which has been attributed to ingestion of fodder or drinking water contaminated with oocysts (McAllister et al., 2005, 2000). Pregnant dams may be

exposed almost at the same time (point-source exposure) to oocysts, which can lead to exogenous transplacental transmission and abortion (Trees and Williams, 2005). Data supporting this view has been obtained from several studies: (1) Epidemic curves observed in outbreaks of *N. caninum*-associated abortion indicate a point-source exposure (Arnold, 2013; McAllister et al., 2000, 1996; Yaeger et al., 1994); This suggests that exposure occurred in a short period, most likely from the same source (Thrusfield and Christley, 2018); (2) Low avidity antibodies, which can be found in primary infections (Aguado-Martínez et al., 2005; Björkman et al., 2005, 1999; Schares et al., 2002a), were observed in a high percentage of dams from herds with outbreaks of *N. caninum*-associated abortion (Aguado-Martínez et al., 2008; Basso et al., 2010; Björkman et al., 2003; Jenkins et al., 2000; McAllister et al., 2000; Williams et al., 2009); Additionally, researchers in Germany analysing DNA extracts from the brain of *N. caninum*-infected foetuses from five herds with outbreaks of abortion, found a common *N. caninum*-microsatellite marker in each individual herd, further supporting the hypothesis of a recent infection from a common point source (Basso et al., 2010); (3) A lack of association between the antibody status of dams and their progeny has been reported in abortion outbreaks, which suggests that postnatal transmission is the main route of transmission in these herds (Dijkstra et al., 2001a; Patitucci et al., 1999; Thurmond et al., 1997; Waldner et al., 1999); (4) Epidemiological studies demonstrated that the presence of farm dogs increases the risk of farms experiencing an abortion storm, supporting the hypothesis of transmission to cattle by ingestion of oocysts shed by dogs (Bartels et al., 1999; Dijkstra et al., 2002b; Hobson et al., 2005).

1.4.2 Endemic abortion

In herds facing endemic *N. caninum* abortions, seropositive animals are often found in familiar clusters, demonstrating the major importance of endogenous transplacental transmission, which propagates the infections to successive generations (Björkman et al., 1996; Davison et al., 1999a; Frössling et al., 2005; Schares et al., 1998). In endemically-infected herds, a strong association between the serostatus of dams and their daughters (i.e., the progeny of seropositive dams is often positive, while most daughters of seronegative dams do not reveal specific *N. caninum* antibodies) is reported, which shows that endogenous transplacental transmission is the major route of transmission in those herds (Bergeron et al., 2000; Björkman et al., 2003, 1996; Davison et al., 1999a; Dijkstra et al., 2001a; Frössling et al., 2005; Hall et al., 2005; Schares et al., 1998; Thurmond et al., 1997; Wouda et al., 1998b).

There is evidence that persistent infections can be acquired vertically (Anderson et al., 1997) or postnatally (Dijkstra et al., 2008; Moen et al., 1998) (Figure 2) and those chronically infected cows may remain infected for life (Trees et al., 1999). Irrespective of the origin of the primary infection (vertical or horizontal), recrudescence of persistent infections can occur in pregnant dams resulting in endogenous transplacental transmission and in some cases abortion (Anderson et al., 1997; Björkman et al., 2003; Dijkstra et al., 2008; Guy et al., 2001; Stenlund et al., 1999; Trees and Williams, 2005; Williams et al., 2009). Thus, an initial episode of postnatal exposure of pregnant cattle can later result in a high rate of endogenous transplacental transmission in those same

cows (Björkman et al., 2003; Dijkstra et al., 2008), possibly maintaining high within-herd prevalence for a long time (Schares and Conraths, 2001).

It is well known that cattle with serum antibodies against *N. caninum* are more likely to abort than their uninfected seronegative counterparts. Reichel et al. (2013) reviewing studies from 10 countries obtained a median increase risk of abortion of 3.5 (ranging from 1.3 to 40.0) in dairy cattle, meaning that seropositive dams have approximately a 3.5-fold increased risk of abortion compared to seronegative dams. Persistently infected seropositive dams are also more likely to experience repetitive abortion than seronegative dams (Asmare et al., 2013; Mazuz et al., 2014; Pabón et al., 2007).

Mixed patterns with both endogenous and exogenous transplacental transmission playing a role in *N. caninum* infections may occur in the field (Dubey et al., 2017b). Theoretical models indicate that at least a low rate of horizontal transmission is important to maintain high seroprevalence in endemically infected herds (French et al., 1999). Seroepidemiological studies have reported low rates of seroconversion, suggesting a low level of horizontal transmission in endemically-infected herds (Davison et al., 1999b; Frössling et al., 2005; Hietala and Thurmond, 1999; Paré et al., 1997, 1996; Schares et al., 1998). A study in The Netherlands based on a random sample of herds and adjusting probabilities of vertical and horizontal transmission also found a low incidence of horizontal transmissions (Bartels et al., 2007). Thus, horizontal transmission within endemic herds may occur in a way that only individual cattle are infected at each time or small and specific groups of cattle are infected at once (McAllister, 2016).

1.5 Clinical signs in cattle

Abortion is the main clinical sign in adult cattle. *N. caninum* may cause foetal resorption, mummification, abortion, premature birth and stillbirth (Dubey and Lindsay, 1996). Nonetheless, most foetuses infected *in utero* are born congenitally infected but clinically healthy (Guy et al., 2001; Paré et al., 1997; Thurmond and Hietala, 1997; Wouda et al., 1998b). Abortions may occur from the third month of gestation to term, but most *N. caninum*-associated abortion occurs during mid-gestation from five to seven months (Dubey and Lindsay, 1996; McAllister, 2016). Following abortion, retained foetal membranes and metritis may occur (Arnold, 2013; Asmare et al., 2013). Neosporosis does not seem to be a significant cause of early embryonic loss (López-Gatius et al., 2004b). Most foetuses dying in utero between three and eight months of gestation are expelled, showing moderate autolysis (Dubey et al., 2006). Rarely, foetuses may die before five months of gestation and be mummified and retained in the uterus (Anderson et al., 1997; Barr et al., 1993; Dubey et al., 2006).

Clinical signs other than abortion are rare and have only been observed in cattle younger than 2 months old (Dubey, 2003). Clinically affected calves may have neurologic signs including ataxia, decreased patellar reflexes, loss of conscious proprioception, and tetraparalysis (Dubey et al., 2006; Micheloud et al., 2015; Wouda, 2007). The hind limbs and/or forelimbs may be flexed or hyperextended (Barr et al., 1993; Parish et al., 1987). Such calves may have normal size or be underweighted (McAllister, 2016). Exophthalmia and an asymmetrical appearance of eyes have been documented (Bryan et al., 1994;

O'Toole and Jeffrey, 1987). Rarely, birth defects such as scoliosis, hydrocephalus or a narrowing of the spinal cord may occur (Dubey and de Lahunta, 1993).

1.6 Pathogenesis of bovine neosporosis

It is well known that not all *N. caninum*-infected cows' abort. However, the exact mechanisms which determine whether a foetus dies or survives are not fully understood, but several hypotheses have been proposed (Dubey et al., 2006; Marugan-Hernandez, 2017; Williams and Trees, 2006). In pregnant cows, *N. caninum* is thought to reach the placenta via circulation and invade the maternal caruncular septa in the placentome before spreading to the foetus (Dubey et al., 2006; Innes, 2007). When naïve pregnant cows are infected by oocyst ingestion, the sporozoites probably infect the intestinal cells and differentiate into tachyzoites which are able to spread across the body and reach the placenta (Dubey et al., 2006; Williams et al., 2009). In the case of persistently infected cows, bradyzoites are thought to differentiate into tachyzoites (recrudescence) which spread via circulation to the gravid uterus (Williams et al., 2009). Parasite multiplication in the placenta or foetal tissues may cause direct tissue damage (Buxton et al., 2002). Thus, foetal damage and possibly abortion may occur due to primary tissue damage by parasite multiplication in the foetus, or due to insufficient oxygen/nutrition, secondary to placental damage (Dubey et al., 2006). Alternatively, placental damage may cause production of maternal prostaglandins causing luteolysis, and as a result uterine contractions and abortion (Baetz et al., 1981; Engeland et al., 1996; Foley et al., 1993). Furthermore, the maternal immune response to control the multiplication of *N. caninum*, with production of pro-inflammatory cytokines (Th1-type immune responses), is known to be detrimental to pregnancy maintenance and may cause foetal expulsion (Innes et al., 2002; Maley et al., 2006; Monney and Hemphill, 2014; Quinn et al., 2002; Rosbottom et al., 2008). The above-referred mechanisms are probably connected, but some may be more important depending on the stage of gestation (Dubey et al., 2006).

1.6.1 Maternal immune responses

The first line of defence against invading pathogens in cattle is mediated by innate immune mechanisms involving Natural Killer (NK) cells (Klevar et al., 2007). NK cells were shown to be capable to lyse *N. caninum*-infected fibroblasts and produce interferon-gamma (IFN-γ) (Boysen et al., 2006). It has been suggested that the early expression of IFN-γ may be important to create an appropriate cytokine environment for the induction of the adaptive immune response through a Th1-type response (Innes, 2007; Monney and Hemphill, 2014). A Th1-type response is known to be important to limit parasite multiplication (Innes et al., 2002) and *N. caninum* stimulates a Th1-type response, associated with infiltration of CD4+ T-cells and IFN-γ production in cattle (Maley et al., 2006; Rosbottom et al., 2007; Williams et al., 2000). In naturally infected cattle, IFN-γ expression during pregnancy was shown to have a protective effect against abortion (Almería et al., 2009b; López-Gatius et al., 2007). Additionally, CD4+ T-cells were shown to have a cytotoxic effect on *N. caninum*-infected cells (Staska et al., 2003).

As stated above, a pro-inflammatory (Th1-type) response is induced after infection with *N. caninum*, which is important to control parasite multiplication (Williams and Trees, 2006). Conversely, Th1-type responses and particularly a strong IFN-γ response may cause foetal death in early gestation (Maley et al., 2006; Quinn et al., 2002) and during mid-gestation (Almería et al., 2010). Thus, an inflammatory response in the periphery and the lymph nodes may be important to contain parasite proliferation, whereas at the maternofoetal interface may cause fetopathy (Innes, 2007). During pregnancy, upregulation of anti-inflammatory (Th2-type) cytokines, such as IL-10, IL-4 and transforming growth factor beta (TGF-β), is important to enable the dam to carry the semi-allogenic foetus without immunological rejection (Entrican, 2002; Innes et al., 2002). These regulatory cytokines are particularly expressed at the maternofoetal interface and counteract the more pro-inflammatory cytokines but may allow parasite proliferation resulting in congenital infection (Entrican, 2002; Innes, 2007; Monney and Hemphill, 2014). Thus, an active maternal immune response, with expression of both pro- and anti-inflammatory cytokines is probably important to ensure the success of the pregnancy in *N. caninum*-infected dams (Almería et al., 2003; Rosbottom et al., 2011).

Specific antibody production may also be important in protective immunity against *N. caninu*m (Innes, 2007). In persistently-infected dams, *N. caninum*-specific antibody fluctuations have been reported during pregnancy (Guy et al., 2001; Paré et al., 1997; Stenlund et al., 1999). Increasing antibody titres probably reflect a greater exposure of parasite antigens to the immune system, caused by the recrudescence of chronic infections with parasite multiplication in the host (Innes et al., 2005; Stenlund et al., 1999). Thus, an increase in antibody levels during mid-gestation in *N. caninum* chronically infected dams is thought to indicate recrudescence of infection (Stenlund et al., 1999). Moreover, antibodies may play an important role to prevent tachyzoite invasion of host cells (Haldorson et al., 2006; Innes et al., 2002).

The ratio of specific IgG_1 and IgG_2 may vary with clinical status (Dubey and Schares, 2011). In non-pregnant cows, cows delivering uninfected calves, and also in one cow that aborted after *N. caninum* infection, no significant differences were found between IgG_1 and IgG_2 titres (Guy et al., 2001). Conversely, in cows naturally infected with *N. caninum* delivering congenitally infected calves, the reciprocal titre of IgG_2 was significantly higher than that of IgG_1 7 months after insemination (Guy et al., 2001). In cows persistently infected with *N. caninum,* a predominant IgG_2 pattern was found in almost all aborting dams, but the non-aborting cattle showed either a dominating IgG_1 or a dominating IgG_2 response (Almería et al., 2009b). However, the predominance of IgG_2 antibodies alone, if not associated with high IFN-γ production, may not be sufficient to protect against *N. caninum*-associated abortion (Almería et al., 2009b; Santolaria et al., 2011). It has been suggested that the prominent IgG_2 indicates a Th1-type response bias (associated with IgG_2) in the dam experiencing transplacental transmission of *N. caninum*, in contrast with the expected Th2-type response bias due to the immunomodulation associated with pregnancy maintenance (Dubey et al., 2017b). However, the IFN-γ production in pregnant and non-pregnant cattle was often not associated with IgG_1/IgG_2 ratios, suggesting that the production of bovine IgG_2 may be

regulated by factors other than IFN-γ (Andrianarivo et al., 2001, 2000; Moore et al., 2005).

The consequences of *N. caninum* infection are different depending on whether the primoinfection occurred before birth (transplacental transmission), or postnatally (Dubey et al., 2017b). Heifers infected *in utero* can remain persistently infected and experience recrudescence of infection during gestation, which may result in abortion or endogenous transplacental transmission (Williams et al., 2003). Postnatal infection of dams during gestation can result in exogenous transplacental transmission and later, during subsequent pregnancies, recrudescence of infection and endogenous transplacental transmission may occur in those same cows (Björkman et al., 2003; Dijkstra et al., 2008). Conversely, when naïve cattle were experimentally challenged with *N. caninum* prior to pregnancy, endogenous transplacental transmission did not occur during the following gestation (Innes et al., 2001; Williams et al., 2000). Consequently, dams infected during gestation or cattle infected prior to birth are not able to develop a good protective immunity against endogenous transmission (Dubey et al., 2017b; Innes et al., 2005). However, persistently infected cattle can acquire protective immunity to prevent exogenous transplacental transmission (McAllister et al., 2005, 2000; Williams et al., 2003).

1.6.2 Foetal immune responses

The foetal immune responses are also a critical factor to determine the outcome of the *N. caninum* infection (Innes et al., 2002). Ruminants have a syndesmochorial placentation, which does not allow the transfer of immunoglobulins and cytokines (Osburn et al., 1982), not even when the placenta has been damaged by an infectious process (Dubey et al., 1987). Therefore, immune responses detected in the foetus are most likely due to *in utero* synthesis by the foetus itself (Dubey et al., 2007). The immunocompetence of the foetus is known to develop progressively throughout gestation (Innes, 2007). Mitogenic cellular responses can be found after 12 weeks of gestation in foetal blood cells and after 14 weeks in foetal spleen and thymus cells (Innes, 2007; Innes et al., 2005; Monney and Hemphill, 2014). A *N. caninum* specific antibody response can be found at least from day 100 of gestation (Bartley et al., 2012). The progressive maturation of the foetal immune system has been demonstrated by the absence of inflammatory lesions accompanying specific *N. caninum* necrotic lesions in early pregnancy (Gibney et al., 2008; Macaldowie et al., 2004), while infection during mid or late pregnancy results in a non-purulent inflammatory infiltrate, fewer lesions and a reduction of parasite dissemination in the foetus (Collantes-Fernández et al., 2006; Gibney et al., 2008; Maley et al., 2003). Furthermore, *N. caninum* was detected in the brain, heart, kidney and lung in foetuses aborted in the first and second trimester, whereas in the third trimesters, lesions were mainly only detected in the brain (Collantes-Fernández et al., 2006). These findings suggest that parasite growth may be progressively controlled by the foetal immune system as pregnancy progresses (Buxton et al., 2002; Dubey et al., 2006; Innes et al., 2005). However, foetal immunocompetence may develop at a different rate from animal to animal (Gibney et al., 2008) and the rudimentary immune response that starts to develop during mid-gestation may not be

adequate to prevent abortion, because most abortions are reported during this period (Bartley et al., 2012; Dubey et al., 2006). In the third trimester, the foetus is capable of mounting a competent immune response against the parasite and may develop into a clinically normal, but congenitally infected calf (Dubey et al., 2006; Williams et al., 2000).

Based on the observations that fewer lesions and a reduction of parasite dissemination occur in mid and late gestation as compared to early pregnancy (Gibney et al., 2008; Macaldowie et al., 2004; Maley et al., 2003), it has been suggested that the immunological maturity of the foetus is more important to determine the outcome of the infection than maternal immune responses (Gibney et al., 2008). However, a more recent study showed that in late pregnancy the maternal immune response may play a critical role in limiting the initial infection, at a peripheral level or locally at the placenta, which along with the foetal immune response may prevent parasite proliferation and foetal mortality (Benavides et al., 2012).

1.6.3 Risk of abortion and transmission relative to gestational stage

The outcome of pregnancy is related to the period in which the primary infection or reactivation occurs (Innes et al., 2002; Williams and Trees, 2006). *N. caninum*-associated abortions are normally diagnosed from 3 to 8 months of gestation (Dubey et al., 2006). The risk of transplacental transmission to the foetus increases with gestational age (Dubey et al., 2006; Innes et al., 2005), but the consequences for the foetus are probably more severe in early gestation than later in gestation (Innes, 2007). Experimental infections within the first trimester of gestation caused foetal loss, whereas infections in the last trimester of gestation do not cause foetal infection, or the infected foetus is born alive (Dubey et al., 2006; Gondim and McAllister, 2022). Similarly, in naturally infected cattle, recrudescence of infection within the first 5 months of gestation results in foetal death, while recrudescence later in gestation causes congenital infection but does cause foetal death (Guy et al., 2001).

1.7 Diagnosis of bovine neosporosis

To diagnose bovine neosporosis both clinical history and epidemiological data are valuable, but to achieve a definite diagnosis the assistance of a veterinary diagnostic laboratory is needed (Anderson et al., 2000; Ortega-Mora et al., 2006). To establish a cause–effect relationship between *N. caninum* and abortion it is recommended to demonstrate the infection in the dam and the aborted foetus, optimally by: (1) histological examination, (2) detection of the *N. caninum* genome by PCR, (3) detection of specific antibodies in individual animals, and (4) epidemiological evidence (Dubey et al., 2017b; Pereira-Bueno et al., 2003; Sager et al., 2001). This comprehensive diagnostic approach is ideal because: (1) in most cases *N. caninum* congenitally infected calves and chronically infected dams are asymptomatic (Paré et al., 1996) and, (2) the presence of the parasite itself or DNA is not unambiguous evidence that *N. caninum* caused the abortion (Jenkins et al., 2002; Thurmond et al., 1999). Results of foetal histopathological and immunohistological examinations are considered to have low positive predictive value (Thurmond et al., 1999) and additional information regarding lesion severity, serologic evidence of infection, gestational age, herd status, and exclusion of other

possible abortifacients may help to differentiate *N. caninum* foetal infection from abortion due to *N. caninum* (Anderson et al., 2000; Thurmond et al., 1999).

Apart from epidemiological data, including the epidemiological pattern of abortion (endemic or epidemic), and the clinical history, the presence of risk factors associated with *N. caninum* abortions should also be analysed (Ortega-Mora et al., 2006). It is known that the presence and number of dogs in contact with cattle is associated with the occurrence of abortion outbreaks (Bartels et al., 1999; Dijkstra et al., 2002b; Hobson et al., 2005). The abundance of coyotes was found to be associated with the seroprevalence of *N. caninum* in beef calves (Barling et al., 2000) and other wild canids were identified as definitive hosts of *N. caninum* (Dubey et al., 2011; Gondim et al., 2004c; King et al., 2010). Other risk factors such as farm location and production system, cattle age, climatic factors and causes of immunosuppression should also be considered (Bartels et al., 1999; Haddad et al., 2005; Schares et al., 2004b; Thurmond et al., 2005; Wouda et al., 1999).

1.7.1 In vivo diagnosis

1.7.1.1 Detection of specific antibodies

Techniques able to detect specific antibodies are the most useful tools for the diagnosis of *N. caninum* infection *in vivo* (Dubey and Schares, 2006; Ortega-Mora et al., 2006). After *N. caninum* infection IgM antibodies appear prior to IgG antibodies. Specific IgM levels increase after infection and peak after 2 weeks, declining posteriorly (De Marez et al., 1999), whereas specific IgG levels increase after experimental primary infection up to 3–6 months (De Marez et al., 1999; Dubey et al., 1996; Schares et al., 2000, 1999c; Williams et al., 2000). Cows may remain infected for life and maintain specific antibodies, although antibody levels may drop below the detection limit of some (less sensitive) tests causing false negative results (Dijkstra et al., 2003; Wouda et al., 1998a). Antibody level may fluctuate with infection stage (transient/persistent), stage of gestation and age (Dubey and Schares, 2006; Roelandt et al., 2015).

A variety of serological techniques including indirect fluorescent antibody tests (IFATs), enzyme-linked immunosorbent assays (ELISAs), immunoblotting (IB) and direct agglutination tests (DATs) have been described and validated. Numerous ELISAs are commercially available (Table 1). Serological tests generally show a good level of agreement in the interpretation of test results (positive or negative), but significant discordances are sometimes observed (Campero et al., 2018; von Blumröder et al., 2004; Wapenaar et al., 2007). There is no serological technique that can be considered a “gold standard” and thus a combination of different techniques (using the result of the majority of tests) is frequently used to classify individual sera as seropositive or seronegative (Álvarez-García et al., 2013; Campero et al., 2018; Jenkins et al., 2005; Schares et al., 2000; von Blumröder et al., 2004). Immunoblotting is often recommended to confirm weakly or borderline positive results in ELISA (Álvarez-García et al., 2002; Staubli et al., 2006).

1.7.1.1.1 Indirect Fluorescent Antibody Test

The IFAT was the first serological test developed for the detection of antibodies to *N. caninum* (Dubey et al., 1988b). In the IFAT culture-derived *N. caninum* tachyzoites are typically air-dried or fixed to microscopic slides (Buxton et al., 1997; Conrad et al., 1993b; Schares et al., 1998). It mainly detects antibodies directed to antigens present on the tachyzoite surface, because intact tachyzoites are used as antigen (Björkman and Uggla, 1999). Performance of the test requires training and experience and the result depends to a certain degree on the reader's interpretation (Björkman and Uggla, 1999; Ortega-Mora et al., 2006). When bright and unbroken tachyzoite membrane fluorescence is observed, the IFAT is regarded as positive (Paré et al., 1995a). A partial (only "polar" or "cap") fluorescence is considered a non-specific reaction and thus it should be regarded as a negative result (Paré et al., 1995a).

The cut-off titre in IFAT differs between laboratories from 1:100 (Dubey et al., 1997) to 1:640 (Conrad et al., 1993b; McNamee et al., 1996) for adult bovines and from 1:16 to 1:80 for foetal serology (Álvarez-García et al., 2003). In a comparative study of different serological techniques frequently used in Europe, a cut-off of 1:200 demonstrated high sensitivity and specificity for adult bovines (von Blumröder et al., 2004). The IFAT outcome may be influenced by the reader, the fluorescence microscope, the protocol, and the reagents used, so there is no general cut-off recommended (Björkman and Uggla, 1999; Dubey et al., 2017a). Given the above mentioned differences among laboratories, direct comparisons of IFAT results are very difficult (Björkman and Uggla, 1999; Haddad et al., 2005). IFAT is time-consuming and expensive compared with ELISA, and requires specialized equipment (e.g., fluorescence microscope). Therefore, is not routinely used for the detection of antibodies to *N. caninum* in cattle (Haddad et al., 2005).

1.7.1.1.2 Direct Agglutination Test

In the DAT intact tachyzoites agglutinate in the presence of specific IgG antibodies (Ortega-Mora et al., 2006). Therefore, species-specific antibodies are not needed, which makes the test suitable for studies in wildlife (Almería, 2013; Dubey and Thulliez, 2005). DATs developed for the detection of antibodies to *N. caninum* (Packham et al., 1998; Romand et al., 1998) were called *Neospora* agglutination test (NAT). In the test, whole fixed tachyzoites are used as antigen and sera are treated with 2-mercaptoethanol to remove nonspecific IgM or IgM-like substances (Desmonts and Remington, 1980). The 2-mercaptoethanol destroys both specific and non-specific IgM antibodies, therefore the NAT detects only IgG antibodies (Romand et al., 1998). Although being a simple technique, NAT has a low sensitivity (65-66%) as compared to other serological techniques used to detect specific antibodies to *N. caninum* in cattle (Ghalmi et al., 2009; Wapenaar et al., 2007).

1.7.1.1.3 Enzyme-Linked Immunosorbent Assays

Many serological tests used for antibody detection utilize culture-derived *N. caninum* tachyzoites of either bovine or canine *N. caninum* isolates as source of antigen (Ortega-Mora et al., 2006). Most indirect ELISAs are based on antigen lysates of whole

tachyzoites prepared by detergent solubilization or sonification (Alvarez-García et al., 2003; Osawa et al., 1998; Paré et al., 1995; Wouda et al., 1998a). To enrich antigen preparations with specific antigens native tachyzoite antigens purified by immunoaffinity (Schares et al., 2000) and tachyzoite antigens incorporated into immune stimulating complexes (ISCOMs) (Björkman et al., 1997) have been used in ELISA. Recombinant antigens have also been described as sources of antigen for ELISA (reviewed by Dubey et al., 2017a). Particularly, two ELISAs using recombinant NcGRA7 and NcSAG4 proteins as source of antigen for the detection of antibodies during acute infection (tachyzoite replication), and chronic infection (bradyzoites), respectively were developed (Aguado-Martínez et al., 2008). The presence of antibodies in serum against both recombinant proteins may indicate reactivation of infection (Aguado-Martínez et al., 2008). Competitive inhibition ELISAs (cELISAs) using monoclonal or polyclonal antibodies can detect antibodies to specific epitopes (Baszler et al., 2001, 1996; Novoa et al., 2020; Sinnott et al., 2015). These ELISAs have the advantage that they can be applied to different host species (McGarry et al., 2000). However, subsequent validation studies are needed to determine their diagnostic performance for each host species (Dubey et al., 2017a).

ELISAs have the advantage that the reaction can be determined objectively by quantification of the colour development using an ELISA reader (Dubey et al., 2017a). It can be easily automated and allows the examination of a large number of samples on a single ELISA plate (Ortega-Mora et al., 2006). Since it requires a plate reader to analyse the reactions, this technique has limited use in the field or in poorly equipped laboratories (Ghalmi et al., 2014).

Milk of infected cattle contains specific IgG antibodies to *N. caninum* and is suitable for the detection of infection in cows (Moskwa et al., 2003). Serum in-house (Björkman et al., 1997; Chatziprodromidou and Apostolou, 2018; Schares et al., 2005a) and commercial (Byrem et al., 2012; Enachescu et al., 2014; González-Warleta et al., 2011; Schares et al., 2004a; Walsh et al., 2013) ELISAs were adapted for the examination of individual milk samples. The ISCOM ELISA and the native p38-ELISA showed a high level of agreement (characterised by *kappa (*κ) values) between individual serum and milk results (Björkman et al., 1997; Schares et al., 2005a). However, agreement between milk and serum samples, as characterized by *κ* values, may vary between 0.52 and 0.94, depending on the ELISA test (Dubey et al., 2017b). Milk is easier to collect than blood, and milk sampling is non-invasive (Björkman et al., 1997; Ortega-Mora et al., 2006). Furthermore, ELISAs validated for bulk milk testing may constitute an inexpensive tool for monitoring seroprevalence in dairy herds (Bartels et al., 2005; Chanlun et al., 2002; Enachescu et al., 2014; Frössling et al., 2006; González-Warleta et al., 2011; Schares et al., 2004a; Varcasia et al., 2006). Bulk milk seems to be suitable to detect a 15% or higher intra-herd seroprevalence of *N. caninum* in lactating cows (Bartels et al., 2005; Enachescu et al., 2014).

Trademark	Antigen preparation	Type	References
BIOVET BOVICHEK *N. caninum*	Sonicate lysate of tachyzoites	iELISA[b]	Paré et al. (1995b) Wu et al. (2002) Waldner et al. (2004) Wapenaar et al. (2007) Alvarez-García et al. (2013)
Bio-X Diagnostics MonoScreen AbELISA *N. caninum* BIO K 192	NcSRS2 purified protein captured by a monoclonal antibody	iELISA	Ghalmi et al. (2009) Alvarez-García et al. (2013) Roelandt et al. (2015)
Bio-X Diagnostics MonoScreen AbELISA *N. caninum* BIO K 451	*N. caninum* protein captured by a monoclonal antibody	iELISA	—
IDEXX *Neospora* Ab test (former Dr. Bommeli test)	Detergent lysate of tachyzoites	iELISA	Paré et al. (1995b) Von Blumröder et al. (2004) Alvarez-García et al. (2013)
IDEXX *Neospora* X2 Ab test	Sonicate lysate of tachyzoites	iELISA	Paré et al. (1995b) Wouda et al. (1998a) Schares et al. (1999a) Wu et al. (2002) Von Blumröder et al. (2004) Wapenaar et al. (2007) Alvarez-García et al. (2013)
IDVET ID Screen *N. caninum* competition	Sonicate lysate of tachyzoites	cELISA[c]	—
IDVET ID Screen *N. caninum* indirect	Sonicate lysate of tachyzoites	iELISA	Alvarez-García et al. (2013)
INGENASA INgezim *Neospora* 3.0	—[a]	iELISA	—
SVANOVIR *N. caninum* ISCOM ELISA	Tachyzoite proteins incorporated into immunostimulating complexes (ISCOMs)	iELISA	Björkman et al. (1997) Frössling et al. (2003) Frössling et al. (2006) Alvarez-García et al. (2013)
VMRD *N. caninum* antibody test cELISA	Surface protein antigen (GP65) captured by a monoclonal antibody	cELISA	Baszler et al. (1996) Baszler et al. (2001) Wapenaar et al. (2007) Alvarez-García et al. (2013)

Table 1. Characteristics of a number of ELISAs commercially available for serological diagnosis of *N. caninum* infection in cattle.

[a] no information.

[b] indirect ELISA.

[c] competitive inhibition ELISA.

1.7.1.1.4 Immunoblots

Immunoblots are valuable in detecting specific antibodies to *N. caninum* by applying reduced (Barta and Dubey, 1992; Baszler et al., 1996; Harkins et al., 1998) or nonreduced antigens (Atkinson et al., 2000a; Barta and Dubey, 1992; Bjerkås et al., 1994; Paré et al., 1995b; Stenlund et al., 1997). Stronger reactions can be observed under nonreduced conditions (Barta and Dubey, 1992), which indicates that the epitopes involved in the *N. caninum* specific antibody response are mainly conformational (Dubey and Schares, 2006). IB has been used for the identification of immunodominant antigens (IDAs) of *N. caninum* (Atkinson et al., 2000b). The most commonly reported IDAs under nonreduced conditions have a relative molecular weight of around 17–19, 29–30, and 35–37 kDa

(Dubey et al., 2017a). The pattern of recognized antigens may be different depending on the stage of infection (Aguado-Martínez et al., 2005; Tomioka et al., 2003). One commonly used cut-off for a positive result is the identification of two or more of four tachyzoite IDAs (Schares et al., 1999c, 1999a). IB is an important tool to characterize reference panels of sera for the validation of new tests (Álvarez-García et al., 2003; Atkinson et al., 2000a; Schares et al., 2000, 1999c, 1999a) and as *a posteriori* test to confirm inconclusive results (Álvarez-García et al., 2002; Staubli et al., 2006). Due to being very time-consuming IB is not routinely used for screening herd sera (Ortega-Mora et al., 2006).

1.7.1.1.5 Avidity assays

Serological avidity tests rely on the fact that the avidity of specific IgG antibodies increases over time after primary infection, which to a certain extent allows to determine the time point and duration of infection (Björkman et al., 2005, 1999). In the avidity ELISAs an incubation step with urea (6M or 8M) is added, so the low affinity antibodies are eluted, but the high avidity antibodies remain bound to the antigen (Aguado-Martínez et al., 2005; Björkman et al., 1999; Sager et al., 2003; Schares et al., 2002a). The antibody titres obtained in the avidity test wells (with urea) and in the control wells (without urea) are used to calculate the IgG avidity index (Jenum et al., 1997). In the comparison of the IgG avidity ELISA tests used in four European laboratories a moderate agreement between the assays used by the different laboratories was observed (Björkman et al., 2006). An avidity Western blot has also been developed (Aguado-Martínez et al., 2005). In field studies, low avidity antibodies have been associated with recent infections, whereas high avidity antibodies are related to chronic infections (Björkman et al., 2003; Frössling et al., 2005; Jenkins et al., 2000; McAllister et al., 2000; Schares et al., 2002a). In naturally infected cattle, high avidity antibodies can be observed in individuals infected for more than 6 months (Björkman et al., 2003, 1999). Low avidity antibodies were found in a high percentage of aborting dams from herds with outbreaks of *N. caninum*-associated abortion, which suggests that a recent primary infection was the cause of the abortions (Basso et al., 2010; Björkman et al., 2003; Jenkins et al., 2000; McAllister et al., 2000; Schares et al., 2002a). Therefore, avidity tests are valuable tools, which may help to determine the predominant route of *N. caninum*-infections within the herd (Dubey and Schares, 2006). However, individual animals can maintain low avidity antibodies for years after infections, so caution should be taken when evaluating avidity antibody responses of individual animals (Björkman et al., 2003).

1.7.1.2 Other methodologies

Apart from serological techniques other diagnostic tools are available for the *in vivo* diagnosis of *N. caninum*, although they have only been used for research purposes (Ortega-Mora et al., 2006). A nested PCR which allows the detection of *N. caninum* DNA in the blood of seropositive cattle has been described (Ferre et al., 2005; Okeoma et al., 2004). *N. caninum* DNA can also be identified in the milk of lactating seropositive cows (Moskwa et al., 2003) and in the semen of bulls (Caetano-da-Silva et al., 2004; Ferre et al., 2005; Ortega-Mora et al., 2003).

1.7.2 Diagnosis in the aborted foetus

Preferably, in cases of abortion diagnostic samples should include the aborted foetus (one or more) and include the placenta (with cotyledons) and a serum sample from the aborting dam (Anderson et al., 2000; McAllister, 2016; Ortega-Mora et al., 2006). Although degenerative or inflammatory lesions may be found through foetal tissues, they are most common in the central nervous system (CNS), heart, skeletal muscle, liver, and placenta (Dubey et al., 2006; Dubey and Schares, 2006). Therefore, if it is not possible to submit the entire foetus, samples from the brain, heart, and liver should be submitted (Ortega-Mora et al., 2006). McAllister (2016) provided a list of suggested tissues to send for the diagnosis of bovine abortion. Foetal tissues should be collected as soon as possible to avoid autolysis (Ortega-Mora et al., 2006), though it may be possible to obtain an accurate diagnosis from autolyzed or mummified foetuses (McAllister, 2016). Aborted foetuses are often autolysed or mummified, but other gross lesions are rare in *N. caninum* abortions (Dubey et al., 2006). Minute pale to dark foci of necrosis and hydrocephalus may occur in the brain (Dubey et al., 1998; Fioretti et al., 2003). White linear foci may be observed in the skeletal muscles and heart (Anderson et al., 2000; Dubey and Lindsay, 1996). Focal areas of discolouration in placental cotyledons have also been described (Fioretti et al., 2003).

1.7.2.1 Histological examination

The routine histopathological examination of foetal tissue sections after staining with haematoxylin and eosin (H&E) may provide a presumptive diagnosis of protozoal infection (Anderson et al., 2000). As stated earlier, histopathological lesions characteristic of neosporosis can be most commonly found in foetal CNS, heart, skeletal muscle, and liver, but the brain is the most consistently affected (Dubey et al., 2006; Dubey and Lindsay, 1996). The most common observations are multifocal non-suppurative encephalitis, which is characterized by multifocal non-suppurative infiltration with or without occasional foci of necrosis, and multifocal non-suppurative myocarditis, characterized by focal infiltration of mononuclear cells with minimal necrosis (Anderson et al., 2000; Dubey et al., 2006; Jenkins et al., 2002). Other histological lesions diagnostically valuable are focal non-suppurative myositis and non-suppurative portal hepatitis, consisting of periportal infiltrations of mononuclear cells with focal hepatic necrosis, and focal non-suppurative interstitial pneumonia (Anderson et al., 2000; Barr et al., 1990; Wouda et al., 1997b). Periportal hepatitis may be more common and severe in epidemic abortion than in endemic *N. caninum*-associated abortion (Collantes-Fernández et al., 2006; Wouda et al., 1997b). The identification of *N. caninum* in tissues stained by H&E is often difficult (Dubey and Schares, 2006). The number of parasites found in bovine foetal tissues is usually low (Dubey et al., 2006), and tachyzoites are often dead or autolytic, being difficult to locate well-preserve tachyzoites (Dubey and Lindsay, 1996). Tissue cysts are also sparse and usually not associated with histological lesions (Wouda et al., 1997b). Therefore, a definitive diagnosis of neosporosis is often only possible by applying parasite detection techniques such as immunohistochemistry or PCR (van Maanen et al., 2004).

1.7.2.2 Immunohistological examination

Immunohistochemical staining to detect *N. caninum* in tissue sections was first performed by Lindsay and Dubey (1989b). As stated before, it is difficult to demonstrate *N. caninum* organisms in H&E-stained tissues and immunohistochemistry (IHC) using antibodies specific to *N. caninum* is an effective method to identify both tissue cysts and tachyzoites (Anderson et al., 2000). Both monoclonal (Cole et al., 1994; Razmi et al., 2010; Uzêda et al., 2013) and polyclonal antibodies (Lindsay and Dubey, 1989b; van Maanen et al., 2004) can be applied to detect *N. caninum* by IHC, but cross-reactivity of rabbit-derived polyclonal antibodies with *T. gondii* and *Sarcocystis* spp. has been reported (Uzêda et al., 2013; van Maanen et al., 2004). However, cross-reactivity of *N. caninum* antibodies with other related Apicomplexan parasites may not be a major concern because *T. gondii* and *Sarcocystis* spp. are rarely associated with bovine abortion (Dubey and Schares, 2006). The use of a single monoclonal antibody to detect *N. caninum* in IHC may result in low sensitivity as compared to IHC applying polyclonal antibodies, but a combination of several monoclonal antibodies was shown to have a synergic effect, which results in an analytical sensitivity similar to the rabbit-derived polyclonal antibodies (Uzêda et al., 2013).

N. caninum is more frequently described by IHC in the brain and heart than in other organs (Dubey and Schares, 2006; Wouda et al., 1997b). Due to the focal distribution of parasites in naturally-infected tissues, the ability to detect *N. caninum* by IHC depends on the number of sections analysed and time spent on microscopic examination (Ortega-Mora et al., 2006). Immunohistochemical staining was shown to be less sensitive than PCR in detecting *N. caninum* in foetal tissues, when monoclonal (Razmi et al., 2010; Reitt et al., 2007) or polyclonal antibodies are used (van Maanen et al., 2004).

1.7.2.3 PCR

PCR techniques can be used to amplify specific-*N. caninum* DNA sequences extracted from bovine fresh, frozen, formalin-fixed, or paraffin-embedded foetal tissues (Baszler et al., 1999; Ellis et al., 1999; Gottstein et al., 1998; Sager et al., 2001). However, the sensitivity of the technique is reduced in the formalin-fixed tissues (Dubey and Schares, 2006). PCR has the advantage of being highly sensitive and specific (Álvarez-García et al., 2007; Dubey et al., 2017b), but the presence of *N. caninum* DNA in a foetus is only indicative of infection and does not prove that the parasite caused the abortion (Jenkins et al., 2002). To conclude that *N. caninum* is the likely cause of abortion, apart from the detection of the parasite, the histological lesions should be typical of *N. caninum* and not compatible with life (Dubey and Schares, 2006).

In *N. caninum*-associated abortion foetal tissues are often autolytic, which may impair the diagnosis by routine histopathological examination and IHC (Dubey and Lindsay, 1996; Dubey and Schares, 2006). PCR techniques can be useful when foetuses are autolytic, and PCRs protocols based on the amplification of small fragments are believed to be less affected by autolytic processes (Dubey and Schares, 2006; Ellis et al., 1999; Ortega-Mora et al., 2006). Samples from the brain and placenta, followed by the heart, lung, and kidneys are considered optimal for PCR detection (Dubey et al., 2017b).

N. caninum DNA can also be amplified from foetal amniotic and allantoic fluids (Gibney et al., 2008; Ho et al., 1997; McInnes et al., 2006).

Several assays have been developed for the detection of *N. caninum* DNA from conventional endpoint PCRs (Holmdahl and Mattsson, 1996; Müller et al., 1996; Payne and Ellis, 1996) to quantitative PCRs (Collantes-Fernández et al., 2002; Liddell et al., 1999). In a study comparing PCR tests developed by different laboratories no association between the PCR format (single or nested) and diagnostic sensitivity was found (van Maanen et al., 2004). In the above-mentioned interlaboratory comparison, a moderate to good agreement, characterized by κ values, was observed between the different PCR tests. The DNA extraction procedure, and particularly the thorough homogenization of samples, was considered a critical step to maximize sensitivity, due to the focal distribution of the parasite in the CNS (van Maanen et al., 2004).

1.7.2.4 Foetal serology

In cattle, there is no transfer of antibodies from the dam to their foetus, because ruminants have a syndesmochorial placentation (Osburn et al., 1982). Thus, the presence of antibodies against *N. caninum* in foetal serum or body fluids is indicative of foetal infection (Björkman and Uggla, 1999). The bovine foetus is known to develop immunocompetence during the fifth month of gestation (Osburn et al., 1982). Detection of specific *N. caninum* antibodies in foetal sera and serosanguinous fluids can be accomplished by IFAT, ELISA, and IB (Barr et al., 1995; Conrad et al., 1993b; Otter et al., 1997; Slotved et al., 1999; Söndgen et al., 2001, 2001). Although foetal blood, pleural and peritoneal fluids, and abomasal content may contain specific antibodies to *N. caninum*, the peritoneal fluid is better than other body fluids for the serological diagnosis (Dubey, 2003). An appropriate cut-off value, different from the cut-off applied for the detection of antibodies to *N. caninum* in adult cattle, should be employed (Álvarez-García et al., 2003). The IFAT cut-off titre for foetal serology may vary from 1:16 to 1:80 (Álvarez-García et al., 2003).

Foetal serology is considered to have low sensitivity when IFAT or ELISA tests are applied (Dubey and Schares, 2006; Jenkins et al., 2002). Immunoblotting increases the specificity and sensitivity of foetal serology (Álvarez-García et al., 2002; Söndgen et al., 2001), but is not often used in routine diagnosis. The low sensitivity of foetal serology may be due to low antibody titres in foetal fluids, or autolysis, which may cause degradation of foetal immunoglobulins (Wouda et al., 1997a). The antibody titre in the foetus's body fluids is influenced by the stage of gestation and foetal immunocompetence (Wouda et al., 1997a), the interval between infection and foetal death (Söndgen et al., 2001) and the level of exposure to parasite antigens (Dubey, 2003). Consequently, a negative result in foetal serology does not exclude the possibility of a *N. caninum* infection (Björkman and Uggla, 1999; Jenkins et al., 2002). On the other hand, the presence of antibodies against *N. caninum* does not prove the parasite infection caused the abortion (Dubey and Schares, 2006), because most *N. caninum*-infected calves are born clinically healthy (Paré et al., 1996).

1.8 Control of bovine neosporosis

After *N. caninum* has been identified as the causative agent of the reproductive problems in a specific farm, it is advisable to estimate the prevalence of *N*. *caninum* in the herd, followed by a cost–benefit analysis of the available control options (Conraths et al., 2007; Lindsay and Dubey, 2020). To reduce the costs of *N. caninum*-related problems different control options have been proposed. Due to regional differences, differences in seroprevalence, and associated risk factors, some may be more advisable than others in each specific situation (Dubey et al., 2007). Based on data from New Zealand and Australian dairies and using economical models, it has been suggested that when the levels of infection within the herd are low (below a threshold of 18–21%), the option to "live with the disease" may be optimal economically (Reichel and Ellis, 2006). To date, there is no chemotherapy available for bovine neosporosis and the only licensed vaccine was withdrawn from the market due to lack of efficiency to prevent *N. caninum*-associated abortion (Dubey et al., 2007; Dubey and Schares, 2011; Lindsay and Dubey, 2020). The main objective of control programmes in *N. caninum*-infected herds is to decrease vertical transmission by reducing the number of seropositive dams and/or decrease the risk of horizontal transmission by avoiding oocyst contamination (Dubey et al., 2007). Standard biosecurity measures are vital to diminish the prevalence of the infection or to avoid the introduction of infection in *N. caninum*-free herds (Benavides et al., 2014).

1.8.1 Managing endogenous transplacental transmission

"Testing and culling" *N. caninum* seropositive cows is a strategy that prevents transmission from dams to their progeny (Larson et al., 2004; Thurmond and Hietala, 1995) and should be considered when endogenous transplacental transmission is the main route of infection (Dubey et al., 2017b). However, culling is not economically viable when the prevalence of *N. caninum* infections is very high (Reichel and Ellis, 2009). Different 'test and cull' strategies have been recommended and effectively employed in the field: (1) test and cull seropositive dams or seropositive dams that fail to calve; (2) test and inseminate the daughters of seropositive dams with beef bull semen only; and (3) test and exclude the daughters of seropositive dams as potential replacements (Conraths et al., 2007; Dubey et al., 2007; Hall et al., 2005; Larson et al., 2004). These measures when combined with actions to avoid re-infection can effectively reduce the prevalence of *N. caninum* within the herd and consequently the risk of abortion (Frössling et al., 2005). Testing for *N. caninum* antibodies before the introduction of purchased cattle is also an important measure, especially in *N. caninum*-free closed herds (Dubey et al., 2007; Reichel et al., 2014). The appropriate cut-off of the serological test should be selected to maximize sensitivity, in order to identify all *N. caninum*-infected cattle (Reichel and Ellis, 2002).

In areas with a high prevalence of *N. caninum* control programmes need to overcome problems such as the inadequate number of seronegative cows available for breeding replacements, reducing selection by genetic merit and a potential increase in replacement costs (Eiras et al., 2011)

Embryo transfer has been shown to effectively prevent endogenous transplacental transmission of *N. caninum* (Baillargeon et al., 2001). Embryos from seropositive cows can be successfully protected from congenital transmission by transfer to seronegative recipient dams (Landmann et al., 2002). Only uninfected seronegative dams should be selected as recipients since congenital infection and abortion are possible if recipient cows are seropositive (de Oliveira et al., 2010). This technique is expensive and may thus be reserved for embryos from genetically valuable seropositive cows (Reichel and Ellis, 2002).

Artificial insemination of seropositive dairy cows with semen from beef bulls reduces the rate of *N. caninum*-associated abortion (López-Gatius et al., 2005a,b; Yániz et al., 2010). Crossbreeding may have a beneficial effect on placental function which might reduce abortion risk (Almería et al., 2009a). The risk of abortion due to *N. caninum* is reduced in crossbreed pregnancies, especially when Limousine breed semen is used (Almería et al., 2009a; Yániz et al., 2010).

1.8.2 Prevention or reduction of exogenous transmission

To prevent exogenous transmission, it is crucial to prevent exposure to potentially infected definitive hosts, and particularly, exposure to feed and water contaminated with oocysts (Dubey et al., 2007; Reichel et al., 2014). Domestic dogs and other canids that can act as definitive hosts should be prevented from defecating in cattle barns, feed, and pasture (Lindsay and Dubey, 2020). Feedstuffs used in mixed rations can be protected by fences or maintained in bins, silos, or behind closed doors (McAllister, 2016). Measures to prevent domestic dogs and other canids from ingesting tissues of intermediary hosts (such as aborted foetuses, placentas, brain, and other potentially infected tissues) should also be implemented (Cavalcante et al., 2011; Dijkstra et al., 2001b; Reichel et al., 2014). Bovine carcasses and all potentially-infected tissues should be safely disposed (Dubey et al., 2007). Removal of all dogs from the farm may be considered to prevent postnatal infections, but in some situations the presence of dogs may be important to reduce the number of other wild canids (Gondim et al., 2004b; McAllister, 2016; Rosypal and Lindsay, 2005). Stray dogs should also be controlled (Schares et al., 2004b). Puppies can shed more oocysts after infection than adult dogs (Gondim et al., 2005), so it is advisable to avoid pregnant bitches or raising pups near pregnant cattle (Reichel et al., 2014).

Although the importance of rodents and poultry as sources for intermediary host infection has not been established, control of rodents and poultry may be important to reduce dogs' infection risk (Dubey et al., 2007; Jenkins et al., 2007; Reichel et al., 2014).

1.9 Monoclonal antibodies (mAbs) and Hybridoma cells

A monoclonal antibody (mAb) consists of an antibody with unique specificity originated from a single B-cell clone and is specific for a single epitope (Nelson, 2000). Because these antibodies bind to a single antigenic epitope, they usually have high specificity (Hnasko and Stanker, 2015). These mAbs can potentially be used for biomedical research, diagnosis, and biotreatment (Ossipow and Fischer, 2014). MAbs consist of a homogenous pool of antibodies (unlike polyclonal antibodies) with similar proprieties regarding binding affinity, isotype, and epitope (Hnasko and Stanker, 2015).

Milstein and Kohler (1975) were the first to develop a method for the scale production of mAbs *in vivo*, developing the "hybridoma" cell technology. In 1984, they were awarded the Nobel Prize in Physiology or Medicine for their work (Bayer, 2019). The fusion of lymphoblasts from an immunized mouse with mouse myeloma cells (the basic concept of hybridoma) allows the production of virtually unlimited quantities of mAbs (Ossipow and Fischer, 2014), which are a continuous renewable resource (Hnasko and Stanker, 2015). More recently, other cloning techniques have been developed for the production of monoclonal antibodies (Ossipow and Fischer, 2014).

1.9.1 Productions of hybridomas

MAbs were previously produced in the Institute for Epidemiology, Friedrich Loeffler-Institut (FLI) Germany, as described by Schares et al. (1999a) and Aguado-Martínez et al. (2010). BALB/c mice were immunized by an intraperitoneal injection of 10^4 freshly isolated *N. caninum* tachyzoites on day 0. To obtain mAbs 9/12-12 and 8/1-10, mice were immunized with the recombinant proteins rNcGRA7 and rNcSAG4, respectively (Aguado-Martínez et al., 2010). On day 180 (3 days before fusion), the mice were boosted with 10^5 freshly isolated *N. caninum* tachyzoites by subcutaneous injection. Mice immunized with recombinant proteins rNcGRA7 and rNcSAG4 were instead boosted for three consecutive days prior to fusion with 50 ng of the recombinant proteins by intravenous injection in the tail vein. Boosting intended to increase the polyclonal response, stimulate immunoglobulin class switching and generate higher-affinity antibodies (Nelson, 2000). After the last immunization, the lymphoblasts from BALB/c mice were fused with Sp2/0 myeloma cells. The latter were chosen because of their deficiency in the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT), which allows the selection of fused hybridomas and the removal of unfused myeloma cells (which could compete with the fused hybridomas), using a selective medium containing hypoxanthine, aminopterin, and thymidine (HAT). This selection is based on the fact that aminopterin blocks the "de novo" pathway of nucleotide biosynthesis, and because the unfused myeloma cells do not have the HGPRT enzyme that allows an alternative pathway for purine biosynthesis, only successfully fused myelomas will survive (because the salvage pathway is provided by B-splenocytes) (Nelson, 2000; Ossipow and Fischer, 2014). The supernatant of hybridoma cells was screened by immunofluorescence and immunoblotting. Positive hybridomas were cloned by limiting dilution and re-cloned at least twice to reach and confirm clonicity (Figure 3).

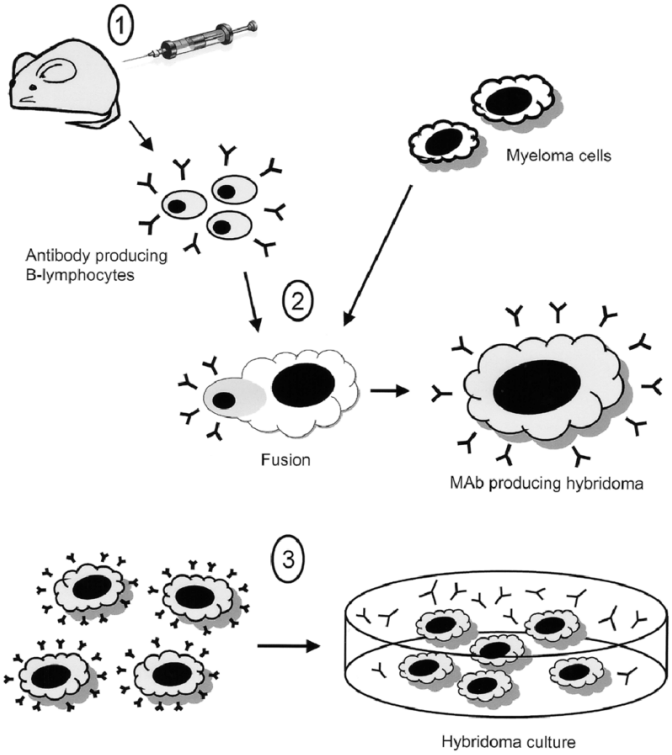

Figure 3. Schematic representation of the hybridoma cell technique used to obtain monoclonal antibodies (mAbs). (1) Immunization of mice with N. *caninum* tachyzoites to stimulate the development of antibody-producing B- cells. (2) Antibody-producing B-lymphocytes are isolated from the spleen and fused with myeloma cells to create an immortal cell line (hybridoma). (3) Hybridomas producing identical antibodies (monoclonal antibodies) are grown in culture. Adapted from Koppe et al. (2005).

1.9.2 Characterization of the mAbs

The mAbs produced by the hybridoma cells were characterized by their immunoglobulin class, using a commercial isotyping kit (Serotec, Oxford, UK) and by their reactivity against *N. caninum* antigen using immunofluorescence, immunoprecipitation of surface-biotinylated antigens and immunoelectron microscopy (Table 2).

MAb designation	Localisation of antigen (additional information)	Stage specificity	Isotype	Reference
3.10.5	Surface	Tachyzoite	IgG_{2a}	Schares, unpublished
4.15.15	Surface (SRS2)	Tachyzoite	IgG_{2a}	Schares, 2000
5.2.9	Surface	Tachyzoite	IgG_1	Schares, unpublished
5.2.15	Surface	Tachyzoite	IgG_1	Schares, 1999
4.11.5	Dense granules	Tachyzoite	IgG_{2a}	Schares, 1999
9/12-12	Dense granules (GRA7)	Tachyzoite	IgG_{2a}	Aguado-Martínez, 2010
4.7.12	Surface	Tachyzoite	IgG_2	Schares, 1999
8/1-10	Surface (SAG4)	Bradyzoite	IgG_1	Aguado-Martínez, 2010

Table 2. Characterization of the eight mAbs obtained by hybridoma cell techniques regarding clone type (heavy chain class), antigen localization, and stage specificity determined by immunofluorescence, immunoprecipitation of surface-biotinylated antigens, and immunoelectron microscopy.

In total, eight mAbs were analyzed in this study for their potential to establish a *N. caninum*-competitive ELISA. All mAbs were directed against *N. caninum* tachyzoite antigens, except mAb 8/1-10, which was generated against a bradyzoite-specific antigen (SAG4). Five (mAbs 3.10.5, 4.7.12, 4.15.15, 5.2.9, and 5.2.15) reacted against the tachyzoite surface and two (4.11.5 and 9/12-12) showed a dense granule pattern of staining in immunofluorescence. Four of the mAbs were IgG_{2a} and three were IgG_1 (Table 2).

2. Materials and Methods

2.1 Parasite culture

The NC-1 strain of *N. caninum* (Dubey et al., 1988b), the RH strain of *T. gondii* (Sabin, 1941) and the BbEvora03 strain of *Besnoitia besnoiti* (Cortes et al., 2006) were maintained in cell culture of MARC-145 cells and Dulbecco's Modified Eagle's Medium (DMEM) containing 5% foetal bovine serum (FBS) and 1% antibiotic (Penicillin and Streptomycin - Life Technologies, New York, USA) at 37°C/5% CO_2. Harvesting was performed according to a previously described protocol (Dubey et al., 2017d; Schares et al., 1999c).

One day before harvesting, the culture medium supplemented with FBS was replaced with a DMEM medium free of FBS. This procedure reduces false-positive reactions in serological assays since commercial FBS frequently contains antibodies to *N. caninum* (Dubey and Schares, 2006; Torres and Ortega, 2006). The rupture of the host cell monolayer was enhanced by scraping off the cells with a plastic cell scraper and vigorous pipetting, releasing the tachyzoites. The tachyzoites were then filtered through a 5 µm filter (Sartorius Lab Instruments, Göttingen, Germany) to remove cell culture proteins and debris and washed with cold phosphate-buffered saline (PBS, pH 7,2) five times by centrifugation (2000 × *g*, 10 min, 4°C, no brake). After each wash, the supernatant was discarded, and the cells were resuspended in PBS. After the last wash, tachyzoites were counted using a Neubauer chamber (NanoEntek, Seoul, Korea) and used immediately (to prepare IFAT slides) or stored as pellets at -80°C until used for immunoblotting or for ELISA antigen preparation.

2.2 Monoclonal antibody production

Hybridoma cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Illinois, USA) supplemented with 1% vitamin solution (Biochrome, Berlin, Germany), 2% non-essential amino acids solution (Biochrome, Berlin, Germany), and 5% FBS, and split at least twice a week. The supernatant of the cultures was collected using a 0.2 µm filter (Sartorius Lab Instruments, Göttingen, Germany) and stored at 8°C until use.

2.3 SDS-PAGE and Immunoblotting

In this study, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques were used to evaluate the reactivity of the *N. caninum* mAbs against *N. caninum* antigens and to identify possible cross-reactions with antigens from other Apicomplexa-related parasites (*B. besnoiti* and *T. gondii*). The same techniques were also employed to test field bovine sera to validate the cELISA.

The technique consists of the separation of tachyzoite proteins by SDS-PAGE, followed by the transfer of the proteins to polyvinylidene fluoride (PVDF) membranes, which bind in the same pattern as that formed in the gel (i.e., forming a replica of the original gel pattern) (Björkman et al., 2007; Gordon et al., 1985). The remaining unoccupied binding sites are blocked with a non-immunoreacting substance, preventing non-specific binding of reagents to the membrane. This procedure is also relevant for promoting renaturation of antigenic sites (Towbin and Gordon, 1984).

For immunodetection, the blotted membranes are probed with test sera (or hybridoma supernatant) and subsequently incubated with anti-species conjugates (ex. anti-bovine or anti-mouse), and the reactions are visualized using a substrate (Björkman et al., 2007; Gordon et al., 1985).

The protocol followed is based on published protocols (Dubey et al., 2017d; Schares et al., 2001, 1999b).

2.3.1.1 Reagents and Materials for SDS-PAGE and antigen preparation

- *Sample buffer (non-reduced conditions)* (5x): 3.75 g tris(hydroxymethyl) aminomethane (Tris), 10 g sodium dodecyl sulfate (SDS), 35 mL glycerol, and 25 mg bromophenol blue; The pH was adjusted to 6.8 with HCl and distilled water was added up to 100 mL.
- *Sample buffer (reduced conditions)* (5x):3.75 g Tris, 10 g SDS, 35 mL glycerol, 25 mg β-mercaptoethanol (βME), and 25 mg bromophenol blue; The pH was adjusted to 6.8 with HCl and distilled water was added up to 100 mL.
- *Solution A:* 6.06 g Tris, 4 mL 10% (w/v) SDS. The pH was adjusted to 6.8 with HCl and 100 mL of distilled water was added.
- *Solution B:* 18.17 g Tris, 4 mL 10% (w/v) SDS. The pH was adjusted to 8.8 with HCl and 100 mL of distilled water was added.
- *Solution C:* 30 g acrylamide, 0.8 g N,N'-methylenbis(acrylamide); distilled water was added up to 100 mL.
- *Electrophoresis buffer (5x):* 15.2 g Tris, 72.1 g glycine, 5 g SDS, distilled water was added up to 1000 mL.
- Ammonium persulfate 40% (w/v) in distilled water (APS).
- N, N, N', N'-tetramethylethylenediamine (TEMED).
- Low molecular weight marker (Protein Test Mixture 6 for SDS PAGE; Serva Electrophoresis GmbH, Heidelberg, Germany) containing:
    - Phosphorylase B 97.4 kD (Mr)
    - Albumin bovine (BSA) 67.0 kD (Mr)
    - Albumin egg 45.0 kD (Mr)
    - Carbonic anhydrase 29.0 kD (Mr)
    - Trypsin inhibitor (soybean) 21.0 kD (Mr)
    - Cytochrome C 12.5 kD (Mr)
    - Trypsin inhibitor (bovine lung) 6.5 kD (Mr)
- Tachyzoite pellets were obtained from cell culture, as previously described in section 2.1.
- Mini-gel SDS-PAGE system (Bio-Rad Laboratories, California, USA).
- Block Heater (Eppendorf, Hamburg, Germany).

2.3.1.2 Buffers and Materials for antigen transfer to PVDF membranes

- *Stock transfer buffer* (10× stock; 25 mM Tris base, 192 mM glycine): Tris—30.3 g, glycine—144.1 g; distilled water, was added up to 1000 mL.
- *Transfer buffer* (1×; with 0.1% [w/v] SDS and 10% [v/v] methanol): 100 mL stock transfer buffer (10×), 10 mL 10% (w/v) SDS, 100 mL methanol, and distilled water was added up to 1000 mL.

- Methanol.
- India ink staining solution (Hancock and Tsang, 1983).
- PBS-T-G: PBS with 0.05% (v/v) Tween 20 and 2% (v/v) liquid fish gelatin (Serva, Heidelberg, Germany).
- Polyvinylidenfluorid (PVDF) membranes (Immobilon-P, Millipore, Darmstadt, Germany).
- Filter paper (Hahnemühle FineArt, Dassel, Germany).
- Electric transfer system (Trans-Blot Turbo Transfer System Bio-Rad, California, USA).

2.3.1.3 Buffers for Immunoblot

- TBS (Tris — 6 g, NaCl—8,7 g, double distilled was added to 1000 mL), and the pH was adjusted to 7.4 with HCl.
- TBS-Tween (TBS-T): TBS with 0.05% (v/v) Tween 20.
- PBS-T-G: PBS with 0.05% (v/v) Tween 20, 2% (v/v) liquid fish gelatin (Serva, Heidelberg, Germany).
- *Conjugate:* Peroxidase-conjugated rabbit anti-bovine IgG [H+L] and peroxidase-conjugated rabbit anti-mouse IgG/IgM [H+L] (Dianova, Hamburg, Germany) were used for testing cattle or hybridoma supernatant, respectively.
- *Substrate:* 30 mg of 4-chloro-1-naphthol (Sigma-Aldrich, USA), 10 mL of methanol, 40 mL of TBS, and 50 µL of 30% H_2O_2.

2.3.2 Execution

The initial step is to prepare the SDS-polyacrylamide separation gel solution, which was prepared according to Table 3 with a concentration of 12.5% (w/v). The gel was added between the glasses, and distilled water was added to overlay the gel. The gel was left to stand until polymerization was completed (approximately 30 minutes to one hour). After this period, the stacking gel was prepared (according to Table 3) and added on top of the separation gel, a comb was then carefully inserted when the stacking gel was still liquid. The comb was removed after 15-30 min (when the stacking gel was polymerized).

	Separation Gel, 12.5% (w/v)	**Stacking Gel, 2% (w/v)**
Solution A	—	1.5 mL
Solution B	2.5 mL	—
Solution C	4.2 mL	0.7 mL
Distilled Water	3.3 mL	3.6 mL
APS	25 µl	8 µl
TEMED	10 µl	16 µl

Table 3. Composition of Separation and Stacking Gels for SDS-PAGE. The volume of separation gel is sufficient for 2 minigels (60 × 70 × 1 mm) and the volume of stacking gel is sufficient for 4 minigels.

For the production of each minigel (60 × 70 × 1 mm), tachyzoite pellets obtained from cell culture were diluted in 80 µL of distilled water and 20 µL of sample buffer (5x) and heated for 10 min at 94°C. For both the reducing and non-reducing conditions, samples

containing 8×10^7 *N. caninum* tachyzoites, 8×10^6 *B. besnoiti* tachyzoites, and 2×10^8 *T. gondii* tachyzoites were used (Table 4).

The gel was placed inside an electrophoresis chamber (Bio-Rad, California, USA), which was then filled with 1x electrophoresis buffer. The antigen (100 µL) was loaded into the preparative slot (Table 4), and the low molecular weight standard was loaded into the narrow slot (5 µL). Electrophoresis was performed at 120 V, until the bromophenol front left the gel.

Western blotting procedure:

The separated antigens were transferred using an electric transfer system, and the PVDF membranes were activated in methanol and placed in 1x transfer buffer. The transfer unit was assembled with three filters, one PVDF membrane, one gel, and another three filters, avoiding air bubbles between them. Semi-dry electro-blot was performed at 25 V/gel for 30 min.

Two strips were then cut from the membrane, one containing the molecular weight marker and a small part of the antigen section, and stained with India ink solution (Hancock and Tsang, 1983). The unoccupied protein-binding sites of the blotted membranes were blocked by incubation at room temperature for 30 min in PBS-T-G. The membranes were left to dry at room temperature and stored at -20°C until used.

Antibody detection procedure:

Stripes 3-4 mm wide were cut from the blotted PVDF membranes and incubated in PBS-T-G for 10 min. To assess the reactivity of mAbs against antigens blotted onto PVDF membranes, stripes were incubated with undiluted hybridoma supernatant for one hour. The controls used when hybridoma supernatant was tested are listed in Table 4. To test unknown field bovine sera, stripes were probed with test sera diluted in PBS-T-G (1:100) along with a negative control bovine serum (1:100 dilution) and a positive control (1:50 dilution). The negative control serum was derived from a cow with an immunoblot (1:100 dilution) and IFAT (titre 1:<25) negative result, and the positive control from Heifer 44 (Schares et al., 1999c), an experimentally infected animal. After five washing steps with TBS-T, stripes were incubated with conjugate (1:500 dilution in TBS) for one hour. After three and two washing steps with TBS-T and TBS, respectively, the reactions were visualized by incubation of each stripe in the 4-chloro-1-naphtol substrate.

Antigen blotted into PVDF membranes	Concentration (tachyzoites/lane)	Positive control (dilution)	Negative Control (dilution)
N. caninum	8 x 10^7	*N. caninum* – experimentally infected mouse (1:100) *N. caninum* – experimentally infected bovine (1:100)	Hybridoma cell culture medium (undiluted)
T. gondii	2 x 10^8	*T. gondii* – experimentally infected mouse (1:10)	
B. besnoiti	8 x 10^6	*B. besnoiti* – positive immunized mouse (1:100) *B. besnoiti* – naturally infected bovine (1:100)	

Table 4. Description of the controls and concentration of antigen used to study the reactivity of mAbs against *N. caninum, T. gondii* and *B. besnoiti* antigens by immunoblotting.

2.3.3 Interpretation of immunoblot results

When bovine sera were tested for *N. caninum* antibodies prepared under non-reducing conditions, reactions against the five previously well-documented (Björkman et al., 2007; Dubey and Schares, 2006; Schares et al., 2001, 1999d, 1998; Staubli et al., 2006) immunodominant antigens (IDAs) were recorded. These IDAs have relative molecular weight of 17-19 kDa, 29 kDa, 30 kDa, 33 kDa, and 37 kDa.

Reactions against non-reduced antigens are stronger, given that the major epitopes of these IDAs are destroyed if the antigen is desaturated under reducing conditions (when βME is used in the SDS-PAGE sample buffer), diminishing the reactivity of bovine sera in the immunoblot. Thus, it is assumed that conformational epitopes are predominantly responsible for *N. caninum*-specific antibody response (Björkman et al., 2007). Fewer cross-reactions between sera of animals infected with *T. gondii* or *Sarcocystis* sp. were observed with non-reduced antigens than with reduced antigens (Dubey and Schares, 2006). For these reasons, only antigens prepared under non-reduced conditions were used to test bovine sera.

If two or more IDAs could be recognized, bovine serum samples were considered positive for the presence of specific *N. caninum* antibodies. If only one IDA was recognized, the test was considered inconclusive. When no reaction was recognized against the five IDAs, the samples were deemed negative (Schares et al., 1999a).

2.4 Immunofluorescence

The indirect fluorescent antibody test (IFAT) detects *N. caninum* antibodies contained in test sera that are able to bind to intact tachyzoites attached to microscope slides. After incubation with the test sera, secondary fluorescein-labelled anti-species antibodies are added, and the reactions are read under a fluorescence microscope.

This protocol has been previously described by Dubey et al. (2017d) and Schares et al. (1998).

2.4.1 Reagents and Materials

- PBS (NaCl—8 g, KH_2PO_4—0.2 g), Na_2HPO_4 12 H_2O—2.9 g, and KCl (0.2 g), and the pH was adjusted to 7.2 with HCl.
- 4 × *fluorescence assay rinse buffer* (FA): Na_2CO_3—11.4 g, $NaHCO_3$—33.6 g, NaCl—8.5 g, double-distilled was added to 1000 mL. The pH of the solution was adjusted to 9.0.
- Ethanol.
- Acetone.
- Evans Blue solution.
- *Conjugate:* Fluorescein isothiocyanate (FITC) dog anti-bovine IgG (Dianova, Hamburg, Germany).
- *Anti-fading-buffer:* 3.5 g 1,4 diazobicyclo [2,2,2]-octane in 90 mL glycerol and 10 mL PBS.
- Printed slides and large coverslips.
- Fluorescence microscope (Nikon Eclipse Ti-U, Nikon, Düsseldorf, Germany).

2.4.2 Execution

N. caninum tachyzoites were obtained from cell culture as described previously (section 2.1). The printed microscope slides were washed in 96% ethanol and left to dry. Then, 10 µL of the parasite suspension was added to each well, and the slides were air-dried. After this step, the slides were frozen at -20°C until used.

Slides were fixed with ice-cold acetone for 5 min and incubated in PBS for 10 min. After aspiration of excess fluid, serum samples diluted in PBS were added to the wells (10 µL). Based on past experience with this protocol, materials, and reagents (i.e., antigen preparation, anti-species conjugate, conjugate dilution, and fluorescence microscope), a dilution of 1:200 (Schares et al., 1999a) was selected as the cut-off. The same serum samples used as controls in the immunoblot technique were included in every ten slides. The slides were incubated for 30 min at 37°C in a moist chamber and subsequently rinsed with FA buffer, followed by incubation for 10 min with the same buffer. After a 10 min incubation in PBS, the excess fluid was removed and 10 µL of conjugate was added to each well and incubated for 30 min at 37°C in a moist chamber. The conjugate was diluted 1:50 in PBS and 0.05% Evans blue. Incubation with the FA buffer was repeated, followed by a 10 min incubation in PBS. The slides were dried and mounted with an anti-fading buffer and coverslips.

2.4.3 Interpretation of IFAT slides

Slides were visualized under a fluorescence microscope (Nikon Eclipse Ti-U, Nikon, Düsseldorf, Germany). A 100-200x magnification was used to interpret the results, and a higher magnification (400x) was used for confirmation, when necessary. A sample was considered positive only if bright continuous peripheral fluorescence of tachyzoites was present (Figure 4). A sole fluorescence of the apical part of the tachyzoite (“cap” or “polar” staining) was considered non-specific, because it can occur due to cross-reactions with other Apicomplexa parasites (Paré et al., 1995a). Since the results can be

influenced by the person who reads the test (Dubey et al., 2017b) the results were interpreted blinded and always by the author.

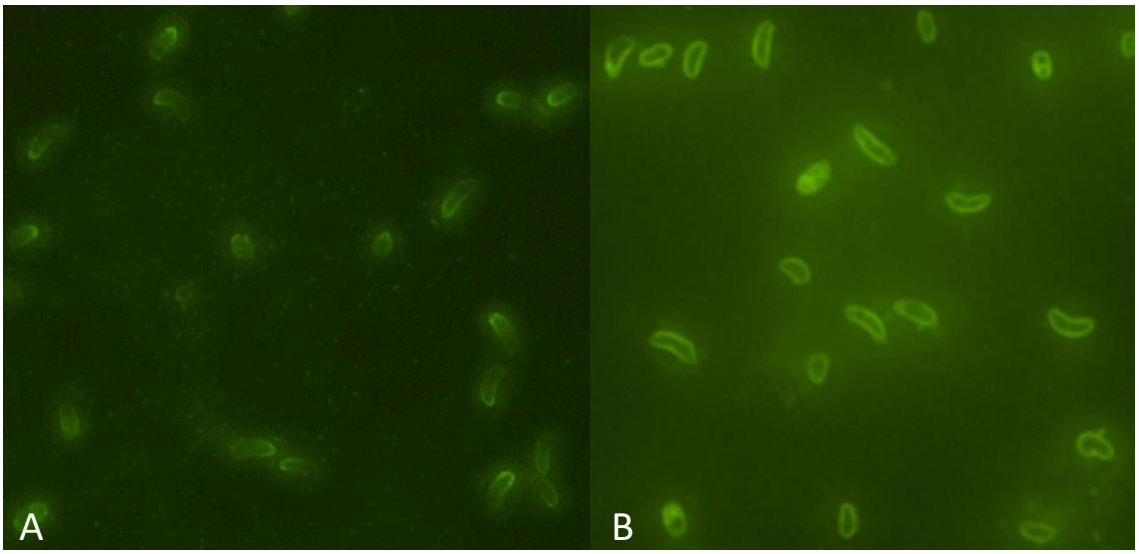

Figure 4. Immunofluorescence antibody test (IFAT) for anti-*N. caninum* IgG using air-dried, acetone-fixed tachyzoites, detecting antibodies to the tachyzoite surface (400× magnification). (A) Negative bovine serum (1:200 dilution) presenting apical (non-specific) fluorescence. (B) Positive bovine serum (1:200 dilution) presenting total (specific) peripheral fluorescence.

2.5 Establishing a mAb-based competitive ELISA

2.5.1 Checkerboard titrations by indirect ELISA

The enzyme-linked immunosorbent assay (ELISA) is a technique used to detect and quantify antigens or antibodies attached to a solid surface (Lin, 2015). In general terms, an enzyme-linked antibody binds to a surface-attached antigen and the subsequently added substrate is hydrolysed by the enzyme (Ausubel, 1987; Lin, 2015). The amount of substrate hydrolysed is determined with a spectrophotometer, which is correlated (proportional) to the amount of antibodies present in the sample (Ausubel, 1987).

This experiment aimed to determine the optimal assay conditions for establishing a *N. caninum* mAb-based competitive ELISA. Therefore, the first step was to determine the optimal concentration of *N. caninum* tachyzoite lysate (antigen preparation) used to coat ELISA plates by serial dilution of the antigen preparation. The second step was to establish a linear range of the ELISA titration curves for each mAb by serially diluting the hybridoma supernatant. The protocol used for the indirect ELISA was adapted from Dubey et al. (2017d).

2.5.1.1 Reagents, buffers and materials

- PBS (NaCl—8 g, KH_2PO_4—0.2 g, Na_2HPO_4 12 H_2O—2.9 g, KCl—0.2 g), pH was adjusted to 7.2 with HCl.
- *Coating buffer:* 100 mM sodium bicarbonate/carbonate, pH 8.3.
- *Washing buffer:* PBS-T:0.05% (v/v) Tween 20 in PBS.
- *Blocking solution:* PBS-T-G: PBS with 0.05% (v/v) Tween 20, 2% (v/v) liquid fish gelatin (Serva, Heidelberg, Germany), used as blocking, sample, and conjugate dilution buffers.

- *N. caninum* mAbs (hybridoma supernatant).
- *N. caninum* tachyzoites harvested from cell culture.
- Peroxidase-conjugated (Affinity Purified) Sheep Anti-Mouse IgG Fcγ Fragment Specific (Jackson ImmunoResearch Laboratories, West Grove, USA).
- *Substrate solution:*
  - *Substrate buffer*: 0.2 M sodium acetate / 0.2 M citric acid solution (247.5 ml + 2.5 ml + 250 ml distilled water)
  - Hydrogen peroxide (H_2O_2) 30%
  - Tetramethyl benzidine (TMB) (Sigma-Aldrich, Taufkirchen, Germany).
  - Preparation (per plate): 10 mL substrate buffer, 100 µL TMB, 1.2 µL H_2O_2, 30%.
- *Stop Solution:* 2 M Sulfuric acid (H_2SO_4).
- Nunc-Immuno™ MicroWell™ 96 well solid plates (Nunc-Immuno (Polisorb), Thermo Fisher Scientific, Langenselbold, Germany).
- ELISA reader (Tecan Sunrise, Tecan, Crailsheim, Germany).
- Ultrasonic Device (VibraCell, Bioblock Scientific, Germany).
- Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Illinois USA).

All solutions were freshly prepared, apart from the blocking and substrate buffers.

2.5.1.2 Preparation of the antigen

N. caninum tachyzoite pellets obtained as described above (cell-culture derived tachyzoites, chapter 2.1) were resuspended in PBS to a final concentration of 1×10^8 tachyzoites/mL and lysed by three freezing-thawing cycles (−50°C/+37°C) and subsequent sonification on ice (90 s, 50% active cycle, output control level 2). After sonification, the suspension was centrifuged at 10,000 × *g* at 4°C for 30 min. The supernatant was then collected, and the protein content (44.82 µg/mL) was determined using the Pierce™ BCA Protein Assay Kit, according to manufacturer instructions and with bovine serum albumin (BSA) as the standard. Aliquots of antigen preparation were stored at -80°C until use.

2.5.1.3 Execution of the ELISA assay

The *N. caninum* tachyzoite lysate (antigen) was serially diluted (two-fold) in coating buffer from 1:50 to 1:800 and used to sensitize a 96-well microtiter ELISA plate (100 µL per well) at 37°C for one hour. All dilutions were prepared in duplicates. The wells were washed three times with PBS-T and incubated with 300 µL per well of blocking solution (PBS-T-G) at 37°C for 30 min. The blocking solution (an immunologically inert substance) binds to free spaces on the plate surface (unbound sites) preventing non-specific binding (Crowther, 2009). The blocking solution was removed and 100 µL per well of undiluted hybridoma supernatant containing the *N. caninum* mAbs was applied. After a washing step (three-times with PBS-T) an anti-mouse peroxidase-conjugate diluted 1:500 (in blocking buffer) was added for 30 min at 37°C. The unbound conjugate was removed, and the wells were again washed tree-times with PBS-T and two times with distilled

water. Substrate solution was added (100 µl per well). The reaction was stopped after 15 min, by the addition of 2 M sulfuric acid, 50 µl/well. Optical density (OD) was measured at 450 nm on the ELISA microplate reader.

The 8/1-10 mAb, a specific bradyzoite antibody (anti-NcSAG4) (Aguado-Martínez et al., 2010), was used as the negative control. The mean OD value of each dilution were calculated, and the results are expressed in Figure 7.

After the antigen titration, the following experiment intended to determine the optimal dilutions of the hybridoma supernatants containing each of the mAbs. For this purpose, a 96-well microtiter ELISA plate was sensitized with the *N. caninum* tachyzoite lysate (antigen) diluted as previously determined (1:100). The rest of the protocol is similar to the one described above, with the exception that serial dilutions of the hybridoma supernatant were used (two-fold dilutions). After the measurement in the microplate reader, the mean OD values were calculated. These values were then expressed in a graph (Figure 8).

2.5.2 Establishment of competitive ELISAs

All mAbs were tested under the optimal conditions established previously by the indirect ELISA (iELISA) to study the effect of serial diluting a positive control on the inhibition characteristics of each mAb. This experiment thus allowed to establish the specific ELISA titration curves for each mAb, which was important to define which mAbs showed a sigmoidal 'dose-response' curve and could potentially be used to test larger sets of sera. The protocol is identical to the indirect ELISA protocol described above, with a few modifications. The workflow is represented in Figure 5.

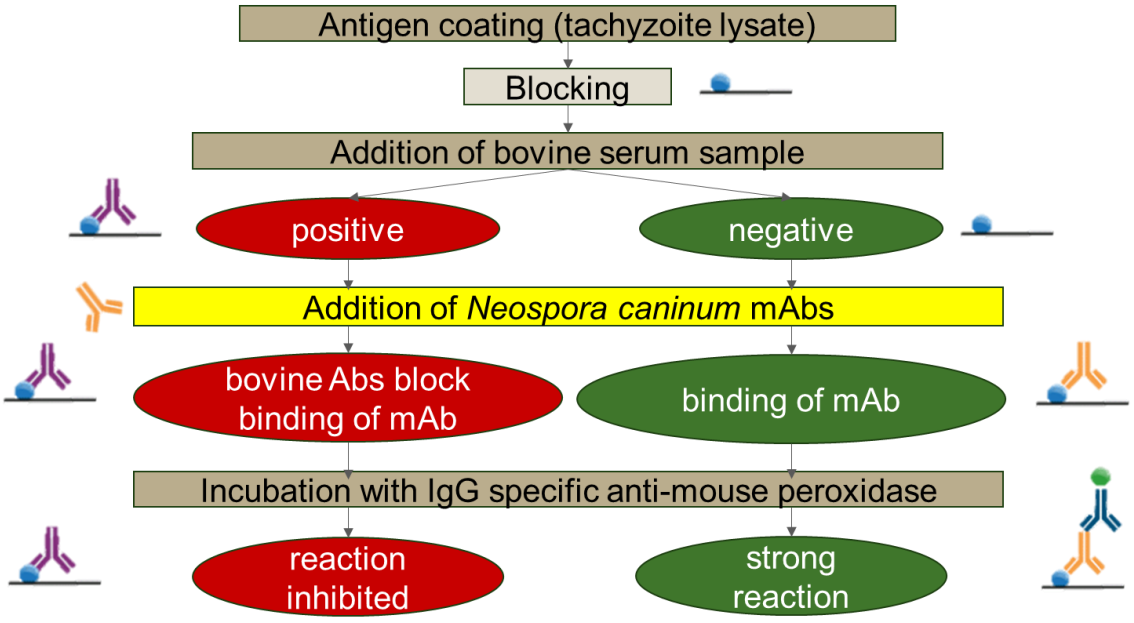

Figure 5. Schematic workflow of blocking ELISA using mouse monoclonal antibodies (mAbs) to detect specific reactivity of bovine antibodies (bovine Abs) against *N. caninum* antigen. Adapted from Schares et al. (2020) and https://m.mcpcourse.com/difference-between-competitive-and-noncompetitive-elisa/, accessed 12/12/2022.

In the competitive ELISA, antibodies present in bovine serum are detected by their capacity to block the binding of mAbs to their specific epitope: a higher titre of antibodies in a specific serum leads to a higher percentage of epitopes being blocked, so a weak signal is expected; whereas, the absence of antibodies in a serum sample leaves all epitopes free for the mAbs biding, leading to a strong signal after the incubation with specific anti-mouse IgG conjugated with a peroxidase enzyme and substrate (Figure 5).

2.5.2.1 Reagents, buffers and materials

- PBS (NaCl—8 g, KH_2PO_4—0.2 g, Na_2HPO_4 12 H_2O—2.9 g, KCl—0.2 g), pH was adjusted to 7.2 with HCl.
- *Coating buffer:* 100 mM sodium bicarbonate/carbonate, pH 8.3.
- *Washing buffer:* PBS-T:0.05% (v/v) Tween 20 in PBS.
- *Blocking solution:* PBS-T-G: PBS with 0.05% (v/v) Tween 20, 2% (v/v) liquid fish gelatin (Serva, Heidelberg, Germany), used as blocking, sample, and conjugate dilution buffers.
- *N. caninum* mAbs (hybridoma supernatant).
- *N. caninum* tachyzoites harvested from cell culture.
- *N. caninum* tachyzoite lysate (antigen), containing 44.82 µg/mL of protein.
- Peroxidase-conjugated (Affinity Purified) Sheep Anti-Mouse IgG Fcγ Fragment Specific (Jackson ImmunoResearch Laboratories, West Grove, USA).
- *Substrate solution:*
    - *Substrate buffer*: 0.2 M sodium acetate / 0.2M citric acid solution (247.5 ml + 2.5 ml + 250 ml distilled water)
    - Hydrogen peroxide (H_2O_2) 30%
    - Tetramethyl benzidine (TMB) (Sigma-Aldrich, Taufkirchen, Germany).
    - Preparation (per plate): 10 mL substrate buffer, 100 µL TMB, 1.2 µL H_2O_2, 30%
- *Stop Solution:* 2 M Sulfuric acid (H_2SO_4).
- Nunc-Immuno™ MicroWell™ 96 well solid plates (Nunc-Immuno (Polisorb), Thermo Fisher Scientific, Langenselbold, Germany).
- ELISA reader (Tecan Sunrise, Tecan, Crailsheim, Germany).

2.5.2.2 Execution of the ELISA assay

A 1:100 dilution in coating buffer of the antigen preparation was used to sensitize 96-well microtiter ELISA plates (100 µL per well) at 37°C for one hour. The wells were washed three times with PBS-T and incubated with a blocking solution (300 µL per well) at 37°C for 30 min. The blocking solution was removed and the serially diluted positive control (two-fold, from 1:2 up to 1:2048) and negative (1:2 dilution) controls were added (100 µL per well). The controls were diluted in blocking buffer (PBS-T-G) and all dilutions were prepared in quadruplicate. The negative control (NC) serum was obtained from a cow with an immunoblot (1:100 dilution) and IFAT (titre 1:<25) negative result. A post-

infection serum from heifer 44 (Schares et al., 1999c) was used as the positive control (PC).

The wells were washed three-times with PBS-T and 100 µL of hybridoma supernatant containing the *N. caninum* mAbs was added per well. MAbs were diluted in blocking buffer (as listed in section 3.2.1). The unbound antibodies were washed away (tree-times with PBS-T) and an anti-mouse peroxidase-conjugate diluted in blocking buffer (1:500) was added for 30 min at 37°C. The unbound conjugate was removed, and the wells were again washed tree-times with PBS-T and two-times with distilled water. Substrate solution was added (100 µl per well). The reaction was stopped after 15 min, by the addition of 2 M sulfuric acid, 50 µl per well. Optical density (OD) was measured at 450 nm on the ELISA microplate reader. The test results were interpreted by calculating the mean OD values and using those values to determine the Sample-to-Control-Inhibition (SCI): SCI = $(1-((OD_{PC}-OD_{PC\ (1:2)}/(OD_{NC}-OD_{PC\ (1:2)}))))$ x 100. The values were plotted together to construct a graph (Figure 9).

2.6 Validation and characterization of the mAb-based ELISA

One of the *N. caninum* anti-tachyzoite surface mAbs (3.10.5) was selected to test field sera since no cross-reactivity was shown against tachyzoite antigens of *B. besnoiti* and *T. gondii* blotted into PVDF membranes (Table 9), demonstrated the highest reactivity against the *N. caninum* tachyzoite antigen (Figure 7 and Figure 8) and apparently the highest analytic sensitivity (Figure 9). Another anti-surface mAb (5.2.9) was also tested in a subset of sera, revealing similar results (not shown), so the experiments were followed with the 3.10.5 mAb.

The materials and the protocol used in this analysis were the same as described previously for the competitive ELISA, but this time field sera of unknown status were tested. A total of 645 bovine sera were used to determine the optimal cut-off and to estimate the diagnostic sensitivity and specificity of the novel cELISA test based on the 3.10.5 mouse monoclonal antibody (Table 5).

Serum from a cow with an immunoblot (1:100 dilution) and IFAT (titre 1:<25) *N. caninum*-negative result was used as a negative control (dilution 1:2). A post-infection serum from heifer 44 (Schares et al., 1999c) was used as a positive control (dilution 1:2). The same serum in a different dilution (1:30) was also used as a weak positive control. A post-infection *N. caninum*-positive serum from cow 24 (Schares et al., 1999c) was included as a positive control (1:2 dilution). All dilutions of controls were prepared in quadruplicate. The test results were interpreted as described before, by calculating SCI: SCI = $(1-((OD_{Sample}-OD_{PC1\ (1:2)}\ /\ (OD_{NC}-OD_{PC1\ (1:2)}))))$ x 100.

2.6.1 Serological reference tests and reference panel

A set of sera (Table 5) was analysed and characterized by two previously established and validated in-house assays: p38-ELISA (Schares et al., 2000), p38-avidity-ELISA (Schares et al., 2002a), both based on the NcSRS2. The latter was used to differentiate animals regarding the stage of *N. caninum* infection as acute (low avidity) or chronically infected (high avidity). Both, p38-ELISA and p38-avidity-ELISA showed an optimal

performance in interlaboratory comparisons (Björkman et al., 2006; von Blumröder et al., 2004). Immunoblot and IFAT, performed as described in the previous sections (2.3 and 2.4), were also used to further characterize the referred set of samples.

Herd Problem	Serostatus	Number of Sera (details)	Total number of sera (reference)
Neosporosis (NcHerd1)	Nc-positive	121 (including 14 with abortion)	121 (reference positive)
	Nc-negative	111	111 (reference negative)
Neosporosis (NcHerd2)	Nc-positive low avidity	53 (including 5 with abortion)	
	Nc-positive high avidity	25 (including 3 with abortion)	78 (reference positive)
	Nc-negative	155	155 (reference negative)
Besnoitosis	Bb-positive	50	48 (reference negative), 2 (reference positive)
	Bb-negative	20	20 (reference negative)
Sarcocystosis	Suspected *Sarcocystis* spp. infections[a]	110	104 (reference negative), 6 (reference positive)
Total			645 (reference total), 207 (reference positive), 438 (reference negative)

Table 5. Set of bovine sera used to validate the *N. caninum* mAb-based cELISA. *N. caninum* antibodies were assessed by *N. caninum* tachyzoite immunoblot, IFAT, p38-ELISA and p38-avidity-ELISA. *B. besnoiti* antibodies were detected using IFAT, *B. besnoiti* tachyzoite and bradyzoite immunoblot (Schares et al., 2010). A majority criterion was used to classify positive and negative reference sera regarding the presence of specific *N. caninum* antibodies.

[a] Serological status of individual animals was unknown, but frequent cases of eosinophilic myositis were recorded at slaughter.

The samples originated from adult cattle since the animals under six months were excluded to avoid interference with maternal antibodies (Conraths and Gottstein, 2007; Dubey et al., 2017b; Hietala and Thurmond, 1999). In one of the sampled herds (NcHerd1) the abortion rate increased in recent years and several samplings were made (as described in section 2.7.1). Only one sample from each animal of the NcHerd1 was selected randomly to be included in the reference panel.

Because serological differences between herds with epidemic and endemic *N. caninum*-associated abortion have been reported (Schares et al., 2000, 1999d) a cattle herd with an epidemic outbreak of abortion (NcHerd2) was also used to estimate the diagnostic sensitivity of the mAb-based cELISA. Bovine sera samples from NcHerd2 included acute (low avidity) and chronically *N. caninum*-infected animals (high avidity) and sera from animals with recent abortions.

To assess potential causes of cross-reactions, sera from Apicomplexa parasites related to *N. caninum* were also included in the reference panel. One herd with recorded bovine besnoitiosis cases was used: these samples were characterized by *B. besnoiti* tachyzoite and bradyzoite immunoblot tests (Schares et al., 2010) and *B. besnoiti* immunofluorescence antibody test (Schares et al., 2010). Fifty of those samples were regarded as *B. besnoiti*-positive and 20 sera were *B. besnoiti*-negative.

Another 110 sera samples from a cattle herd with several cases of Sarcocystosis diagnosed at slaughter over the recent years were used to validate the novel cELISA. Samples from 13 animals with eosinophilic myositis were sent to FLI, and PCR examinations revealed the presence of *Sarcocystis cruzi* and *Sarcocystis bovifelis* in 10 of 13 of these animals after sequencing (Schares, unpublished).

2.6.2 Classical approach to estimate diagnostic characteristics of the novel cELISA

There is no "gold standard" for the diagnosis of *N. caninum* infections (Dubey and Schares, 2006). Thus, a common approach to overcome this problem is to combine several serological tests and obtain reference results for each sample using a majority criterion (Álvarez-García et al., 2013; Campero et al., 2018; Jenkins et al., 2005; Schares et al., 2000; von Blumröder et al., 2004). Following this approach, sera were regarded as reference-positive when at least two of the three serological reference tests were positive (i.e., immunoblot ≥ 2 bands recorded, IFAT positive (1:200 dilution) and p38-ELISA positive (0.04 index value as cut-off)). The complete description of sera used to validate the cELISA and the respective reference classification is shown in Table 5.

2.6.3 A Bayesian latent class analysis to estimate diagnostic characteristics of the novel cELISA

An alternative approach to estimate the cELISA performance, which does not rely on a "gold standard" was also used. A Bayesian latent class analysis (BLCA) was performed using the Gibbs sampler. The method makes use of existing information to define prior probabilities and in the statistical procedure both observed data and prior probabilities are considered to define the posterior probabilities (Enøe et al., 2000; Frössling et al., 2003). For this reason, the BLCA is a valuable tool to evaluate the diagnostic performance of one test when imperfect tests are used as reference (Cheung et al., 2021; Enøe et al., 2000). Unlike the previous approach, in the BLCA each sample is not classified as "positive" or "negative" but is instead assumed to have a probability of being "positive", taking into account the combination of the results given by the tests, prior knowledge of the characteristics of the tests used, and prior information of prevalence in the sampled populations (Cheung et al., 2021; Collins and Huynh, 2014).

The Bayesian model requires at least two different populations with different prevalences of the disease or the infection. Thus, each of the sampled herds (Table 5) was considered a different population. Another prerequisite is the assumption that the behaviour of the test (regarding sensitivity and specificity) is constant in all populations (Hui and Walter, 1980). The final assumption was that the serological tests (immunoblot, p38-ELISA, IFAT and cELISA) are not independent of each other, because they are based on the same biological process (Gardner et al., 2000) i.e., the detection of specific antibodies to – at least partially – the same antigens (i.e. antigens of *N. caninum* tachyzoites).

In Gibbs sampling, prior probabilities are defined as beta distributions (Enøe et al., 2000). The priors can be uninformative (a distribution which gives the same probability to all possibilities) or informative (when a prior estimate of the probable value and range

for a given value is known) and usually based on expert opinion or relevant published literature (Cheung et al., 2021). A non-informative prior (characterized by a distribution Beta (1,1)) was selected for the prevalence of *N. caninum* antibodies in the herds with *N. caninum*-associated abortion problems (NcHerd1 and 2), given that a wide range of prevalence estimations has been reported for different case herds (Dubey et al., 2017b; Dubey and Schares, 2006). Based on a study in which seroprevalences for *N. caninum* were compared between several European countries, including Germany (Bartels et al., 2006), a seroprevalence of 4.1% (95% CI: 3.1% - 5.0%) in the beef herds with *B. besnoiti* or *Sarcocystis* spp. infections was considered, originating a distribution Beta (31.88, 855.24).

Prior information regarding the sensitivity and specificity of the serological tests was also included in the model. These priors were based on a study in which several serological tests frequently used in Europe were compared using the same set of samples (von Blumröder et al., 2004) and another study comparing the performance of immunoblot and ELISA test to detect specific *N. caninum* antibodies (Staubli et al., 2006). No prior information regarding the diagnostic characteristics of the particular IFAT used was available and is known that IFAT tests performed in different laboratories are not comparable (Dubey and Schares, 2006). However, since they were based on the same principle and the same cut-off dilution was applied, the diagnostic characteristics of another IFAT test (Álvarez-García et al., 2003) which were estimated in the referred interlaboratory comparison (von Blumröder et al., 2004) were considered to estimate the IFAT prior information.

The prior information regarding test performance of the serological tests included in the model is listed in Table 6. A non-informative prior was used for the diagnostic characteristics of the novel cELISA (Beta (1,1)).

Serological test (reference)		**Median (95% CI)**	**Distribution**
Immunoblot (Staubli et al. 2006)	Sensitivity (Ss)	98.47% (94.59% – 99.81%)	Beta (64.94, 1.34)
	Specificity (Sp)	100% (89.72% – 100%)	Beta (13.91, 0.33)
p38-ELISA (Blumröder et al. 2004)	Sensitivity (Ss)	98.98% (96.38% - 99.88%)	Beta (98.33, 1.34)
	Specificity (Sp)	99.08% (97.33% – 99.81%)	Beta (168.51, 1.89)
IFAT (Blumröder et al. 2004)	Sensitivity (Ss)	98.50% (96.80% - 100%)	Beta (228.68, 3.81)
	Specificity (Sp)	95.10% (92.80% – 97.50%)	Beta (290.22, 15.27)

Table 6. Diagnostic characteristics of the reference tests and the respective Beta distributions used as priors in the Bayesian latent class analysis.

2.7 Application of the mAb-based ELISA to study a herd with *N. caninum*-associated abortion

2.7.1 Characterization of the study population (NcHerd1)

A dairy farm located close to Rostock in the state of Mecklenburg-Western Pomerania, Germany was experiencing an increasing rate of abortions per year since 2019. This increase in the abortion rate was attributed by the local veterinary services as being *N. caninum* related. FLI was contacted to examine sera from adult cattle collected on the farm and to support the efforts to reduce the impact of Neosporosis.

From 2016 to 2018 only one abortion per year was recorded, but since then the number of abortions increased (Table 7):

Year	2022	2021	2020	2019	2018	2017	2016
No of Abortions	10	12	7	8	1	1	1

Table 7. Number of abortions occurred in the studied farm (NcHerd1) over 7 years.

The Farm population consisted of about 120 adult cows and a total population of approximately 200 animals. All the animals were from the Holstein Friesian breed. The young calves were kept in pens separate from the milking cows (adult group) and some of the heifers were kept on the farm for replacement. The population was closed since 1990, meaning that no animals were introduced from outside. In terms of housing, milking cows were kept indoors during winter and had access to pasture during summer in different groups. No record of these groups was kept. The animals were fed with silage, hay, and total mixed ration. Two dogs were present on the farm and had access to the stables, pasture, and the places where the fodder was stored. The farm was located close to a village and outside dogs from neighbouring houses had free access to trails around the fences where the animals grazed. Before parturition, pregnant dams were moved to an open shed are regularly checked.

2.7.2 Examination of sera from the farm with suspected *N. caninum*-associated abortion (NcNerd1)

Since the abortion problems started, blood samples were obtained and sent to the FLI for examination (Table 8).

Number of samples per animal	Number of animals (number of samples)
1	118 (118)
2	68 (136)
3	68 (204)
Total	**254 (458)**

Table 8. Description of the samples obtained from the herd with suspected *N. caninum*-associated abortion (NcHerd1), during the three years of the study.

In 2020, 133 animals were sampled, in 2021 two separate samplings were made, the first in April (129 animals) and the second in July (69 animals, mainly comprising the heifers). In 2022 only one sampling was made and blood was collected from 127 animals. Some animals were sampled tree-times, others twice and others just once (Table 8). No aborted foetuses or placentas were received for examination.

2.7.3 Age-related differences

The age-related seroprevalences are considered an important indicator of the possible routes of transmission of *N. caninum* infection within the farm in the year(s) prior to sampling (Schares et al., 1998; Wouda, 2007).

With the information collected from the farmer and the Herkunftssicherungs- und Informationssystem für Tiere (HIT) (i.e. the Identification and Information System for Animals), it was possible to divide the animals into different age groups (Dijkstra et al., 2001a; López-Gatius et al., 2004a; Schares et al., 1998; Wouda et al., 1999). For each year three or four age groups were formed (<2 years, 2-3 years, 4-5 years and ≥6 years). The criteria to define age groups were the following: (1) find groups that were meaningful considering the animals that were housed together; (2) have a similar number of animals in each group, so they could more easily be compared.

2.7.4 Comparison of dam-daughter serostatus

The serological results of dams and daughters were plotted together, and the relations were established (i.e., positive dams with positive daughters, negative dams with negative daughters, positive dams with negative daughters and negative dams with positive daughters). The rationale was to find if there was a significant association between the serological status of dams and their daughters. To this analysis, whenever a test suggested a seroconversion after the birth of the daughter, the dam/daughter was considered negative since seroconversions were assumed to be related to post-natal infection. In total, data of 118 dam-daughter pairs were analysed by Pearson's chi-square test, for each serological test. The pairs were then separated into three different groups: one with all daughters born before 2019 (n=44); a second that included all daughters born in 2019 (n=35) and a third group with daughters born in 2020 (n=35) and 2021 (n=4).

2.8 Statistics

The computer program R, version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) and the R package ''optimal.cutpoints" (López-Ratón et al., 2014) were used to define an optimal cut-off point for the cELISA and the respective diagnostic sensitivity and specificity (using the majority criterion as reference) including 95% confidence intervals (95% CI). Figures were assembled with the R package "ggplot2" (Wickham, 2016).

For the BLCA, the R package "PriorGen" (Kostoulas, 2018) was used to generate beta distributions, based on the prevalence of *N. caninum* antibodies and the diagnostic characteristics of the serological tests obtained from literature. The model was

estimated with Gibbs sampling methods in the JAGS (Just Another Gibbs Sampler) (Plummer, 2003) using the R package “runjags” (Denwood, 2016).

To analyse contingency tables, the Fisher’s Exact test and Chi-squared test were performed using the R commands “fisher.test” and “chisq.test”, respectively. To correct for multiple testing the Bonferroni correction was applied (Bland, 2015). The R package ‘‘epitools” (Sergeant, 2018) was used to determine the Odds Ratio with 95% confidence intervals. Results in cELISAs for different mAb and positive control dilutions were compared in the Kruskal–Wallis rank sum test using the R command ‘‘kruskal.test”. The Wilcoxon rank sum test with continuity correction to compare SCI values between different groups of animals was performed using the command ‘‘wilcox.test” in R.

To study whether p38-ELISA and cELISA values were correlated, the Person correlation test was performed using the R command “cor.test”. A R^2 lower than 0.5 was considered an indication of a low correlation between test results, while a R^2 between 0.5 and 0.69 indicated a moderate relationship. If R^2 values were between 0.7 and 0.89 a high correlation was assumed. If R^2 was higher than 0.9, a very high correlation was assumed (Asuero et al., 2006; Ratner, 2009).

To determine the level of agreement between tests, *kappa* (κ) statics was performed using a prevalence and bias-adjusted *kappa* (Byrt et al., 1993) with the command “epi.kappa” (from R package ‘‘epiR” (Stevenson et al., 2015)). The results were interpreted as follows: slight agreement (< 0.2), fair agreement (0.2 - 0.4), moderate agreement (0.4 - 0.6), substantial agreement (0.6 - 0.8), almost perfect agreement (>0.8) (Sim and Wright, 2005). For this purpose, the 0.04 index value was used as cut-off for the p38-ELISA (von Blumröder et al., 2004) and immunoblot inconclusive results (one band recognized) were regarded as negative (Schares et al., 2000; Staubli et al., 2006).

3. Results

3.1 SDS-PAGE and Immunoblotting

The reactivity of the monoclonal antibodies and their specificity for *N. caninum* antigens was assessed by immunoblotting with non-reduced and reduced antigens originated from *N. caninum, B. besnoiti* and *T. gondii* tachyzoites. The relative molecular weight of the antigen bands recognized were compared with the marker proteins which were run under the same conditions as the antigen (Figure 6).

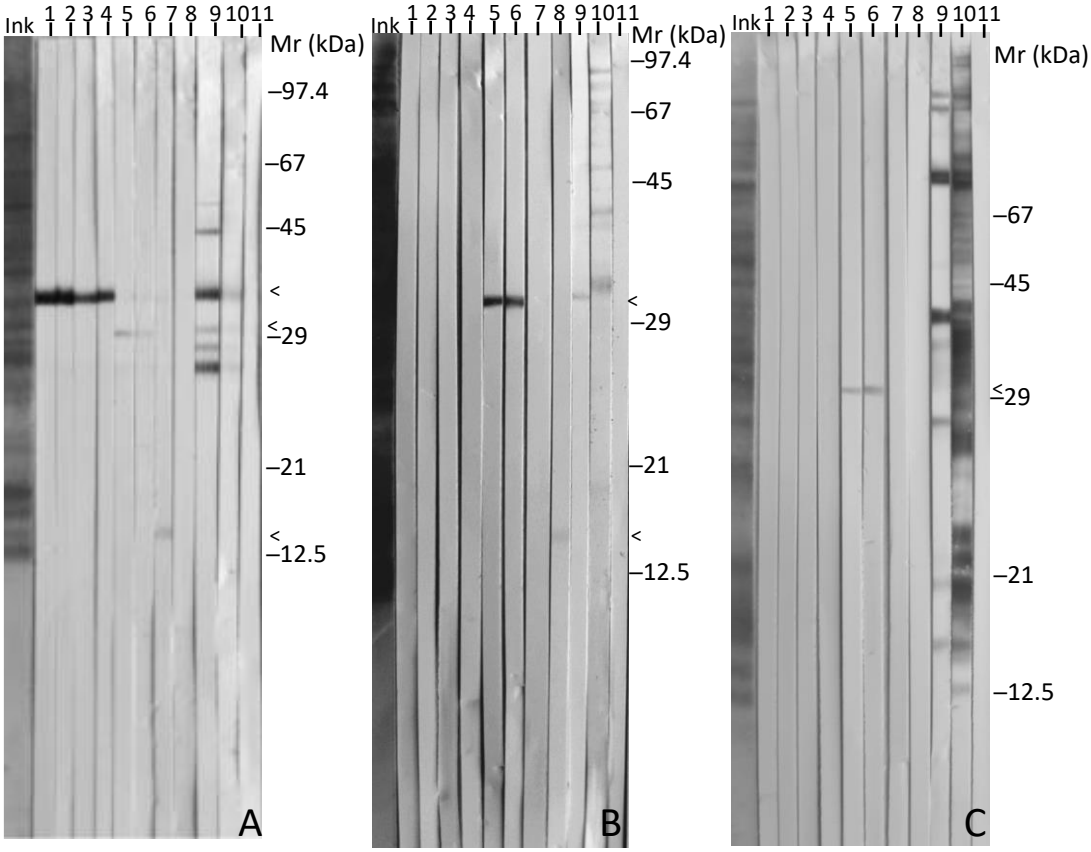

Figure 6. Immunoblot reactions against non-reduced (A) and reduced (B) *N. caninum* tachyzoite antigen, and non-reduced *B. besnoiti* tachyzoite antigen (C), probed with monoclonal antibodies 3.10.5 (1), 4.15.15 (2), 5.2.9 (3), 5.2.15 (4), 4.11.5 (5), 9/12-12 (6), 4.7.12 (7), 8/1-10 (8), mouse positive serum (9), bovine positive serum (10), and hybridoma-negative control (11). More information about SDS-PAGE preparation and control is available in Table 4. Antigen bands recognized by the mAbs are marked (<). Mr=relative molecular weight.

When *N. caninum* tachyzoites prepared under non-reducing conditions were probed with mAbs 3.10.5, 4.15.15, 5.2.9, and 5.2.15 a strong reaction against a 38 kDa molecule was observed. Monoclonal antibodies 4.11.5 and 9/12-12 recognized a 33 kDa band in both and non-reduced *N. caninum* tachyzoites. Interestingly, a band with similar relative molecular weight was also recognized by 4.11.5 and 9/12-12 mAbs when *B. besnoiti* tachyzoites were treated under non-reducing conditions, but not with reducing conditions. Similarly to what was described by Schares (1999b), mAb 4.7.12 recognized a 19 kDa molecule. Surprisingly, mAb 8/1-10 also recognized a 19 kDa molecule in reduced *N. caninum* tachyzoite preparation. These results are expressed in Figure 6 and Table 9.

No reactions were visualized when reduced *B. besnoiti* tachyzoites, and both reduced and non-reduced *T. gondii* tachyzoites were probed with the monoclonal antibodies (not shown).

		N. caninum		***B. besnoiti***	
mAb number	**mAb designation**	**Nonreduced antigen (kDa)**	**Reduced antigen (kDa)**	**Nonreduced antigen (kDa)**	**Reduced antigen (kDa)**
1	**3.10.5**	38	–[a]	–	–
2	**4.15.15**	38	–	–	–
3	**5.2.9**	38	–	–	–
4	**5.2.15**	38	–	–	–
5	**4.11.5**	33	33	33	–
6	**9/12-12**	33	33	33	–
7	**4.7.12**	19	–	–	–
8	**8/1-10**	–	19	–	–

Table 9. Characterization of the molecular weights (kDa) of *N. caninum* and *B. besnoiti* tachyzoite antigens by mouse monoclonal antibodies in the immunoblot after SDS-PAGE under reducing and non-reducing conditions. [a] No reactivities observed.

3.2 Establishing a mAb-based competitive ELISA

3.2.1 Checkerboard titrations by indirect ELISA

To establish a mAb-based cELISA, iELISA assays were performed to assess the working concentration of each component of the test. An anti-mouse conjugate (peroxidase-conjugated sheep anti-mouse IgG Fcγ, fragment specific) which according to the manufacturer exhibits minimal cross-reaction with bovine serum proteins was selected, since cross-reactions when other anti-mouse conjugates were applied were observed in the past (Schares, personal communication). Based on prior experience with this anti-species conjugate a dilution of 1:500 was selected for the first experiments.

The initial experiment aimed to assess the reactivity of the mAbs against the crude antigen (tachyzoite lysate) used to sensitize the ELISA plates and also to determine the optimal single dilution of the antigen preparation, which would be used to detect reactions by mAbs. The results are displayed in Figure 7. Serial dilutions of mAb 8/1-10 (negative control) were performed to evaluate the background obtained at various dilutions of hybridoma supernatant, as suggested by Crowther (2009).

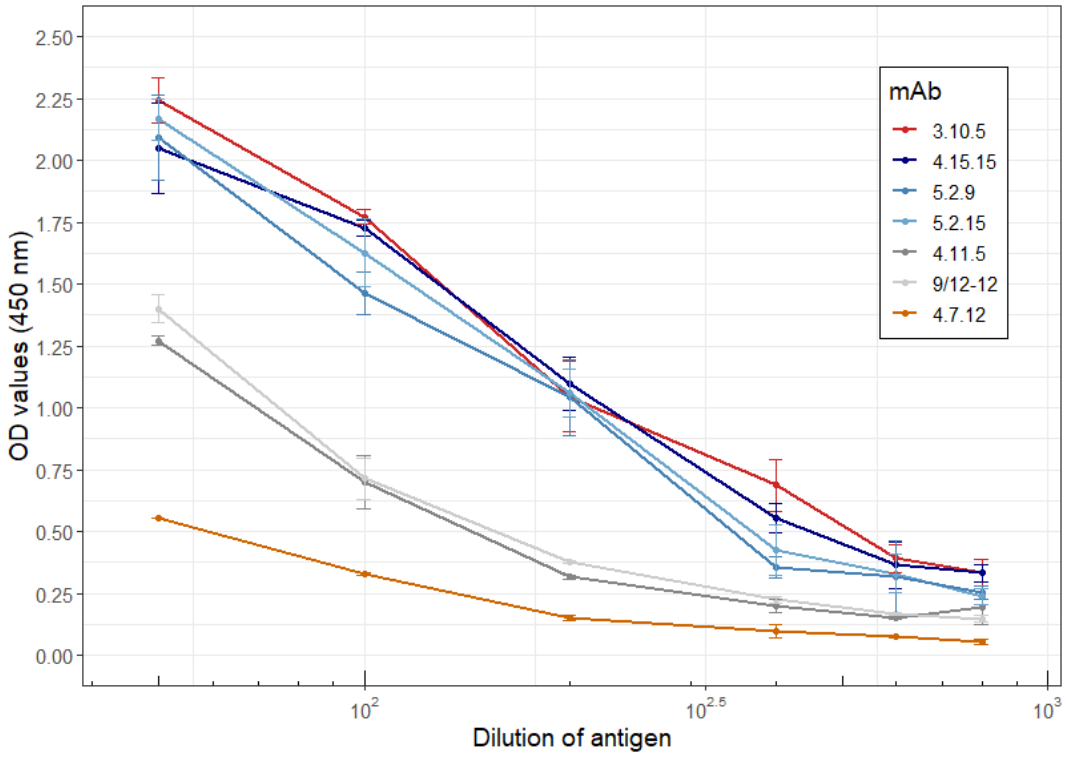

Figure 7. Effect of the dilution of antigen on the OD values obtained by indirect ELISA. These values allow the establishment of the specific ELISA titration curves (represented) for each mAb. Standard deviation (SD) is represented in the form of error bars.

The OD values obtained for each mAb were compared with the background (determined by mAb 8/1-10) by calculating a ratio. The highest ratio was obtained at a dilution of 1:100. This point was in the linear range of the ELISA titration curves and was therefore selected as the optimal dilution of antigen (tachyzoite lysate). With this antigen dilution, a final concentration of protein of 0.44 µg/mL is obtained, which is in the range proposed by Ausubel (1987) of 0.2 to 10 µg/mL.

Having established the antigen dilution, the next step was to establish the optimal dilution of hybridoma supernatant for each mAb, increasing the sensitivity of the ELISA test. An iELISA assay was performed with this purpose, carried out with serial dilutions of hybridoma supernatant for each of the seven mAbs that reacted against tachyzoite antigen. The results are expressed in Figure 8.

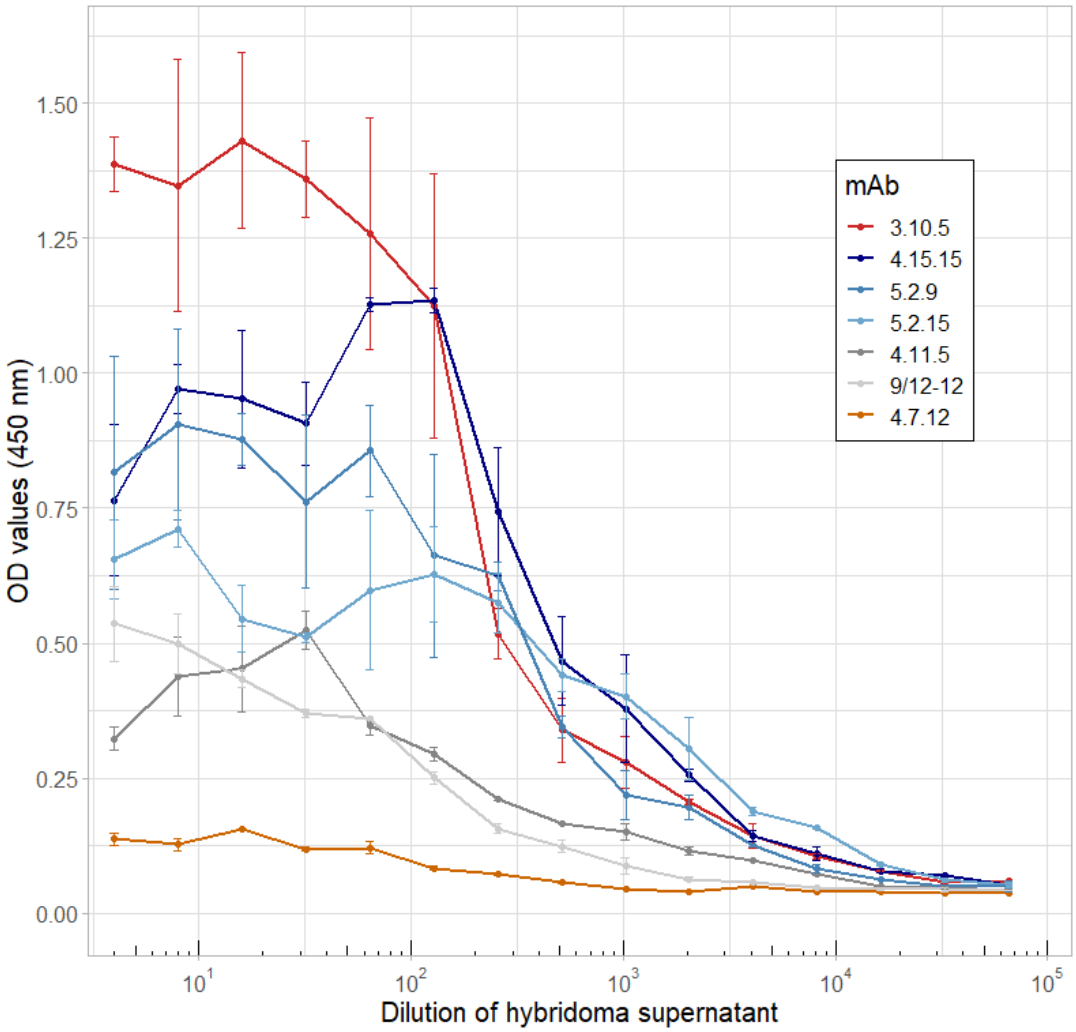

Figure 8. Effect of the dilution of hybridoma supernatant on the OD values obtained by indirect ELISA. These results allow the establishment of the specific ELISA titration curves (represented) for each mAb. Standard deviation (SD) is represented in the form of error bars.

The shape of the curves and their sigmoidal nature (Figures 4 and Figure 8) can be explained by the fact that when the antibodies are reacting maximally with the antigen, there is no increase in colour even if the antigen/antibody concentration is increased (plateau, top of the individual graphs), i.e. the threshold of saturation was reached. The other non-linear region is obtained at the bottom of the graphs, where the OD values are similar to the background OD, so the colour development represents nonspecific adsorption/binding. Between these two regions of the graph, a linear region can be obtained, at which OD values corresponded to the dilution of antigen/antibodies (Crowther, 2009).

A point in the linear region of the specific titration curves of each mAb was selected as a single dilution of hybridoma supernatant to be used as a screening dilution to establish cELISAs with each mAb. For mAbs 3.10.5, 4.15.15, 5.2.9, and 5.2.15 a hybridoma supernatant dilution of 1:100 was chosen. A dilution of 1:10 was selected for mAbs 4.11.5 and 9/12-12 and mAb 4.7.12 was applied undiluted.

The iELISA assays allowed the optimization of reagent dilution (antigen and hybridoma supernatant), by checkerboard titrations. This information was vital to the development of the cELISAs, ensuring that no reagents were in excess, which could lead to nonspecific attachment and higher backgrounds. At the other extreme, if the reagents were too diluted no reactions could be detected (Crowther, 2009).

3.2.2 Analytical sensitivity of the cELISAs

After optimization of the working reagents by applying the mAbs in an iELISA, competitive ELISAs employing the monoclonal antibodies and using the conditions established previously were performed. The titration of a positive control bovine serum allowed the study of the inhibition characteristics of this particular serum in the dilution range. It was assessed whether the use of different mAbs had an impact on the inhibition characteristics of the positive control serum (Figure 9).

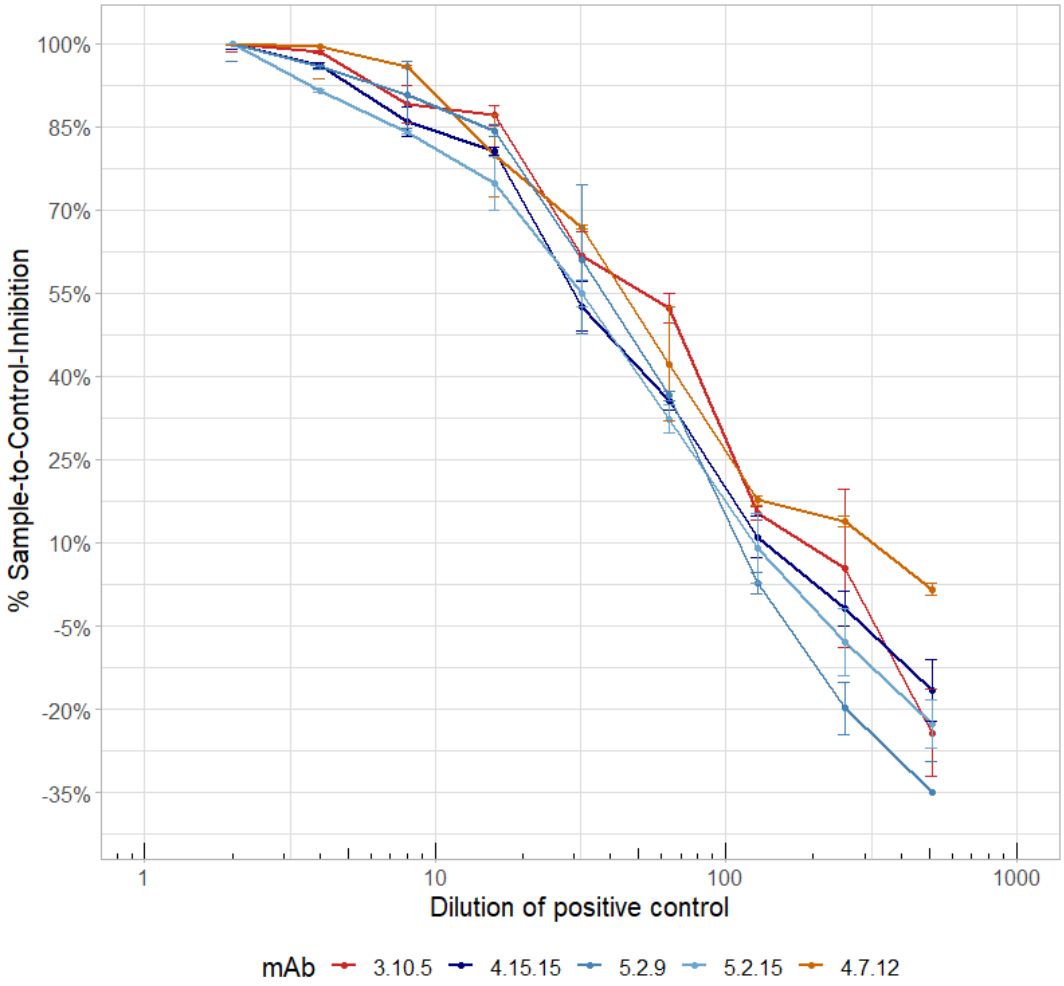

Figure 9. Analytical sensitivity of the competitive ELISAs established by using mouse monoclonal antibodies. Only those mAbs showing a sigmoidal curve shape were represented. Standard deviation (SD) is represented in the form of error bars.

Figure 9 shows the inhibition characteristics of a bovine-positive control serum when different mAbs were applied in a competitive ELISA. Mouse monoclonal antibodies

reacting against dense granules by immunofluorescence (4.11.5 and 9/12-12) were not represented since they showed no linearity and were, for that reason, excluded as possible candidates for the establishment of a competitive ELISA. The positive control was able to inhibit the reaction of the represented mAbs by more than 50% even in a 1:32 dilution. The reaction of the 3.10.5 mAb was inhibited by more than 50% even in a 1:64 positive control dilution. However, no statistically significant differences (p-value >0.05, Kruskal–Wallis chi-squared) were found between the different mAbs.

It was further assessed whether different dilutions of one of the mAbs (3.10.5) had an effect on the inhibition characteristics of the positive control serum, i.e., if one particular dilution of hybridoma supernatant allowed a higher sensitivity of the competitive ELISA when mAb 3.10.5 was employed.

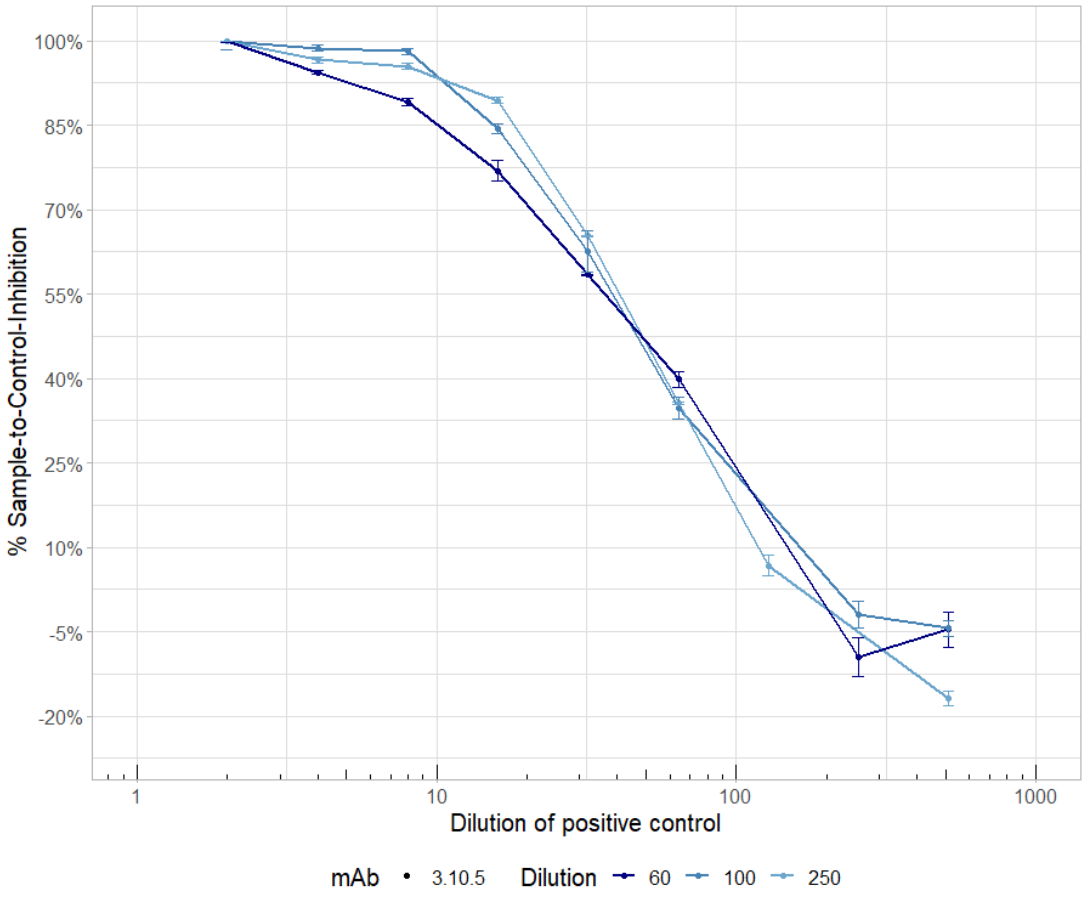

Figure 10. Analytical sensitivity of the competitive ELISA using mAb 3.10.5 as determined by titration of the positive control serum. Three hybridoma supernatant dilutions in the linear range of mAb 3.10.5 were compared. Standard deviation (SD) is represented in the form of error bars.

The dilutions that characterised the linear range of mAb 3.10.5 (1:60, 1:100, 1:250) were compared to clarify whether different dilutions of hybridoma supernatant could affect the inhibition characteristics of the positive bovine control serum (Figure 10). Once again, positive control was able to inhibit the reaction of the mAb by more than 50% even in a 1:32 dilution. At the positive control serum dilution of 1:512, the mAb dilutions 1:60 and 1:100 were apparently more sensitive. No statistically significant differences (p-value >0.05, Kruskal–Wallis chi-squared) were found between the

different hybridoma supernatant dilutions, so the previously selected dilution (1:100) was henceforward used.

3.2.3 Validation and characterization of the mAb-based ELISA

The diagnostic characteristics (sensitivity and specificity) of the cELISA using mAb 3.10.5 were determined by testing a total of 645 samples from herds with bovine neosporosis (both acute and chronic cases), besnoitiosis and sarcocystosis (Table 5). All samples were analysed with three other reference tests to establish a reference classification (positive or negative) for these samples.

Sera collected from animals with chronic and acute neosporosis, in which at least two of the serological reference tests revealed the presence of specific *N. caninum* antibodies (p38-ELISA positive, reciprocal IFAT titre ≥200, immunoblot ≥2 bands), were used to define an appropriate cut-off value and to assess the diagnostic sensitivity of the novel ELISA test. Bovine sera from herds with cases of sarcocystosis, besnoitiosis and neosporosis (acute and chronic), in which no reference test was positive (p38-ELISA positive, reciprocal IFAT titre ≥200, immunoblot ≥2 bands) or only one was positive, were used to define an appropriate cut-off and the diagnostic specificity of the ELISA.

Based on the population analysed, the Youden index was calculated (using the R package ‘‘optimal.cutpoints”), and an “optimal” cut-off was obtained at 38% SCI. With this cut-off, four reference positive sera tested negative in the novel mAb based-ELISA (false-negatives) and ten reference negative tested positive (false-positive results). Interestingly, from the false-positive results, only one serum was negative in all reference tests: six were regarded as positive by p38-ELISA (and four of them being inconclusive in immunoblot), two by IFAT (being inconclusive in immunoblot) and another by immunoblot. The selected cut-off results in a sensitivity of 98.1% (95% CI: 95.1%- 99.5%) and specificity of 97.7% (95% CI: 95.8% - 99.9%), relative to the reference sera.

When a 38% SCI cut-off is applied, the mAb-based cELISA almost perfectly separates negative- from positive-reference sera (Figure 11):

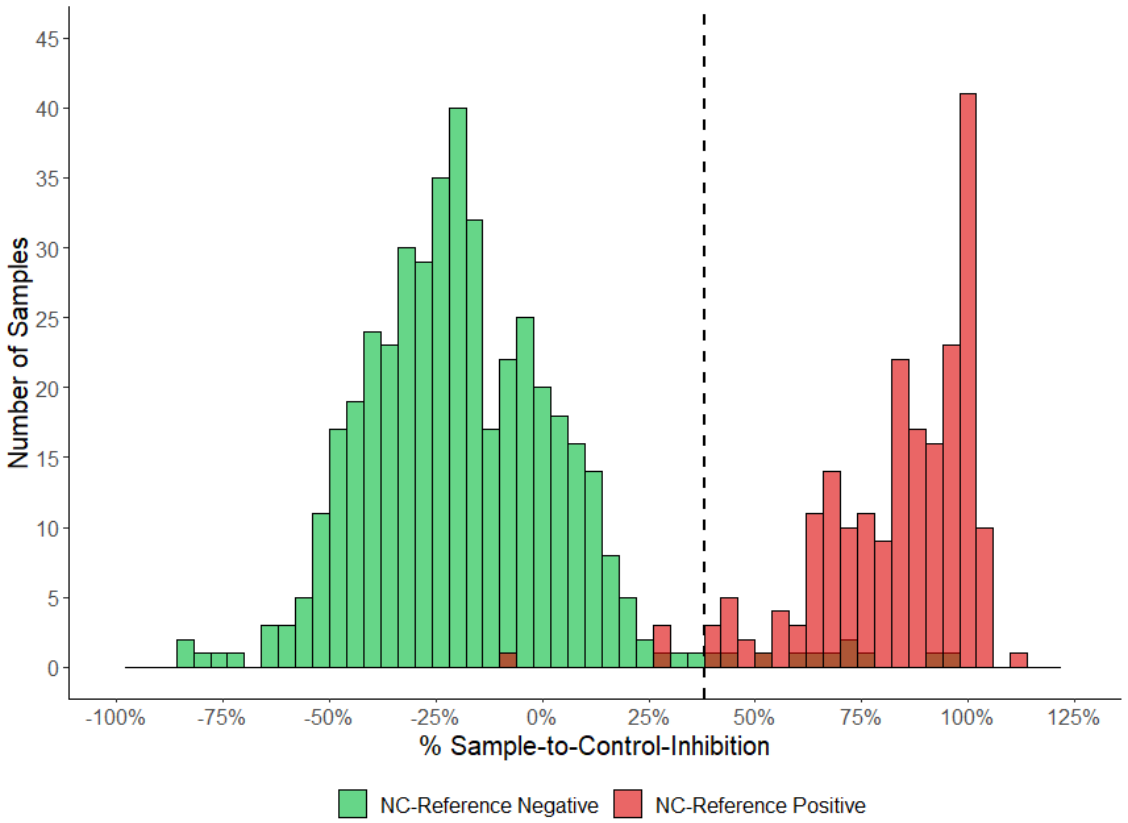

Figure 11. Sample-to-Control-Inhibition (SCI) values (%) obtained for the reference-positive (red) and reference-negative (green) sera (Table 5). The dashed line represents the selected cut-off (38% SCI), which almost perfectly separates positive from negative-reference sera. Most positive results show values higher than 75% of inhibition, whereas the negative-reference sera have values around -25%, with a higher dispersion.

A model based on Bayesian statistics was developed to estimate the specificity and sensitivity of the novel cELISA in a range of cut-off values (Figure 12). The Youden index was calculated for each of the points and the highest value was obtained with a cut-off point of 37% SCI. This cut-off point results in a sensitivity of 98.5% (95% CI: 96.5%- 99.9%) and specificity of 98.4% (95% CI: 97.1% - 99.4%) as determined by the BLCA model.

In the receiver operating characteristics (ROC) plot obtained from the BLCA (Figure 12) even when extremely high or low SCI values are applied as cut-off, estimates for sensitivity vary between 87-98% and 95-99% for specificity. Overall, specificity increases slightly with increasing cut-off values, contrarily to sensitivity which is approximately stable between cut-off values of 15% and 37% SCI, starting to decrease after this point. A plateau between the cut-off values of 32% and 37% was observed.

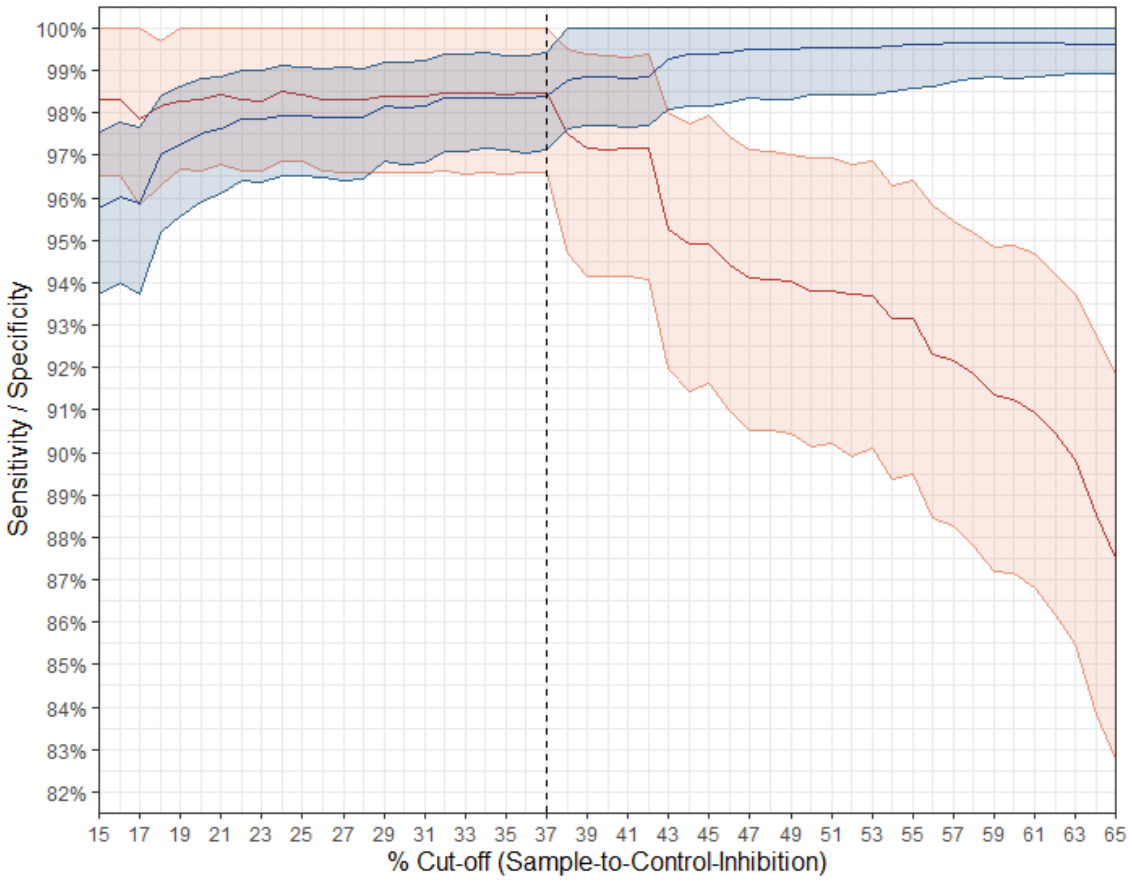

Figure 12. Two-graph receiver operating characteristics (TG-ROC) plot with sensitivity (red) and specificity (blue) at different cut-off values, as estimated by Gibbs sampling. The shaded area represents the 95% confidence limits and the dashed line represents the "optimal" cut-off (37%), defined by the Youden index.

Comparing the cut-off values determined by the two approaches, only one sample from the reference panel changes from negative to positive when a cut-off value of 37% (determined by the BLCA) is applied instead of 38% (determined by reference classification based on the majority). This sample is derived from NcHerd1 and was regarded as positive by IFAT and inconclusive by immunoblot. The estimated diagnostic characteristics of the cELISA are slightly higher when the BLCA was used. Henceforward, the cut-off value of 37% SCI was applied for further analyses.

3.2.4 Analytical specificity

Reference-negative sera obtained from the herd with besnoitiosis (n=68) cases yielded SCI values that were not statistically significantly different to reference-negative sera obtained from herds with *N. caninum*-associated abortion. When only *B. besnoiti*-positive reference sera (n=48) were considered, the SCI values were statistically significantly lower (p-value = 0.004, Wilcoxon rank sum test) than those obtained for the reference-negative from *N. caninum* herds. However, SCI values of reference-negative sera from the herd with sarcocystosis cases (n=104) were slightly higher than those obtained from reference-negative from *N. caninum* herds ($p<0.001$, Wilcoxon rank sum test). The highest SCI value observed for the reference-negative population of the herd

with *Sarcocystis* spp. infections was 38%, but 75% of the samples from this herd yielded SCI values below 6% and only 3 samples were above 25% (Figure 13).

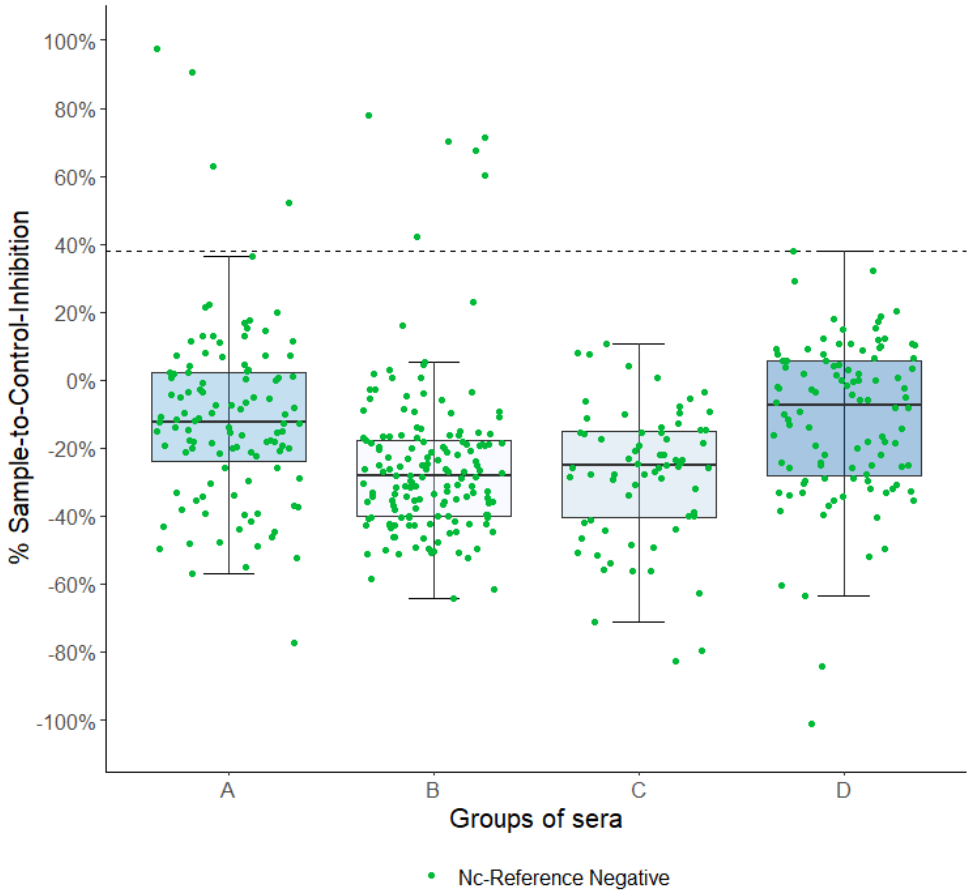

Figure 13. Comparison between Sample-to-Control-Inhibition (SCI) values obtained in the cELISA of reference-negative sera, collected from herds *N. caninum*-associated abortions (NcHerd1 (A) and NcHerd2 (B)), the herd with besnoitiosis cases (C), and the herd with suspected *Sarcocystis* spp. infections (D). The dashed line represents the cut-off (37% SCI).

3.2.5 Comparison of the cELISA with reference tests

3.2.5.1 Comparison with Immunoblot results

When reference sera (n=645) were grouped according to their number of recognized *N. caninum*-specific immunodominant bands (IDAs) in immunoblot, the SCI values tended to increase with an increasing number of recognized IDAs (Figure 14). When no bands were recognized in the immunoblot (n=402), almost all reference samples were below the selected cut-off (n=396), whereas when two or more bands were recognized in the immunoblot (n=210), at least 75% of the reference sera were above the selected cut-off (Figure 14). SCI values of sera recognizing one or more immunodominant bands differed statistically significantly (p-value<0.001, Wilcoxon rank sum test) from reference sera recognizing none of the five immunodominant bands.

A direct comparison between the immunoblot results and the cELISA results applying the selected cut-off, reveals an almost perfect agreement (κ=0.91; 95% CI: 0.87-0.94). A total of 30 reference sera samples (n=645) show divergent results in both tests (Figure

14): thirteen sera tested positive in immunoblot, but negative in the cELISA and another seventeen sera tested negative in the immunoblot but positive in the cELISA.

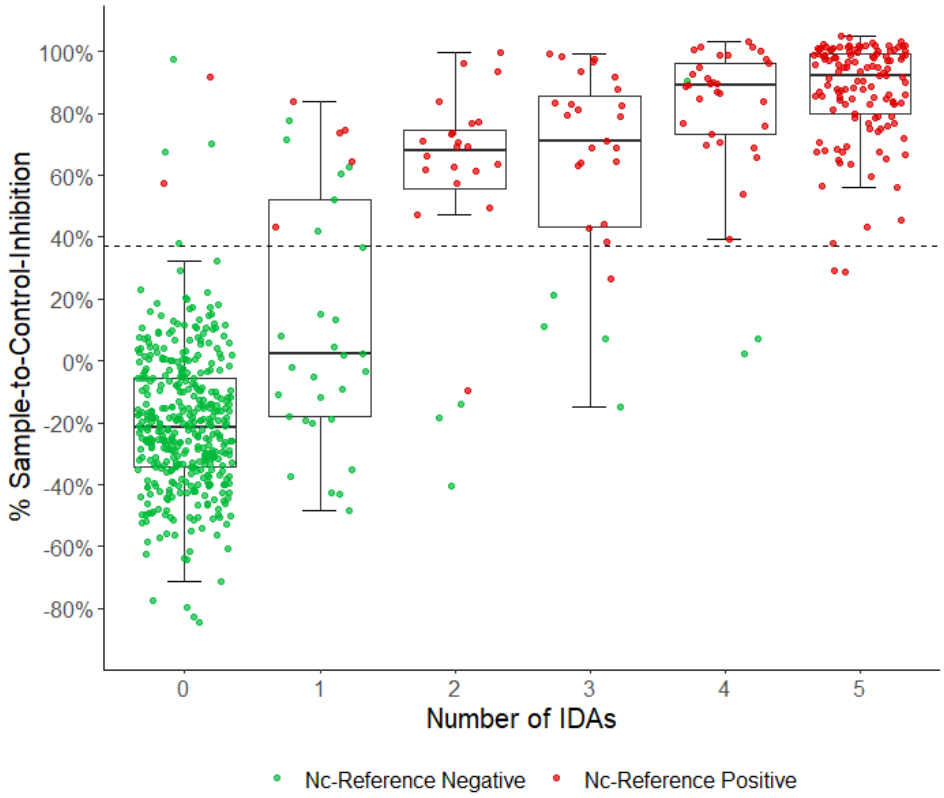

Figure 14. Comparison of the cELISA (Sample-to-Control-Inhibition, SCI) with immunoblot (number of *N. caninum*-specific IDAs) results, when reference sera samples (n=645) were grouped according to their number of recognized immunodominant bands (IDAs). The reference-positive sera are represented in red and the reference-negative sera in green. The dashed line represents the selected cut-off (37% SCI), showing a clear separation between clear negative immunoblot results (0 bands recognized) and positive immunoblot results (≥2 bands recognized).

3.2.5.2 Comparison with p38-ELISA results

To compare the reference p38-ELISA to the cELISA and determine whether the results of both tests were correlated, a linear regression analysis was performed (Figure 15). The correlation was considered statistically significant ($p<0.001$), and its strength, determined by Pearson correlation coefficient ($R^2=0.69$), was in the threshold between a moderate ($0.5< R^2 >0.7$) and a high correlation ($R^2 >0.7$). When reference sera results of both testes were compared directly, an almost perfect agreement was achieved ($\kappa=0.9$; 95% CI: 0.86-0.93). In total 32 reference sera (n=645) yielded contradictory results in the two tests (Figure 15): sixteen sera were classified as positive by p38-ELISA, but were negative by cELISA and another sixteen sera tested negative in the p38-ELISA but positive in the cELISA.

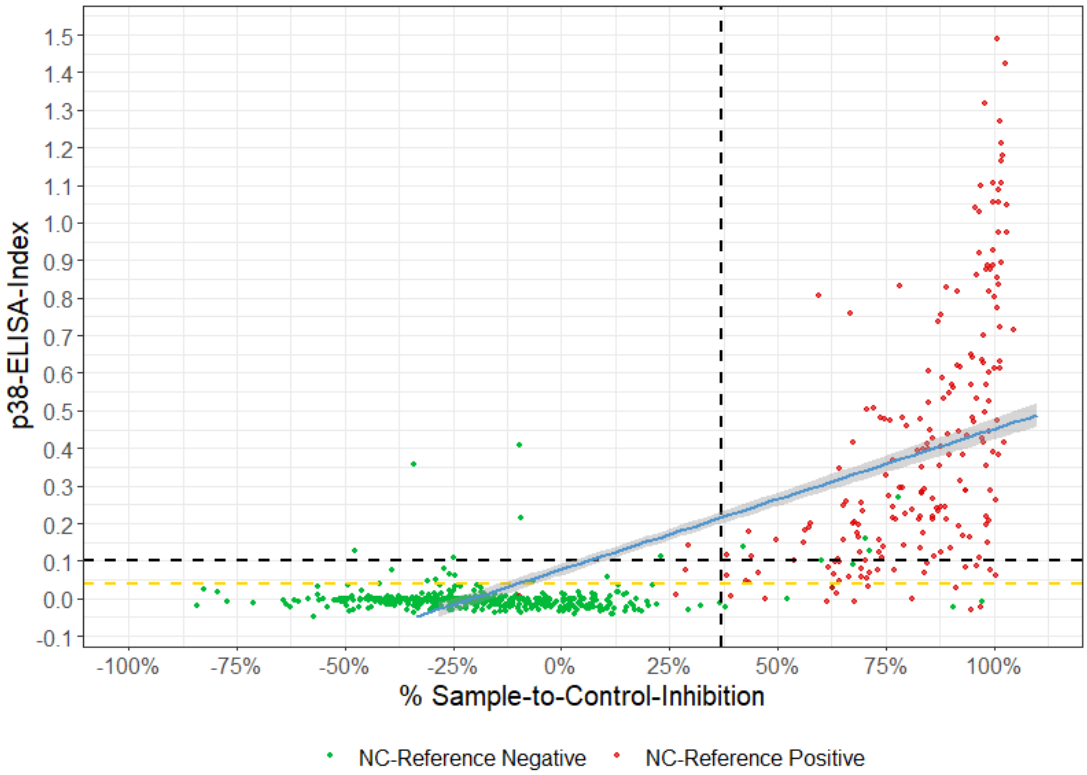

Figure 15. Correlation of the Sample-to-Control-Inhibition (SCI) of the cELISA with the p38-ELISA index values. The reference-positive sera are represented in red and the reference-negative sera in green. The blue line represents the linear regression line (R^2 =0.69) and the shaded area is the 95% confidence limit. The black horizontal dashed line represents the cut-off (0.1 index value) of the p38-ELISA, whereas the yellow dashed line represents the inconclusive cut-off (0.04 index value). The vertical dashed line represents the selected cut-off of the cELISA (37% SCI).

3.2.5.3 Comparison with IFAT

Appling *κ*-statistics, only a substantial agreement between the novel Nc-cELISA and IFAT was revealed (κ=0.78; 95% CI: 0.73-0.83). A direct comparison between the two serological tests shows 69 conflicting results: 29 sera tested positive in IFAT and negative in the cELISA, whereas 40 sera were regarded as negative by IFAT, but positive by cELISA. These sera consisted of 31 which were reference-positive and tested false-negative in IFAT; nine tested negative in IFAT but false-positive in the cELISA; another 27 sera, which were reference-negative, tested false-positive in IFAT and correct-positive in the cELISA; two reference-positive tested correct-positive in IFAT but false-negative in the cELISA.

3.2.6 Comparison between aborting and non-aborting dams

The results showed in this section were obtained considering the reference-positive sera from herds with *N. caninum*-associated abortion (NcHerd1 and NcHerd2, Table 5) collected from dams older than 2 years (n=172). When aborting and non-aborting reference-positive dams were compared, the aborting dams (n=20) showed statistically significantly higher SCI values (p-value=0.001, Wilcoxon rank sum test) than non-aborting dams (n=152). Restricting the analysis to the NcHerd1, the group of aborting dams (n=12) showed statistically significantly higher SCI values (p-value=0.007, Wilcoxon rank sum test) compared with the non-aborting group (n=82). Likewise, if only samples from NcHerd2 were considered, the aborting dams (n=8) showed higher SCI

values as compared to non-aborting dams (n=70), but this difference was not statistically significant (p-value>0.05, Wilcoxon rank sum test).

A similar finding was obtained when within the animals with an abortion risk (i.e., "at risk" in the following) aborting dams (n=8) were compared with the at risk non-aborting group (n=16) from the herd with an epidemic abortion pattern (NcHerd2), but this time the differences were statistically significant (p-value=0.009, Wilcoxon rank sum test). The results presented in this section were similar to what has been reported by others (Schares et al., 2000, 1999a).

Comparing reference-positive sera from the two herds with *N. caninum*-associated abortion, the SCI values tend to be higher for the NcHerd1 (n=94), than for the NcHerd2 (epidemic herd) (n=78) and this difference was statistically significant (p-value<0.001, Wilcoxon rank sum test). This finding is also in agreement with previous studies (Schares et al., 2000, 1999a).

3.3 Antibody responses of dams experimentally infected with *N. caninum*

The antibody profiles of experimentally infected cattle (Schares et al., 1999c) obtained by p38-ELISA (Schares et al., 2000) were compared with those obtained with the newly developed competitive ELISA (Figure 16).

The p38-ELISA indices increased stably over time in all three cows, until the second-month post-infection. Cow 24 maintained a slow and stable increase until the end of the observation period, whereas heifer 44 and 49 showed a peak after approximately 30- and 60-days post-infection, respectively, and a subsequent decline. Heifer 44 showed another peak in the p38-ELISA index values after 300 days post-infection. The fluctuation in the antibody levels of heifers 44 and 49 was attributed to the stages of the reproductive cycle (Schares et al., 2000; Stenlund et al., 1999). The p38-ELISA indices of the three animals were considered positive after 14 days post-infection (Figure 16).

Different antibody profiles of the three experimentally infected animals in the newly developed cELISA were obtained as compared to those obtained in the p38-ELISA. Heifers 44 and 49 yielded increasing SCI values in the cELISA until a plateau was reached after 60 to 90 days post-infection. Both heifers maintained SCI values above 80% until the end of the observation period. Fluctuations in the antibody levels of heifers 44 and 49 during the period of the experiment, as demonstrated by p38-ELISA, were not detectable when the cELISA was employed. Cow 24 showed increasing SCI values during the period of the study, being positive after 60 days post-infection, in contrast with heifer 44 and 49 which yielded positive-SCI values after 15 days post-infection (Figure 16).

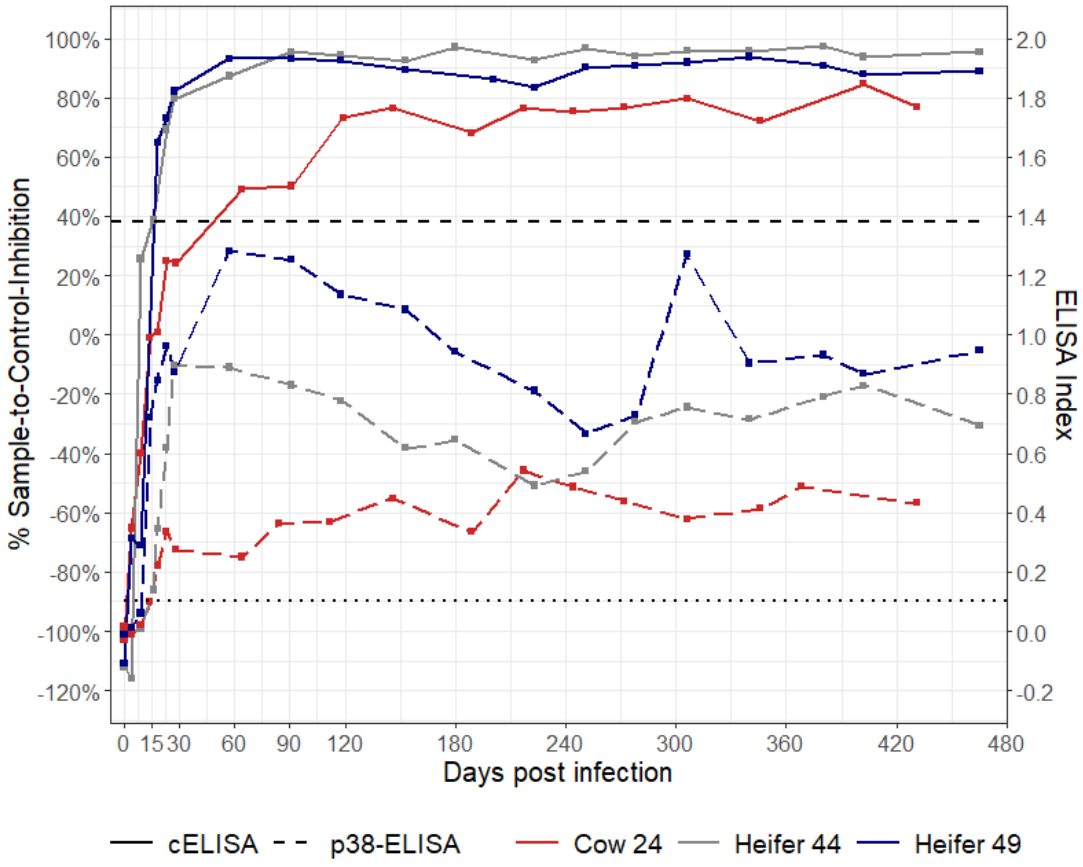

Figure 16. Antibody profiles of three experimentally infected cattle (Cow 24, Heifer 44, and Heifer 49) as determined by p38-ELISA (ELISA indices) and cELISA (Sample-to-Control-Inhibition, SCI). All animals were infected on day 0 of the experiment. The black horizontal dashed and dotted lines represent the cELISA and p38-ELISA cut-off points, respectively.

3.4 Application of the mAb-based ELISA to study a herd with *N. caninum* associated abortion

3.4.1 Analysis of abortions that occurred in 2021

During the year 2021, twelve abortions were recorded on the farm (Figure 17). Since more information was available for this year, abortions were studied to determine its pattern (epidemic or endemic) and the possible source of infection (oocyst-derived infection of naïve dams or recrudescence of infection in chronically infected dams). Eight of these abortions occurred in two months (from 22.05.2021 to 19.07.2021) and the affected cows were pregnant for five to seven months. At the first sampling (2020), only some (n=8) of the cows that aborted during 2021 were sampled. Two were negative in all tests apart from IFAT, one was negative in both p38-ELISA and cELISA, but positive in immunoblot (two IDAs) and IFAT. The other dam was positive in immunoblot (four IDAs) but negative on all the other tests. Two were positive in all tests. One was positive in all tests apart from IFAT and a last one was both positive in the cELISA and IFAT, but negative in p38-ELISA and immunoblot. At the second sampling (April 2021) all dams were positive in all tests, apart from one. The negative dam at the second sampling was only deemed positive by one test (IFAT, in the first sampling).

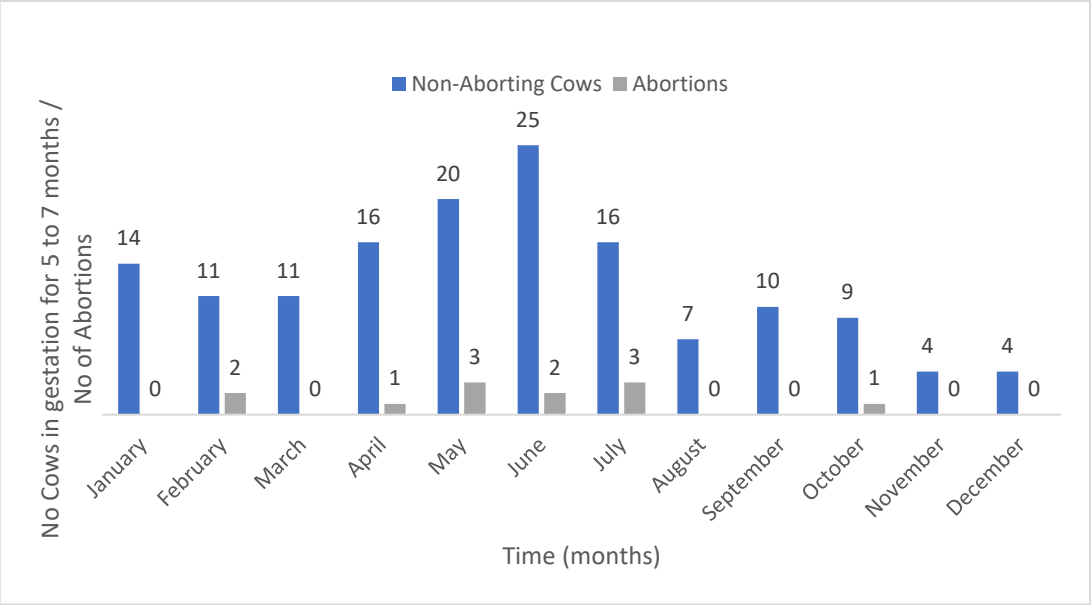

Figure 17. Frequency of abortions (per month) in 2021 compared with the number of non-aborting cows in a period of gestation from five to seven months.

The "positivity" of the aborting dams (i.e., the presence of *N. caninum* antibodies in the serum) does not allow a definitive diagnosis of *N. caninum* as the cause of the abortion because only a small percentage of infected dams abort and the majority of calves born from *N. caninum*-infected dams are born healthy (but persistently infected) (Dubey et al., 2017b; Ortega-Mora et al., 2006). Aborted foetuses and placentas were not available for examination, so the presence of *N. caninum* in foetuses was not possible to determine. For these reasons, an epidemiologic approach was taken, using the "herd-based analysis" (Kinsel, 1999; Thurmond et al., 1997). This approach aimed to determine whether the presence of specific *N. caninum* antibodies in sera was an indicator of abortion, i.e., if the proportion of seropositive animals was significantly higher in aborting dams than in non-aborting dams.

Both patterns of abortion were considered: dams were regarded as "at risk" for endemic abortion if they had been in gestational stage during the period in which the abortions occurred (Dubey et al., 2017b), i.e. from February to October 2021; dams pregnant for 58-200 days at the first day of the epidemic were considered "at risk" for epidemic abortion (Dubey et al., 2017b). The p-value and Odds Ratio were calculated considering both possible abortion patterns (Table 10 and Table 11).

When all the period of increased abortion rate was considered (assuming an endemic abortion pattern), 118 dams were included in the "at risk" group. It was only possible to determine the serostatus of 112 dams, since six had not been sampled. The estimated abortion rate for this period was 10% (12/118). The relationship between *N. caninum* infection and abortion was considered statistically significant independently of the selected test and high Odds Ratio's were obtained with a range from 7.54 to 12.19 (Table 10).

Test	% Aborting (n)	% Non-aborting (n)	Odds Ratio (95 % CI)	p-value (Fisher's Exact)
IB	92% (11/12)	59% (59/100)	7.54 (1.02 -336)	0.029
p38-ELISA	92% (11/12)	48% (48/100)	11.72 (1.59-521)	0.005
IFAT	92% (11/12)	47% (47/100)	12.19 (1.65-542)	0.004
cELISA	92% (11/12)	47% (47/100)	12.19 (1.65-542)	0.004

Table 10. Seropositivity rates (IB, p38-ELISA, IFAT and cELISA) and estimated odd ratios (with 95% confidence limits) for aborting and non-aborting (at-risk) cows, considering an endemic pattern of abortion.

Restricting the analysis to the period where the majority of abortions occurred (from 22.05.2021 to 19.07.2021) an abortion rate of 18% is obtained. Several authors cited by Dubey et al., (2007) defined epidemic abortion as if more than 15% of cows at risk abort within 4 weeks (Schares et al., 1999a), 12.5% within 8 weeks (Wouda et al., 1999) and 10% within 6 weeks (Moen et al., 1998). Therefore, this period of 8 weeks, where most of the abortions occurred in the NcHerd1, matches the definition of epidemic abortion. A total of 44 dams were pregnant for 58-200 days on the first day of the abortion outbreak (22.05.2022) and were included in the "at risk" group of dams, but serum samples were only available for 42 dams. The relationship between *N. caninum* infection and abortion was only considered statistically significant by p38-ELISA and IFAT and the lower confidence limit of the Odds Ratio was only higher than 1 for IFAT (Table 11).

Test	% Aborting (n)	% Non-aborting (n)	Odds Ratio (95 % CI)	p-value (Fisher's Exact)
IB	88% (7/8)	62% (21/34)	4.21 (0.45 -210)	0.233*
p38-ELISA	88% (7/8)	44% (15/34)	8.47 (0.92-419)	0.047
IFAT	88% (7/8)	35% (12/34)	12.09 (1.31-602)	0.015
cELISA	88% (7/8)	50% (17/34)	6.73 (0.73-333)	0.109*

Table 11. Seropositivity rates (IB, p38-ELISA, IFAT and cELISA) and estimated odd ratios (with 95% confidence limits) for aborting and non-aborting (at-risk) cows, considering an epidemic pattern of abortion. *p-value not significant.

When aborting and non-aborting groups were compared (Figure 18) by means of their SCI values in the cELISA, aborting cows yielded statistically significant higher values than non-aborting cows (p-value=0.031, Wilcoxon rank sum test). A similar finding was observed when p38-ELISA indices were compared (p-value=0.018, Wilcoxon rank sum test).

The avidity values of antibodies present in sera from the aborting animals (n=12) were determined by p38-avidity-ELISA (Schares et al., 2002a), by adding an incubation step with 6M urea to the conventional p38-ELISA. Any of the dams presented avidity index values that might indicate the presence of low avidity antibodies (<55%).

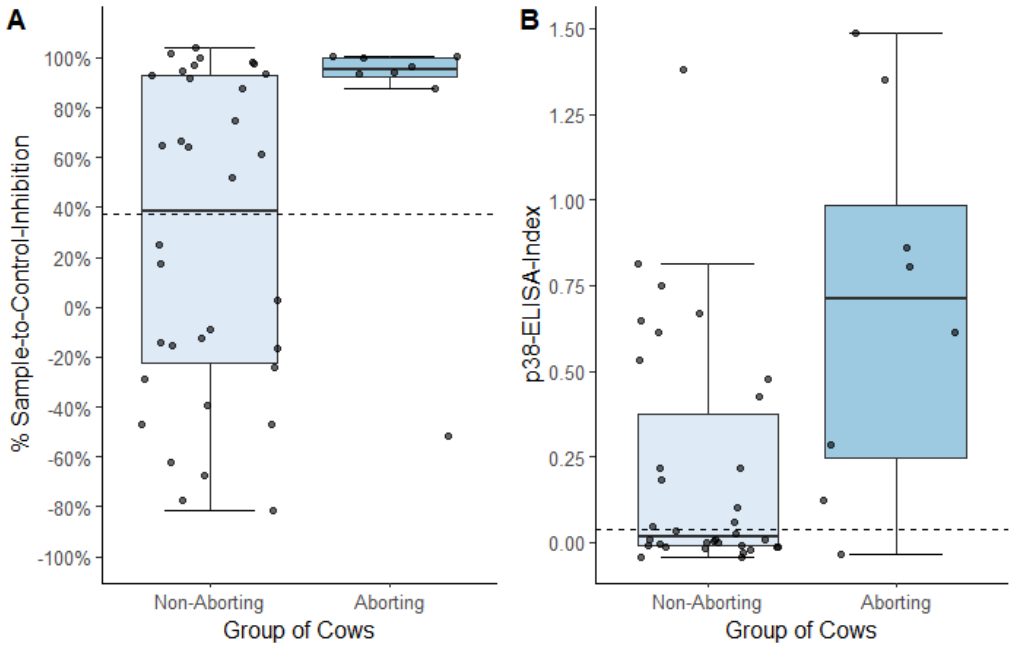

Figure 18. Comparison of the Sample-to-Control-Inhibition (SCI) in cELISA (A) and p38-ELISA indices (B) between aborting and non-aborting groups of cows in 2021, considering an epidemic pattern of abortion. The dashed lines represent the cut-off values of each test.

3.4.2 Age-related differences

For each *N. caninum* serological test performed, the sampled cows and heifers from the NcHerd1 were plotted against the year of birth. This procedure was followed for the different years of sampling and the results are shown in Table 12.

Sampling	Age (years)	% IB-positives (n)	% p38-ELISA-positives (n)	% IFAT-positives (n)	% cELISA-positives (n)
2020	2-3	40% (25/61)	40% (24/61)	66% (40/61)	48% (29/61)
	4-5	31% (16/43)	26% (11/43)	56% (24/43)	37% (16/43)
	≥6	31% (9/29)	10% (3/29)	68% (20/29)	31% (9/29)
	Total	38% (50/133)	29% (38/133)	63% (84/133)	41% (79/133)
2021	<2	42% (27/65)	40% (26/65)	32% (21/65)	40% (26/65)
	2-3	79% (44/56)	73% (41/56)	63% (35/56)	75% (42/56)
	4-5	58% (29/50)	50% (25/50)	40% (20/50)	52% (26/50)
	≥6	56% (15/27)	37% (10/27)	40% (11/27)	44% (12/27)
	Total	58% (115/198)	52% (102/198)	44% (87/198)	54% (106/198)
2022	2-3	60% (31/52)	54% (28/52)	40% (25/52)	58% (30/52)
	4-5	60% (21/35)	54% (19/35)	54% (19/35)	62% (22/35)
	≥6	45% (18/40)	45% (18/40)	40% (16/40)	48% (19/40)
	Total	55% (70/127)	51% (65/127)	47% (60/127)	56% (71/127)

Table 12. Percentages of N. *caninum* antibodies in the NcHerd1 determined by IB, p38-ELISA, IFAT and cELISA. The values were stratified according to the age of the animals on the day of the sampling.

When the percentage of positive animals was stratified according to the age of the animals, values varied between 10% and 79% depending on the selected test and year of sampling. The total percentage of positive animals varied between 29% and 58%. For all tests (apart from IFAT), increasing age seemed to be associated with a lower number of positives, especially in 2020, however, a statistically significant difference in the

seroprevalences of different age groups was only found for p38-ELISA (p-value=0.02, Pearson's chi-square test). In the 2021 samplings, the age group 2-3 years showed a higher percentage of positives when compared with other age groups, and this difference was considered statistically significant, regardless of the selected test (highest p-value=0.008, Pearson's chi-square test). In the year 2022, the age group where the oldest cows (>6 years) were included continued to have a lower percentage of positive animals, but the differences were not statistically significant (p-value>0.05, Pearson's chi-square test).

When the abortions that occurred in 2021 (n=12) were plotted against the year of birth of at-risk dams (data not shown), the results revealed six abortions in the age group 2-3 years, three in age group 4-5 years and two in the oldest age group (>6 years). Considering only the abortions of the presumed epidemic outbreak (n=8) the results revealed four abortions in the age group 2-3 years, two in age group 4-5 years and another two in the oldest age group (>6 years). No statistically significant differences (p-value >0.05, Pearson's chi-square test) were found between the different age groups for both an endemic and epidemic pattern of abortion.

3.4.3 Comparison of dam-daughter serostatus

A comparison of the serological status between dam and daughter pairs was performed to establish whether the proportion of seropositive daughters born to seropositive mothers is higher than the proportion of seropositive daughters born to seronegative mothers. This information could be interpreted as an indication of the predominate route of transmission of *N. caninum* infection within the herd.

The overall analysis of the dam-daughter pairs (n=118) revealed a statistically significant association between the serological status of dams and daughters when immunoblot (p-value=0.009, Pearson's chi-square test), p38-ELISA (p-value=0.035, Pearson's chi-square test), and cELISA (p-value=0.014, Pearson's chi-square test) were performed, but not significant when IFAT results were considered (p-value>0.05, Pearson's chi-square test). Any test showed a significant association when the dam-daughter serology was restricted to the group of pairs in which the daughters were born before 2019 (n=44) or to the group of pairs in which the daughters were born in 2019 (n=35) (p-value>0.05, Pearson's chi-square test). However, in the last group of pairs (n=39), in which daughters born in 2020/21 were included, 86% to 93% (depending on the selected serological test) of seropositive daughters had a seropositive mother and the relationship was considered statistically significant when immunoblot, p38-ELISA and cELISA (p-value<0.001, Fisher's Exact test) were applied but not IFAT (p-value>0.05, Fisher's Exact test). The Odds Ratios of seropositive daughters having a seropositive mother was 50.6 (95%CI: 6.13-808) for immunoblot, 16.5 (95%CI: 2.76-138) for p38-ELISA and 76 (95%CI: 7.6-4080) for cELISA, as compared to seronegative daughters.

4. Discussion

4.1 Development of a competitive ELISA based on mAb 3.10.5

In this study, a *N. caninum* competitive ELISA based on mouse monoclonal antibody reactions against crude *N. caninum* antigen was successfully developed. Serological techniques can be applied *in vivo* and are particularly important in cases of *N. caninum*-associated abortion (Dubey et al., 2017b; Dubey and Schares, 2006) due to the absence of clinical signs in adult cows other than abortion (Dubey and Schares, 2011; McAllister, 2016). Particularly, ELISA assays enable the rapid determination of antibody levels in serum and are the most suitable serological test for a complete herd analysis, since this technique allows the examination of a larger number of samples at the same time as compared to other serological techniques (Björkman et al., 2007, 1997; Dubey et al., 2017b; Ortega-Mora et al., 2006).

Serological tests which include crude *N. caninum* tachyzoites as antigen can potentially have problems regarding their specificity because of the relatedness of *N. caninum* to other parasites, particularly *Sarcocystis* spp. (Baszler et al., 1996; Dubey et al., 1996). This can be a problem due to the importance and high prevalence of cattle infected with *Sarcocystis* sp. worldwide, as demonstrated by examinations of tissues obtained at abattoirs (Dubey et al., 2015; Dubey and Rosenthal, 2022). Cross-reactivity with *B. besnoiti* could also constitute a problem since both share cattle as the major host and co-infections in regions where both parasites are endemic seem to be frequent (Jacquiet et al., 2010). Cross-reactivity in *B. besnoiti* ELISAs and immunoblots with *N. caninum*-infected animals (especially those presenting high titres) have been reported (Gondim et al., 2017; Schares et al., 2010). The cELISAs using mAbs as the method for detecting specific antibodies have the advantage of mAb epitope-specific binding, potentially overcoming problems reported by others with non-specific binding and cross-reactivities (Baszler et al., 1996; Björkman et al., 1997; Williams et al., 1997).

The specificity of the monoclonal antibodies for *N. caninum* antigen was first studied by immunoblotting. Reactions against both reduced and non-reduced *T. gondii* tachyzoites were not observed by immunoblotting, however, two of the mAbs (4.11.5 9/12-12) reacted against *B. besnoiti* tachyzoites previously prepared by SDS-PAGE under non-reducing conditions. These mAbs showed dense granule-like staining by immunofluorescence (Table 2) and mAb 9/12-12 was generated against a recombinant NcGRA7 protein. Cross-reactivity of *N. caninum*-infected cattle with *B. besnoiti* antigens has been described in the past, in which a band with a similar 33 KDa molecular range was recorded (Schares et al., 2010). This observation seems to suggest that *N. caninum* and *B. besnoiti* share similar epitopes. The fact that no cross-reactions with *T. gondii* were observed is also in agreement with previous observations that cross-reactions between *T. gondii* and *N. caninum* are minimal (Dubey, 2003), especially regarding reactions against immunodominant antigens (Gondim et al., 2017).

Before the establishment of cELISAs with the *N. caninum* mAbs, experiments to determine the optimal concentration of reagents (antigen and hybridoma supernatant)

were performed by iELISA. During these experiments, different levels of optimal reactivity were obtained depending on the mAb tested with the antigen solution. The fact that different levels of optimal reactivity (referred to as plateau in the results) were observed in both, the antigen (Figures 4) and the hybridoma-specific titration curves (Figure 8) could perhaps be explained by the different number of reactive antigenic sites, and the quantities and specificities of antibody population present in the hybridoma supernatant (Crowther, 2009). In the titration curves of the hybridoma supernatant, an initial increase in the OD values was visible, instead of a complete plateau as expected. This may be explained by the so called prozone or "hook" effect, caused by the excessively high concentrations of the mAbs, resulting in a decrease in signal (Lin, 2015). This effect is more common, but not exclusive, to one-stage sandwich assays (Wu and Christensen, 1991). In assays with sequential incubations, only a low percentage of antibodies may bind bivalently to the antigen, resulting in a decrease of OD for the highest antibody concentrations (Vos et al., 1987).

Monoclonal antibody 3.10.5 reacted against a 38 kDa surface antigen of *N. caninum* tachyzoites (Table 2 and Table 9) and cross-reactions were neither observed against *T. gondii* tachyzoites nor against *B. besnoiti* tachyzoites (Table 9). Furthermore, mAb 3.10.5 showed OD values higher than 1 OD unit in indirect ELISA when plates were coated with crude tachyzoite antigen (Figure 8). Thus, this reactivity was in the range of 1-1.5 OD units, recommended by Crowther (2009b) which was considered important to obtain a high relation between specific colour development and background. However, the titration curves of a positive control bovine serum (Figure 9) showed no statistically significant differences regarding the sensitivity of cELISAs based on different anti-surface mAbs. This might be an indication that other anti-surface mAbs (other than 3.10.5) were also suitable candidates to establish a cELISA able to detect *N. caninum*-infections in cattle. The fact that the two mAbs, which react against *N. caninum* dense granule proteins (4.11.5 and 9/12-12), did not show a linear range in the titration curves in cELISA experiments was surprising, because it has been shown that dense granule proteins are expressed in *N. caninum* tachyzoites (Wang et al., 2021) and a linear range was observed during the experiments when antigen (Figure 7) and hybridoma supernatant (Figure 8) were titrated. During previous experiments (data not shown), a similar problem characterized by high background levels was observed when a different blocking and sample dilution buffer (PSB-T with horse serum) was used. Therefore, it is possible to speculate that the selected blocking buffer (PBS-T-G) is not optimal to determine the effect of the positive control serum on the inhibition characteristics of the two referred mAbs (4.11.5 and 9/12-12).

4.1.1 Validation and characterization of the mAb-based ELISA

The serological tests that have been used to detect *N. caninum* antibodies were not validated based on the recovery of the viable parasite, thus the cut-offs selected can only be considered presumptive (Dubey et al., 2007). Consequently, there is no gold standard to discriminate between a true-positive and a true-negative in a given group of cattle sera (Dubey et al., 2017b; Dubey and Schares, 2006). To overcome this problem three previously published serological tests were applied to define the positive and

negative population, using a majority criterion. The results revealed high diagnostic sensitivity (98.1%) and specificity (97.7%) for the detection of specific *N. caninum* antibodies, using a cut-off of 38% SCI, which maximized Youden's index. The distribution of SCI values by reference classification of sera (Figure 11) shows a clear distinction between the two reference populations, with a small overlap between them. This was interpreted as a good indicator of the ability of the test to discriminate between true-positive and true-negative results (Crowther, 2009).

In contrast to this approach, a gold-standard-free method of selecting an appropriate cut-off was also applied, based on Bayesian statistics. As far as the author knows, there are only two publications which follow this method to select an appropriate cut-off value and determine the respective sensitivity and specificity of *N. caninum*-ELISAs (Frössling et al., 2003; Roelandt et al., 2015) but BLCA are becoming increasingly popular when reference tests are imperfect (Cheung et al., 2021). The data obtained from the Bayesian model suggests that every cut-off value between 32% and 37% SCI would result in a diagnostic sensitivity and specificity >98%, but Youden's index was maximized at the cut-off point of 37% SCI. As expected, this range corresponds to the area of separation between the reference-positive and negative populations in Figure 11, since the same reference sera were used to assess the diagnostic characteristics of the novel cELISA by the two methods.

Different cut-off values should be selected depending on the purpose of the test (Álvarez-García et al., 2007; Greiner and Gardner, 2000). Based on the BLCA, the cut-off of 37% SCI seems appropriate for monitoring herds with reproduction problems or to screen populations with unknown infectious status, maximizing both diagnostic sensitivity and specificity (>98%) (Frössling et al., 2003). In eradication schemes, higher cut-off values for evaluating the culling of highly valuable animals are recommended (Frössling et al., 2003; Ortega-Mora et al., 2006). A more stringent cut-off in order to obtain maximum specificity may be of use for diagnosing *N. caninum*-associated abortions (Álvarez-García et al., 2003; Reichel and Pfeiffer, 2002; Schares et al., 1999a). A cut-off value of 43% SCI would maximize specificity (>99%), maintaining a high sensitivity (95%), which may fit these two purposes. The confidence limits separate at the cut-off value of 43% SCI (Figure 12), so the selected cut-off should not be higher than 43% SCI to maintain high sensitivity. High sensitivity is needed when a more rapid eradication is intended (even with the risk of having more false-positive results), or to examine individual cattle before purchase into a *N. caninum*-free herd (Álvarez-García et al., 2002; Dubey and Schares, 2006; Frössling et al., 2003; Reichel and Pfeiffer, 2002). In the BLCA model, a plateau between the cut-off values of 32% and 37% SCI is observed. Thus, every cut-off value between these two points would maximize diagnostic sensitivity (>98%), maintaining high specificity (>98%).

Due to the test design which does not require host species-specific conjugates other than an anti-mouse conjugate, the newly developed cELISA can potentially be applied for screening *N. caninum* antibodies in different host species other than cattle (McGarry et al., 2000). However, further validation studies are needed to estimate the sensitivity

and specificity of the test when applied to those species, which may result in adjustments to the cut-off value (Dubey et al., 2017a).

4.1.2 Analytical specificity of the cELISA based on mAb 3.10.5

Once the competitive ELISA based on mAb 3.10.5 was established, the specificity of this particular mAb was confirmed by testing field sera originating from herds with confirmed besnotiosis cases and suspected *Sarcocystis* spp. infections (Table 5). Reference-negative sera originated from these two herds were compared with reference-negative sera from the herds with neosporosis (section 3.2.4 and Figure 13). The differences in SCI values were not statistically significantly different when the comparison was made to the herd with besnoitiosis cases. This represents further confirmation that the epitope recognized by mAb 3.10.5 is not present in *B. besnoiti*. However, the group of animals from the *Sarcocystis* spp.-infected herd yielded SCI values statistically significantly higher than those of the negative reference population in the neosporosis herds. It has been shown that antibodies against bovine *Sarcocystis* spp. do not present considerable cross-reactions when *N. caninum*-immunodominant antigens are used (Gondim et al., 2017) thus this finding could perhaps be attributed to stearic interference of cross-reacting antibodies to other *N. caninum* antigens in the crude (tachyzoite lysate) antigen preparation as suggested by Baszler et. al (1996). Only one sample yielded a SCI value high enough to reach the threshold of positivity (Figure 13) but since the serostatus of individual animals regarding *Sarcocystis* spp. infections were unknown, further studies need to determine whether the specificity of the cELISA would benefit from the use of a different antigen preparation (ex. purified antigen).

4.1.3 Comparison of the cELISA with reference tests

The newly developed cELISA showed a high level of agreement with reference serological tests by κ-statistics. An almost perfect agreement was obtained between cELISA and both, the p38-ELISA and immunoblot, and the lowest agreement was obtained with IFAT. The high agreement between the novel cELISA and the other two reference tests (p38-ELISA and immunoblot) reflects the similarities regarding positive/negative classification. However, κ-statistics do not indicate which test is more accurate or what kind of errors either test is producing. Compared to the reference standard, IFAT showed a high number of false-positive results (n=29) and an even higher number of false-negative results (n=33), suggesting that the lack of agreement between cELISA and IFAT is more likely due to false-positive and false-negative results of IFAT. The subjective evaluation of fluorescence in the IFAT technique might have played a role in these discrepancies.

Regression analysis revealed a moderate to high coefficient of determination between cELISA and p38-ELISA. A very high coefficient of determination was never expected due to the different technical aspects of the two ELISAs. The p38-ELISA is based on an affinity-purified 38 KDa surface antigen of *N. caninum* tachyzoites and biotinylated anti-bovine antibodies are used to amplify the signal, whereas in the competitive ELISA, there are a limited number of epitopes available, thus the inhibition of mAb binding only increases until the threshold of 100%. These differences might also help to explain the

observations in the scatterplot (Figure 15), in which strong positive sera exhibited increasing index values in p38-ELISA but SCI values around 100% in the cELISA.

4.1.4 Comparison between aborting and non-aborting dams

Given that serological tests should be previously validated for the application in which they will be used (Greiner and Gardner, 2000), the suitability of novel cELISA to examine herds with abortion problems was investigated. Using the cELISA, it was possible to confirm previous observations in which dams experiencing abortion due to neosporosis showed higher antibody titres than their non-aborting counterparts (Almería et al., 2009a; Dubey et al., 1997; González-Warleta et al., 2011; Hernandez et al., 2002; Kashiwazaki et al., 2004; López-Gatius et al., 2005b; McAllister et al., 1996; Quintanilla-Gozalo et al., 2000; Schares et al., 2000; Stenlund et al., 1999; Wouda et al., 1999; Yániz et al., 2010). The antibody titres are indirect indicators of exposure to parasite antigens i.e., increasing parasite activity and multiplication in the host (Innes et al., 2005). The fact that aborting dams show higher ELISA values most likely indicates that they are more exposed to *N. caninum* antigens, having higher specific antibody levels than non-aborting infected dams (Dubey et al., 2017b). These observations seem to suggest that the novel Nc-cELISA is suitable to examine herds experiencing abortions when neosporosis is suspected, but further studies with samples from other herds are needed.

Similarly to what was reported in the past for other ELISA systems (Schares et al., 2000, 1999d) sera obtained from herds with epidemic abortion demonstrate lower ELISA values as compared to sera from endemic abortion herds, which could suggest that different cut-offs for herds with epidemic and endemic abortion patterns are needed. This approach was not taken in this study since only two herds (one with presumably endemic cases and the other with an epidemic pattern of abortion) were analysed, because of the risk of defining a cut-off only suitable for a particular set of samples derived from the same herd.

4.1.5 Antibody responses of dams experimentally infected with *N. caninum*

The antibody profiles obtained by cELISA of three experimental infected cows were compared with those obtained by p38-ELISA. Interestingly, the antibody fluctuations of heifer 44 and 49 observed when p38-ELISA was implemented were not observed when those animals were tested in the cELISA. A possible explanation is the differences in test design, as discussed previously. Cow 24, which presented a slow and stable increase in antibody titres over the period of the experiment, was only regarded as positive by the cELISA after two months of infection. This finding was unexpected and may reveal that the novel cELISA is not as sensitive as p38-ELISA in the detection of acute *N. caninum*-infections. However, this apparent lack of sensitivity was not evident when field low-avidity sera (Table 5) were tested, since only two false-negative results were obtained. Thus, further studies need to elucidate the diagnostic sensitivity of the novel cELISA in larger sets of sera derived from animals with acute *N. caninum*-infections.

4.2 Application of the mAb-based ELISA to study a herd with *N. caninum* associated abortion

After validation and characterization, the newly develop Nc-cELISA based in mAb 3.10.5 was used to study a herd (NcHerd1) with suspicion of *N. caninum*-associated abortion. Based on the data that was possible to obtain from the farmer and HIT the abortions that occurred in 2021 were studied to establish if these abortions could be linked with *N. caninum* infections and, if so, speculate about the possible sources of infection and the predominant pattern of abortion. The causal relationship between abortion and *N. caninum* infection was possible to establish independently of the serological test used (Table 10) and high odds ratio revealed that seropositive cows were 7.5-12-times more likely to abort than seronegative cows. However, this casual relation was not clear if the period of abortion was restricted to a possible outbreak of epidemic abortion (Table 11), even considering the high rate of abortions (18%) and the fact that only one of the aborting cows (n=8) didn't have evidence of *N. caninum* antibodies in serum. This could perhaps be explained by the lack of power of the statistical test since only 34 non-aborting cows could be included. A more stringent cut-off for the cELISA (i.e., 43% SCI, as discussed above) would not affect the statistical association because any of the "at risk" dams showed SCI between 38% and 43% SCI. As expected, if a more stringent cut-off was applied in the p38-ELISA (0.1 index value), a lower p-value would be observed for the relationship between abortion and *N. caninum* infection (p-value=0.02, Fisher's Exact test).

Because the individual serum specific *N. caninum* antibody titre is a good indicator of the abortion risk (Quintanilla-Gozalo et al., 2000), a comparison between SCI values of aborting and non-aborting cows, as suggested by McAllister et al. (2016), as a way to establish the causal relationship between *N. caninum*-infection and abortion was performed. Statistically significantly higher SCI values in the clinically affected animals were observed as compared to non-aborting animals, suggesting that *N. caninum* was the cause of the abortions. The same finding was obtained by the comparison between p38-ELISA index values from the two groups of animals, supporting the hypothesis that *N. caninum* infections are the cause of the abortions.

No indication for the presence of low avidity antibodies (<55% avidity index) was found in the aborting group of animals (n=12), suggesting that these dams were not recently exposed to *N. caninum* by the horizontal route at the time of sampling (Björkman et al., 1999; Dijkstra et al., 2003; Jenkins et al., 2000; McAllister et al., 2000; Schares et al., 2002a).

When the seropositive animals were separated into different age groups, a particular age cluster was revealed, showing a significantly higher percentage of seropositive animals as compared to other age groups (2-3 years, in 2021 sampling). The animals from this age cluster were born between 2018 and 2019 (the abortion problems started in 2019) and it could be speculated that these animals became horizontally infected, probably by access to fodder or water contaminated with oocysts when they were still separated from the adult cows. Since 61% (n=34) of the animals included in this age

group were younger than 2 years old (in 2021) serum samples were not collected in the first year of the study, which could explain the fact that the same positive age cluster is not evident in the first sampling. Furthermore, from the 28 dam-daughter pairs that were possible to establish with the referred group of animals (n=34) included as daughters, only four (14%) of the seropositive daughters had seropositive mothers in both p38-ELISA and immunoblot. The cELISA and IFAT tests recognized seven (25%) and eight (29%) seropositive daughters with seropositive mothers, respectively. These findings corroborate the hypothesis of post-natal infection of the daughters (Dijkstra et al., 2001a; Schares et al., 1998; Thurmond et al., 1997). Nevertheless, it cannot be excluded that animals from other age groups might have been infected in recent years (and particularly during the study period) since some seroconversions during the study period were noted by all the tests and the overall apparent seroprevalence increased (Table 12).

The lack of association between the serological status of dam-daughter pairs which were composed of daughters born in 2019 and before, also supports the idea of a recent introduction of *N. caninum* in the herd due to horizontal transmission (Dijkstra et al., 2001a; Schares et al., 1998; Thurmond et al., 1997). Due to the strong association between seropositivity of mothers and daughters, it is reasonable to assume that oocyst derived-infections no longer took place or at least became less important as a vertical, endogenous transplacental transmission became the predominant route of infection for the daughters born after 2020 (the first year of this study). In fact, the odds of a seropositive daughter born after 2020 having a seropositive mother were 16.5-76 times higher as compared to a seronegative daughter. Unfortunately, these assumptions cannot be further verified since there is not enough information to establish dam-daughter pairs for the subsequent period.

The age group of 2-3 years in 2021 was more affected by abortion than the other age groups (see results), which probably is a consequence of the higher percentage of *N. caninum* seropositive cows in this group. It is well known that seropositive dams have an increased risk of abortion compared to seronegative dams (López-Gatius et al., 2004a, 2004b; Moen et al., 1998; Pabón et al., 2007; Paré et al., 1997; Stenlund et al., 2003; Weston et al., 2005). However, the differences between the age groups were not considered statistically significant.

Taking into account the findings previously discussed, three hypotheses arose to explain the high rate of abortions (18%) that occurred in a short period (8 weeks) of the year 2021, which could theoretically be considered an outbreak of epidemic abortion: (1) an accumulation by chance of endemic abortion cases in a short period of time (Thurmond et al., 1997) i.e. if only by chance a high proportion of *N. caninum*-infected cows were in the same stage of gestation and therefore more susceptible to an abortion caused by *N. caninum* due to the endogenous transplacental transmission; (2) a synchronized recrudescence of *N. caninum* infections derived from an immunosuppressive event in a significant number of animals (Atkinson et al., 2000a; Dijkstra et al., 2001a; Wouda et al., 1999) also caused by endogenous transplacental

transmission; or, (3) an oocyst derived infection of several naïve animals causing the infection of the foetus by the exogenous transplacental transmission (Trees and Williams, 2005; Williams et al., 2009).

A possible explanation for the first hypothesis when a high number of endemic abortions occur in a short period, resulting in a pattern similar to epidemic abortion, was suggested by McAllister (2016) as the practice of seasonal breeding instead of year-round breeding.

The sampling of 2021 took place in April which means that seven out of eight aborting cows were seropositive for at least 45 days when the first abortion of the cluster took place. Furthermore, three of these cows (the other five were not tested previously) were regarded as seropositive by at least two serological tests in the previous year. A previous infection and particularly the presence of specific *N. caninum* antibodies in serum has been shown to have a protective effect on horizontal (oocyst-derived) natural (McAllister et al., 2005, 2000) and experimental infections (Innes et al., 2001; Williams et al., 2003). Another factor to consider is that horizontal infections probably became less important to maintain *N. caninum* infections in the herd in 2020 and 2021 (see above). The avidity values provide information on the period between infection and sampling (Björkman et al., 1999; Jenkins et al., 2000; Schares et al., 2002a). In this case, sampling occurred 45 days before the first abortion of the cluster, therefore evidence of recent (acute) *N. caninum*-infections (low avidity sera) would be expected in the case of an oocyst-derived infection of naïve cattle. So, it seems unlikely that these abortions were caused by exogenous transplacental transmission as a result of an oocyst-derived infection.

The period when these abortions occurred matches the peak when more cows were pregnant for 5 to 7 months (Figure 17), precisely the period when most of the abortions related to an *N. caninum* infection occur (Dubey, 2003; McAllister, 2016). This evidence seems to support the hypothesis of an epidemic-like pattern caused by the accumulation of endemic abortions in a short period as a result of a high number of cows being more susceptible to *N. caninum* abortion in this period. It has been demonstrated that the time when cattle become infected or when the recrudescence of persistent infections occurs is critical to determine the outcome of the infection (Innes et al., 2005, 2001; Macaldowie et al., 2004; Maley et al., 2003; Williams et al., 2000). So, the hypothesis of a synchronized recrudescence of *N. caninum* infections related to an immunosuppressive event cannot be fully excluded since abortions caused by such an event would probably also occur in the period when the cows are more susceptible to *N. caninum*-associated abortion.

A study in The Netherlands identified “Feeding of moldy maize-silage to dairy cows during summer” or “Feeding of remnant fodder to heifers during summer” as risk factors for epidemic *N. caninum* abortions. The given explanation for these risk factors was the deleterious effects of mycotoxins on the bovine immune system which might lead to recrudescence infections in persistently infected cows (Bartels et al., 1999; Thurmond et al., 1997; Wouda et al., 1999). The effect of mycotoxin intoxication in enhancing the

reactivation of chronic infection was shown in mice with chronic toxoplasmosis (Venturini et al., 1996), but such an effect in cattle chronically infected with *N. caninum* remains to be proven directly.

Another factor that might influence reproductive performance is heat stress, especially during early pregnancy (García-Ispierto et al., 2006; López-Gatius et al., 2003). However, a hot environment alone seems not to directly affect the risk of *N. caninum*-associated abortion (García-Ispierto et al., 2006; López-Gatius et al., 2003; Yániz et al., 2010), but might contribute to oocyst sporulation and therefore indirectly to infection of naïve cattle (Bartels et al., 1999; López-Gatius et al., 2005a; Sanderson et al., 2000; Wouda et al., 1999). Heat stress also affects the immune system by increasing cortisol concentrations (Bagath et al., 2019). Hyperthermia is associated with shift towards the more anti-inflammatory Th2-type cytokines, which may increase the susceptibility to disease (Bagath et al., 2019).

Another environmental factor—rainfall during the second trimester of gestation—was considered a risk factor for *N. caninum*-associated abortion (López-Gatius et al., 2005a; Yániz et al., 2010). The authors of the two studies speculated that increased rainfall maybe be associated with the augment of direct stress, elevating heat production in response to cold temperatures, and indirect stress, due to behavioural stress, reduction of food quality, and hygiene deterioration (López-Gatius et al., 2005a). Such stress may lead to immune suppression increasing the susceptibility to infections or recrudescence of chronic infections (López-Gatius et al., 2005a; Roth, 1985; Sternberg, 1997).

Concurrent agents other than *N. caninum* were suggested to contribute to *N. caninum* abortion outbreaks (Björkman et al., 2000; Thurmond et al., 1995). Particularly, in a Swedish study, a relationship between seropositivity to *N. caninum* and bovine viral diarrhoea virus (BVDV) in aborting cows in herds with abortion problems was found (Björkman et al., 2000). A logistic regression model with data from Swiss dairy herds identified positive BVDV serology as a putative risk factor for *N. caninum*-associated abortion at the herd level (Hässig and Gottstein, 2002). Nonetheless, the case-control analysis revealed that herds experiencing *N. caninum*-associated abortions were less often positive for antibodies against BVDV (Hässig and Gottstein, 2002). The possible explanation for the association between *N. caninum*-associated abortion and BVBV infections might be because the latter is known to cause immunosuppression (Walz et al., 2020). However, a case-control retrospective study analysing Dutch herds experiencing abortion storms showed a negative relationship between seropositivity to *N. caninum* and BVDV among the aborting dams (Bartels et al., 1999). One of the factors for contradictory results in serological risk factor analysis is that both vaccination and infection can induce antibodies and often it is not clear whether antibodies are present due to infection or vaccination (Dubey et al., 2017b). Moreover, a study analysing dairy herds in Ontario, Canada, associated vaccination against infectious agents, including other abortifacients such as *Leptospira* sp. and BVDV, as a protective factor for the occurrence of *N. caninum*-associated abortion (Hobson et al., 2005). It was hypothesised

that vaccination might reduce the level of stress in a herd and for that reason reduced the risk of *N. caninum*-associated abortions (Hobson et al., 2005).

Similarly to other infectious agents causing abortion, *N. caninum*-associated abortions are most likely multifactorial and a result of an imbalance between parasite multiplication and the effectiveness of both maternal and foetal immune response (Innes et al., 2001). The mechanism responsible for the recrudescence of persistent infections is not fully understood (Dubey et al., 2017b; Dubey and Schares, 2006; Williams et al., 2009). The reactivation might be expressed by the conversion of bradyzoites into tachyzoites which spread across the placenta and into the foetus (Williams et al., 2009) and an increase in specific *N. caninum* antibody titres during mid-gestation was interpreted as a manifestation of this reactivation in chronically infected adult cows (Guy et al., 2001; Paré et al., 1997; Stenlund et al., 1999). Immunity to *N. caninum* seems to be cell-mediated rather than humoral (Innes et al., 2005) and it is known that proinflammatory cytokines (Th1-type) such as IFN-γ are important in the protective cell-mediated response against *N. caninum* (Innes et al., 2001). However, during gestation this Th1-type response at the materno-foetal interface appears to be detrimental to pregnancy maintenance (Entrican, 2002; Innes et al., 2002). So, the regulatory Th2-type cytokine response, such as IL-10, IL-4 and TGF-β, is enhanced during this period especially at the placenta, in order to counteract the inflammatory responses induced by the Th1-type cytokines (Entrican, 2002; Innes et al., 2005). So, it could be hypothesized that this complex balance between the natural immunomodulation to maintain gestation and the proinflammatory immune response to control infection might be compromised by an external immunosuppressive event, originating recrudescence of infection and disease. A possible role of immune suppression in the reactivation of chronic infections causing epidemic outbreaks of *N. caninum*-abortions has been suggested in the past (Atkinson et al., 2000a; Dijkstra et al., 2001a; Wouda et al., 1999), but not definitely proven (Dubey et al., 2017b; Dubey and Schares, 2006). Cyst rupture and the release of parasites in blood was shown in immunosuppressed mice chronically infected with *T. gondii* (Ferguson et al., 1991; Johnson, 1992). The administration of high doses of corticosteroids to *N. caninum* experimentally infected dogs resulted in the reactivation of chronic infections (Dubey and Lindsay, 1990). It is thus possible to speculate that a similar process may also occur in cattle persistently infected with *N. caninum* (López-Gatius et al., 2005a).

The origin of the epidemic outbreak that occurred in the studied herd was not definitively determined. Further studies are needed to elucidate the complex host-parasite relations and particularly the mechanisms beyond the recrudescence of chronic infections and abortion.

Conclusion

Serological techniques can be applied *in vivo* and are particularly important in cases of *N. caninum*-associated abortion due to the absence of clinical signs in adult cows other than abortion. Accurate serological techniques are essential to design and successfully implement control measures to decrease the impact of *N. caninum*-associated abortion or to prevent its introduction into *Neospora*-free herds. ELISA assays constitute a rapid, simple, and cost-effective method for the detection of specific antibodies. Because of this, ELISA assays are the preferred method for complete herd analysis. Competitive inhibition ELISAs based on mAbs can potentially increase the specificity of serological tests because mAbs bind to a single antigenic epitope.

In this study, a *N. caninum* cELISA based on the mouse monoclonal antibody 3.10.5 and reactions against crude *N. caninum* antigen was successfully developed. The specificity of the mAb for *N. caninum* antigens was previously assessed by immunoblotting with non-reduced and reduced antigens originated from *N. caninum, B. besnoiti* and *T. gondii* tachyzoites, and no cross-reactions were observed. Sera from cattle infected with *N. caninum* were used to access diagnostic test sensitivity and sera from cattle infected with *B. besnoiti,* and *Sarcocystis* spp. were used to access the diagnostic test specificity. Because there is no "gold standard" test for the diagnosis of *N. caninum* infections, a BLCA was performed using the Gibbs sampler to estimate the diagnostic characteristics of the cELISA. The results revealed a sensitivity of 98.5% (95% CI: 96.5% - 99.9%) and a specificity of 98.4% (95% CI: 97.1% - 99.4%) when a cut-off value of 37% SCI was applied. However, further studies may be needed to further validate the diagnostic characteristics of the test. The newly developed cELISA may constitute a valuable tool for the diagnosis of N. caninum infections, with the potential to be applied in different host species. It may be applied in herd analysis for the diagnosis and control of neosporosis in cattle, or in epidemiology studies.

The cELISA was used to analyse cattle sera from a herd with suspected *N. caninum*-associated abortions and determine whether these abortions could be attributed to neosporosis. Neosporosis was considered the likely cause of the abortions, regardless of the abortion pattern considered. It was not possible to determine the origin of the presumed epidemic outbreak of abortion that occurred in the studied herd, but the hypotheses of an epidemic-like pattern caused by the accumulation of endemic abortions in a short period, or a synchronized recrudescence of *N. caninum* infections related to an immunosuppressive event seem more plausible. Further studies are needed to elucidate the complex host-parasite relations and particularly the mechanisms beyond the recrudescence of chronic infections and abortion.

References

Aguado-Martínez, A., Álvarez-García, G., Arnaiz-Seco, I., Innes, E., Ortega-Mora, L.M., 2005. Use of avidity enzyme-linked immunosorbent assay and avidity Western blot to discriminate between acute and chronic *Neospora caninum* infection in cattle. *J. Vet. Diagn. Invest.* 17, 442–450. https://doi.org/10.1177/104063870501700506

Aguado-Martínez, A., Álvarez-García, G., Fernández-García, A., Risco-Castillo, V., Arnaiz-Seco, I., Rebordosa-Trigueros, X., Navarro-Lozano, V., Ortega-Mora, L.M., 2008. Usefulness of rNcGRA7- and rNcSAG4-based ELISA tests for distinguishing primo-infection, recrudescence, and chronic bovine neosporosis. *Vet. Parasitol.* 157, 182–195. https://doi.org/10.1016/j.vetpar.2008.08.002

Aguado-Martínez, A., Álvarez-García, G., Schares, G., Risco-Castillo, V., Fernández-García, A., Marugán-Hernández, V., Ortega-Mora, L., 2010. Characterisation of NcGRA7 and NcSAG4 proteins: Immunolocalisation and their role in the host cell invasion by *Neospora caninum* tachyzoites. *Acta Parasitol.* 55. https://doi.org/10.2478/s11686-010-0056-9

Almería, S., De Marez, T., Dawson, H., Araujo, R., Dubey, J.P., Gasbarre, L.C., 2003. Cytokine gene expression in dams and foetuses after experimental *Neospora caninum* infection of heifers at 110 days of gestation. *Parasite Immunol.* 25, 383–392. https://doi.org/10.1046/j.1365-3024.2003.00645.x

Almería, S., López-Gatius, F., García-Ispierto, I., Nogareda, C., Bech-Sàbat, G., Serrano, B., Santolaria, P., Yániz, J.L., 2009a. Effects of crossbreed pregnancies on the abortion risk of *Neospora caninum*-infected dairy cows. *Vet. Parasitol.* 163, 323–329. https://doi.org/10.1016/j.vetpar.2009.04.026

Almería, S., Nogareda, C., Santolaria, P., Garcia-Ispierto, I., Yániz, J.L., López-Gatius, F., 2009b. Specific anti-*Neospora caninum* IgG1 and IgG2 antibody responses during gestation in naturally infected cattle and their relationship with gamma interferon production. *Vet. Immunol. Immunopathol.* 130, 35–42. https://doi.org/10.1016/j.vetimm.2009.01.003

Almería, S., Araujo, R., Tuo, W., López-Gatius, F., Dubey, J.P., Gasbarre, L.C., 2010. Fetal death in cows experimentally infected with *Neospora caninum* at 110 days of gestation. *Vet. Parasitol.* 169, 304–311. https://doi.org/10.1016/j.vetpar.2009.12.044

Almería, S., 2013. *Neospora caninum* and wildlife. ISRN *Parasitology* 2013, 947347. https://doi.org/10.5402/2013/947347

Álvarez-García, G., Pereira-Bueno, J., Gómez-Bautista, M., Ortega-Mora, L.M., 2002. Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. *Vet. Parasitol.* 107, 15–27. https://doi.org/10.1016/s0304-4017(02)00091-2

Álvarez-García, G., Collantes-Fernández, E., Costas, E., Rebordosa, X., Ortega-Mora, L.M., 2003. Influence of age and purpose for testing on the cut-off selection of serological methods in bovine neosporosis. *Vet. Res.* 34, 341–352. https://doi.org/10.1051/vetres:2003009

Álvarez-García, G., Ortega-Mora, L.M., Dijkstra, T., Wouda, W., 2007. 3.1 Neosporosis: Diagnostic applications and recommended diagnostic schemes, in: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), *Protozoal Abortion in Farm*

Ruminants: Guidelines for Diagnosis and Control, CABI Books. CABI International, pp. 89–98. https://doi.org/10.1079/9781845932114.0042

Álvarez-García, G., García-Culebras, A., Gutiérrez-Expósito, D., Navarro-Lozano, V., Pastor-Fernández, I., Ortega-Mora, L.M., 2013. Serological diagnosis of bovine neosporosis: A comparative study of commercially available ELISA tests. *Vet. Parasitol.* 198, 85–95. https://doi.org/10.1016/j.vetpar.2013.07.033

Alves Neto, A.F., Bandini, L.A., Nishi, S.M., Soares, R.M., Driemeier, D., Antoniassi, N.A.B., Schares, G., Gennari, S.M., 2011. Viability of sporulated oocysts of *Neospora caninum* after exposure to different physical and chemical treatments. *J. Parasitol.* 97, 135–139. https://doi.org/10.1645/GE-2571.1

Anderson, M.L., Reynolds, J.P., Rowe, J.D., Sverlow, K.W., Packham, A.E., Barr, B.C., Conrad, P.A., 1997. Evidence of vertical transmission of *Neospora* sp. infection in dairy cattle. *J. Am. Vet. Med. Assoc.* 210, 1169–1172.

Anderson, M.L., Andrianarivo, A.G., Conrad, P.A., 2000. Neosporosis in cattle. Anim Reprod Sci 60–61, 417–431. https://doi.org/10.1016/s0378-4320(00)00117-2

Andrianarivo, A.G., Rowe, J.D., Barr, B.C., Anderson, M.L., Packham, A.E., Sverlow, K.W., Choromanski, L., Loui, C., Grace, A., 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. *Int. J. Parasitol*. 30, 985–990. https://doi.org/10.1016/s0020-7519(00)00088-6

Andrianarivo, A.G., Barr, B.C., Anderson, M.L., Rowe, J.D., Packham, A.E., Sverlow, K.W., Conrad, P.A., 2001. Immune responses in pregnant cattle and bovine fetuses following experimental infection with *Neospora caninum*. *Parasitol. Res*. 87, 817–825. https://doi.org/10.1007/s004360100442

Arnold, L.M., 2013. Investigation of an abortion epidemic due to *Neospora caninum* in a beef herd on pasture. *Bov. Pract.* 47, 1–6. https://doi.org/10.21423/bovine-vol47no1p1-6

Asmare, K., Regassa, F., Robertson, L.J., Skjerve, E., 2013. Seroprevalence of *Neospora caninum* and associated risk factors in intensive or semi-intensively managed dairy and breeding cattle of Ethiopia. *Vet. Parasitol.* 193, 85–94. https://doi.org/10.1016/j.vetpar.2012.11.025

Asuero, A.G., Sayago, A., González, A.G., 2006. The Correlation Coefficient: An Overview. *Crit. Rev. Anal. Chem.* 36, 41–59. https://doi.org/10.1080/10408340500526766

Atkinson, R., Cook, R., Reddacliff, L., Rothwell, J., Broady, K., Harper, P., Ellis, J., 2000a. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a dairy cattle herd. *Aust. Vet. J.* 78, 262–266. https://doi.org/10.1111/j.1751-0813.2000.tb11752.x

Atkinson, R., Harper, P.A., Reichel, M.P., Ellis, J.T., 2000b. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. *Parasitol. Today* 16, 110–114. https://doi.org/10.1016/s0169-4758(99)01604-x

Ausubel, F.M., 1987. *Current Protocols in Molecular Biology*. Greene Pub. Associates and Wiley-Interscience.

Baetz, A.L., Leiting, S.E., Bryner, J., Barnett, D., 1981. Prepartum changes of plasma concentrations of prostaglandin F and 13,14-dihydro-15-ketoprostaglandin metabolites in pregnant animals exposed to *Sarcocystis cruzi* or *Campylobacter fetus*. *Am. J. Vet. Res.* 42, 22–24.

Bagath, M., Krishnan, G., Devaraj, C., Rashamol, V.P., Pragna, P., Lees, A.M., Sejian, V., 2019. The impact of heat stress on the immune system in dairy cattle: A review. Research in Veterinary Science 126, 94–102. https://doi.org/10.1016/j.rvsc.2019.08.011

Baillargeon, P., Fecteau, G., Paré, J., Lamothe, P., Sauvé, R., 2001. Evaluation of the embryo transfer procedure proposed by the International Embryo Transfer Society as a method of controlling vertical transmission of *Neospora caninum* in cattle. *J. Am. Vet. Med. Assoc.* 218, 1803–1806. https://doi.org/10.2460/javma.2001.218.1803

Bandini, L.A., Neto, A.F.A., Pena, H.F.J., Cavalcante, G.T., Schares, G., Nishi, S.M., Gennari, S.M., 2011. Experimental infection of dogs (*Canis familiaris*) with sporulated oocysts of *Neospora caninum*. *Vet. Parasitol.* 176, 151–156. https://doi.org/10.1016/j.vetpar.2010.10.047

Barling, K.S., Sherman, M., Peterson, M.J., Thompson, J.A., McNeill, J.W., Craig, T.M., Adams, L.G., 2000. Spatial associations among density of cattle, abundance of wild canids, and seroprevalence to *Neospora caninum* in a population of beef calves. *J. Am. Vet. Med. Assoc.* 217, 1361–1365. https://doi.org/10.2460/javma.2000.217.1361

Barr, B.C., Anderson, M.L., Blanchard, P.C., Daft, B.M., Kinde, H., Conrad, P.A., 1990. Bovine fetal encephalitis and myocarditis associated with protozoal infections. *Vet. Pathol.* 27, 354–361. https://doi.org/10.1177/030098589002700508

Barr, B.C., Conrad, P.A., Breitmeyer, R., Sverlow, K., Anderson, M.L., Reynolds, J., Chauvet, A.E., Dubey, J.P., Ardans, A.A., 1993. Congenital *Neospora* infection in calves born from cows that had previously aborted *Neospora*-infected fetuses: four cases (1990-1992). *J. Am. Vet. Med. Assoc.* 202, 113–117.

Barr, B.C., Conrad, P.A., Sverlow, K.W., Tarantal, A.F., Hendrickx, A.G., 1994. Experimental fetal and transplacental *Neospora* infection in the nonhuman primate. *Lab. Invest.* 71, 236–242.

Barr, B.C., Anderson, M.L., Sverlow, K.W., Conrad, P.A., 1995. Diagnosis of bovine fetal *Neospora* infection with an indirect fluorescent antibody test. *Vet. Rec.* 137, 611–613.

Barta, J.R., Dubey, J.P., 1992. Characterization of anti-*Neospora caninum* hyperimmune rabbit serum by western blot analysis and immunoelectron microscopy. *Parasitol. Res.* 78, 689–694. https://doi.org/10.1007/BF00931522

Bartels, C.J.M., Wouda, W., Schukken, Y.H., 1999. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in The Netherlands (1995 to 1997). *Theriogenology* 52, 247–257. https://doi.org/10.1016/S0093-691X(99)00126-0

Bartels, C.J.M., van Maanen, C., van der Meulen, A.M., Dijkstra, T., Wouda, W., 2005. Evaluation of three enzyme-linked immunosorbent assays for detection of antibodies to *Neospora caninum* in bulk milk. *Vet. Parasitol.* 131, 235–246. https://doi.org/10.1016/j.vetpar.2005.05.011

Bartels, C.J.M., Arnaiz-Seco, J.I., Ruiz-Santa-Quitera, A., Björkman, C., Frössling, J., von Blumröder, D., Conraths, F.J., Schares, G., van Maanen, C., Wouda, W., Ortega-Mora, L.M., 2006. Supranational comparison of *Neospora caninum* seroprevalences in cattle in Germany, The Netherlands, Spain and Sweden. *Vet. Parasitol.* 137, 17–27. https://doi.org/10.1016/j.vetpar.2005.12.016

Bartels, C.J.M., Huinink, I., Beiboer, M.L., van Schaik, G., Wouda, W., Dijkstra, T., Stegeman, A., 2007. Quantification of vertical and horizontal transmission of *Neospora caninum* infection in Dutch dairy herds. *Vet. Parasitol.* 148, 83–92. https://doi.org/10.1016/j.vetpar.2007.06.004

Bartley, P.M., Wright, S.E., Maley, S.W., Macaldowie, C.N., Nath, M., Hamilton, C.M., Katzer, F., Buxton, D., Innes, E.A., 2012. Maternal and foetal immune responses of cattle following an experimental challenge with *Neospora caninum* at day 70 of gestation. *Vet. Res.* 43, 38. https://doi.org/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. *J. Parasitol.* 87, 612–618. https://doi.org/10.1645/0022-3395(2001)087[0612:FIONCF]2.0.CO;2

Basso, W., Schares, S., Minke, L., Bärwald, A., Maksimov, A., Peters, M., Schulze, C., Müller, M., Conraths, F.J., Schares, G., 2010. Microsatellite typing and avidity analysis suggest a common source of infection in herds with epidemic *Neospora caninum*-associated bovine abortion. *Vet. Parasitol.* 173, 24–31. https://doi.org/10.1016/j.vetpar.2010.06.009

Basso, W., Moré, G., Quiroga, M.A., Balducchi, D., Schares, G., Venturini, M.C., 2014. *Neospora caninum* is a cause of perinatal mortality in axis deer (*Axis axis*). *Vet. Parasitol.* 199, 255–258. https://doi.org/10.1016/j.vetpar.2013.10.020

Baszler, T.V., Knowles, D.P., Dubey, J.P., Gay, J.M., Mathison, B.A., McElwain, T.F., 1996. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 34, 1423–1428. https://doi.org/10.1128/jcm.34.6.1423-1428.1996

Baszler, T.V., Gay, L.J.C., Long, M.T., Mathison, B.A., 1999. Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. *J. Clin. Microbiol.* 37, 4059–4064.

Baszler, T.V., Adams, S., Vander-Schalie, J., Mathison, B.A., Kostovic, M., 2001. Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. *J. Clin. Microbiol.* 39, 3851–3857. https://doi.org/10.1128/JCM.39.11.3851-3857.2001

Bayer, V., 2019. An Overview of Monoclonal Antibodies. Seminars in Oncology Nursing 35, 150927. https://doi.org/10.1016/j.soncn.2019.08.006

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

Benavides, J., Collantes-Fernández, E., Ferre, I., Pérez, V., Campero, C., Mota, R., Innes, E., Ortega-Mora, L.M., 2014. Experimental ruminant models for bovine neosporosis: what is known and what is needed. *Parasitology* 141, 1471–1488. https://doi.org/10.1017/S0031182014000638

Bergeron, N., Fecteau, G., Paré, J., Martineau, R., Villeneuve, A., 2000. Vertical and horizontal transmission of *Neospora caninum* in dairy herds in Québec. *Can. Vet. J.* 41, 464–467.

Bergeron, N., Fecteau, G., Villeneuve, A., Girard, C., Paré, J., 2001a. Failure of dogs to shed oocysts after being fed bovine fetuses naturally infected by *Neospora caninum*. *Vet. Parasitol.* 97, 145–152. https://doi.org/10.1016/s0304-4017(01)00396-x

Bergeron, N., Girard, C., Paré, J., Fecteau, G., Robinson, J., Baillargeon, P., 2001b. Rare detection of *Neospora caninum* in placentas from seropositive dams giving birth to full-term calves. *J. Vet. Diagn. Invest.* 13, 173–175. https://doi.org/10.1177/104063870101300216

Bień, J., Moskwa, B., Cabaj, W., 2010. In vitro isolation and identification of the first *Neospora caninum* isolate from European bison (*Bison bonasus bonasus* L.). *Vet. Parasitol.* 173, 200–205. https://doi.org/10.1016/j.vetpar.2010.06.038

Bjerkås, I., Mohn, S.F., Presthus, J., 1984. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Z. Parasitenkd.* 70, 271–274. https://doi.org/10.1007/BF00942230

Bjerkås, I., Dubey, J.P., 1991. Evidence that *Neospora caninum* is identical to the *Toxoplasma*-like parasite of Norwegian dogs. *Acta Vet. Scand.* 32, 407–410. https://doi.org/10.1186/BF03546972

Bjerkås, I., Jenkins, M.C., Dubey, J.P., 1994. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. *Clin. Diagn. Lab. Immunol.* 1, 214–221.

Björkman, C., Johansson, O., Stenlund, S., Holmdahl, O.J., Uggla, A., 1996. *Neospora* species infection in a herd of dairy cattle. *J. Am. Vet. Med. Assoc.* 208, 1441–1444.

Björkman, C., Holmdahl, O.J.M., Uggla, A., 1997. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. *Vet. Parasitol.* 68, 251–260. https://doi.org/10.1016/S0304-4017(96)01076-X

Björkman, C., Näslund, K., Stenlund, S., Maley, S.W., Buxton, D., Uggla, A., 1999. An IgG avidity ELISA to discriminate between recent and chronic *Neospora caninum* infection. *J. Vet. Diagn. Invest.* 11, 41–44. https://doi.org/10.1177/104063879901100106

Björkman, C., Uggla, A., 1999. Serological diagnosis of *Neospora caninum* infection. Int *J. Parasitol.* 29, 1497–1507. https://doi.org/10.1016/s0020-7519(99)00115-0

Björkman, C., Alenius, S., Manuelsson, U., Uggla, A., 2000. *Neospora caninum* and bovine virus diarrhoea virus infections in Swedish dairy cows in relation to abortion. *Vet. J.* 159, 201–206. https://doi.org/10.1053/tvjl.1999.0446

Björkman, C., McAllister, M.M., Frössling, J., Näslund, K., Leung, F., Uggla, A., 2003. Application of the *Neospora caninum* IgG avidity ELISA in assessment of chronic reproductive losses after an outbreak of neosporosis in a herd of beef cattle. *J. Vet. Diagn. Invest.* 15, 3–7. https://doi.org/10.1177/104063870301500102

Björkman, C., Gondim, L.F.P., Näslund, K., Trees, A.J., McAllister, M.M., 2005. IgG avidity pattern in cattle after ingestion of *Neospora caninum* oocysts. *Vet. Parasitol.* 128, 195–200. https://doi.org/10.1016/j.vetpar.2004.11.030

Björkman, C., Álvarez-García, G., Conraths, F.J., Mattsson, J.G., Ortega-Mora, L.M., Sager, H., Schares, G., 2006. *Neospora caninum* IgG avidity tests: An interlaboratory comparison. *Vet. Parasitol.* 140, 273–280. https://doi.org/10.1016/j.vetpar.2006.04.030

Björkman, C., Sager, H., Schares, G., 2007. 3.1 Neosporosis: Diagnostic techniques - Serology, in: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), *Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control*, CABI Books. CABI International, pp. 63–88. https://doi.org/10.1079/9781845932114.0042

Bland, M., 2015. An introduction to medical statistics, Fourth edition. ed, Oxford medical publications. Oxford University Press, Oxford.

Blumröder, D., Stambusch, R., Labohm, R., Klawonn, W., Dräger, K., Fasen, W., Conraths, F., Schares, G., 2006. Potenzielle Risikofaktoren für den serologischen Nachweis von *Neospora caninum* Infektionen in Rinderherden in Rheinland-Pfalz. (Potential risk factors for the serological detection of *Neospora caninum*-infections in cattle herds in Rhineland-Palatinate (Germany)). *Tierärztl. Prax.* 34, 141–147.

Boulton, J.G., Gill, P.A., Cook, R.W., Fraser, G.C., Harper, P.A., Dubey, J.P., 1995. Bovine *Neospora* abortion in north-eastern New South Wales. *Aust. Vet. J.* 72, 119–120. https://doi.org/10.1111/j.1751-0813.1995.tb15026.x

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

Bryan, L., Gajadhar, A., Dubey, J., Haines, D., 1994. Bovine neonatal encephalomyelitis associated with a *Neospora* sp. protozoan. *Can. Vet.* 35, 111–3.

Buxton, D., Caldow, G.L., Maley, S.W., Marks, J., Innes, E.A., 1997. Neosporosis and bovine abortion in Scotland. *Vet. Rec.* 141, 649–651.

Buxton, D., McAllister, M.M., Dubey, J.P., 2002. The comparative pathogenesis of neosporosis. *Trends Parasitol.* 18, 546–552. https://doi.org/10.1016/s1471-4922(02)02414-5

Byrem, T.M., Bartlett, P.C., Donohue, H., Voisinet, B.D., Houseman, J.T., 2012. Performance of a commercial serum ELISA for the detection of antibodies to *Neospora caninum* in whole and skim milk samples. *Vet. Parasitol.* 190, 249–253. https://doi.org/10.1016/j.vetpar.2012.06.005

Byrt, T., Bishop, J., Carlin, J.B., 1993. Bias, prevalence and kappa. *J. Clin. Epidemiol.* 46, 423–429. https://doi.org/10.1016/0895-4356(93)90018-v

Caetano-da-Silva, A., Ferre, I., Collantes-Fernández, E., Navarro, V., Aduriz, G., Ugarte-Garagalza, C., Ortega-Mora, L.M., 2004. Occasional detection of *Neospora caninum* DNA in frozen extended semen from naturally infected bulls. *Theriogenology* 62, 1329–1336. https://doi.org/10.1016/j.Theriogenology.2004.01.010

Calero-Bernal, R., Horcajo, P., Hernández, M., Ortega-Mora, L.M., Fuentes, I., 2019. Absence of *Neospora caninum* DNA in human clinical samples, Spain. *Emerg. Infect. Dis.* 25, 1226–1227. https://doi.org/10.3201/eid2506.181431

Campero, L.M., Moreno-Gonzalo, J., Venturini, M.C., Moré, G., Dellarupe, A., Rambeaud, M., Echaide, I.E., Valentini, B., Campero, C.M., Moore, D.P., Cano, D.B., Fort, M., Mota, R.A., Serrano-Martínez, M.E., Cruz-Vázquez, C., Ortega-Mora, L.M., Álvarez-García, G., 2018. An Ibero-American inter-laboratory trial to evaluate serological tests for the detection of anti-*Neospora caninum* antibodies in cattle. *Trop. Anim. Health Prod.* 50, 75–84. https://doi.org/10.1007/s11250-017-1401-x

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

Cavalcante, G.T., Monteiro, R.M., Soares, R.M., Nishi, S.M., Alves Neto, A.F., Esmerini, P. de O., Sercundes, M.K., Martins, J., Gennari, S.M., 2011. Shedding of *Neospora caninum* oocysts by dogs fed different tissues from naturally infected cattle. *Vet. Parasitol.* 179, 220–223. https://doi.org/10.1016/j.vetpar.2011.02.026

Cedillo, C.J.R., Martínez, M.J.J., Santacruz, A.M., Banda, R.V.M., Morales, S.E., 2008. Models for experimental infection of dogs fed with tissue from fetuses and neonatal cattle naturally infected with *Neospora caninum*. *Vet. Parasitol.* 154, 151–155. https://doi.org/10.1016/j.vetpar.2008.02.025

Chanlun, A., Näslund, K., Aiumlamai, S., Björkman, C., 2002. Use of bulk milk for detection of *Neospora caninum* infection in dairy herds in Thailand. *Vet. Parasitol.* 110, 35–44. https://doi.org/10.1016/S0304-4017(02)00315-1

Chatziprodromidou, I.P., Apostolou, T., 2018. Diagnostic accuracy of enzyme-linked immunosorbent assay (ELISA) and immunoblot (IB) for the detection of antibodies against *Neospora caninum* in milk from dairy cows. *Epidemiol. Infect.* 146, 577–583. https://doi.org/10.1017/S0950268817002916

Cheung, A., Dufour, S., Jones, G., Kostoulas, P., Stevenson, M.A., Singanallur, N.B., Firestone, S.M., 2021. Bayesian latent class analysis when the reference test is imperfect. *Rev. Sci. Tech.* OIE 40. https://doi.org/10.20506/rst.40.1.3224

Cole, R.A., Lindsay, D.S., Dubey, J.P., Toivio-Kinnucan, M.A., Blagburn, B.L., 1994. Characterization of a murine monoclonal antibody generated against *Neospora caninum* tachyzoites by use of western blot analysis and immunoelectron microscopy. *Am. J. Vet. Res.* 55, 1717–1722.

Collantes-Fernández, E., Zaballos, Á., Álvarez-García, G., Ortega-Mora, L.M., 2002. Quantitative detection of *Neospora caninum* in bovine aborted fetuses and experimentally infected mice by real-time PCR. Journal of clinical microbiology 40, 1194–1198.

Collantes-Fernández, E., Rodríguez-Bertos, A., Arnáiz-Seco, I., Moreno, B., Aduriz, G., Ortega-Mora, L.M., 2006. Influence of the stage of pregnancy on *Neospora caninum* distribution, parasite loads and lesions in aborted bovine foetuses. *Theriogenology* 65, 629–641. https://doi.org/10.1016/j.Theriogenology.2005.06.003

Collins, J., Huynh, M., 2014. Estimation of diagnostic test accuracy without full verification: a review of latent class methods. *Statist. Med.* 33, 4141–4169. https://doi.org/10.1002/sim.6218

Conrad, P.A., Barr, B.C., Sverlow, K.W., Anderson, M., Daft, B., Kinde, H., Dubey, J.P., Munson, L., Ardans, A., 1993a. In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. *Parasitol.* 106, 239–249. https://doi.org/10.1017/s0031182000075065

Conrad, P.A., Sverlow, K., Anderson, M., Rowe, J., BonDurant, R., Tuter, G., Breitmeyer, R., Palmer, C., Thurmond, M., Ardans, A., Dubey, J.P., Duhamel, G., Barr, B., 1993b. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. *J. Vet. Diagn. Invest.* 5, 572–578. https://doi.org/10.1177/104063879300500412

Conraths, F.J., Gottstein, B., 2007. 3.1 Neosporosis: General considerations, in: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), *Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control*, CABI Books. CABI International, pp. 42–45. https://doi.org/10.1079/9781845932114.0042

Conraths, F.J., Schares, G., Ortega-Mora, L.M., Gottstein, B., 2007. 5.1 Neosporosis: Options for control, in: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), *Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control*, CABI Books. CABI International, pp. 277–281. https://doi.org/10.1079/9781845932114.0276

Corbellini, L.G., Smith, D.R., Pescador, C.A., Schmitz, M., Correa, A., Steffen, D.J., Driemeier, D., 2006. Herd-level risk factors for *Neospora caninum* seroprevalence in dairy farms in southern Brazil. *Prev. Vet. Med.* 74, 130–141. https://doi.org/10.1016/j.prevetmed.2005.11.004

Cortes, H.C.E., Reis, Y., Waap, H., Vidal, R., Soares, H., Marques, I., Pereira da Fonseca, I., Fazendeiro, I., Ferreira, M.L., Caeiro, V., Shkap, V., Hemphill, A., Leitão, A., 2006. Isolation of *Besnoitia besnoiti* from infected cattle in Portugal. *Vet. Parasitol.* 141, 226–233. https://doi.org/10.1016/j.vetpar.2006.05.022

Crowther, J.R., 2009. *The ELISA Guidebook*, Methods in Molecular Biology. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-60327-254-4

Davison, H.C., French, N.P., Trees, A.J., 1999a. Herd-specific and age-specific seroprevalence of *Neospora caninum* in 14 British dairy herds. *Vet. Rec.* 144, 547–550. https://doi.org/10.1136/vr.144.20.547

Davison, H.C., Otter, A., Trees, A.J., 1999b. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. Int *J. Parasitol.* 29, 1683–1689. https://doi.org/10.1016/s0020-7519(99)00129-0

Davison, H.C., Guy, C.S., McGarry, J.W., Guy, F., Williams, D.J., Kelly, D.F., Trees, A.J., 2001. Experimental studies on the transmission of *Neospora caninum* between cattle. *Res. Vet. Sci.* 70, 163–168. https://doi.org/10.1053/rvsc.2001.0457

Davidson, M.J., Huaman, J.L., Pacioni, C., Stephens, D., Hitchen, Y., Carvalho, T.G., 2022. Active shedding of *Neospora caninum* detected in Australian wild canids in a nonexperimental context. *Transbound. Emerg. Dis.* 69, 1862–1871. https://doi.org/10.1111/tbed.14170

De Marez, T., Liddell, S., Dubey, J.P., Jenkins, M.C., Gasbarre, L., 1999. Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses. Int *J. Parasitol.* 29, 1647–1657. https://doi.org/10.1016/s0020-7519(99)00154-x

de Oliveira, V.S.F., Álvarez-García, G., Ortega-Mora, L.M., Borges, L.M.F., da Silva, A.C., 2010. Abortions in bovines and *Neospora caninum* transmission in an embryo transfer center. *Vet. Parasitol.* 173, 206–210. https://doi.org/10.1016/j.vetpar.2010.06.028

Denwood, M.J., 2016. runjags: An R package providing interface utilities, model templates, parallel computing methods and additional distributions for MCMC models in JAGS. *J. Stat. Softw.* 71, 1–25. https://doi.org/10.18637/jss.v071.i09

Desmonts, G., Remington, J.S., 1980. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. *J. Clin. Microbiol.* 11, 562–568. https://doi.org/10.1128/jcm.11.6.562-568.1980

Dijkstra, T., Barkema, H.W., Eysker, M., Wouda, W., 2001a. Evidence of post-natal transmission of *Neospora caninum* in Dutch dairy herds. Int *J. Parasitol.* 31, 209–215. https://doi.org/10.1016/S0020-7519(00)00160-0

Dijkstra, T., Eysker, M., Schares, G., Conraths, F.J., Wouda, W., Barkema, H.W., 2001b. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. Int *J. Parasitol.* 31, 747–752. https://doi.org/10.1016/s0020-7519(01)00230-2

Dijkstra, T., Barkema, H.W., Björkman, C., Wouda, W., 2002a. A high rate of seroconversion for *Neospora caninum* in a dairy herd without an obvious increased incidence of abortions. *Vet. Parasitol.* 109, 203–211. https://doi.org/10.1016/s0304-4017(02)00303-5

Dijkstra, T., Barkema, H.W., Eysker, M., Hesselink, J.W., Wouda, W., 2002b. Natural transmission routes of *Neospora caninum* between farm dogs and cattle. *Vet. Parasitol.* 105, 99–104. https://doi.org/10.1016/s0304-4017(02)00010-9

Dijkstra, T., Barkema, H.W., Hesselink, J.W., Wouda, W., 2002c. Point source exposure of cattle to *Neospora caninum* consistent with periods of common housing and feeding and related to the introduction of a dog. *Vet. Parasitol.* 105, 89–98. https://doi.org/10.1016/s0304-4017(02)00009-2

Dijkstra, T., Barkema, H.W., Eysker, M., Beiboer, M.L., Wouda, W., 2003. Evaluation of a single serological screening of dairy herds for *Neospora caninum* antibodies. *Vet. Parasitol.* 110, 161–169. https://doi.org/10.1016/S0304-4017(02)00323-0

Dijkstra, T., Lam, T.J.G.M., Bartels, C.J.M., Eysker, M., Wouda, W., 2008. Natural postnatal *Neospora caninum* infection in cattle can persist and lead to endogenous transplacental infection. *Vet. Parasitol.* 152, 220–225. https://doi.org/10.1016/j.vetpar.2007.12.034

Dubey, J.P., Hughes, H.P., Lillehoj, H.S., Gamble, H.R., Munday, B.L., 1987. Placental transfer of specific antibodies during ovine congenital toxoplasmosis. *Am. J. Vet. Res.* 48, 474–476.

Dubey, J.P., Carpenter, J.L., Speer, C.A., Topper, M.J., Uggla, A., 1988a. Newly recognized fatal protozoan disease of dogs. *J. Am. Vet. Med. Assoc.* 192, 1269–1285.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988b. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *J. Am. Vet. Med. Assoc.* 193, 1259–1263.

Dubey, J.P., Leathers, C.W., Lindsay, D.S., 1989. *Neospora caninum*-like protozoon associated with fatal myelitis in newborn calves. *J. Parasitol.* 75, 146–148. https://doi.org/10.2307/3282954

Dubey, J.P., Lindsay, D.S., 1990. Neosporosis in dogs. *Vet. Parasitol.* 36, 147–151. https://doi.org/10.1016/0304-4017(90)90103-I

Dubey, J.P., Koestner, A., Piper, R.C., 1990. Repeated transplacental transmission of *Neospora caninum* in dogs. J. Am. Vet. Med. Assoc. 197, 857–860.

Dubey, J.P., de Lahunta, A., 1993. Neosporosis associated congenital limb deformities in a calf. *Appl. Parasitol.* 34, 229–233.

Dubey, J.P., Lindsay, D.S., 1996. A review of *Neospora caninum* and neosporosis. *Vet. Parasitol.* 67, 1–59. https://doi.org/10.1016/s0304-4017(96)01035-7

Dubey, J.P., Lindsay, D.S., Adams, D.S., Gay, J.M., Baszler, T.V., Blagburn, B.L., Thulliez, P., 1996. Serologic responses of cattle and other animals infected with *Neospora caninum*. *Am. J. Vet. Res.* 57, 329–336.

Dubey, J.P., Jenkins, M.C., Adams, D.S., McAllister, M.M., Anderson-Sprecher, R., Baszler, T.V., Kwok, O.C.H., Lally, N.C., Bjorkman, C., Uggla, A., 1997. Antibody Responses of Cows during an Outbreak of Neosporosis Evaluated by Indirect Fluorescent Antibody Test and Different Enzyme-Linked Immunosorbent Assays. *J. Parasitol.* 83, 1063. https://doi.org/10.2307/3284363

Dubey, J.P., Abbitt, B., Topper, M.J., Edwards, J.F., 1998. Hydrocephalus associated with *Neospora caninum* infection in an aborted bovine fetus. *J. Comp. Pathol.* 118, 169–173. https://doi.org/10.1016/s0021-9975(98)80010-8

Dubey, J.P., 1999. Recent advances in *Neospora* and neosporosis. *Vet. Parasitol.* 84, 349–367. https://doi.org/10.1016/s0304-4017(99)00044-8

Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkås, I., Björkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modrý, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J.L., Lindsay, D.S., 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. Int. J. Parasitol. 32, 929–946. https://doi.org/10.1016/S0020-7519(02)00094-2

Dubey, J.P., 2003. Review of *Neospora caninum* and neosporosis in animals. Korean *J. Parasitol.* 41, 1. https://doi.org/10.3347/kjp.2003.41.1.1

Dubey, J.P., Thulliez, P., 2005. Prevalence of Antibodies to *Neospora caninum* in Wild Animals. *J. Parasitol.* 91, 1217–1218. https://doi.org/10.1645/GE-576R.1

Dubey, J.P., Schares, G., 2006. Diagnosis of bovine neosporosis. *Vet. Parasitol.* 140, 1–34. https://doi.org/10.1016/j.vetpar.2006.03.035

Dubey, J.P., Buxton, D., Wouda, W., 2006. Pathogenesis of bovine neosporosis. *J. Comp. Pathol.* 134, 267–289. https://doi.org/10.1016/j.jcpa.2005.11.004

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and Control of Neosporosis and *Neospora caninum*. *Clin. Microbiol. Rev.* 20, 45.

Dubey, J.P., Schares, G., 2011. Neosporosis in animals—The last five years. *Vet. Parasitol.* 180, 90–108. https://doi.org/10.1016/j.vetpar.2011.05.031

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

Dubey, J.P., Jenkins, M.C., Ferreira, L.R., Choudhary, S., Verma, S.K., Kwok, O.C.H., Fetterer, R., Butler, E., Carstensen, M., 2014. Isolation of viable *Neospora caninum* from brains of wild gray wolves (*Canis lupus*). *Vet. Parasitol.* 201, 150–153. https://doi.org/10.1016/j.vetpar.2013.12.032

Dubey, J.P., Calero-Bernal, R., Rosenthal, B.M., Speer, C.A., Fayer, R., 2015. Sarcocystosis of Animals and Humans, Second Edition. Taylor & Francis.

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G., 2017a. General Biology, in: *Neosporosis in Animals.* CRC Press, pp. 7–114. https://doi.org/10.1201/9781315152561-3

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G., 2017b. Neosporosis in Cattle, in: *Neosporosis in Animals.* CRC Press, pp. 133–259. https://doi.org/10.1201/9781315152561-5

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G., 2017c. Neosporosis in Dogs, in: *Neosporosis in Animals.* CRC Press, pp. 261–315. https://doi.org/10.1201/9781315152561-6

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G., 2017d. Techniques, in: *Neosporosis in Animals.* CRC Press, pp. 115–132. https://doi.org/10.1201/9781315152561-4

Dubey, J.P., Rosenthal, B.M., 2022. Bovine sarcocystosis: *Sarcocystis* species, diagnosis, prevalence, economic and public health considerations, and association of *Sarcocystis* species with eosinophilic myositis in cattle. Intern *J. Parasitol.* https://doi.org/10.1016/j.ijpara.2022.09.009

Eiras, C., Arnaiz, I., Álvarez-García, G., Ortega-Mora, L.M., Sanjuánl, M.L., Yus, E., Diéguez, F.J., 2011. *Neospora caninum* seroprevalence in dairy and beef cattle from the northwest region of Spain, Galicia. *Prev. Vet. Med.* 98, 128–132. https://doi.org/10.1016/j.prevetmed.2010.10.014

Ellis, J.T., McMillan, D., Ryce, C., Payne, S., Atkinson, R., Harper, P.A., 1999. Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA. *Int J. Parasitol.* 29, 1589–1596. https://doi.org/10.1016/s0020-7519(99)00144-7

Enachescu, V., Ionita, M., Mitrea, I.L., 2014. Comparative study for the detection of antibodies to *Neospora caninum* in milk and sera in dairy cattle in southern Romania. *Acta Parasitol*. 59, 5–10. https://doi.org/10.2478/s11686-014-0202-x

Engeland, I.V., Waldeland, H., Kindahl, H., Ropstad, E., Andresen, O., 1996. Effect of *Toxoplasma gondii* infection on the development of pregnancy and on endocrine foetal-placental function in the goat. *Vet. Parasitol.* 67, 61–74.

Enøe, C., Georgiadis, M.P., Johnson, W.O., 2000. Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. *Prev. Vet. Med.* 45, 61–81. https://doi.org/10.1016/S0167-5877(00)00117-3

Entrican, G., 2002. Immune regulation during pregnancy and host–pathogen interactions in infectious abortion. *J. Comp. Pathol.* 126, 79–94. https://doi.org/10.1053/jcpa.2001.0539

Ferguson, D.J., Graham, D.I., Hutchison, W.M., 1991. Pathological changes in the brains of mice infected with *Toxoplasma gondii*: a histological, immunocytochemical and ultrastructural study. *Int. J. Exp. Pathol.* 72, 463–474.

Ferre, I., Aduriz, G., Del-Pozo, I., Regidor-Cerrillo, J., Atxaerandio, R., Collantes-Fernández, E., Hurtado, A., Ugarte-Garagalza, C., Ortega-Mora, L.M., 2005. Detection of *Neospora caninum* in the semen and blood of naturally infected bulls. *Theriogenology* 63, 1504–1518. https://doi.org/10.1016/j.Theriogenology.2004.07.002

Fioretti, D.P., Pasquali, P., Diaferia, M., Mangili, V., Rosignoli, L., 2003. *Neospora caninum* infection and congenital transmission: serological and parasitological study of cows up to the fourth gestation. *J. Vet. Med. B* 50, 399–404. https://doi.org/10.1046/j.1439-0450.2003.00686.x

Foley, G.L., Schlafer, D.H., Elsasser, T.H., Mitchell, M., 1993. Endotoxemia in pregnant cows: comparisons of maternal and fetal effects utilizing the chronically catheterized fetus. *Theriogenology* 39, 739–762.

French, N.P., Clancy, D., Davison, H.C., Trees, A.J., 1999. Mathematical models of *Neospora caninum* infection in dairy cattle: transmission and options for control. *Int*. *J. Parasitol.* 29, 1691–1704. https://doi.org/10.1016/s0020-7519(99)00131-9

Frössling, J., Bonnett, B., Lindberg, A., Björkman, C., 2003. Validation of a *Neospora caninum* iscom ELISA without a gold standard. *Prev. Vet. Med.* 57, 141–153. https://doi.org/10.1016/S0167-5877(02)00216-7

Frössling, J., Uggla, A., Björkman, C., 2005. Prevalence and transmission of *Neospora caninum* within infected Swedish dairy herds. *Vet. Parasitol.* 128, 209–218. https://doi.org/10.1016/j.vetpar.2004.12.006

Frössling, J., Lindberg, A., Björkman, C., 2006. Evaluation of an iscom ELISA used for detection of antibodies to *Neospora caninum* in bulk milk. *Prev. Vet. Med.* 74, 120–129. https://doi.org/10.1016/j.prevetmed.2005.11.012

García-Ispierto, I., López-Gatius, F., Santolaria, P., Yániz, J.L., Nogareda, C., López-Béjar, M., De Rensis, F., 2006. Relationship between heat stress during the peri-implantation period and early fetal loss in dairy cattle. *Theriogenology* 65, 799–807. https://doi.org/10.1016/j.Theriogenology.2005.06.011

Gardner, I.A., Stryhn, H., Lind, P., Collins, M.T., 2000. Conditional dependence between tests affects the diagnosis and surveillance of animal diseases. *Prev. Vet. Med.* 45, 107–122. https://doi.org/10.1016/S0167-5877(00)00119-7

Ghalmi, F., China, B., Kaidi, R., Losson, B., 2009. Evaluation of a SRS2 sandwich commercial enzyme-linked immunosorbent assay for the detection of anti-*Neospora caninum* antibodies in bovine and canine sera. *J. Vet. Diagn. Invest.* 21, 108–111. https://doi.org/10.1177/104063870902100116

Ghalmi, F., China, B., Jenkins, M., Azzag, N., Losson, B., 2014. Comparison of different serological methods to detect antibodies specific to *Neospora caninum* in bovine and canine sera. *J. Vet. Diagn. Invest.* 26, 136–140. https://doi.org/10.1177/1040638713515480

Gibney, E.H., Kipar, A., Rosbottom, A., Guy, C.S., Smith, R.F., Hetzel, U., Trees, A.J., Williams, D.J.L., 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. Int *J. Parasitol.* 38, 579–588. https://doi.org/10.1016/j.ijpara.2007.09.015

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

Gondim, L.F.P., McAllister, M.M., Anderson-Sprecher, R.C., Björkman, C., Lock, T.F., Firkins, L.D., Gao, L., Fischer, W.R., 2004a. Transplacental transmission and abortion in cows administered *Neospora caninum* oocysts. *J. Parasitol.* 90, 1394–1400. https://doi.org/10.1645/GE-359R

Gondim, L.F.P., McAllister, M.M., Mateus-Pinilla, N.E., Pitt, W.C., Mech, L.D., Nelson, M.E., 2004b. Transmission of *Neospora caninum* between wild and domestic animals. *J. Parasitol.* 90, 1361–1365. https://doi.org/10.1645/GE-341R

Gondim, L.F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004c. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 34, 159–161. https://doi.org/10.1016/j.ijpara.2004.01.001

Gondim, L.F.P., McAllister, M.M., Gao, L., 2005. Effects of host maturity and prior exposure history on the production of *Neospora caninum* oocysts by dogs. *Vet. Parasitol.* 134, 33–39. https://doi.org/10.1016/j.vetpar.2005.06.011

Gondim, L.F.P., Mineo, J.R., Schares, G., 2017. Importance of serological cross-reactivity among *Toxoplasma gondii, Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti*. *Parasitology* 144, 851–868. https://doi.org/10.1017/S0031182017000063

Gondim, L.F.P., McAllister, M.M., 2022. Experimental *Neospora caninum* infection in pregnant cattle: different outcomes between inoculation with tachyzoites and oocysts. *Front. Vet. Sci.* 9.

González-Warleta, M., Castro-Hermida, J.A., Carro-Corral, C., Mezo, M., 2011. Anti-*Neospora caninum* antibodies in milk in relation to production losses in dairy cattle. *Prev. Vet. Med.* 101, 58–64. https://doi.org/10.1016/j.prevetmed.2011.04.019

González-Warleta, M., Castro-Hermida, J.A., Calvo, C., Pérez, V., Gutiérrez-Expósito, D., Regidor-Cerrillo, J., Ortega-Mora, L.M., Mezo, M., 2018. Endogenous transplacental transmission of *Neospora caninum* during successive pregnancies across three generations of naturally infected sheep. *Vet. Res.* 49, 106. https://doi.org/10.1186/s13567-018-0601-3

Gordon, J., Rordorf, Ch., Rosenthal, M., Sun, Y.Z., 1985. Immunoblotting and Dot Immunobinding, in: Habermehl, K.-O. (Ed.), *Rapid Methods and Automation in Microbiology and Immunology*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 103–114. https://doi.org/10.1007/978-3-642-69943-6_14

Gottstein, B., Hentrich, B., Wyss, R., Thür, B., Busato, A., Stärk, K.D.C., Müller, N., 1998. Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. *Int. J. Parasitol.* 28, 679–691. https://doi.org/10.1016/S0020-7519(98)
00006-X

Greiner, M., Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. *Prev. Vet. Med.* 45, 3–22.

Guido, S., Katzer, F., Nanjiani, I., Milne, E., Innes, E.A., 2016. Serology-based diagnostics for the control of bovine neosporosis. *Trends Parasitol.* 32, 131–143. https://doi.org/10.1016/j.pt.2015.11.014

Guy, C.S., Williams D.J.L., Kelly, D.F., McGarry, J.W., Guy, F., Björkman, C., Smith, R.F., Trees, A.J., 2001. *Neospora caninum* in persistently infected, pregnant cows: spontaneous transplacental infection is associated with an acute increase in maternal antibody. *Vet. Rec.* 149, 443–449. https://doi.org/10.1136/vr.149.15.443

Haddad, J.P.A., Dohoo, I.R., VanLeewen, J.A., 2005. A review of *Neospora caninum* in dairy and beef cattle — a Canadian perspective. *Can. Vet. J.* 46, 230–243.

Haldorson, G.J., Stanton, J.B., Mathison, B.A., Suarez, C.E., Baszler, T.V., 2006. *Neospora caninum*: Antibodies directed against tachyzoite surface protein NcSRS2 inhibit parasite attachment and invasion of placental trophoblasts in vitro. *Exp. Parasitol.* 112, 172–178. https://doi.org/10.1016/j.exppara.2005.11.004

Hall, C.A., Reichel, M.P., Ellis, J.T., 2005. *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. *Vet. Parasitol.* 128, 231–241. https://doi.org/10.1016/j.vetpar.2004.12.012

Hancock, K., Tsang, V.C.W., 1983. India ink staining of proteins on nitrocellulose paper. Analytical Biochemistry 133, 157–162. https://doi.org/10.1016/0003-2697(83)90237-3

Harkins, D., Clements, D.N., Maley, S., Marks, J., Wright, S., Esteban, I., Innes, E.A., Buxton, D., 1998. Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and with *Toxoplasma gondii*. *J. Comp. Pathol.* 119, 45–55. https://doi.org/10.1016/s0021-9975(98)80070-4

Hässig, M., Gottstein, B., 2002. Epidemiological investigations of abortions due to *Neospora caninum* on Swiss dairy farms. *Vet. Rec.* 150, 538–542. https://doi.org/10.1136/vr.150.17.538

Hemphill, A., 1999. The host-parasite relationship in neosporosis. *Adv. Parasitol.* 43, 47–104. https://doi.org/10.1016/S0065-308X(08)60241-9

Hemphill, A., Fuchs, N., Sonda, S., Hehl, A., 1999. The antigenic composition of *Neospora caninum*. *Int. J. Parasitol.* 29, 1175–1188. https://doi.org/10.1016/s0020-7519(99)00085-5

Hemphill, A., Vonlaufen, N., Naguleswaran, A., 2006. Cellular and immunological basis of the host-parasite relationship during infection with *Neospora caninum*. *Parasitology* 133, 261–278. https://doi.org/10.1017/S0031182006000485

Hernandez, J., Risco, C., Donovan, A., 2002. Risk of abortion associated with *Neospora caninum* during different lactations and evidence of congenital transmission in dairy cows. *J. Am. Vet. Med. Assoc.* 221, 1742–1746. https://doi.org/10.2460/javma.2002.221.1741

Hietala, S.K., Thurmond, M.C., 1999. Postnatal *Neospora caninum* transmission and transient serologic responses in two dairies. *Int. J. Parasitol.* 29, 1669–1676. https://doi.org/10.1016/S0020-7519(99)00102-2

Hnasko, R.M., Stanker, L.H. (2015). Hybridoma Technology. In: Hnasko, R. (Ed.), *ELISA: Methods and Protocols*, Methods in Molecular Biology. Springer, New York, NY, pp. 15–27. https://doi.org/ 10.1007/978-1-4939-2742-5_2

Ho, M.S., Barr, B.C., Rowe, J.D., Anderson, M.L., Sverlow, K.W., Packham, A., Marsh, A.E., Conrad, P.A., 1997. Detection of *Neospora* sp. from infected bovine tissues by PCR and probe hybridization. *J. Parasitol.* 83, 508–514. https://doi.org/10.2307/3284418

Hobson, J.C., Duffield, T.F., Kelton, D., Lissemore, K., Hietala, S.K., Leslie, K.E., McEwen, B., Peregrine, A.S., 2005. Risk factors associated with *Neospora caninum* abortion in Ontario Holstein dairy herds. *Vet. Parasitol.* 127, 177–188. https://doi.org/10.1016/j.vetpar.2004.09.025

Holmdahl, O.J.M., Mattsson, J.G., 1996. Rapid and sensitive identification of *Neospora caninum* by in vitro amplification of the internal transcribed spacer 1. *Parasitology* 112, 177–182.

Hui, S.L., Walter, S.D., 1980. Estimating the error rates of diagnostic tests. *Biometrics* 36, 167–171.

Innes, E.A., Wright, S.E., Maley, S., Rae, A., Schock, A., Kirvar, E., Bartley, P., Hamilton, C., Carey, I.M., Buxton, D., 2001. Protection against vertical transmission in bovine

neosporosis. *Int. J. Parasitol.* 31, 1523–1534. https://doi.org/10.1016/S0020-7519(01)00284-3

Innes, E.A., Andrianarivo, A.G., Björkman, C., Williams, D.J.L., Conrad, P.A., 2002. Immune responses to *Neospora caninum* and prospects for vaccination. *Trends Parasitol.* 18, 497–504. https://doi.org/10.1016/s1471-4922(02)02372-3

Innes, E.A., Wright, S., Bartley, P., Maley, S., Macaldowie, C., Esteban-Redondo, I., Buxton, D., 2005. The host–parasite relationship in bovine neosporosis. *Vet. Immunol. Immunopathol.* 108, 29–36. https://doi.org/10.1016/j.vetimm.2005.07.004

Innes, E.A., 2007. The host-parasite relationship in pregnant cattle infected with *Neospora caninum*. *Parasitology* 134, 1903–1910. https://doi.org/10.1017/S0031182007000194

Jacquiet, P., Liénard, E., Franc, M., 2010. Bovine besnoitiosis: Epidemiological and clinical aspects. *Vet. Parasitol.* 174, 30–36. https://doi.org/10.1016/j.vetpar.2010.08.013

Jenkins, M.C., Caver, J.A., Björkman, C., Anderson, T.C., Romand, S., Vinyard, B., Uggla, A., Thulliez, P., Dubey, J.P., 2000. Serological investigation of an outbreak of *Neospora caninum*-associated abortion in a dairy herd in southeastern United States. *Vet. Parasitol.* 94, 17–26. https://doi.org/10.1016/S0304-4017(00)00373-3

Jenkins, M., Baszler, T., Björkman, C., Schares, G., Williams, D., 2002. Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. *Int. J. Parasitol.* 32, 631–636. https://doi.org/10.1016/S0020-7519(01)00363-0

Jenkins, M.C., Fetterer, R., Schares, G., Björkman, C., Wapenaar, W., McAllister, M., Dubey, J.P., 2005. HPLC purification of recombinant NcGRA6 antigen improves enzyme-linked immunosorbent assay for serodiagnosis of bovine neosporosis. *Vet. Parasitol.* 131, 227–234. https://doi.org/10.1016/j.vetpar.2005.05.005

Jenkins, M.C., Parker, C., Hill, D., Pinckney, R.D., Dyer, R., Dubey, J.P., 2007. *Neospora caninum* detected in feral rodents. *Vet. Parasitol.* 143, 161–165. https://doi.org/10.1016/j.vetpar.2006.08.011

Jenum, P.A., Stray-Pedersen, B., Gundersen, A.G., 1997. Improved diagnosis of primary *Toxoplasma gondii* infection in early pregnancy by determination of antitoxoplasma immunoglobulin G avidity. *J. Clin. Microbiol.* 35, 1972–1977. https://doi.org/10.1128/jcm.35.8.1972-1977.1997

Johnson, L.L., 1992. SCID mouse models of acute and relapsing chronic *Toxoplasma gondii* infections. *Infect. Immun.* 60, 3719–3724.

Kashiwazaki, Y., Gianneechini, R.E., Lust, M., Gil, J., 2004. Seroepidemiology of neosporosis in dairy cattle in Uruguay. *Vet. Parasitol.* 120, 139–144. https://doi.org/10.1016/j.vetpar.2004.01.001

King, J.S., Šlapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T., Windsor, P.A., 2010. Australian dingoes are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 40, 945–950. https://doi.org/10.1016/j.ijpara.2010.01.008

King, J.S., Jenkins, D.J., Ellis, J.T., Fleming, P., Windsor, P.A., Šlapeta, J., 2011. Implications of wild dog ecology on the sylvatic and domestic life cycle of *Neospora caninum* in Australia. The Veterinary Journal 188, 24–33. https://doi.org/10.1016/j.tvjl.2010.03.002

Kinsel, M.L., 1999. An epidemiologic approach to investigating abortion problems in dairy herds, in: *American Association of Bovine Practitioners Conference Proceedings.* pp. 152–160.

Klevar, S., Kulberg, S., Boysen, P., Storset, A.K., Moldal, T., Björkman, C., Olsen, I., 2007. Natural killer cells act as early responders in an experimental infection with *Neospora caninum* in calves. *Int. J. Parasitol.* 37, 329–339. https://doi.org/10.1016/j.ijpara.2006.11.002

Köhler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–497. https://doi.org/10.1038/256495a0

Koppe, M.J., Bleichrodt, R.P., Oyen, W.J.G., Boerman, O.C., 2005. Radioimmunotherapy and colorectal cancer. *Br. J. Surg.* 92, 264–276. https://doi.org/10.1002/bjs.4936

Kostoulas, P., 2018. PriorGen: Generates prior distributions for proportions. R package version 1. https://cran.r-project.org/web/packages/PriorGen/index.html.

Koyama, T., Kobayashi, Y., Omata, Y., Yamada, M., Furuoka, H., Maeda, R., Matsui, T., Saito, A., Mikami, T., 2001. Isolation of *Neospora caninum* from the brain of a pregnant sheep. *J. Parasitol.* 87, 1486–1488. https://doi.org/10.1645/0022-3395(2001)087[1486:IONCFT]2.0.CO;2

Landmann, J.K., Jillella, D., O'Donoghue, P.J., McGowan, M.R., 2002. Confirmation of the prevention of vertical transmission of *Neospora caninum* in cattle by the use of embryo transfer. *Aust. Vet. J.* 80, 502–503. https://doi.org/10.1111/j.1751-0813.2002.tb12475.x

Larson, R.L., Hardin, D.K., Pierce, V.L., 2004. Economic considerations for diagnostic and control options for *Neospora caninum*-induced abortions in endemically infected herds of beef cattle. *J. Am. Vet. Med. Assoc.* 224, 1597–1604. https://doi.org/10.2460/javma.2004.224.1597

Liddell, S., Jenkins, M.C., Dubey, J.P., 1999. A competitive PCR assay for quantitative detection of *Neospora caninum*. *Int. J. Parasitol.* 29, 1583–1587.

Lin, A.V., 2015. Indirect ELISA, in: Hnasko, R. (Ed.), *ELISA: Methods and Protocols,* Methods in Molecular Biology. Springer, New York, NY, pp. 51–59. https://doi.org/10.1007/978-1-4939-2742-5_5

Lindsay, D.S., Dubey, J.P., 1989a. *Neospora caninum* (Protozoa: Apicomplexa) infections in mice. *J. Parasitol.* 75, 772–779.

Lindsay, D.S., Dubey, J.P., 1989b. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. *Am. J. Vet. Res.* 50, 1981–1983.

Lindsay, D.S., Dubey, J.P., 1990. Infections in mice with tachyzoites and bradyzoites of *Neospora caninum* (Protozoa: Apicomplexa). *J. Parasitol.* 76, 410–413.

Lindsay, D.S., Dubey, J.P., Duncan, R.B., 1999a. Confirmation that the dog is a definitive host for *Neospora caninum*. *Vet. Parasitol.* 82, 327–333. https://doi.org/10.1016/s0304-4017(99)00054-0

Lindsay, D.S., Upton, S.J., Dubey, J.P., 1999b. A structural study of the *Neospora caninum* oocyst. *Int. J. Parasitol.* 29, 1521–1523. https://doi.org/10.1016/s0020-7519(99)00121-6

Lindsay, D.S., Dubey, J.P., 2020. Neosporosis, toxoplasmosis, and sarcocystosis: An update. *Vet. Clin. N. Am. - Food Anim. Pract.* 36, 205–222. https://doi.org/10.1016/j.cvfa.2019.11.004

López-Gatius, F., Yániz, J.L., Madriles-Helm, D., 2003. Effects of body condition score and score change on the reproductive performance of dairy cows: a meta-analysis. *Theriogenology* 59, 801–812. https://doi.org/10.1016/S0093-691X(02)01156-1

López-Gatius, F., López-Béjar, M., Murugavel, K., Pabón, M., Ferrer, D., Almería, S., 2004a. *Neospora*-associated abortion episode over a 1-year period in a dairy herd in north-east Spain. *J. Vet. Med.* B 51, 348–352. https://doi.org/10.1111/j.1439-0450.2004.00779.x

López-Gatius, F., Pabón, M., Almería, S., 2004b. *Neospora caninum* infection does not affect early pregnancy in dairy cattle. *Theriogenology* 62, 606–613. https://doi.org/10.1016/j.Theriogenology.2003.11.002

López-Gatius, F., García-Ispierto, I., Santolaria, P., Yániz, J.L., López-Béjar, M., Nogareda, C., Almería, S., 2005a. Relationship between rainfall and *Neospora caninum*-associated abortion in two dairy herds in a dry environment. *J. Vet. Med. B* 52, 147–152. https://doi.org/10.1111/j.1439-0450.2005.00831.x

López-Gatius, F., Santolaria, P., Yániz, J.L., Garbayo, J.M., Almería, S., 2005b. The use of beef bull semen reduced the risk of abortion in *Neospora*-seropositive dairy cows. *J. Vet. Med. B* 52, 88–92. https://doi.org/10.1111/j.1439-0450.2004.00818.x

López-Gatius, F., Almería, S., Donofrio, G., Nogareda, C., García-Ispierto, I., Bech-Sàbat, G., Santolaria, P., Yániz, J.L., Pabón, M., de Sousa, N.M., Beckers, J.F., 2007. Protection against abortion linked to gamma interferon production in pregnant dairy cows naturally infected with *Neospora caninum*. *Theriogenology* 68, 1067–1073. https://doi.org/10.1016/j.Theriogenology.2007.08.006

López-Ratón, M., Rodríguez-Álvarez, M.X., Cadarso-Suárez, C., Gude-Sampedro, F., 2014. OptimalCutpoints: An R package for selecting optimal cutpoints in diagnostic tests. *J. Stat. Softw.* 61, 1–36. https://doi.org/10.18637/jss.v061.i08

Macaldowie, C., Maley, S.W., Wright, S., Bartley, P., Esteban-Redondo, I., Buxton, D., Innes, E.A., 2004. Placental pathology associated with fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. *J. Comp. Pathol.* 131, 142–156. https://doi.org/10.1016/j.jcpa.2004.02.005

Mainar-Jaime, R.C., Thurmond, M.C., Berzal-Herranz, B., Hietala, S.K., 1999. Seroprevalence of *Neospora caninum* and abortion in dairy cows in northern Spain. *Vet. Rec.* 145, 72–75. https://doi.org/10.1136/vr.145.3.72

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., Innes, E.A., 2003. The Pathogenesis of Neosporosis in Pregnant Cattle: Inoculation at Mid-gestation. Journal of Comparative Pathology 129, 186–195. https://doi.org/10.1016/S0021-9975(03)00032-X

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., Innes, E.A., 2006. Characterization of the immune response in the placenta of cattle experimentally infected with *Neospora caninum* in early gestation. *J. Comp. Pathol.* 135, 130–141. https://doi.org/10.1016/j.jcpa.2006.07.001

Marugan-Hernandez, V., 2017. *Neospora caninum* and bovine neosporosis: Current vaccine research. *J. Comp. Pathol.* 157, 193–200. https://doi.org/10.1016/j.jcpa.2017.08.001

Mazuz, M.L., Fish, L., Reznikov, D., Wolkomirsky, R., Leibovitz, B., Savitzky, I., Golenser, J., Shkap, V., 2014. Neosporosis in naturally infected pregnant dairy cattle. *Vet. Parasitol.* 205, 85–91. https://doi.org/10.1016/j.vetpar.2014.06.009

McAllister, M.M., Huffman, E.M., Hietala, S.K., Conrad, P.A., Anderson, M.L., Salman, M.D., 1996. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. *J. Vet. Diagn. Invest.* 8, 355–357. https://doi.org/10.1177/104063879600800313

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 28, 1473–1478.

McAllister, M.M., Björkman, C., Anderson-Sprecher, R., Rogers, D.G., 2000. Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. *J. Am. Vet. Med. Assoc.* 217, 881–887. https://doi.org/10.2460/javma.2000.217.881

McAllister, M.M., Wallace, R.L., Bjorkman, C., Gao, L., Firkins, L.D., 2005. A probable source of *Neospora caninum* infection in an abortion outbreak in dairy cows. The Bovine Practitioner 69–74.

McAllister, M.M., 2016. Diagnosis and control of bovine neosporosis. *Vet. Clin. Food Anim.* 32, 443–463. https://doi.org/10.1016/j.cvfa.2016.01.012

McCann, C.M., McAllister, M.M., Gondim, L.F.P., Smith, R.F., Cripps, P.J., Kipar, A., Williams, D.J.L., 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. *Int. J. Parasitol.* 37, 1631–1639. https://doi.org/10.1016/j.ijpara.2007.05.012

McGarry, J., Guy, F., Trees, A.J., Williams, D., Davison, H.C., Bjorkman, C., 2000. Validation and application of an inhibition ELISA to detect serum antibodies to *Neospora caninum* in different host species. Int *J. Parasitol.* 30, 880–884.

McGarry, J.W., Stockton, C.M., Williams, D.J.L., Trees, A.J., 2003. Protracted shedding of oocysts of *Neospora caninum* by a naturally infected foxhound. *J. Parasitol.* 89, 628–630. https://doi.org/10.1645/0022-3395(2003)089[0628:PSOOON]2.0.CO;2

McInnes, L.M., Ryan, U.M., O'Handley, R., Sager, H., Forshaw, D., Palmer, D.G., 2006. Diagnostic significance of *Neospora caninum* DNA detected by PCR in cattle serum. *Vet. Parasitol.* 142, 207–213. https://doi.org/10.1016/j.vetpar.2006.07.013

McNamee, P.T., Trees, A.J., Guy, F., Moffett, D., Kilpatrick, D., 1996. Diagnosis and prevalence of neosporosis in cattle in Northern Ireland. *Vet. Rec.* 138, 419–420. https://doi.org/10.1136/vr.138.17.419

Melendez, P., Ilha, M., Woldemeskel, M., Graham, J., Coarsey, M., Baughman, D., Whittington, L., Naikare, H., 2021. An outbreak of *Neospora caninum* abortion in a dairy herd from the State of Georgia, United States. *Vet. Med. Sci.* 7, 141–147. https://doi.org/10.1002/vms3.346

Micheloud, J.F., Moore, P.D., Canal, A.M., Lischinsky, L.H., Hecker, Y.P., Canton, G.J., Odriozola, E.R., Odeon, A.C., Campero, C.M., 2015. First report of congenital *Neospora caninum* encephalomyelitis in two newborn calves in the Argentinean Pampas. *J. Vet. Sci. Tech*. 6, 251.

Modrý, D., Václavek, P., Koudela, B., Slapeta, J.R., 2001. Placentophagia — an alternative way for horizontal transmission of *Neospora caninum* in cattle? *Trends Parasitol.* 17, 573–575. https://doi.org/10.1016/s1471-4922(01)02144-4

Moen, A.R., Wouda, W., Mul, M.F., Graat, E.A., van Werven, T., 1998. Increased risk of abortion following *Neospora caninum* abortion outbreaks: a retrospective and prospective cohort study in four dairy herds. *Theriogenology* 49, 1301–1309. https://doi.org/10.1016/S0093-691X(98)00077-6

Monney, T., Hemphill, A., 2014. Vaccines against neosporosis: what can we learn from the past studies? *Exp. Parasitol.* 140, 52–70. https://doi.org/10.1016/j.exppara.2014.02.015

Moore, D.P., Leunda, M.R., Zamorano, P.I., Odeón, A.C., Romera, S.A., Cano, A., de Yaniz, G., Venturini, M.C., 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. *Vet. Parasitol.* 130, 29–39. https://doi.org/10.1016/j.vetpar.2005.03.010

Moskwa, B., Cabaj, W., Pastusiak, K., Bień, J., 2003. The suitability of milk in detection of *Neospora caninum* infection in cows. *Acta Parasitol.* 48, 138–141.

Moskwa, B., Goździk, K., Bień, J., Cabaj, W., 2008. Studies on *Neospora caninum* DNA detection in the oocytes and embryos collected from infected cows. *Vet. Parasitol.* 158, 370–375. https://doi.org/10.1016/j.vetpar.2008.09.018

Moskwa, B., Pastusiak, K., Bien, J., Cabaj, W., 2007. The first detection of *Neospora caninum* DNA in the colostrum of infected cows. *Parasitol. Res.* 100, 633–636. https://doi.org/10.1007/s00436-006-0288-7

Müller, N., Zimmermann, V., Hentrich, B., Gottstein, B., 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *J. Clin. Microbiol.* 34, 2850–2852.

Nelson, P.N., 2000. Demystified: Monoclonal antibodies. *Mol. Pathol.* 53, 111–117. https://doi.org/10.1136/mp.53.3.111

Novoa, M.B., Valentini, B.S., Sarli, M., Torioni-de-Echaide, S.M., Primo, M.E., Echaide, I.E., 2020. Evaluation of a competitive inhibition ELISA based on the recombinant protein tSAG1 to detect anti–*Neospora caninum* antibodies in cattle. *J. Vet. Diagn. Invest.* 32, 401–408. https://doi.org/10.1177/1040638720916711

Okeoma, C.M., Williamson, N.B., Pomroy, W.E., Stowell, K.M., Gillespie, L., 2004. The use of PCR to detect *Neospora caninum* DNA in the blood of naturally infected cows. *Vet. Parasitol.* 122, 307–315. https://doi.org/10.1016/j.vetpar.2004.06.001

Ortega-Mora, L.M., Ferre, I., del-Pozo, I., Caetano-da-Silva, A., Collantes-Fernández, E., Regidor-Cerrillo, J., Ugarte-Garagalza, C., Aduriz, G., 2003. Detection of *Neospora caninum* in semen of bulls. *Vet. Parasitol.* 117, 301–308. https://doi.org/10.1016/j.vetpar.2003.09.015

Ortega-Mora, L., Fernández-García, A., Gómez-Bautista, M., 2006. Diagnosis of bovine neosporosis: Recent advances and perspectives. *Acta Parasitol.* 51, 1–14. https://doi.org/10.2478/s11686-006-0001-0

Osburn, B.I., MacLachlan, N.J., Terrell, T.G., 1982. Ontogeny of the immune system. *J. Am. Vet. Med. Assoc*. 181, 1049–1052.

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

Ossipow, V., Fischer, N. (Eds.), 2014. Monoclonal Antibodies: Methods and Protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-62703-992-5

O'Toole, D., Jeffrey, M., 1987. Congenital sporozoan encephalomyelitis in a calf. *Vet. Rec.* 121, 563–566.

Otter, A., Jeffrey, M., Scholes, S.F., Helmick, B., Wilesmith, J.W., Trees, A.J., 1997. Comparison of histology with maternal and fetal serology for the diagnosis of abortion due to bovine neosporosis. *Vet. Rec.* 141, 487–489. https://doi.org/10.1136/vr.141.19.487

Pabón, M., López-Gatius, F., García-Ispierto, I., Bech-Sàbat, G., Nogareda, C., Almería, S., 2007. Chronic *Neospora caninum* infection and repeat abortion in dairy cows: a 3-year study. *Vet. Parasitol.* 147, 40–46. https://doi.org/10.1016/j.vetpar.2007.03.017

Packham, A.E., Sverlow, K.W., Conrad, P.A., Loomis, E.F., Rowe, J.D., Anderson, M.L., Marsh, A.E., Cray, C., Barr, B.C., 1998. A modified agglutination test for *Neospora caninum*: development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.* 5, 467–473. https://doi.org/10.1128/CDLI.5.4.467-473.1998

Paré, J., Hietala, S., Thurmond, M., 1995a. Interpretation of an Indirect Fluorescent Antibody Test for Diagnosis of *Neospora* sp. Infection in Cattle. *J. Vet. Diagn. Invest.* 7, 273–275. https://doi.org/10.1177/104063879500700222

Paré, J., Hietala, S.K., Thurmond, M.C., 1995b. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. *J. Vet. Diagn. Invest.* 7, 352–359. https://doi.org/10.1177/104063879500700310

Paré, J., Thurmond, M.C., Hietala, S.K., 1996. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. *Can. J. Vet. Res.* 60, 133–139.

Paré, J., Thurmond, M.C., Hietala, S.K., 1997. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. *J. Parasitol.* 83, 82. https://doi.org/10.2307/3284321

Paré, J., Fecteau, G., Fortin, M., Marsolais, G., 1998. Seroepidemiologic study of *Neospora caninum* in dairy herds. *J. Am. Vet. Med. Assoc.* 213, 1595–1598.

Parish, S.M., Maag-Miller, L., Besser, T.E., Weidner, J.P., McElwain, T., Knowles, D.P., Leathers, C.W., 1987. Myelitis associated with protozoal infection in newborn calves. *J. Am. Vet. Med. Assoc.* 191, 1599–1600.

Patitucci, A.N., Charleston, W.A., Alley, M.R., O'Connor, R.J., Pomroy, W.E., 1999. Serological study of a dairy herd with a recent history of *Neospora* abortion. *N. Z. Vet. J.* 47, 28–30. https://doi.org/10.1080/00480169.1999.36104

Payne, S., Ellis, J., 1996. Detection of *Neospora caninum* DNA by the polymerase chain reaction. *Int. J. Parasitol.* 26, 347–351. https://doi.org/10.1016/0020-7519(96)00030-6

Pereira-Bueno, J., Quintanilla-Gozalo, A., Pérez-Pérez, V., Espi-Felgueroso, A., Álvarez-García, G., Collantes-Fernández, E., Ortega-Mora, L.M., 2003. Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. *Vet. Parasitol.* 111, 143–152. https://doi.org/10.1016/s0304-4017(02)00361-8

Peters, M., Lütkefels, E., Heckeroth, A.R., Schares, G., 2001. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of

naturally infected dogs and cattle. *Int. J. Parasitol.* 31, 1144–1148. https://doi.org/10.1016/s0020-7519(01)00221-1

Piagentini, M., Moya-Araujo, C.F., Prestes, N.C., Sartor, I.F., 2012. *Neospora caninum* infection dynamics in dairy cattle. *Parasitol. Res.* 111, 717–721. https://doi.org/10.1007/s00436-012-2891-0

Plummer, M., 2003. JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling. *Proceedings of the 3rd International Workshop on Distributed Statistical Computing* (Vol 124, N. 125. 10 pp. 1-10).

Quinn, H.E., Ellis, J.T., Smith, N.C., 2002. *Neospora caninum*: a cause of immune-mediated failure of pregnancy? *Trends Parasitol.* 18, 391–394. https://doi.org/10.1016/s1471-4922(02)02324-3

Quintanilla-Gozalo, A., Pereira-Bueno, J., Seijas-Carballedo, A., Costas, E., Ortega-Mora, L.M., 2000. Observational studies in *Neospora caninum* infected dairy cattle: Relationship infection-abortion and gestational antibody fluctuations. *Int*. *J. Parasitol.* 30, 900–906.

Ratner, B., 2009. The correlation coefficient: Its values range between +1/−1, or do they? *J. Target. Meas. Anal. Mark.* 17, 139–142. https://doi.org/10.1057/jt.2009.5

Razmi, G.R., Zarea, H., Naseri, Z., 2010. A survey of *Neospora caninum*-associated bovine abortion in large dairy farms of Mashhad, Iran. *Parasitol. Res.* 106, 1419–1423. https://doi.org/10.1007/s00436-010-1820-3

Reichel, M.P., Pfeiffer, D.U., 2002. An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle. *Vet. Parasitol.* 107, 197–207. https://doi.org/10.1016/s0304-4017(02)00086-9

Reichel, M.P., Ellis, J.T., 2002. Control options for *Neospora caninum* infections in cattle — Current state of knowledge. *N. Z. Vet. J.* 50, 86–92. https://doi.org/10.1080/00480169.2002.36288

Reichel, M.P., Ellis, J.T., 2006. If control of *Neospora caninum* infection is technically feasible does it make economic sense? *Vet. Parasitol.* 142, 23–34. https://doi.org/10.1016/j.vetpar.2006.06.027

Reichel, M.P., Ellis, J.T., 2009. *Neospora caninum* – How close are we to development of an efficacious vaccine that prevents abortion in cattle? *Int. J. Parasitol.* 39, 1173–1187. https://doi.org/10.1016/j.ijpara.2009.05.007

Reichel, M.P., Alejandra Ayanegui-Alcérreca, M., Gondim, L.F.P., Ellis, J.T., 2013. What is the global economic impact of *Neospora caninum* in cattle — The billion dollar question. *Int. J. Parasitol.* 43, 133–142. https://doi.org/10.1016/j.ijpara.2012.10.022

Reichel, M.P., McAllister, M.M., Pomroy, W.E., Campero, C., Ortega-Mora, L.M., Ellis, J.T., 2014. Control options for *Neospora caninum* – is there anything new or are we going backwards? *Parasitol.* 141, 1455–1470. https://doi.org/10.1017/S0031182014000158

Reitt, K., Hilbe, M., Voegtlin, A., Corboz, L., Haessig, M., Pospischil, A., 2007. Aetiology of bovine abortion in Switzerland from 1986 to 1995 — A retrospective study with emphasis on detection of *Neospora caninum* and *Toxoplasma gondii* by PCR. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.* 54, 15–22. https://doi.org/10.1111/j.1439-0442.2007.00913.x

Rodrigues, A. a. R., Gennari, S.M., Aguiar, D.M., Sreekumar, C., Hill, D.E., Miska, K.B., Vianna, M.C.B., Dubey, J.P., 2004. Shedding of *Neospora caninum* oocysts by dogs

fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. *Vet. Parasitol.* 124, 139–150. https://doi.org/10.1016/j.vetpar.2004.07.007

Roelandt, S., Van der Stede, Y., Czaplicki, G., Van Loo, H., Van Driessche, E., Dewulf, J., Hooyberghs, J., Faes, C., 2015. Serological diagnosis of bovine neosporosis: a Bayesian evaluation of two antibody ELISA tests for in vivo diagnosis in purchased and abortion cattle. Veterinary Record 176, 598–598. https://doi.org/10.1136/vr.102872

Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. *Parasitology Res.* 84, 50–53. https://doi.org/10.1007/s004360050355

Romero, J.J., Perez, E., Dolz, G., Frankena, K., 2002. Factors associated with *Neospora caninum* serostatus in cattle of 20 specialised Costa Rican dairy herds. *Prev. Vet. Med.* 53, 263–273. https://doi.org/10.1016/s0167-5877(01)00290-2

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

Rosbottom, A., Gibney, E.H., Guy, C.S., Kipar, A., Smith, R.F., Kaiser, P., Trees, A.J., Williams, D.J.L., 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. *Infect. Immun.* 76, 2352–2361. https://doi.org/10.1128/iai.01780-06

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

Rosypal, A.C., Lindsay, D.S., 2005. The sylvatic cycle of *Neospora caninum*: where do we go from here? *Trends Parasitol.* 21, 439–440. https://doi.org/10.1016/j.pt.2005.08.003

Roth, J.A., 1985. Cortisol as Mediator of Stress-Associated Immunosuppression in Cattle, in: Moberg, G.P. (Ed.), *Animal Stress*. Springer New York, New York, NY, pp. 225–243. https://doi.org/10.1007/978-1-4614-7544-6_13

Sabin, A.B., 1941. Toxoplasmic encephalitis in children. *J. Am. Med. Assoc.* 116, 801–807. https://doi.org/10.1001/jama.1941.02820090001001

Sager, H., Fischer, I., Furrer, K., Strasser, M., Waldvogel, A., Boerlin, P., Audigé, L., Gottstein, B., 2001. A Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. *Vet. Parasitol.* 102, 1–15. https://doi.org/10.1016/s0304-4017(01)00524-6

Sager, H., Gloor, M., Björkman, C., Kritzner, S., Gottstein, B., 2003. Assessment of antibody avidity in aborting cattle by a somatic *Neospora caninum* tachyzoite antigen IgG avidity ELISA. *Vet. Parasitol.* 112, 1–10. https://doi.org/10.1016/S0304-4017(02)00416-8

Sanderson, M.W., Gay, J.M., Baszler, T.V., 2000. *Neospora caninum* seroprevalence and associated risk factors in beef cattle in the northwestern United States. *Vet. Parasitol.* 90, 15–24. https://doi.org/10.1016/S0304-4017(00)00234-X

Santolaria, P., Almería, S., Martínez-Bello, D., Nogareda, C., Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Pabón, M., Yániz, J.L., López-Gatius, F., 2011. Different

humoral mechanisms against *Neospora caninum* infection in purebreed and crossbreed beef/dairy cattle pregnancies. *Vet. Parasitol.* 178, 70–76. https://doi.org/10.1016/j.vetpar.2010.12.018

Schares, G., Peters, M., Wurm, R., Bärwald, A., J. Conraths, F., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Vet. Parasitol.* 80, 87–98. https://doi.org/10.1016/S0304-4017(98)00195-2´

Schares, G., Conraths, F.J., Reichel, M.P., 1999a. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. *Int. J. Parasitol.* 29, 1659–1667. https://doi.org/10.1016/S0020-7519(99)00104-6

Schares, G., Dubremetz, J.F., Dubey, J.P., Bärwald, A., Loyens, A., Conraths, F.J., 1999b. *Neospora caninum*: Identification of 19-, 38-, and 40-kDa surface antigens and a 33-kDa dense granule antigen using monoclonal antibodies. *Exp. Parasitol.* 92, 109–119. https://doi.org/10.1006/expr.1999.4403

Schares, G., Rauser, M., Zimmer, K., Peters, M., Wurm, R., Dubey, J.P., de Graaf, D.C., Edelhofer, R., Mertens, C., Hess, G., Conraths, F.J., 1999c. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. *J. Parasitol.* 85, 688–694.

Schares, G., Rauser, M., Söndgen, P., Rehberg, P., Bärwald, A., Dubey, J.P., Edelhofer, R., Conraths, F.J., 2000. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. *Int. J. Parasitol.* 30, 1123–1130. https://doi.org/10.1016/s0020-7519(00)00092-8

Schares, G., Conraths, F.J., 2001. Placentophagia – an alternative way for horizontal transmission of *Neospora caninum* in cattle? Trends in *Parasitol.* 17, 574–575. https://doi.org/10.1016/S1471-4922(01)02145-6

Schares, G., Heydorn, A., Cüppers, A., Conraths, F., Mehlhorn, H., 2001. Cyclic transmission of *Neospora caninum*: serological findings in dogs shedding oocysts. *Parasitol. Res.* 87, 873–877. https://doi.org/10.1007/s004360100459

Schares, G., Bärwald, A., Staubach, C., Söndgen, P., Rauser, M., Schröder, R., Peters, M., Wurm, R., Selhorst, T., Conraths, F.J., 2002a. p38-avidity-ELISA: Examination of herds experiencing epidemic or endemic *Neospora caninum*-associated bovine abortion. *Vet. Parasitol.* 106, 293–305. https://doi.org/10.1016/S0304-4017(02)00103-6

Schares, G., Heydorn, A., Cüppers, A., Mehlhorn, H., Geue, L., Peters, M., Conraths, F.J., 2002b. In contrast to dogs, red foxes (*Vulpes vulpes*) did not shed *Neospora caninum* upon feeding of intermediate host tissues. *Parasitol. Res.* 88, 44–52. https://doi.org/10.1007/s004360100496

Schares, G., Bärwald, A., Staubach, C., Wurm, R., Rauser, M., Conraths, F.J., Schroeder, C., 2004a. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. *Vet. Parasitol.* 120, 55–63. https://doi.org/10.1016/j.vetpar.2003.11.016

Schares, G., Bärwald, A., Staubach, C., Ziller, M., Klöss, D., Schröder, R., Labohm, R., Dräger, K., Fasen, W., Hess, R.G., Conraths, F.J., 2004b. Potential risk factors for bovine *Neospora caninum* infection in Germany are not under the control of the farmers. *Parasitology* 129, 301–309. https://doi.org/10.1017/s0031182004005700

Schares, G., Bärwald, A., Conraths, F.J., 2005a. Adaptation of a surface antigen-based ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. *J. Vet. Med. B* 52, 45–48. https://doi.org/10.1111/j.1439-0450.2004.00819.x

Schares, G., Pantchev, N., Barutzki, D., Heydorn, A.O., Bauer, C., Conraths, F.J., 2005b. Oocysts of *Neospora caninum*, Hammondia heydorni, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. *Int. J. Parasitol.* 35, 1525–1537. https://doi.org/10.1016/j.ijpara.2005.08.008

Schares, G., Losson, B., 2007. 3.1 Neosporosis: Dog, in: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), *Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control*, CABI Books. CABI International, pp. 98–103. https://doi.org/10.1079/9781845932114.0042

Schares, G., Basso, W., Majzoub, M., Rostaher, A., Scharr, J.C., Langenmayer, M.C., Selmair, J., Dubey, J.P., Cortes, H.C., Conraths, F.J., Gollnick, N.S., 2010. Comparative evaluation of immunofluorescent antibody and new immunoblot tests for the specific detection of antibodies against *Besnoitia besnoiti* tachyzoites and bradyzoites in bovine sera. *Vet. Parasitol.* 171, 32–40. https://doi.org/10.1016/j.vetpar.2010.03.017

Schares, G., Nascimento, D., Bärwald, A., Jutras, C., Rivard, S., Brodeur, V., DeNotta, S.L., Basso, W., Conraths, F.J., 2020. First highly sensitive and specific competitive ELISA for detection of bovine besnoitiosis with potential as a multi-species test. *Int. J. Parasitol.* 50, 389–401. https://doi.org/10.1016/j.ijpara.2019.12.010

Sergeant, E.S.G., 2018. Epitools epidemiological calculators. http://epitools.ausvet.com.au.

Serrano, E., Ferre, I., Osoro, K., Aduriz, G., Mateos-Sanz, A., Martínez, A., Atxaerandio, R., Hidalgo, C.O., Ortega-Mora, L.M., 2006. Intrauterine *Neospora caninum* inoculation of heifers. *Vet. Parasitol.* 135, 197–203. https://doi.org/10.1016/j.vetpar.2005.10.003

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

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

Sim, J., Wright, C.C., 2005. The kappa statistic in reliability studies: use, interpretation, and sample size requirements. *Phys. Ther.* 85, 257–268.

Sinnott, F.A., Monte, L.G., Collares, T.F., De Matos, B.M., Pacheco, D.B., Borsuk, S., Andreotti, R., Hartleben, C.P., 2015. Blocking ELISA using recombinant NcSRS2 protein for diagnosing bovine neosporosis. *Curr. Microbiol.* 70, 429–432. https://doi.org/10.1007/s00284-014-0737-y

Slotved, H.C., Jensen, L., Lind, P., 1999. Comparison of the IFAT and ISCOM-ELISA response in bovine foetuses with *Neospora caninum* infection. *Int. J. Parasitol.* 29, 1165–1174. https://doi.org/10.1016/s0020-7519(99)00095-8

Söndgen, P., Peters, M., Bärwald, A., Wurm, R., Holling, F., Conraths, F.J., Schares, G., 2001. Bovine neosporosis: immunoblot improves foetal serology. *Vet. Parasitol.* 102, 279–290. https://doi.org/10.1016/S0304-4017(01)00543-X

Speer, C.A., Dubey, J.P., McAllister, M.M., Blixt, J.A., 1999. Comparative ultrastructure of tachyzoites, bradyzoites, and tissue cysts of *Neospora caninum* and *Toxoplasma gondii*. *Int. J. Parasitol.* 29, 1509–1519. https://doi.org/10.1016/s0020-7519(99)00132-0

Staska, L.M., McGuire, T.C., Davies, C.J., Lewin, H.A., Baszler, T.V., 2003. *Neospora caninum*-infected cattle develop parasite-specific CD4+ cytotoxic T lymphocytes. *Infect. Immun.* 71, 3272–3279. https://doi.org/10.1128/IAI.71.6.3272-3279.2003

Staubli, D., Nunez, S., Sager, H., Schares, G., Gottstein, B., 2006. *Neospora caninum* immunoblotting improves serodiagnosis of bovine neosporosis. *Parasitol. Res.* 99, 648–658. https://doi.org/10.1007/s00436-006-0207-y

Stenlund, S., Björkman, C., Holmdahl, O.J., Kindahl, H., Uggla, A., 1997. Characterization of a Swedish bovine isolate of *Neospora caninum*. *Parasitol. Res.* 83, 214–219. https://doi.org/10.1007/s004360050236

Stenlund, S., Kindahl, H., Magnusson, U., Uggla, A., Björkman, C., 1999. Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with *Neospora caninum*. *Vet. Parasitol.* 85, 227–234. https://doi.org/10.1016/S0304-4017(99)00120-X

Stenlund, S., 2000. *Neospora caninum* in Cattle in Sweden: Isolation of the Parasite and Studies of Its Transmission. Swedish University of Agricultural Sciences Uppsala; Doctoral Thesis.

Stenlund, S., Kindahl, H., Uggla, A., Björkman, C., 2003. A long-term study of *Neospora caninum* infection in a Swedish dairy herd. *Acta Vet. Scand.* 44, 63–71. https://doi.org/10.1186/1751-0147-44-63

Sternberg, E.M., 1997. Neural-immune interactions in health and disease. *J. Clin. Invest*. 100, 2641–2647. https://doi.org/10.1172/JCI119807

Stevenson, M., Stevenson, M.M., BiasedUrn, I., 2015. Package 'epiR.' Tools for the analysis of epidemiological data R package version 0.9–62.

Thrusfield, M., Christley, R., 2018. *Veterinary Epidemiology*. Wiley.

Thurmond, M., Hietala, S., 1995. Strategies to control *Neospora* infection in cattle. *Bov. Pract.* 1995, 60–63. https://doi.org/10.21423/bovine-vol1995no29p60-63

Thurmond, M.C., Anderson, M.L., Blanchard, P.C., 1995. Secular and Seasonal Trends of *Neospora* Abortion in California Dairy Cows. *J. Parasitol.* 81, 364. https://doi.org/10.2307/3283817

Thurmond, M.C., Hietala, S.K., 1997. Effect of congenitally acquired *Neospora caninum* infection on risk of abortion and subsequent abortions in dairy cattle. *Am. J. Vet. Res.* 58, 1381–1385.

Thurmond, M.C., Hietala, S.K., Blanchard, P.C., 1997. Herd-based diagnosis of *Neospora caninum*-induced endemic and epidemic abortion in cows and evidence for congenital and postnatal transmission. *J. Vet. Diagn. Invest.* 9, 44–49. https://doi.org/10.1177/104063879700900108

Thurmond, M.C., Hietala, S.K., Blanchard, P.C., 1999. Predictive values of fetal histopathology and immunoperoxidase staining in diagnosing bovine abortion caused by *Neospora caninum* in a dairy herd. *J. Vet. Diagn. Invest.* 11, 90–94. https://doi.org/10.1177/104063879901100115

Thurmond, M.C., Branscum, A.J., Johnson, W.O., Bedrick, E.J., Hanson, T.E., 2005. Predicting the probability of abortion in dairy cows: a hierarchical Bayesian logistic-survival model using sequential pregnancy data. *Prev. Vet. Med.* 68, 223–239. https://doi.org/10.1016/j.prevetmed.2005.01.008

Tomioka, Y., Sawada, M., Ochiai, K., Umemura, T., 2003. *Neospora caninum* antigens recognized by mouse IgG at different stages of infection including recrudescence. *J. Vet. Med. Sci*. 65, 745–747. https://doi.org/10.1292/jvms.65.745

Torres, M.P., Ortega, Y.R., 2006. *Neospora caninum* antibodies in commercial fetal bovine serum. *Vet. Parasitol.* 140, 352–355. https://doi.org/10.1016/j.vetpar.2006.03.032

Towbin, H., Gordon, J., 1984. Immunoblotting and dot immunobinding — Current status and outlook. Journal of Immunological Methods 72, 313–340. https://doi.org/10.1016/0022-1759(84)90001-2

Trees, A.J., Davison, H.C., Innes, E.A., Wastling, J.M., 1999. Towards evaluating the economic impact of bovine neosporosis. *Int. J. Parasitol.* 29, 1195–1200. https://doi.org/10.1016/s0020-7519(99)00093-4

Trees, A.J., McAllister, M.M., Guy, C.S., McGarry, J.W., Smith, R.F., Williams, D.J.L., 2002. *Neospora caninum*: oocyst challenge of pregnant cows. *Vet. Parasitol.* 109, 147–154. https://doi.org/10.1016/s0304-4017(02)00234-0

Trees, A.J., Williams, D.J.L., 2005. Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. *Trends Parasitol.* 21, 558–561. https://doi.org/10.1016/j.pt.2005.09.005

Uggla, A., Stenlund, S., Holmdahl, O.J., Jakubek, E.B., Thebo, P., Kindahl, H., Björkman, C., 1998. Oral *Neospora caninum* inoculation of neonatal calves. *Int. J. Parasitol.* 28, 1467–1472. https://doi.org/10.1016/s0020-7519(98)00110-6

Uzêda, R.S., Costa, K.D.S., Santos, S.L., Pinheiro, A.M., De Almeida, M.A.O., McAllister, M.M., Gondim, L.F.P., 2007. Loss of infectivity of *Neospora caninum* oocysts maintained for a prolonged time. *Korean J. Parasitol.* 45, 295–299. https://doi.org/10.3347/kjp.2007.45.4.295

Uzêda, R.S., Schares, G., Ortega-Mora, L.M., Madruga, C.R., Aguado-Martinez, A., Corbellini, L.G., Driemeier, D., Gondim, L.F.P., 2013. Combination of monoclonal antibodies improves immunohistochemical diagnosis of *Neospora caninum*. *Vet. Parasitol.* 197, 477–486. https://doi.org/10.1016/j.vetpar.2013.07.008

van Maanen, C., Wouda, W., Schares, G., von Blumröder, D., Conraths, F.J., Norton, R., Williams, D.J.L., Esteban-Redondo, I., Innes, E.A., Mattsson, J.G., Björkman, C., Fernández-García, A., Ortega-Mora, L.M., Müller, N., Sager, H., Hemphill, A., 2004. An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. *Vet. Parasitol.* 126, 351–364. https://doi.org/10.1016/j.vetpar.2004.08.016

Varcasia, A., Capelli, G., Ruiu, A., Ladu, M., Scala, A., Bjorkman, C., 2006. Prevalence of *Neospora caninum* infection in Sardinian dairy farms (Italy) detected by ISCOM ELISA on tank bulk milk. *Parasitol. Res.* 98, 264–267. https://doi.org/10.1007/s00436-005-0044-4

Venturini, M.C., Quiroga, M.A., Risso, M.A., Lorenzo, C.D., Omata, Y., Venturini, L., Godoy, H., 1996. Mycotoxin T-2 and aflatoxin B1 as immunosuppressors in mice chronically infected with *Toxoplasma gondii*. *J. Comp. Pathol.* 115, 229–237. https://doi.org/10.1016/s0021-9975(96)80081-8

Vianna, M.C.B., Sreekumar, C., Miska, K.B., Hill, D.E., Dubey, J.P., 2005. Isolation of *Neospora caninum* from naturally infected white-tailed deer (*Odocoileus virginianus*). *Vet. Parasitol.* 129, 253–257. https://doi.org/10.1016/j.vetpar.2004.02.031

von Blumröder, D., Schares, G., Norton, R., Williams, D.J.L., Esteban-Redondo, I., Wright, S., Björkman, C., Frössling, J., Risco-Castillo, V., Fernández-García, A., Ortega-Mora, L.M., Sager, H., Hemphill, A., van Maanen, C., Wouda, W., Conraths, F.J., 2004. Comparison and standardisation of serological methods for the diagnosis of *Neospora caninum* infection in bovines. *Vet. Parasitol.* 120, 11–22. https://doi.org/10.1016/j.vetpar.2003.12.010

Vos, Q., Klasen, E.A., Haaijman, J.J., 1987. The effect of divalent and univalent binding on antibody titration curves in solid-phase ELISA. *J. Immunol. Methods* 103, 47–54. https://doi.org/10.1016/0022-1759(87)90240-7

Waldner, C.L., Janzen, E.D., Henderson, J., Haines, D.M., 1999. Outbreak of abortion associated with *Neospora caninum* infection in a beef herd. *J. Am. Vet. Med. Assoc.* 215, 1485–1490, 1448–1449.

Waldner, C.L., Cunningham, G., Campbell, J.R., 2004. Agreement between three serological tests for *Neospora caninum* in beef cattle. *J. Vet. Diagn. Invest.* 16, 313–315. https://doi.org/10.1177/104063870401600409

Walsh, R.B., Kelton, D.F., Hietala, S.K., Duffield, T.F., 2013. Evaluation of enzyme-linked immunosorbent assays performed on milk and serum samples for detection of neosporosis and leukosis in lactating dairy cows. *Can. Vet.. J.* 54, 347–352.

Walz, P.H., Chamorro, M.F., M. Falkenberg, S., Passler, T., van der Meer, F., R. Woolums, A., 2020. Bovine viral diarrhea virus: An updated American College of Veterinary Internal Medicine consensus statement with focus on virus biology, hosts, immunosuppression, and vaccination. *J. Vet. Intern. Med.* 34, 1690–1706. https://doi.org/10.1111/jvim.15816

Wang, F., Wang, X., Song, X., Ma, L., Yang, J., Liu, Q., Liu, J., 2021. Function of *Neospora caninum* dense granule protein 7 in innate immunity in mice. *Parasitol. Res.* 120, 197–207. https://doi.org/10.1007/s00436-020-06961-4

Wapenaar, W., Barkema, H.W., VanLeeuwen, J.A., McClure, J.T., O'Handley, R.M., Kwok, O.C.H., Thulliez, P., Dubey, J.P., Jenkins, M.C., 2007. Comparison of serological methods for the diagnosis of *Neospora caninum* infection in cattle. *Vet. Parasitol.* 143, 166–173. https://doi.org/10.1016/j.vetpar.2006.08.007

Weston, J.F., Williamson, N.B., Pomroy, W.E., 2005. Associations between pregnancy outcome and serological response to *Neospora caninum* among a group of dairy heifers. *N. Z. Vet. J.* 53, 142–148. https://doi.org/10.1080/00480169.2005.36492

Wickham, H., 2016. *ggplot2: Elegant Graphics for Data Analysis.* Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Williams, D.J.L., McGarry, J., Guy, F., Barber, J., Trees, A.J., 1997. Novel ELISA for detection of *Neospora* -specific antibodies in cattle. Veterinary Record 140, 328–331. https://doi.org/10.1136/vr.140.13.328

Williams, D.J.L., Guy, C.S., McGarry, J.W., Guy, F., Tasker, L., Smith, R.F., MacEachern, K., Cripps, P.J., Kelly, D.F., Trees, A.J., 2000. *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. *Parasitology* 121, 347–358. https://doi.org/10.1017/s0031182099006587

Williams, D.J.L., Guy, C.S., Smith, R.F., Guy, F., McGarry, J.W., McKay, J.S., Trees, A.J., 2003. First demonstration of protective immunity against foetopathy in cattle with latent *Neospora caninum* infection. *Int. J. Parasitol.* 33, 1059–1065. https://doi.org/10.1016/s0020-7519(03)00143-7

Williams, D.J.L., Hartley, C.S., Björkman, C., 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. https://doi.org/10.1017/S0031182009990588

Williams, D.J.L., Trees, A.J., 2006. Protecting babies: vaccine strategies to prevent foetopathy in *Neospora caninum*-infected cattle. *Parasite Immunol.* 28, 61–67. https://doi.org/10.1111/j.1365-3024.2005.00809.x

Wouda, W., Dubey, J.P., Jenkins, M.C., 1997a. Serological diagnosis of bovine fetal neosporosis. *J. Parasitol.* 83, 545–547.

Wouda, W., Moen, A.R., Visser, I.J., van Knapen, F., 1997b. 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. *J. Vet. Diagn. Invest.* 9, 180–185. https://doi.org/10.1177/104063879700900212

Wouda, W., Brinkhof, J., van Maanen, C., de Gee, A.L.W., Moen, A.R., 1998a. Serodiagnosis of neosporosis in individual cows and dairy herds: A comparative study of three enzyme-linked immunosorbent assays. *Clin. Diagn. Lab. Immuno*l. 5, 711–716. https://doi.org/10.1128/CDLI.5.5.711-716.1998

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

Wouda, W., Bartels, C.J., Moen, A.R., 1999. Characteristics of *Neospora caninum*-associated abortion storms in diary herds in The Netherlands (1995 to 1997). *Theriogenology* 52, 233–245. https://doi.org/10.1016/s0093-691x(99)00125-9

Wouda, W., 2007. 3.1 Neosporosis: Biology, transmission and clinical signs, in: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), *Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control*, CABI Books. CABI International, pp. 46–53. https://doi.org/10.1079/9781845932114.0042

Wu, J.T., Christensen, S.E., 1991. Effect of different test designs of immunoassays on "hook effect" of CA 19-9 measurement. *J. Clin. Lab. Anal*. 5, 228–232. https://doi.org/10.1002/jcla.1860050314

Wu, J.T., Dreger, S., Chow, E.Y.W., Bowlby, E.E., 2002. Validation of 2 commercial *Neospora caninum* antibody enzyme linked immunosorbent assays. *Can. J. Vet. Res.* 66, 264–271.

Yaeger, M.J., Shawd-Wessels, S., Leslie-Steen, P., 1994. *Neospora* abortion storm in a midwestern dairy. *J. Vet. Diagn. Invest.* 6, 506–508. https://doi.org/10.1177/104063879400600424

Yániz, J., López-Gatius, F., García-Ispierto, I., Bech-Sàbat, G., Serrano, B., Nogareda, C., Sanchez-Nadal, J., Almería, S., Santolaria, P., 2010. Some factors affecting the abortion rate in dairy herds with high incidence of *Neospora*-associated abortions are different in cows and heifers. *Reprod. Domest. Anim.* 45, 699–705. https://doi.org/10.1111/j.1439-0531.2008.01337.x